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CURRENT OPINION

Implications of Non-Specific Effects for Testing, Approving, and Regulating Vaccines

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Abstract

The current framework for testing and regulating vaccines was established before the realization that vaccines, in addition to their effect against the vaccine-specific disease, may also have "non-specific effects" affecting the risk of unrelated diseases. Accumulating evidence from epidemiological studies shows that vaccines in some situations can affect all-cause mortality and morbidity in ways that are not explained by the prevention of the vaccine-targeted disease. Live attenuated vaccines have sometimes been associated with decreases in mortality and morbidity that are greater than anticipated. In contrast, some non-live vaccines have in certain contexts been associated with increases in all-cause mortality and morbidity. The non-specific effects are often greater for female than male individuals. Immunological studies have provided several mechanisms that explain how vaccines might modulate the immune response to unrelated pathogens, such as through trained innate immunity, emergency granulopoiesis, and heterologous T-cell immunity. These insights suggest that the framework for the testing, approving, and regulating vaccines needs to be updated to accommodate non-specific effects. Currently, non-specific effects are not routinely captured in phase I–III clinical trials or in the post-licensure safety surveillance. For instance, an infection with *Streptococcus pneumoniae* occurring months after a diphtheria-tetanus-pertussis vaccination would not be considered an effect of the vaccination, although evidence indicates it might well be for female individuals. Here, as a starting point for discussion, we propose a new framework that considers the non-specific effects of vaccines in both phase III trials and post-licensure.

Key Points

The existing framework for testing, approving, and regulating vaccines does not consider that vaccines have broad effects on the immune system that may alter the risk of unrelated infections.

It is now clear that vaccines can have important non-specific effects that can sometimes be very beneficial and sometimes harmful. In current practice, this can go unnoticed.

We propose a new framework for testing, approving, and regulating vaccines, with phase III trials, which should collect data on all symptoms arising during the follow-up, and with phase IV trials designed to assess vaccine effects on overall health.

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1 Introduction: Current Framework for Testing, Approving, and Regulating Vaccines

Vaccines are described as biological preparations that induce immunity towards a specific pathogen by the induction of pathogen-specific antibody-producing B-cells, B-memory cells, T-memory cells, or a combination of cellular responses, that remember the pathogen and respond quickly upon infectious challenge. It is well known that vaccines may cause frequent but generally mild adverse reactions, such as pain at the injection site, redness, soreness, and perhaps fever or fatigue in the days after vaccination. It is also accepted that vaccines, in rare circumstances, may cause serious adverse reactions that can occur weeks to months after vaccination.

The current clinical testing and approval process is built on the following generally accepted concepts. During a phase I trial, small groups of healthy volunteers receive the candidate vaccine. In phase II, the vaccine is given to individuals with characteristics matching those for whom

the new vaccine is intended. In phase III, the vaccine is given to thousands of participants in a randomized and blinded manner, with both an intervention and a control group, testing for efficacy and safety. Efficacy is typically the primary objective; safety is most commonly a secondary objective. Efficacy is typically assessed by comparing the vaccinated and the control groups with respect to the occurrence of the vaccine-specific disease and/or correlates of protection against the clinical disease. No standardized protocols exist for how phase III trials should collect data on safety, but there are some guidelines [1]. Typically, safety data are collected and reported in two groups. "Solicited" adverse events (AEs) are expected events related to reactogenicity, such as pain, redness, and swelling at the injection site, and are typically collected up to 2 weeks after a vaccination. "Unsolicited" events are unexpected events that are spontaneously reported by the participant. They are typically collected for up to 4 weeks after the last dose. Furthermore, participants are followed for serious AEs (SAEs; deaths and hospitalizations for any cause) and any pre-specified AEs of special interest for 6 months after the last dose. For vaccines that contain new adjuvants, it is recommended that there should be a follow-up for at least 12 months after the last dose to allow for the documentation of any autoimmune diseases or other immune-mediated AEs [1].

Rare AEs typically do not manifest in the clinical trial programs, and even if they do, there are usually too few cases to draw conclusions of causality. For example, if a vaccine caused a serious adverse reaction in 1 in 10,000 cases, it would take a study with 30,000 subjects to have a 95% chance of detecting even one case [2]. Therefore, after a vaccine has come on the market, there is a reporting system where vaccine providers and the public can report health problems ("post-licensure safety surveillance"). If there is doubt about the real-life effectiveness and safety of a vaccine, regulators can also require that a phase IV trial, a post-authorization safety study, be carried out [3]. This framework has worked well to deliver numerous new vaccines to the market; vaccines that were effective against the specific disease the vaccine was to target and for which we have reasonable assurance that the vaccine is not associated with frequent serious events that would shift the benefit/risk balance.

However, it is now evident that vaccines may affect the risk of other diseases in ways that were not foreseen when the current framework was established. Here, we propose that a new framework for testing, approving, and regulating vaccines is needed. This framework includes an assessment of vaccine effects on infections other than the target infection, and on overall health (such as all-cause mortality, all-cause hospitalization, or all-cause consultation rates).

In the following, we provide a background for this proposition, which is based on the discovery of the non-specific effects of vaccines. Subsequently, we outline the contours of the proposed framework, as a starting point for discussion.

1.1 Observations of Non-Specific Effects of Vaccines: Epidemiological Studies

Historically, there is anecdotal evidence that the smallpox vaccine reduced the risk of a number of other diseases [4]. Calmette, co-inventor of the Bacillus Calmette-Guérin (BCG) vaccine, noted that mortality was reduced by 75% among BCG vaccinated children in Paris, much more than could be explained by prevention of tuberculosis; he speculated that the vaccine may have additional benefits, strengthening the general resistance against other infections [5]. In the 1960s and 1970s, the Russian virologist Voroshilova conducted large trials of live enteroviruses, including oral polio vaccine, and found that they significantly reduced the risk of influenza infection [6].

In the 1980s, when the Danish-Guinean field station Bandim Health Project started a systematic investigation into the overall health effects of routinely used childhood vaccines, it became clear that most vaccines affected all-cause mortality and morbidity more than explained by prevention of the target disease. These effects were termed the "non-specific effects" of vaccines [7].

A pattern emerged with differences in effects between live attenuated vaccines and non-live vaccines. The live attenuated vaccines have broadly beneficial non-specific effects [8], beneficial non-specific effects that are seen while they are the most recently administered vaccine. For example, African children, who receive live vaccines, have considerably lower all-cause mortality compared with children who do not receive the live vaccines, and the difference is not explained by differences in mortality due to the vaccine-targeted infection [8]. Because the mortality in such settings is mainly due to infectious diseases, this suggests that the vaccines decrease susceptibility to unrelated infections or their severity, and where it has been possible to stratify by causes of deaths, studies have shown a particular effect against infectious deaths [9, 10]. Lower-than-anticipated all-cause mortality has been observed for four live vaccines: measles-containing vaccine, smallpox vaccine, BCG vaccine, and oral polio vaccine [8]. The initial data came from observational studies. It is difficult to test already approved vaccines in randomized trials, but in some situations, it has been possible, for example, by randomizing children to receive the vaccine at different ages, allowing an unbiased comparison over the time window between when the early group and the late group were vaccinated. Such randomized trials have largely corroborated the beneficial non-specific

effects of the BCG vaccine [9, 11], measles vaccine [12, 13], and oral polio vaccine [14]. However, the findings have not always been consistent [15–17], interpreted as possibly due to differences in vaccine strains, some strains having stronger immunological effects than others[18], or due to interactions with other vaccines that varied in frequency between trial settings [19]. Thus, non-specific effects are context dependent [20].

In contrast to the live vaccines, some non-live vaccines, though protective against the vaccine's target disease, may increase the risk of other infections, particularly in female individuals, in certain contexts. For example, in low-income settings, female individuals who receive a non-live diphtheria-tetanus-pertussis (DTP) vaccine have a 1.5–2 times higher mortality rate than female individuals who have not received the vaccine and a similar increased risk above that of male individuals vaccinated with DTP [21]. This pattern has been observed for six non-live vaccines: [8] DTP vaccine, pentavalent vaccine [22] (DTP plus hepatitis B and *Haemophilus influenzae* type B vaccines), hepatitis B vaccine [23], inactivated polio vaccine[24], H1N1 influenza vaccine [25], and RTS,S malaria vaccine [26]. This has not been consistent in all studies [27, 28], so non-specific effects, positive as well as negative, can be modified—most clearly by sex [8, 21], but also by factors such as the administration of other vaccine types [8].

These non-specific effects are most pronounced when a given vaccine is the most recent vaccine. Most studies have been conducted among children, who usually receive frequent vaccinations, and therefore there are few studies on the duration of the non-specific effects, should no other vaccines be given. However, non-specific effects seem to last at least 6 months [8, 29], and sometimes persist for many years [30, 31]. The non-specific effects of vaccines were initially observed in low-income settings with high mortality due to infectious diseases, but non-specific effects have also been reported in some studies from high-income settings, which assessed the risk of non-targeted infectious disease hospitalizations [32, 33], corroborating that vaccines can affect the risk of unrelated infections.

1.2 Immune Mechanisms Underlying the Non-Specific Effects of Vaccines

Supporting the consistent observations from epidemiological studies, immunological studies have demonstrated at least three vaccine-mediated effects on the immune system that can explain how a vaccine might affect the risk for unrelated infections. First, it has been shown that several vaccines alter the ability of innate immune cells to respond to subsequent unrelated challenges, "trained innate immunity" [34]. Monocytes and natural killer cells from humans vaccinated with BCG show an enhanced production of proinflammatory cytokines, not only upon challenge with *Mycobacterium tuberculosis* (the specific pathogen), but also upon challenge with unrelated pathogens such as *Staphylococcus aureus* and *Candida albicans* [35]. This is mediated via epigenetic changes in the promoters and enhancers of proinflammatory cytokine genes. The clinical implications have been demonstrated: when a BCG vaccine was given to human volunteers before challenge with the live yellow fever vaccine, the yellow fever viral load in the circulation was reduced [36]. Likewise, in human experimental studies, the BCG vaccine modified the course of an experimental malaria infection [37]. In a recent study, intravesical BCG in patients with bladder cancer induced trained immunity and decreased the risk of respiratory tract infections [38]. Innate immune training has been demonstrated for live vaccines such as the BCG vaccine and smallpox vaccine [39] and more recently also the adenovirus-based COVID-19 vaccine [40], and may explain why these vaccines have beneficial non-specific effects. In contrast, several non-live vaccines (DTP vaccine [41, 42], typhoid vaccine [43], and non-replicating smallpox vaccine [39]) have been shown to induce innate immune tolerance towards unrelated pathogenic challenges. The increased innate tolerance towards other pathogens may explain why non-live vaccines are associated with increased susceptibility to other infections. However, the pattern of live vaccines inducing innate immune training and non-live vaccines inducing innate tolerance is not completely consistent because recently some non-live vaccines such as inactivated influenza vaccine have been associated with an induction of trained immunity, although these effects appear dependent on adjuvants in the formulation [44, 45].

Second, it has been shown that the BCG vaccine given to neonates leads to emergency granulopoiesis that expands neutrophil storage pools, thereby releasing them in larger numbers in response to ongoing or subsequent infection with non-vaccine pathogens [46], a plausible explanation for the strong protective effects of the BCG vaccine given at birth on all-cause mortality during the first month of life [9]. Third, vaccines may induce cross-protective T cells that can respond to pathogens unrelated to the vaccine pathogen. For instance, in humans, cross-reactive influenza virus-specific CD8+ T cells can contribute to lymphoproliferation in Epstein–Barr virus-associated infectious mononucleosis [47]

The immunological mechanisms underlying the sex differences in the non-specific effects of vaccines have not yet been fully understood, but it is well documented that male and female individuals have different immune responses to a pathogen challenge, and that they exhibit different dynamics and kinetics [48, 49]. Therefore, differential sex-based outcomes should be anticipated [48, 49].

Though there is still a paucity of studies linking immunological non-specific effects to clinical heterologous effects, it is now clear that vaccines affect the immune system in

additional ways beyond the induction of vaccine-specific immunity. This adds biological plausibility to the epidemiological studies showing that vaccines may affect the risk of unrelated infections. This new knowledge necessitates a re-evaluation of the current framework for testing, approving, and regulating vaccines.

2 Gaps in the Current Practice

2.1 Insufficient Assessment of the Effect of Vaccines on Unrelated Infectious Diseases and All-Cause Mortality and Morbidity

Current phase III trials can capture unrelated infections as AEs, but such events would be "unsolicited" and reported only upon suspicion by the study participant or investigator. If unrelated infections during follow-up lead to death or hospitalization they would be captured as SAEs, but studies would usually not be powered to detect significant differences in SAEs between groups. If there were statistically significant differences in rates of SAEs between treatments, the guidelines stipulate that they should be interpreted with caution unless the trial was designed to address pre-specified hypotheses regarding such endpoints [1]. It is furthermore stipulated that the biological plausibility that SAEs may be related to vaccination should be taken into consideration when deciding on the need for further pre- or post-licensure trials to investigate and quantify the potential risks [1]. With the current view that relevant vaccine-induced effects are solely those that are pathogen specific: if there were a significant difference in the occurrence of SAEs due to unrelated infections in the two groups—either a lower or higher risk in the intervention than in the control group—this would likely be ascribed to chance, as it would be judged biologically implausible that it was due to the vaccine.

Post-licensing, health professionals and the lay public have little notion that unrelated infections occurring perhaps weeks to months post-vaccination could be related to effects following vaccination, and thus there will be no or very limited reporting of such events. Accordingly, it is possible to introduce in the vaccination program a new vaccine that is associated with effects on other infections and on all-cause mortality and morbidity—positive or negative—without these being detected.

As an example, the "high-titer" measles vaccine (HTMV) introduced by the World Health Organization in 1989 in areas with a high incidence of measles infection was fully protective against measles. Its introduction was based on its ability to induce seroconversion also in the presence of maternal antibodies and a lack of adverse reactions when compared with a few hundred children receiving the standard measles vaccine and with 63 unvaccinated children [50]. When independent researchers examined the vaccine's non-specific effects in randomized trials, comparing the HTMV with the standard measles vaccine, the HTMV was associated with a doubling of mortality in female individuals compared with the standard measles vaccine [51]. In response, the World Health Organization withdrew the HTMV in 1992 [52], when these findings had been replicated several times. The results of the meta-analysis carried out afterwards [53] indicated that with the mortality level at that time in Africa, the continued use of the HTMV could have led to up to 500,000 excess female deaths per year in Africa. Had independent researchers not assessed the effect of the HTMV on overall health, the negative non-specific and fatal effects of introducing the HTMV in female individuals would likely have gone unnoticed. Even if an excess mortality had been observed, with the current perception of vaccine mechanisms, little thought would have been given to the possibility that the introduction of the HTMV would be associated with fatal non-specific effects, for a vaccine judged effective and safe. Similarly, current practice does not allow for the detection of beneficial non-specific effects of vaccines.

2.2 Context Independence Versus Context Dependence

Many studies show that female individuals respond with stronger antibody responses but also more side effects than male individuals [54–57]. Though the strength of the immune response may vary by sex and other factors such as age [55–57] and geographical latitude, current clinical practice largely assumes that vaccine effects are context independent, that is, that a correctly applied vaccine will induce specific protection in most individuals. Phase III trials often aim for inclusion of both sexes, but often employ quite narrow inclusion and exclusion criteria. Other health interventions that may affect the immune system, such as other vaccines received before or during a follow-up, are rarely accounted for; concomitantly administered vaccines may be investigated, but only to detect if there is an interference in the generation of immune responses or unacceptable reactogenicity.

As indicated in the Introduction, the non-specific effects of vaccines, in contrast, vary significantly by context [8, 20]. The immune response to a vaccine is not limited to specific B and T cells but is influenced by the state of the immune system at the time of vaccination, including other interventions and external factors that affect the immune system. Such interactions may not be considered by vaccinologists, but are well known in pharmacology, where it

is standard practice to search for interactions, for example, between drugs that affect cytochrome P450 [58]. Non-specific effects can depend strongly on the temporary order of vaccination [8]. For example, a non-live DTP vaccine given after a live measles-containing vaccine is associated with increased all-cause mortality in female individuals, whereas a measles-containing vaccine given after a DTP vaccine is associated with reduced all-cause mortality [27]. Other identified effect modifiers include interventions that affect the immune system, such as vitamin A supplements, and comorbidities affecting immune status. The effect of vitamin A supplementation has been shown to depend more on the vaccines with which it is given than on the degree of vitamin A deficiency, being very beneficial when given at the time of a live measles-containing vaccine, but not at the time of a non-live DTP vaccine to female individuals [59]. Interactions can also occur across generations. Maternal priming with a vaccine may influence her child's non-specific response to the vaccine, for example, a BCG vaccination is significantly more beneficial for the children of women who were themselves BCG vaccinated than in the children of BCG-unvaccinated women [60, 61].

Current practice therefore has two important deficiencies: it does not emphasize systematic assessment of the non-specific effects on unrelated infections and overall health effects, and it rarely considers effect modifiers.

3 Proposed New Framework

To detect if a vaccine has important non-specific effects with consequences for overall health, we propose a new framework for testing, approving, and regulating a vaccine against a disease for which there is not already an existing vaccine (Table 1) [If there is already an approved vaccine against the disease, there will be other considerations: e.g., whether the new vaccine and the approved vaccine should be compared directly in a randomized trial; this would make sense if the approved vaccine had already been assessed for its non-specific effects].

3.1 Phase III Trials

- The trials should include the anticipated target population, as already recommended, but not always followed [62].
- The control group should only have saline, another neutral treatment or no intervention—not another vaccine or adjuvant that might have non-specific effects, thus not being a true control [63].
- The trials should ideally, if possible, be conducted with a sufficiently large study population to rule out, with a prespecified degree of certainty, any major risk of serious outcomes from the vaccine, such as increased all-cause mortality or hospitalization.

Table 1 Current and proposed assessment of vaccine safety

	Current phase III trials	Proposed phase III trials
"Placebo"	Some use another vaccine or the adjuvant	Never use another vaccine or the adjuvant if there is no approved vaccine for the targeted infection(s) [because they may also have non-specific effects]
Adverse events	Solicited adverse events collected within a limited time frame. Deaths/hospitalization collected for full duration of the trial. Assessed for plausibility	All clinical symptoms should be actively asked for and recorded and coded for at least 12 months [everything is plausible]
Coding of adverse events	Diagnoses are preferentially coded before signs and symptoms	Symptoms should be given priority
Outcomes	Typically (symptomatic) infection and/or "correlates of protection"	In addition, all infectious diseases and overall mortality and morbidity (e.g., all-cause consultations, hospitalizations, deaths), by sex and age group, as well as biomarkers of non-specific effects, such as in vitro cytokine and immune cell responses to non-related pathogens
Duration of follow-up	Variable. Sometimes the control group is vaccinated once the vaccine is approved	Blinded follow-up of vaccinated and control subjects for at least 12 months
	Current post-licensure surveillance	Proposed post-licensure surveillance
Comparison groups	Observational studies comparing vaccinated vs unvaccinated; before and after comparisons	Randomized trials individually or by cluster; step-wedged roll-out. Powered to study overall health outcomes in both sexes
Safety	Reporting by general practitioners and citizens	Active follow-up through interviews/registers
Outcomes	"Plausible" adverse events	All infectious diseases. Overall mortality and morbidity, e.g., all-cause consultation, hospitalization, deaths

- A systematic follow-up for all symptoms should be for at least 12 months to register all-cause health outcomes, non-specific effects (positive and negative), and possible AEs. All symptoms occurring during the full duration should be coded by symptom/disease category and by sex and age. They should be reported in the trial publication and to regulatory authorities in sufficient detail to allow for scrutiny by independent researchers.
- Assessment of biological plausibility should include the possibility that an unrelated infection or an increased severity of infection could be due to non-specific effects, for example, an infection with *Streptococcus pneumoniae* occurring months after a DTP vaccination could well be an effect of the vaccine.
- Specific as well as non-specific vaccine effects should be analyzed and reported, applying the intention-to-treat principle from the day of randomization.
- Vaccine trials should systemically register and report other interventions provided during a follow-up that may affect the immune system, for example, if participants received other vaccines. Efficacy and safety should be reported before and after such additional interventions.
- In parallel, it is recommended that appropriate biomarkers for both positive and negative non-specific effects be sought. In the future, such biomarkers could potentially serve as "stop-go" signals already in the phase I/II trials.

3.2 Post-Licensure Testing

Once a vaccine is approved, it should be randomly allocated to ensure that large groups of initially comparable vaccinated and unvaccinated population groups can be followed and compared over time. This would allow the detection of differences in the incidence or severity of other diseases and provide an assessment of the effect of the vaccine on overall health and its cost effectiveness. There are several ways to do this. One way is to introduce the new vaccine in the form of a cluster randomized trial; the randomization unit could be general practice clinics, municipalities, or regions. Such population-based randomized trials have been carried out in Finland [64].

Alternatively, the vaccine can be introduced gradually, in a step-wedge design. For example, starting vaccination in one region and gradually, over months or years, introducing it to other regions.

The final design of the post-licensure assessment (whether it is conducted as a randomized trial or by a step-wedge roll-out or something else) should be triangulated based on prior knowledge from the phase III trial and information about the type of vaccine. Based on the current evidence, if it is a new vaccine type, or if the vaccine is a non-live vaccine, it should prompt a randomized trial. Furthermore, if the phase III trial does not show an effect on all-cause mortality or morbidity that is in line with what was anticipated based on the vaccine's effects against the target disease, and/or if the collected biomarkers and/or immunological studies reveal signals of increased innate immune tolerance, this should prompt a more thorough phase IV evaluation.

Importantly, these post-licensure assessments should include information on the context because, as mentioned, the effect of a vaccine on overall health is dependent on factors such as sex and can be modified by the vaccination status of the recipient, by other vaccines and interventions the recipient receives during a follow-up, and other factors that may influence the immune system. At a minimum, a program should assess the overall health effects of new vaccines separately for female and male individuals. These recommendations would gradually lead to the establishment of a global knowledge database on vaccine interactions with clinical consequences, and ways to test these.

3.3 Economic Considerations

The cost of undertaking a phase III vaccine trial is presently the responsibility of the developer. The prospect of expanding phase III trials to include more participants comes with a considerable associated cost. However, with more robust results this may reduce the need for some post-licensure studies and the additional health benefits beyond the specific disease protection may result in a wider vaccine uptake, inevitably supported by governments and health authorities. Therefore, there is potential for government-private sector co-sponsorship of phase III vaccine trials. Post-licensure evaluation can occur as a phase IV trial, sponsored by the developer in partnership with the government. Indeed, it may be preferable that oversight for post-licensure evaluation be within the sole jurisdiction of health authorities, to ensure authenticity.

3.4 Ethical Considerations

The proposed framework raises some important ethical considerations. Even in situations without any existing vaccines, some would argue that the control group should have some type of intervention. However, as endorsed by a World Health Organization expert panel, placebo use in vaccine trials is ethically acceptable when no efficacious and safe vaccine exists [65].

Larger phase III trials with longer durations could cause delays in the release of a new vaccine. Randomized or step-wedged roll-out of a new vaccine would mean that the vaccine would not be available for all from the date of approval. However, given the reality of production capacity limitations combined with the often slow release of a new vaccine, the

suggested framework is not only realistic, but also probably more advantageous for the overall health of the population.

It is no longer defensible to ignore the accumulating evidence that vaccines have broad effects on the immune system and thereby the risk for other infections and ultimately the risk of all-cause mortality and morbidity. Ignoring vaccine non-specific effects would risk new vaccines increasing all-cause morbidity and even mortality, thereby severely undermining the credibility of vaccine programs. For example, the introduction of the HTMV would have led to major increases in childhood mortality if the non-specific effects had not been detected by independent investigators.

A fear is that these proposed changes to the current framework for testing, approving, and regulating vaccines may open the door to an unwarranted discussion of vaccine safety, therefore strengthening the anti-vaccination movement. However, in our view, it would be devastating if the anti-vaccination movement were given this type of power to define, and possibly restrict, the use of a sound scientific method forward.

We believe the public will understand the logic of this reasoned approach, health-wise, scientifically, and economically. Most likely, these initiatives will address safety concerns and increase trust in health authorities to mitigate vaccine hesitancy and counter the rhetoric of the anti-vaccination movement.

4 Conclusions

Currently, there is a well-developed framework for testing, approving, and regulating vaccines. Yet, with what we know today, we are not optimally testing vaccines before their introduction. There is a growing body of evidence that vaccines have broad effects on the immune system and on the risk of unrelated infections. To optimize vaccine benefits, reduce possible harm, and maintain public trust, it is essential to document that a given vaccine has a net beneficial effect on overall health. With this paper, we hope to start a discussion on how this could best be accomplished.

Declarations

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Authors' contributions CSB wrote the first version of the paper after having discussed the topic with the co-authors, particularly with PA. All co-authors (mentioned in alphabetic order) read and commented on the paper and approved the final version of the manuscript.

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