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## Vaccine

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## Vaccine development: From concept to early clinical testing

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## ABSTRACT

In the 21st century, an array of microbiological and molecular allow antigens for new vaccines to be specifically identified, designed, produced and delivered with the aim of optimising the induction of a protective immune response against a well-defined immunogen. New knowledge about the functioning of the immune system and host pathogen interactions has stimulated the rational design of vaccines. The design toolbox includes vaccines made from whole pathogens, protein subunits, polysaccharides, pathogen-like particles, use of viral/bacterial vectors, plus adjuvants and conjugation technology to increase and broaden the immune response. Processes such as recombinant DNA technology can simplify the complexity of manufacturing and facilitate consistent production of large quantities of antigen. Any new vaccine development is greatly enhanced by, and requires integration of information concerning:

**1. Pathogen life-cycle & epidemiology.** Knowledge of pathogen structure, route of entry, interaction with cellular receptors, subsequent replication sites and disease-causing mechanisms are all important to identify antigens suitable for disease prevention. The demographics of infection, specific risk groups and age-specific infection rates determine which population to immunise, and at what age.

**2. Immune control & escape.** Interactions between the host and pathogen are explored, with determination of the relative importance of antibodies, T-cells of different types and innate immunity, immune escape strategies during infection, and possible immune correlates of protection. This information guides identification and selection of antigen and the specific immune response required for protection.

**3. Antigen selection & vaccine formulation.** The selected antigen is formulated to remain suitably immunogenic and stable over time, induce an immune response that is likely to be protective, plus be amenable to eventual scale-up to commercial production.

**4. Vaccine preclinical & clinical testing.** The candidate vaccine must be tested for immunogenicity, safety and efficacy in preclinical and appropriately designed clinical trials.

This review considers these processes using examples of differing pathogenic challenges, including human papillomavirus, malaria, and ebola.

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**Abbreviations:** APC, antigen presenting cells; CSP, circumsporozoite protein; DHF, dengue haemorrhagic fever; DSS, dengue shock syndrome; GMP, good manufacturing practices; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HIV, human immunodeficiency virus; HPV, human papillomavirus; HSV, herpes simplex virus; MPL, 3-O-de-acylated-4-mono-phosphoryl lipid A; PAMPs, pathogen-associated molecular patterns; PRR, pattern recognition receptors; VLP, virus-like particles; VSV, vesicular stomatitis virus; VZV, varicella zoster virus.

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## 1. Introduction

Natural immunity against a pathogen derives from the integrated activation of the innate and adaptive immune systems (Table 1) [1]. Innate immunity arises after detection of specific pathogen-associated molecular patterns (PAMPs) through a variety of pattern recognition receptors (PRRs) [2]. The PRRs are able to detect common structural and functional features associated with different classes of microorganisms, and depending on the type of PAMP, activate specialised Antigen Presenting Cells (APCs) e.g., dendritic cells. Activation of innate immunity induces expansion

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**Table 1**  
Key characteristics and effectors cells of the innate and adaptive immune response.

Innate immunity: first line of defence	Adaptive immunity: second line of defence
<ul style="list-style-type: none"> <li>• Triggered by damage or threat (recognition of PAMPs)</li> <li>• Rapid response (hours)</li> <li>• Usually No development of immune memory</li> <li>• Pathogen destruction via phagocytosis, killing and release of bioactive mediators</li> <li>• Triggers tissue repair</li> <li>• Stereotypical response</li> <li>• Triggers downstream adaptive responses via antigen-presenting cells</li> <li>• Effector cells: Granulocytes (basophils, neutrophils, eosinophils), Mast cells, Macrophages, Monocytes, Natural killer cells, Dendritic cells</li> </ul>	<ul style="list-style-type: none"> <li>• Activated by pathogen encounter</li> <li>• Slower response (days or weeks)</li> <li>• Large repertoire of effector molecules</li> <li>• Antibody mediated and T-cell mediated destruction</li> <li>• Development of memory</li> <li>• Highly specific and adaptable</li> </ul> <p>• Effector cells: CD4+ T-cells, CD8+ T-cells, B-cells, Plasma cells</p>

PAMPs = pathogen-associated molecular patterns.

of adaptive immune cells targeted to the particular threat through antigen-specific T-cell effector and antibody mechanisms. The immunological memory derived from this antigen-specific response persists and can react more rapidly upon subsequent infection [3].

Immunisation is the strategy of stimulating the host's defence against a specific pathogen to establish immunological memory and thus protect against the consequences of infection. Some vaccines are made of whole viruses or bacteria which contain the microbial elements (PAMPs) that trigger the innate immune response required to initiate a suitable adaptive response. However, a whole-pathogen approach may not be feasible, practically or from a safety perspective, or desirable, especially if the agent is very reactogenic or tumorigenic. In such cases, partial fractionation may reduce reactogenicity or tumorigenicity by removing some pathogen components. Alternatively, recombinant DNA technology and biotechnology, or chemical purification can be used to produce a subunit of the pathogen as the vaccine antigen. The latter approaches require in-depth knowledge of the biology of the pathogen to identify the immunologically-relevant vaccine component(s). Since purified proteins usually demonstrate poor immunogenicity by themselves, adjuvants are used to enhance and modulate the immune responses by providing innate/PAMP triggers, thereby driving a protective response to the pathogenic threat. Combining the correct antigens and adjuvants to optimise the subsequent downstream adaptive immune response is a crucial task in the development of any new vaccine. Here we discuss the key principles and challenges faced in the development of vaccines targeting a diverse set of pathogens from concept to clinical trial in humans.

## 2. Pathogen life-cycle and epidemiology

Detailed knowledge of the biology and structure of the pathogen, its interaction with cellular receptors and its disease-causing mechanisms is important in order to identify antigens suitable for disease prevention. For some microorganisms, characteristics that differentiate commensal from pathogenic forms may need to be identified. Where the key subunit immunogens, e.g., capsule polysaccharides or virus surface proteins, are not conserved, or broad cross-reactive immunity cannot be generated (e.g., pneumococcus or human papilloma virus [HPV], Box 1 [3–5]), it may be necessary to prioritise the most common or the most medically important strains or serogroups. These often vary geographically or temporally: understanding the epidemiology of the disease is crucial to identifying the target antigens. The selection of serotypes is based on complex modelling involving serotype distribution, value and reimbursement, and the number of different subunits the vaccine may realistically contain based on costs and complexity of manufacture [6]. Where the epidemiology indicates a constrained distribution, a vaccine providing relevant – but less broad – strain coverage may be preferred on the grounds of cost

or availability [7]. In some circumstances, such as for seasonal influenza, variability of the key antigens is unavoidable and a new vaccine is made each year.

Knowledge of the route of entry and subsequent replication sites of the pathogen is essential. This is because protection against pathogens entering via the respiratory (influenza, pneumococcus), gastrointestinal (Salmonella) or genital tracts (Herpes simplex virus [HSV] or human immunodeficiency virus [HIV]), or entering the bloodstream by injury/injection (hepatitis B/C) or mosquito bite (Malaria, Box 2 [8–10]), may require different vaccination strategies. To take one example, the immune response after natural malaria infection is considered to be predominantly directed against the blood stage of the pathogen but some vaccines have shown that it is possible to induce effective immunity by targeting the pre-erythrocytic stage, e.g. during sporozoite stage and the liver stage of the pathogen [8–10]. Similarly, prevention of the reactivation of infection may require different strategies to preventing primary infection. Special cases in vulnerable populations include postpartum infections such as group B streptococcus, and antenatal/perinatal infection such as hepatitis B (HBV) and HSV, as well as persistent virus such as Varicella zoster virus (VZV) and cytomegalovirus (CMV).

The clinical manifestations of the disease of interest and potential outcomes in the natural setting will also influence the vaccine requirements. For example, some pathogens, such as pneumococcus, can cause multiple clinical syndromes (invasive disease, pneumonia and otitis media), while dengue virus-associated diseases are substantially more serious when antibodies to one of the four types are already present [11].

Knowledge of the demographics of infection (poverty, overcrowding versus delayed exposure in wealthier countries), specific risk groups and age-specific infection rates determine which population to immunise, and at what age. Having clear diagnostic criteria is fundamental and increasingly these diagnostic approaches include the capacity to identify both pathogen and serotype. Partner diagnostics are now being developed to support new vaccines. If the accuracy of diagnosis is poor, then the frequency of infection may be grossly under- or over-estimated, which has implications for understanding the disease burden to be prevented, and the impact of the vaccine after it is used.

The breadth of challenge for successful vaccine development is illustrated by comparison of the diversity of structure, polymorphism, natural history of infection and the consequence for human health of oncogenic HPV (Box 1), *Plasmodium falciparum* (responsible for the most aggressive malaria) (Box 2), and haemorrhagic Ebolavirus (Box 3 [12–14]).

## 3. Natural immune control & escape

Human pathogens show enormous diversity in their biology, differing in the type of infection they induce (acute, chronic, latent), tissue target (skin or mucosal infections of the gastroin-

**Box 1**

Human papillomavirus (HPV) vaccines.

Pathogen	High risk HPV
Disease	Anogenital & oral pharyngeal cancer
Structure & diversity	<ul style="list-style-type: none"> <li>• 8 kb, double-stranded DNA <i>papillomavirus</i></li> <li>• genome encodes 8 proteins</li> <li>• 55 nm particle of 72 capsomeres composed of major L1 &amp; minor L2 capsid proteins</li> <li>• 12 High Risk oncogenic types(16,18, 31,33, 39,45, 51,52, 56,58 &amp; 59)</li> <li>• 70% of cancer caused by HPV types 16, 18</li> </ul>
Life cycle & epidemiology	<ul style="list-style-type: none"> <li>• Exclusively epithelial; no viraemia</li> <li>• Infection of basal epithelia mainly through sexual activity causing minor trauma. Productive infection linked to terminal differentiation of epithelium with particle release from uppermost apoptotic cells</li> <li>• Risk for cancer from persistent infection leading to viral E6 &amp; E7 driven immortalisation &amp; genetic instability</li> </ul>
Disease Burden	<ul style="list-style-type: none"> <li>• Premalignant cancers only apparent through screening</li> <li>• Cervical cancer most prevalent with 230,000 deaths and &gt;0.5 million new cases/year; mostly in the developing world</li> </ul>
Natural Immune control and escape	<ul style="list-style-type: none"> <li>• Natural immune control and clearance most likely from T-cell immunity against viral early antigens E2,E6 &amp; E7</li> <li>• Neutralising antibodies develop against L1 but late after infection and only at low levels in 50% patients</li> <li>• Insufficient neutralising antibodies in cervico-vaginal secretions and local cellular immunity from lack of antigen-presenting cell activation/ inhibition of effector pathways leading to persistent infection</li> </ul>
Vaccine strategy	<ul style="list-style-type: none"> <li>• Vaccination to induce neutralising antibodies to prevent infection prior to sexual debut in females can impact major disease burden</li> </ul>
Antigen selection	<ul style="list-style-type: none"> <li>• Recombinant HPV L1 can form a virus-like particle (VLP) mimicking key antigenic features of HPV types</li> <li>• L1 only made in terminally differentiated cells so cellular immunity not helpful for clearance of infection</li> <li>• Unknown if natural infection can necessarily boost a vaccinated individual's antibody response in a timely fashion to prevent infection</li> <li>• Animal studies established potential for antibody-mediated protection</li> <li>• Need neutralising antibodies at sufficient levels to impact infection sites to maximise levels &amp; longevity for sexual life protection; which can be optimised by the use of adjuvants</li> </ul>
Vaccine formulation	<ul style="list-style-type: none"> <li>• A quadrivalent vaccine containing VLPs of types 6, 11, 16 and 18 produced in yeast with an aluminium hydroxyphosphate sulphate adjuvant</li> <li>• A bivalent vaccine containing recombinant baculovirus produced HPV 16 &amp; 18 VLPs with aluminium hydroxide + 3-O-de-acylated-4-mono-phosphoryl lipid A (MPL) adjuvant (ASO4). MPL is a detoxified form of bacterial lipopolysaccharide which binds to Toll like receptor-4, a pattern recognition receptor</li> </ul>
Immunogenicity	<ul style="list-style-type: none"> <li>• HPV cannot easily be grown <i>in vitro</i>, nor is it cytopathic</li> <li>• Enzyme-linked immunosorbant assay, Competitive Luminex, pseudo-neutralisation or cervicovaginal murine challenge assays of immunogenicity available are all surrogates for natural infection since there is no known immune correlate of protection</li> <li>• Immunisation schedules in volunteers based on 2 or 3 vaccinations for both vaccines gave 100% sero-conversion and antibody levels many fold higher than natural levels</li> </ul>
Clinical Trial Design & testing	<ul style="list-style-type: none"> <li>• Aim is to prevent cancer but have to use a surrogate endpoint of high grade cervical intraepithelial neoplasia (CIN2/3) in sexually active young women</li> <li>• Both vaccines are safe, give virtually 100% efficacy in protection against the vaccine-targeted types of CIN2/3 in women naïve to the corresponding type at the time of vaccination in early clinical trials support the use of the preferred vaccination strategy. Follow up times limited</li> <li>• Use in adolescents is justified by excellent safety and stronger immune responses</li> <li>• The bivalent ASO4 adjuvant formulation provides for increased cross-protection against oncogenic non-vaccine types; quadrivalent vaccine gives effective protection against HPV 6/11 associated with genital warts [3]</li> </ul>
Future	<ul style="list-style-type: none"> <li>• Nonavalent Vaccine [4] is quadrivalent vaccine plus 5 other HPV types. Licensed but yet to be shown to be more effective than quadrivalent or bivalent in protection versus CIN3</li> <li>• Therapeutic vaccines: for treatment of infections or early cancers by targeting E6 and E7 using a plethora of approaches. Recent DNA vaccine delivered by electroporation met primary clinical endpoint in CIN3 [5]</li> </ul>

testinal, respiratory and urogenital tracts, infections in specific non-mucosal target tissues or organs such as the meninges, blood stream or liver) and location (intracellular versus extracellular). Many pathogens also show considerable genetic diversity within their species or virus type. In most cases, infection is met by a timely innate and subsequent adaptive immune response leading to control and elimination of the infection. However, pathogens often express a number of virulence determinants that allow the pathogen to avoid immune defences, facilitating infectivity and transmission. These include stealthy infection, whereby pathogen-encoded determinants bind to specific cellular receptors allowing cell entry without alerting the immune response (viruses and some bacteria), production of proteins, enzymes and micro-RNAs that inhibit host-pathogen recognition mechanisms and innate immune effector responses (influenza A) [15], and production of structures such as polysaccharide capsules that inhibit immune effectors such as complement (*Neisseria meningitidis*) [16]. Other virulence determinants are structural changes that promote intracellular sequestration (*Escherichia coli*) [17], high rates of mutation that ensure that antibodies stimulated during earlier infection remain ineffective (influenza, HIV, hepatitis C) [18], expression of toxins that cause tissue destruction and modulate

the immune response (pneumococcus) [19], mimicry of host proteins (meningococcus serotype B) [20], and latent stages that remain undetected by the immune system (herpes viruses, HIV, tuberculosis) [21]. Detailed knowledge of immune escape strategies used by individual pathogens is important for developing effective vaccines against them.

A major goal in vaccine development is determining the immune response that must be elicited by vaccination; the so-called 'correlate of protection'. Antibody-mediated neutralisation has traditionally been the major target of vaccines, as many pathogens require receptor-mediated binding to cells and/or fusion, or mediate pathogenicity by producing specific toxins; all of which can represent protective antibody targets. Newer vaccines targeting more complex pathogens are designed to enhance other aspects of the innate and adaptive response. The relative contribution of antibodies, CD4+ and CD8+ T-cells and innate immunity to protection against infection needs to be evaluated and transposed to vaccine-induced primary immunity or to immunotherapy of persistent or recurrent infections. The role of selected T-cell-mediated effector functions, including pro-inflammatory cytokine production and help for antibody-mediated immunity, also needs to be determined.

**Box 2**  
Malaria vaccine.

Pathogen	<i>Plasmodium falciparum</i>
Disease	Malaria
Structure & diversity	<ul style="list-style-type: none"> <li>• 23mb Haploid parasite</li> <li>• Genome encodes 5500 proteins</li> <li>• <i>P. falciparum</i> is the most virulent (98% mortality; most drug resistant) of 5 species of human parasites. Others have slightly different life-cycles with geographic and antigenic diversity</li> </ul>
Life-cycle & epidemiology	<ul style="list-style-type: none"> <li>• Transmission by female anophelene mosquito; 3 life cycle phases</li> <li>• Pre-erythrocytic (invasive sporozoites develop to large schizonts in liver)</li> <li>• Asexual blood stage merozoite invasion &amp; replication in erythrocytes with significant parasitaemia causing clinical symptoms</li> <li>• Sexual stage: male &amp; female gametocytes in blood ingested by mosquito</li> </ul>
Disease Burden	<ul style="list-style-type: none"> <li>• 584,000 deaths; 198 million clinical illnesses, majority in sub-Saharan Africa</li> </ul>
Natural Immune control and escape	<ul style="list-style-type: none"> <li>• Distinct immune control mechanisms at each stage; depending on whether parasite is extra- or intracellular, antibody and/or cellular immunity can provide protection (unknown% of infections)</li> <li>• Abundant genetic polymorphism evolved to escape host immune responses</li> </ul>
Vaccine strategy	<ul style="list-style-type: none"> <li>• Many different strategies being tested. Most advanced targets the pre-erythrocytic stages of the cycle through <i>P. falciparum</i> circumsporozoite protein (CSP) immunity</li> </ul>
Antigen selection	<ul style="list-style-type: none"> <li>• CSP present on sporozoite surface, expressed by early liver forms; exported to the cytoplasm of hepatocytes</li> <li>• CSP conformation has functional properties relating to parasite binding, motility, cell traversal &amp; biochemistry</li> <li>• Based on experience in development of Hepatitis B (HB) vaccine a fusion construct was made with repetitive immuno-dominant B cell epitope in central region (R) &amp; T-cell epitope (T) in C-terminal flanking region from CSP genetically fused to HBs Ag (S) &amp; expressed together with free HBs antigen in yeast. The proteins assemble into VLPs like HBsAg proteins</li> </ul>
Vaccine Formulation (e.g.s)	<ul style="list-style-type: none"> <li>• RTS,S was tested with different adjuvant formulations with AS01 showing the best immunogenicity and efficacy against controlled human malarial infection</li> <li>• AS01 is a liposomal suspension of the immune enhancers MPL and QS21, a natural saponin molecule</li> </ul>
Immunogenicity	<ul style="list-style-type: none"> <li>• Testing in animal models is of limited value</li> <li>• The vaccine's mechanism of action is to induce a specific anti-CSP immune response that prevents initiation of blood-stage infection by killing parasites at the pre-erythrocyte stage. There is no immune correlate of protection</li> <li>• Anti-CSP antibody &amp; CD4 T-cell activation have been correlated in human subjects</li> </ul>
Clinical Trial Design & testing	<ul style="list-style-type: none"> <li>• PATH (Program for Appropriate Technology in Health) Malarial Vaccine initiative plus World Health Organization vaccine technology road map challenge is to license a vaccine that is &gt;50% efficacious for &gt;1 year against severe disease and death</li> <li>• Focus on reducing the burden of <i>P. falciparum</i> disease in infants &amp; children in sub-Saharan Africa</li> <li>• Immunogenicity better with 3 dose schedules</li> <li>• Phase 2 in conditions of natural exposure confirmed vaccine is safe and conferred partial protection</li> <li>• Phase 3 study in 11 centres across 7 countries with various patterns of malarial transmission shown to prevent a proportion of severe malaria occurrence in infants and has been submitted for licensure</li> <li>• Immunisation (×3) of children (5–17 months) &amp; infants (6–12 weeks). Vaccine efficacy against clinical malaria (fever &amp; parasitaemia) at 20 months was 50% or 30% respectively. Lower antibody levels in infants may derive from immaturity of the immune system, passive transfer of CSP, or HBs co-vaccinations</li> </ul>
Future [8–10]	<ul style="list-style-type: none"> <li>• Need for higher efficacy through increased immunogenicity using RTS,S/AS01 alternative immunisation regimes, use of other CSP-based platforms &amp; combination of RTS,S/AS01 with alternative antigens of pre-erythrocytic, blood or sexual stages</li> <li>• Whole <i>P. falciparum</i> sporozoite vaccines</li> <li>• Heterologous prime/boost DNA, rV &amp; or bacteria/rProtein + adjuvant</li> <li>• Extracellular gamete antigens to prevent transfer to mosquito. Requires herd immunity to reduce infections in community</li> </ul>

QS-21: Quillaja saponaria Molina: fraction 21. (Antigenics Inc, a wholly owned subsidiary of Aenus Inc., Lexington, MA, USA).  
MPL: 3-deacylated monophosphoryl lipid.

The relative importance of each immune mechanism varies for any given pathogen. For example, antibodies are thought to play a major role in preventing HBV and influenza infections, whereas T-cells, especially CD4+ T-cells are believed necessary for control of tuberculosis. For pathogens such as HIV and influenza virus which are highly variable, antigenically broad neutralising antibodies capable of targeting common epitopes are now being explored.

Although non-neutralising antibodies can bind to a virus, they were thought not to significantly affect virus capacity to infect cells and replicate. Exceptions are now being identified as antibodies capable of directing immune killer cells to the pathogen-infected target have been found to be important [22]. Enhancing antibodies that result in increased infection have also been reported, so optimising antigen structure is a key aspect of vaccine development.

The uptake and presentation of pathogen-derived antigens to T-cells by APCs (primarily dendritic cells in the primary immune response, and monocyte-derived cells and B-cells in subsequent responses) can be exploited by vaccination [23]. New generation vaccine adjuvants target these APCs via their PRRs, enhancing their ability to present antigen, migrate and stimulate T- and B-cell effector cells [24,25]. These adjuvants can replace and even exceed the effects of natural pathogen PAMPs (e.g., the adjuvanted Herpes zoster vaccine [26]). The clinical outcome to be prevented needs to be defined (systemic infection, mucosal disease, reactivation,

severe disease). For many diseases, data on surrogate markers or correlates of protection may only be attainable after licensure when the vaccine is used in large populations. Nevertheless, immunologic studies in clinical trials of partially effective vaccines are especially important in guiding future improvement of the candidate. Such trials could potentially identify crucial mechanisms by comparing vaccinees with breakthrough infections to those apparently protected.

As examples, the evasion of immunity for oncogenic HPV derives from stealthy infection and virus production without cell death plus viral gene modulation blunting antigen presentation and effector function (Box 1); for *P. falciparum* there is immense genetic polymorphism and a requirement for distinct immune responses at each stage of a very complex life-cycle (Box 2); for Ebola infection, which can neutralise key innate immune defences such as interferon, the impact is so devastating in immune-naïve individuals there is only a low rate of natural immune-mediated survival, although this may be protective in those who do survive (Box 3). For HPV, natural immune clearance is primarily due to cell-mediated immunity whereas for *P. falciparum* and Ebola the characterisation of the key components of natural immunity is incomplete and/or not fully understood. Note that the relative importance of immune mechanisms may differ for protection from disease and for clearance of infection.

**Box 3**

## Ebola vaccine.

Pathogen	<i>Ebola</i>
Disease	Haemorrhagic fever; disseminated intravascular coagulation, multiple organ failure, massive bleeding, shock, death (mortality can reach 60–90%, higher in children, older adults and pregnant women)
Structure & diversity	<ul style="list-style-type: none"> <li>• RNA virus</li> <li>• 19 Kb Genome encoding 7 proteins</li> <li>• Helical structure covered by lipid membrane in which is embedded the glycoprotein</li> </ul>
Life cycle & epidemiology	<ul style="list-style-type: none"> <li>• Spreads within endemic areas via infected body fluids from bats to non-human primates</li> <li>• Humans initially acquire infections from these reservoirs in endemic areas in Africa</li> <li>• Human to human spread via infected body fluids (blood, faeces, saliva, vomit, tears, breast milk, semen)</li> <li>• Isolated from these fluids up to 40 days after onset, incubation period 5–9 days</li> <li>• Progressive strain variation as it spreads</li> <li>• Nosocomial spread via reuse of non-sterile injections, healthcare workers at risk</li> <li>• Occasional imported infections into western countries</li> </ul>
Disease Burden	<ul style="list-style-type: none"> <li>• Only 2600 cases in central Africa up until 2014–15 outbreak of 28,000 cases in Liberia, Guinea and Sierra Leone. Occasional imported cases create great public anxiety requiring considerable health resources to manage</li> </ul>
Natural Immune control and escape	<ul style="list-style-type: none"> <li>• Neutralising antibody is critical</li> <li>• Immune evasion: lymphocyte apoptosis and inhibition of interferon induction</li> <li>• Endothelial damage and activation by EBOV envelope protein leading to disseminated intravascular coagulation</li> <li>• Immunopathology: massive pro-inflammatory cytokine release ('storm')</li> </ul>
Vaccine strategy	<ul style="list-style-type: none"> <li>• Induce neutralising antibodies</li> <li>• Role of T-cells unclear</li> </ul>
Antigen selection	<ul style="list-style-type: none"> <li>• The surface glycoprotein is the target for neutralising antibody &amp; monoclonal antibodies against it (ZMAPP) can prevent &amp; facilitate cure of infection in non-human primates</li> </ul>
Vaccine Formulation (e.g.s)	<ul style="list-style-type: none"> <li>• More than 10 vaccine candidates under development: two hybrid recombinant viruses incorporating EBOV immunogenic glycoprotein are most advanced: chimp adenovirus 3 (cAd3)–EBO Z(NIH-GSK), recombinant vesicular stomatitis virus (rVSV)–ZEBOV (Canadian Dept. Public Health-Merck)</li> </ul>
Immunogenicity	<ul style="list-style-type: none"> <li>• Both advanced vaccine candidates are immunogenic in inducing suprathreshold levels of antibodies in on human primate models (which were used initially to determine correlates of protection) with a single injection. Vaccines induce lower levels of T-cell immunity</li> </ul>
Clinical Trial Design & testing	<ul style="list-style-type: none"> <li>• Phase I trials in Europe and Africa, Phase II–III trials underway in Africa. In African trials, rVSV-ZEBOV showed 75% efficacy in ring vaccination the closing stages of the large 2014–15 epidemic. However high reactogenicity may preclude general prophylactic immunisation</li> <li>• Safety trials planned: 10,000 subjects each for the GSK and Merck vaccines and a placebo</li> </ul>
Future	<ul style="list-style-type: none"> <li>• Await phase III results with cAd3-EBOZ and trials with several other candidates which are in development [12,13]. Problem is the difficulty in testing in humans in view of erratic epidemics [14]. Role of T-cells needs to be clarified</li> </ul>

**4. Antigen selection & vaccine formulation**

Classically, 'protective' antibodies elicited by natural infection are studied to identify the main target(s) of the effective immune response, usually proteins (toxins [such as diphtheria and tetanus or filamentous haemagglutinin in pertussis vaccines]) or carbohydrates (capsular polysaccharides [pneumococcus, meningococcus]). Potential candidates are analysed for suitability as vaccine antigens, including determining their homology with human proteins and potential inherent toxicity. Detoxification may be required before an antigen (for example, pertussis toxin) can safely be administered to humans, but some detoxification methods may destroy epitopes in the process, and impact immunogenicity [27]. Furthermore, purification of antigens away from other viral and bacterial components such as lipids and nucleic acids may exclude PAMPS, altering the nature of the immune response. Initial *in vitro* studies may evaluate antigen-antibody binding capacity and function, and early investigation of the immune response to the candidate antigen may be assessed in animals.

However, immunity is not always mediated via a humoral response (e.g., malaria [Box 2] or tuberculosis); rapid evolution of the pathogen may mean that potential antigens change rapidly (influenza and HIV); the antigen may be similar to human proteins, potentially increasing the risk of autoimmunity (meningococcal serogroup B capsule, group A streptococcal capsule); a complex life-cycle may mean that the host is exposed to different antigens at different times in the life-cycle (malaria, Box 2), or that reactivation occurs in a previously infected host (VZV, [28]). New technologies, such as reverse vaccinology [6], are required to identify candidate antigens for these challenges where the expression of surface structures is predicted, based on inferred protein sequences from the pathogen's genome.

Vaccines need to be efficiently produced and deliverable in a form acceptable to the recipient. The earliest vaccines used whole organisms; either alive (attenuated versions of the pathogen, or related but less virulent organisms that could induce cross-reactive immunity without inducing disease), or dead. Whole organisms have the advantage of being highly immunogenic and typically stimulate a response similar to that generated by natural infection. Unfortunately, they may also generate pathology similar to that induced by the natural infection; and in cases where natural infection does not generate protective immunity, a whole-organism vaccine may be likewise ineffective. There is the secondary limitation that whole organisms are complex mixtures of proteins, lipids and carbohydrates, which greatly complicates production, characterisation and quality control of the final vaccine.

For these reasons, the focus has shifted toward vaccines containing specific antigens, which can be characterised at the molecular level. The properties of the most commonly-used vaccine technologies are shown in Table 2. With the exception of whole organisms, all of these technologies rely on presenting selected, key antigens that will ensure a strong, persistent and broad immune response of the kind needed for protection, while avoiding or minimising reactogenicity. Purified antigens can sometimes be poorly immunogenic, so the first task is to choose a vaccine delivery system that boosts immunogenicity and promotes a protective immune response. It is also essential that the technology selected is economically viable. Since prophylactic vaccines are typically administered to very large numbers of healthy individuals they are among the most price-sensitive of all medical products, and the acceptable price depends greatly on the target population: a vaccine for childhood malaria, most needed in some of the poorest countries in the world, faces different design constraints than one against, for example, zoster, which is targeted primarily toward

**Table 2**  
Toolbox of technologies currently being used or investigated in vaccine design.

Technology	Examples	Advantages	Disadvantages	Limitations
Whole pathogen: live attenuated	<ul style="list-style-type: none"> <li>• Oral poliovirus,</li> <li>• measles-mumps-rubella,</li> <li>• varicella,</li> <li>• influenza,</li> <li>• BCG (Bacillus Calmette-Guérin)</li> </ul>	<ul style="list-style-type: none"> <li>• Mimics natural infection,</li> <li>• effective priming with durable immunity</li> </ul>	<ul style="list-style-type: none"> <li>• Rarely may revert to virulence,</li> <li>• not suitable for some populations (pregnant women, immunocompromised),</li> <li>• may induce mild disease symptoms,</li> <li>• can be difficult to produce consistently</li> </ul>	Not suitable for micro-organisms that do not grow well in culture, or whose characteristics change throughout their life-cycle (parasites) or pathogens with effective immune-evasion or latent stages
Whole pathogen: killed	<ul style="list-style-type: none"> <li>• Inactivated poliovirus,</li> <li>• hepatitis A,</li> <li>• whole-cell pertussis</li> </ul>	<ul style="list-style-type: none"> <li>• Induces broad immune response to multiple antigens</li> </ul>	<ul style="list-style-type: none"> <li>• Multiple doses needed,</li> <li>• Reactogenic,</li> <li>• key epitopes maybe destroyed by the inactivation process</li> </ul>	
Related non-pathogens	<ul style="list-style-type: none"> <li>• Cowpox (small pox)</li> </ul>	<ul style="list-style-type: none"> <li>• Induces broad immune response to multiple antigens</li> </ul>	<ul style="list-style-type: none"> <li>• Not suitable for some populations (pregnant women, immunocompromised),</li> <li>• may induce mild disease symptoms</li> </ul>	Not many micro-organisms are suitable
Purified protein (split or subunit)	<ul style="list-style-type: none"> <li>• Acellular pertussis vaccines</li> <li>• influenza</li> </ul>	<ul style="list-style-type: none"> <li>• Induces a highly specific response,</li> <li>• non-infectious,</li> <li>• low reactogenicity,</li> <li>• synthetic production may ease production</li> </ul>	<ul style="list-style-type: none"> <li>• Correct 3-dimensional structure may be difficult to achieve,</li> <li>• multiple subunits are often necessary,</li> <li>• little cross-reactivity,</li> <li>• potential for escape mutants,</li> <li>• lower immunogenicity requiring adjuvants</li> </ul>	Not suitable for pathogens with characteristics that change throughout their life-cycle (parasites, some chronic or latent infections)
Polysaccharide–protein conjugates	<ul style="list-style-type: none"> <li>• <i>Neisseria meningitidis</i>,</li> <li>• <i>Streptococcus pneumoniae</i>,</li> <li>• <i>Haemophilus influenzae</i> type b</li> </ul>	<ul style="list-style-type: none"> <li>• Conjugation triggers T-cell dependant mechanisms and immune memory</li> </ul>	<ul style="list-style-type: none"> <li>• Technically difficult and costly to produce</li> <li>• finite number of strains possible in one vaccine</li> </ul>	
Pathogen-like particles	<ul style="list-style-type: none"> <li>• Malaria,</li> <li>• hepatitis B,</li> <li>• human papilloma virus</li> </ul>	<ul style="list-style-type: none"> <li>• Can induce enhanced responses compared to natural immunity</li> </ul>		
RNA replicon	<ul style="list-style-type: none"> <li>• Influenza</li> </ul>	<ul style="list-style-type: none"> <li>• Unable to produce infection</li> </ul>	<ul style="list-style-type: none"> <li>• Antigen dissemination may be limited</li> </ul>	
Recombinant DNA technology	<ul style="list-style-type: none"> <li>• Hepatitis B,</li> <li>• human papilloma virus, herpes zoster</li> </ul>	<ul style="list-style-type: none"> <li>• Suitable for pathogens that do not grow in culture</li> <li>• Suitable for pathogens that do not grow well in culture,</li> <li>• efficient to manufacture</li> </ul>	<ul style="list-style-type: none"> <li>• potential for recombination to infectious form</li> <li>• Lower immunogenicity requiring adjuvants or multiple peptides</li> </ul>	
Viral/bacterial vectors	<ul style="list-style-type: none"> <li>• Respiratory syncytial virus,</li> <li>• ebola</li> </ul>	<ul style="list-style-type: none"> <li>• Suitable for pathogens that do not grow well in culture</li> </ul>	<ul style="list-style-type: none"> <li>• Pre-existing antibody may limit response,</li> <li>• different vaccines may be needed for priming and boosting</li> </ul>	Potentially unsuitable for immunocompromised or pregnant women

adults in developed countries who have the resources to purchase it. For these reasons, some delivery modalities (for example, requiring multiple doses, many different components, or separate vaccine technologies for priming and boosting) may theoretically improve outcome but prove to be too complex or expensive to be practical. Formulation becomes key here. Combination vaccines that target several diseases in a single injection may alleviate cost and delivery restrictions, but their complexity imposes a higher development cost and there is the risk of immune interference in which a component of a combination vaccine may prove less effective than when tested in isolation. Likewise, the behaviour of an antigen with different delivery systems is not always predictable, leading to altered antigen availability dose response or epitope recognition [29,30]. In the absence of predictive models for these interactions, there is currently no alternative to time-consuming (and expensive) *in vivo* testing.

Alum, the oldest adjuvant (aluminium hydroxide or aluminium phosphate) has been in use for over 90 years. While alum has demonstrated an excellent safety record [31], it has been assessed primarily on its ability to promote humoral immune responses, with little or no attention paid to the cellular immunity induced [32]. Early emulsions, such as Freund's complete adjuvant, generated highly effective cellular immune responses; but also unacceptable side effects [33].

When molecular mechanisms involved in the induction of the immune response to adjuvants were studied and understood in the 1990s, it was realised that immunogenicity and immunopathology were promoted by different, partially overlapping immune pathways. This allowed for the development of new adjuvants that combined enhanced immunogenicity with acceptable reactogenicity. Several of these are now used in products that are licensed [34,35] and typically include multiple components to stimulate a broad range of immune responses (Table 3 [32,36–40]).

Concurrent with the development of better adjuvants for vaccine delivery, other vehicles that allow the tight association of antigen and immunomodulators have been developed. These include, but are not limited to, toxoids, virosomes, liposomes, immunostimulating complexes (ISCOMS) and micro- or nano-particles. The first two are already used in commercially-available vaccines (Table 3), while the rest have provided promising clinical data (reviewed in [41]). Toxoid-conjugated vaccines, though employing different technologies to those discussed above, utilise the same general

concept; the combination of a detoxified, but immunogenic microbial PAMP able to stimulate innate and T-cell dependent responses, linked to antigenic polysaccharide targets, which can generate strong B-cell responses and antibody production. This approach has been used to generate licensed vaccines against serious invasive bacterial infections (*Haemophilus influenzae type b*, *N. meningitidis*, *Streptococcus pneumoniae*) using toxoids derived from either tetanospasmin of *Clostridium tetani* or the diphtheria toxin of *Corynebacterium diphtheria*, which have been shown to generate more robust and long-lived antibody responses than the polysaccharide targets alone, presumably due to the activation of T-cell help [42]. Other conjugate vaccines, such as a typhoid vaccine using a modified exoprotein A from *Pseudomonas aeruginosa* as the toxoid are under development [43].

Virosomes, liposomes and ISCOMS, although different in structure and immunostimulatory capacity, are built around the concept of a lipid vesicle (the lipids used are themselves weakly immunostimulatory) to which can be added both antigenic targets and immunomodulatory molecules [37]. The physical properties and size of the vesicle can be tailored to requirements depending on the lipid composition and production methods, as can the ionic charge. These factors influence the ability of the delivery system to form depots, bind to antigen-presenting cells and the antigen loading of the delivery system [37]. In many ways, these adjuvants mimic natural enveloped bacteria or viruses, with a lipid envelope and associated proteins. This process is taken a step further by virus-like particles (VLP), where the lipids and antigenic target are derived directly from the pathogen in question, producing a delivery vehicle that resembles a pathogen but without the genes required to initiate infection [44].

## 5. Vaccine preclinical & clinical testing

One of the most challenging aspects of vaccine design remains assessing the efficacy or effectiveness of new vaccine formulations. The clinical assessment of vaccines is discussed in more detail in Preiss et al. in this issue. In some cases, where clear correlates of immunity can be observed (such as for measles, where the link between antibody level and clinical outcome is indisputable [45]), or animal models which accurately mimic human disease, non-clinical estimates of efficacy can be made with a high degree of certainty; unfortunately, while having an immune correlate is desirable, and extremely helpful, they are mostly lacking in the

**Table 3**  
Adjuvants used in currently licensed or approved vaccines.

Adjuvant	Composition	Mechanism of action	Current use
Alum	Aluminium hydroxide or aluminium phosphate	Possible depot effect, directs toward a Th2-like response with increased antibody production [32]	Multiple vaccines, including DTaP, Influenza, etc.
AS01	QS-21 and MPL with liposomes	Induction of a broad population of activated antigen presenting cells, with synergy between QS21 and MPL that acts to enhance the adaptive response [39]	Malaria vaccine, zoster vaccine
AS03	Oil-in-water emulsion with alpha-tocopherol (Vitamin E)	Transient NF-κB, cytokine and chemokine response, increased recruitment of innate immune cells. Enhanced recruitment of innate immune cells at the local draining lymph node. Enhancement of T-cell mediated help of B-cell responses [40]	Influenza (pre-pandemic and pandemic)
AS04	MPL adsorbed onto aluminium hydroxide or aluminium phosphate	Transient NF-κB, cytokine and chemokine response, increased numbers and efficiency of activated dendritic cells leading to enhanced antibody responses [38]	Human papillomavirus vaccine
MF59	Oil-in-water emulsion with squalene	Enhances antigen uptake by APCs, cytokine and chemokine response enhancing the local immune response, increased quantity and diversity of the antibody response [36]	hepatitis B (pre- and haemodialysis patients) Influenza (seasonal and pandemic)
Virosomes	Phospholipids: lecithin, cephalin	The virosome structure functions to enhance uptake by APCs. Stimulates mononuclear cells toward a Th1 cytokine profile [37]	Influenza, hepatitis A

QS-21: Quillaja saponaria Molina: fraction 21. (Antigenics Inc, a wholly owned subsidiary of Agenesis Inc., Lexington, MA, USA).

MPL: 3-deacylated monophosphoryl lipid.

CpG7909: an immunostimulatory nucleotide.

DTaP – combined diphtheria-tetanus acellular pertussis vaccine

early phases of clinical development [46]. Although a specific immune response (induced either by vaccination or natural infection) is generally observed in protected individuals, it is often difficult to identify the precise immune mechanism(s) responsible for efficient protection from the large array of elicited effectors (antibodies, cytokines, T-cells and so on). As an example, the effectiveness of HBV vaccines is measured by the ability to induce an antibody response against the HBV surface antigen (HBsAg) of  $\geq 10$  mIU/ml of blood [45,47] based on the observation that individuals with such a response were protected from infection. Long-term follow-up indicated that in some individuals antibody levels waned with time; but these individuals remained protected from infection [48]. Booster vaccinations revealed that vaccinated individuals displaying low to undetectable levels of serum antibodies were nevertheless able to mount a robust recall response, indicative of a persistent and protective immune memory despite a waning humoral response [49]. The presence of an antibody response after vaccination (or infection) demonstrates that an immune response has been generated, but in this case, there is no direct correlation between the magnitude of the antibody response and the degree of protection. In other words, antibody production in response to vaccination is an indicator of immunogenicity, not efficacy. This may be due to the fine specificity of the protective antibody response, not the total level of antibody produced. Nonetheless, long experience with vaccines such as those against pneumococcus and influenza, has proved that the linkage between immunogenicity and vaccine effect is so robust, that for these vaccines, generation of a sufficiently strong and mature antibody response is accepted for licensure, even if the antibody is only part of the protective immune response [50].

The ultimate test of vaccine efficacy is of course, protection in humans; but when choosing which vaccine candidates to take into clinical trials, other surrogate approaches are needed. The critical points along the preclinical pathway include detailing the host-pathogen interaction, understanding the protective immune mechanisms involved and selecting an appropriate antigen and adjuvant to achieve the desired immune response. Subsequent steps involve the production of the antigen, and a compatible vaccine delivery system, followed by the development of immune readouts and toxicological tests to assess the safety and performance of the candidate vaccine construct under preclinical evaluation.

If a plausible mechanism of protection or suitable surrogate markers can be identified, animal models can be informative with regard to protective effects and antigen recognition, even if they do not replicate the human disease closely enough to be predictive [51]. Non-human primate and rodent models of Ebola infection exist, and since efficacy studies are not feasible in view of the sporadic nature of Ebola outbreaks, evidence gained from animal models has been considered to support vaccine licensure [14]. By contrast, no good animal model for HIV infection has been identified, though infection of non-human primates with simian immunodeficiency virus is a useful surrogate model. However, improvements to this model and the development of a more affordable animal model remains a priority [52]. Animal models have also proven their value for the assessment of vaccine safety and toxicology, even though preclinical toxicology studies are typically relatively small, and powered to identify direct toxic effects [53]. In cases where an effective treatment is available for the disease, human challenge studies where volunteers agree to be exposed after vaccination is the closest possible model to human disease: this approach has been successfully used in malaria vaccine development (Box 2), and is being explored for other diseases that lack suitable animal models such as typhoid fever [54].

Some approaches used for vaccine design and testing are presented in the examples provided in Boxes 1–3.

### 5.1. HPV vaccines

The licensed HPV vaccines are based on VLP technology [44] plus an adjuvant, to generate a stronger antibody response than natural infection. The quadrivalent vaccine uses alum and in the bivalent HPV vaccine, this is further supplemented by the use of detoxified monophosphoryl lipid A (MPL), a modified membrane component common in Gram-negative bacteria, which binds to TLR4 and stimulates strong cell-mediated immunity [35,55]. The combination of MPL and aluminium induces higher antibody levels than aluminium alone [38]. The value of more complex adjuvants can be seen in direct comparisons of the antibody induced by alum-adjuvanted antigens and the combination delivery system and may explain the broad cross-reactivity seen with this vaccine, and its ability to provide protective immune memory significantly superior to that derived from natural infection [24,25,56]. Consistent with this, HPV vaccines' immunogenicity and capacity to induce long lasting responses were initially tested in HPV-naïve young women, in which antibody levels were found to exceed those produced by individuals naturally exposed to HPV. Subsequent clinical trials in HPV-naïve young women used a surrogate endpoint of cancer, high grade cervical intra-epithelial neoplasia (CIN3), to establish efficacy but as yet there is no immune correlate of protection with antibody levels. Additionally, the principle vaccination target group for prevention of cervical cancer is young girls before sexual debut, and thus licencing depended on bridging studies showing greater immunogenicity in this cohort.

Even when there are licenced vaccines against a pathogenic threat, there is almost always room for improvement. Thus HPV VLP vaccines containing more oncogenic types, or even therapeutic vaccines based on HPV oncogenes, which could also be prophylactic, are strategies driving future developments (Box 1).

### 5.2. Malaria vaccines

The first vaccine against *P. falciparum* to successfully complete phase III trials (RTS,S) combines a fusion construct of epitopes from the Circumsporozoite Protein with HBsAg adjuvanted with AS01, a liposomal suspension of the immune enhancers MPL and QS21 (Box 2). The malarial vaccine RTS,S may be licenced based on the available protection data, but there will be a need to further improve the longevity of the immune response and vaccine impact to encourage widespread vaccination: for example, while the vaccine offers some protection from malaria caused by *P. falciparum*, a vaccine against *P. vivax*, the second most important cause of malaria, is also of high priority [57].

### 5.3. Herpes zoster vaccine

For herpes zoster, two vaccine strategies have been shown to stimulate waning cell mediated immunity to earlier infection by the VZV: high dose whole attenuated virus and a subunit adjuvanted vaccine which aims to enhance cell-mediated immunity (Supplementary Box 1). The high efficacy in older people of the subunit VZV/adjuvant vaccine compared with the results from independent trials with the highly concentrated attenuated viral vaccine is very encouraging for overcoming age-related declines in immune responses [26].

### 5.4. Dengue vaccines

There are several candidate dengue virus vaccines in various stages of testing (Supplementary Box 2). A chimeric viral vaccine using yellow fever as a backbone has been licenced in some countries and induces neutralising antibodies to all four serotypes, but optimal use appears to require priming with yellow fever vaccine

one year previously. For dengue virus vaccines adopting a goal of preventing hospitalisation rather than complete prevention of disease may influence future developments [11].

### 5.5. Ebola vaccines

For Ebola there are more than 10 candidate vaccines under development. The most advanced are recombinant adeno- and vesicular-stomatitis virus (VSV) encoding EBOV glycoprotein. Ebola vaccination strategies based on available hybrid recombinant viruses have been catapulted into clinical testing, driven by the impact of the recent devastating Ebola outbreak in several African countries. The rVSV-ZEBOV vaccine induces elevated levels of antibodies in primate models and vaccination trials in Africa show 75% efficacy but with high reactogenicity (Box 3). There are many challenges for comprehensive testing of Ebola vaccines in view of the epidemic nature of the disease. The wider consequences of viral persistence after apparent recovery are not yet fully understood and the analysis of the role of cellular immunity in recovery may be critical to future vaccine design.

## 6. Conclusion

Vaccine development is a complex multidisciplinary activity, combining understanding of host-pathogen interactions at the molecular level, with clinical science, population-level epidemiology and the biomechanical requirements of production. The basis is an understanding of which immune processes shape disease and protection, and how these vary between individuals, risk groups and populations. That knowledge in turn informs the selection of antigenic targets, and the adjuvants/delivery systems used to shape the immune response induced by the vaccine; which in turn determines the manufacturing requirements and the clinical trial design. The ultimate goal is an affordable vaccine that generates strong and lasting immunity with the fewest possible side effects, implemented without the need for expensive cold chains.

## Trademarks

Shingrix, Bexsero and Cervarix are trademarks of the GSK group of companies. MF59 is a trademark of Novartis.

## Contributorship

All authors were involved in the development of this manuscript and gave final approval before submission.

## Disclosures

ALC reports consultancy fees paid to his institution, unrelated to the present work, from the GSK group of companies. NG was previously an employee of the GSK group of companies, declares stock ownership and is also inventor on patents owned by the GSK group of companies. OL has received grants, unrelated to the present work, from the GSK group of companies. LRF, BL and MD are employees of the GSK group of companies, LRF and MD own shares/stock options in the GSK group of companies. RS reports no conflict of interest. PS has received consultancy fees, unrelated to the present work, from the GSK group of companies.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2016.10.016>.

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