Vaccination to protect against infection of the female reproductive tract

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Abbreviations: FRT: Female reproductive tract, HPV: human papillomavirus, HepB: hepatitis B virus, HSV: herpes simplex virus, RTI: reproductive tract infection

Infection of the female genital tract can result in serious morbidities and mortalities from reproductive disability, pelvic inflammatory disease, cancer, to impacts on the foetus such as infant blindness. Whilst therapeutic agents are available to cure or in some cases reduce the disease burden, frequent testing and treatment is required to prevent the occurrence of the severe disease sequelae. Hence, sexually transmitted infections remain a major public health burden with ongoing social and economic barriers to prevention and treatment. Unfortunately, whilst there are two success stories in the development of vaccines to protect against HPV infection of the FRT (Female Reproductive Tract), many serious infectious agents impacting on the female reproductive tract still have no vaccines available. Vaccination to prevent infection of the female reproductive tract is an inherently difficult target, with many impacting factors such as; appropriate vaccination strategys/mechanisms to induce a suitable protective response locally in the genital tract, variation in the local immune responses due to the hormonal cycle, selection of vaccine antigen(s) that confers effective protection against multiple variants of a single pathogen (e.g. the different serovars of *C. trachomatis*), and timing of the vaccine administration prior to infection exposure. In spite of these difficulties there are numerous ongoing efforts to develop effective vaccines against these infectious agents and it is likely this important human health field will see further major developments in the next 5 years.

Infection of the female reproductive tract result represents a serious health burden worldwide

Infections of the female reproductive tract that are associated with high morbidities and mortalities around the world include; human immunodeficiency virus (HIV), human papillomavirus (HPV), Herpes Simplex Virus (HSV), Hepatitis B (HepB), *Chlamydia (C.) trachomatis, Neisseria (N.) gonococcus, Treponema (T.) pallidum* (syphilis), group B streptococcus during pregnancy, and *Trichomanas (T.) vaginalis*. Female reproductive tract infections (RTIs) usually originate in the lower genital tract as vaginitis and cervicitis and may produce symptoms such as abnormal vaginal discharge, genital pain, itching, and a burning feeling with urination. However, for some infections such as *Chlamydia*, a high prevalence of asymptomatic infection occurs, which is a barrier to effective control. RTIs impose a heavy toll on women, if untreated can cause the serious consequences of pelvic inflammatory disease, chronic pelvic pain, infertility, ectopic pregnancy, cervical cancer, menstrual disturbances, pregnancy wastage, low birth weight babies, and increased risk of HIV infection and transmission. A number of these infections can be effectively treated once diagnosed, however, the greatest burden of these treatable infections is in developing countries where access to treatment is limited (Fig 1). The WHO estimates that in 1999 alone 116.5 million curable sexually transmitted infections (STIs) occurred, however, the majority of these were in locations where access to health care is severely limited (e.g. 32 million in sub-saharan Africa). In developing countries reproductive tract infections rank second to maternal morbidity and mortality as a health encumbrance for women [1]. In the near future, the most pressing health impact of these infections is likely to be their role in increasing the infection and transmission rates of the HIV virus [2].

The leading bacterial agents with known established pathological impacts on the FRT (*N. gonorrehoeae*, *C. trachomatis*, *T. palladium*, *Haemophilus ducreyi*) currently cause a combined total of greater than 169 million infections annually in humans [3]. Importantly, it seems that in both developing and developed countries the actual incidence of infections are likely to far exceed those which are diagnosed and treated [4-5]. The antibiotics typically used to treat these bacterial FRT infections include; ceftraxone, ceftriaxone, ciprofloxacin, ofloxacin, all of which are listed on the WHO essential medicines scheme for developing countries at a cost of 0.019-0.32 USD (US $) per dose. However, azithromycin, the most

effective treatment for *Chlamydia* is not currently listed on the WHO essential medicines scheme. These medicines all retain the same relative cost effectiveness in developed countries, costing from approximately $4.80 per dose to $11.50 for a dose of Azithromycin [6]. Even though affordable antibiotic treatment is available for these infections; limited access to testing and treatment, or infrequent testing and treatment, or treatment after pathologies have already developed are major factors in the spread and health burden of these infections. Well implemented and robust vaccination programs have the potential to deliver longer term protection against infection and also prevention of the resultant morbidities. Thus vaccine strategies are likely to deliver a far greater cost:benefit once they can be successfully implemented.

There is also a pressing need for vaccines to target viral STIs from both a population health and economic perspective, but unfortunately the outcomes of recent clinical trials of experimental HIV vaccines have not been encouraging. The infection and disease morbidity rates are far greater for the viral infectious agents compared to bacterial STIs. Worldwide there are; greater than 33 million HIV positive individuals, HSV2 prevalence maybe as high as 20%, 350 million cases of chronic hepatitis cases occur annually, and there are at least 500 000 cases of cervical cancer annually. There are no treatments to cure any of these infections. The treatments which exist to reduce the disease burdens require established health infrastructure and high socioeconomic circumstances. Current HIV antiretroviral treatments, are costly, involve complicated combination therapy regimens, and can result in the development of viral resistance in some cases (reviewed in [7]). The current treatments for HSV are acyclovir, valacyclovir, and penciclovir, which effectively prevent 70-80% of the symptomatic recurrent epiosodes. However, these require multiple doses for up to 10 days at a cost of 65-100 USD to treat a primary infection [8]. HepB treatments include; interferon alfa, pegylated interferon alfa-2a, lamivudine, and adefovir to reduce the liver inflammation and further progression of the chronic disease [9]. Antiviral drug treatment is not recommended for HPV (CDC guidelines, USA), instead participation in cervical cancer screening programs and intervention when pathological lesions are detected is the recommended HPV strategy. Thus, the development of vaccines to prevent viral FRT infection and disease morbidities is imperative to reduce the health burden from these infections. The recent success of vaccines against HPV (Gardasil® and Cervarix®) reinforces the value of vaccination as a means of targeting these infections and associated disease.

The most common protozoan organism that causes disease in the FRT is *Trichomonas*. There are an estimated 170 million cases worldwide annually. The infection can result in symptomatic reproductive tract infection, pelvic inflammatory disease, and has been associated but not casually linked with adverse pregnancy and reproductive capability outcomes in women [10]. The infection is also associated with increased risk of HIV infection [11]. Trichomoniasis is curable with treatments such as metronidazole or tinidazole. The potential for development of a vaccine may be limited due to evidence that repeat infections do not result in protective immunity in humans [12].

Given that the treatments available for all STIs require early diagnosis, usually require ongoing or multiple dosing, do not actually clear the infection in the case of viral STIs and may not be administered in time to reduce the reproductive tract morbidities it is clear that vaccines should be the major focus of efforts to combat these diseases. Indeed, globally vaccines are acknowledged as the most effective health intervention strategy, with the childhood vaccine programs having saved approximately 20 million lives in the past two decades [13]. We review here the issues and strategies involved in developing vaccines to prevent infection of the female reproductive tract, highlight the key successes in the field, and present a perspective on where the next successes may arise.

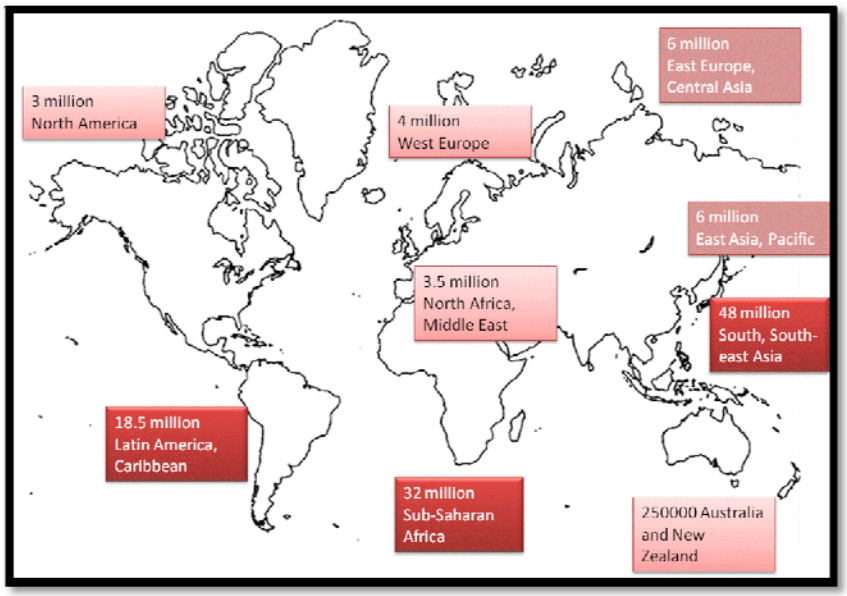

Fig 1. Global prevalence of curable STIs in adults around the world in 1999. This figure has been reproduced using data sourced from the WHO website.

The Female Reproductive Tract

The female reproductive tract is a unique immune compartment in the body. The tissue structure is continually changing, and the hormonal cycle also alters the immune profile of this tissue. Bacterial flora have a critical role in preventing infection in the lower genital tract, whilst limiting damaging ascending infection and facilitating effective conception and reproduction are features of the upper genital tract. Thus, this mucosal location has quite distinct immunological and hormonal factors that can impact dramatically on the design of vaccines to target STIs.

Hormonal regulation of FRT

The changes induced in the immunology of the female reproductive tract by fluctuating hormone levels may present a challenge for the development and delivery of successful vaccines. Progestins and estrogens are the principle hormones acting on the female reproductive tract (FRT). Hormone levels fluctuate naturally during the menstrual cycle but are also influenced by the use of hormonal contraceptives, hormone replacement therapies, pregnancy and chronic stress. These changes in hormone levels cause alterations to immune cell number and function and have the potential to profoundly influence the immunity of the host and the efficacy of any vaccination.

Innate immunity provides the first level of pathogen recognition and defence. The mucosal surfaces of the body including the female reproductive tract frequently encounter and respond to pathogenic attack. Innate immune mechanisms at the mucosal surface include the thickening of the epithelial cell layer and the production of mucins by epithelial cells, and production of antimicrobial products such as secretory leucocyte protease inhibitor (SLPI), cathelicidins and defensins by epithelial cells. It has been reported that β-defensins can directly defend against microbes whereas α-defensins act by allowing immune cells to access vascularised tissues [14]. SLPI is produced by macrophages as well as epithelial cells and is known to have bactericidal effects [15]. The sex hormones present in the reproductive tract regulate the production of these innate immune factors that represent the first line of defence.

Detection of foreign microbes occurs through the sensing of conserved pathogen associated molecular patterns (PAMPs) expressed by pathogens. These PAMPs are recognised by families of receptors such as the Toll like receptors (TLRs) and NOD/CARD receptors present on immune cells and also epithelial cells. Recognition of pathogens such as bacteria and viruses via these receptors signals the immune system to mount a strong response against the foreign pathogen to clear infection. The immune amplifying properties of many adjuvants are based on successful activation of TLRs to stimulate a strong immune response against the vaccine antigen. TLRs are sensitive to the presence of sex hormones and their expression is differentially regulated during the menstrual cycle. Expression of TLRs is reported to be much higher in human endometrial tissue during the progesterone high secretory phase of the menstrual cycle compared to other times [16-17].

Changes in the cellularity of the FRT are detectable at different times during the menstrual cycle when the body is under the influence of different sex hormones. Levels of antibody secreting B cells, NK cells, T cells, macrophages and DC's are reported to be affected by the presence of sex hormones. NK cells are reported to decrease in frequency in the presence of estradiol [18-20]. Macrophages and dendritic cell (DC) populations in the FRT are reported to vary relative to the levels of ovarian hormones present, with frequencies of macrophages and immature DCs highest at the menstrual phase of the cycle [21-22]. The cytolytic activity of CD3 T cells in the human uterine endometrium is influenced by the menstrual cycle with activity detectable during the proliferative, but not the secretory phase [23]. Lymphoid aggregates have been reported in the human uterine endometrium and are linked to the menstrual cycle. These lymphoid aggregates consist of a core of B cells surrounded by CD8 T cells and an outer halo of monocytes and macrophages [24-25]. The observed aggregates were of small size during the proliferative period and were observed to greatly increase in size during the secretory phase, but were absent in post-menopausal women [25]. Fluctuations in the number of antibody secreting B cells in relation to ovarian hormones has been reported in healthy women, with the highest frequency of cells present during the peri-ovulatory stage of the menstrual cycle [26]. It has also been shown in rats that the levels of IgA and IgG in cervicovaginal secretions are under the regulation of sex hormones, with both estradiol and progesterone treatment lowering measured antibody titres [27].

Fluctuations in the levels of sex hormones in the reproductive tract also affects the process of antigen presentation, a critical step in the initiation of a successful immune response. Both professional antigen presenting cells (APCs; Dendritic cells (DCs) and macrophages) and also non-conventional APCs (stromal and epithelial cells) are influenced by the presence of sex hormones. Estrogen, or other ER ligands can directly activate DCs, which express estrogen receptors [28-29], indirectly regulating adaptive immune responses through affects on DC function. *In vitro*, estrogen pre-treatment of human monocyte derived DCs has been shown to cause increased ability to stimulate naïve CD4 T cell responses [30]. Human macrophages have also been shown to express a variant estrogen receptor – meaning estrogen can have direct effects on this cell type also [31]. The presence of progesterone can effectively induce semi-mature tolerogenic DCs and reportedly inhibits mature DC activity – inhibiting the development of an immune response [32].

Collectively, the hormone-induced changes in many aspects of the innate and adaptive immune response can also affect susceptibility to infection by sexually transmitted pathogens. For example, women are less susceptible to chlamydial infection during the progesterone dominant secretory phase of the menstrual cycle and we have demonstrated that this is due to activation of multiple innate immune pathways in progesterone-treated endocervical cell lines and secretory phase primary endocervical cells (Wan et al., submitted, personal communication). Furthermore, Wira and Fahey [33] have identified a window of vulnerability to HIV infection during the normal menstrual cycle. During this period (7-10 days following ovulation) multiple components of the innate, humoral and cell-mediated immune response are suppressed by sex hormones to optimize conditions for procreation, the side affect of which is deceased anti-viral immunity and increased susceptibility to infection.

Hormonal variation can also impact on the efficacy of vaccination, depending on the route of administration. Studies in mice found that nasal vaccine administration could induce neutralizing titres of HPV16 IgG and IgA throughout the estrus cycle, whereas parenteral immunisation could only achieve neutralising antibody titres when administered during diestrus [34]. Human studies have shown that vaginal vaccination elicits variable antibody responses, depending on the timing of vaccination within the menstrual cycle, with administration on days 10 and 24 of the menstrual cycle eliciting better protection and higher antibody titres than patients vaccinated independently of the timing of the menstrual cycle [35]. Nasal vaccination has been shown to be superior to rectal or vaginal immunisation in

the production of cholera toxin B (CTB) specific antibodies after vaccination with CTB, irrespective of the influence of the menstrual cycle [36]. Interestingly, although nasal delivery has proven to be efficient at inducing systemic and mucosal neutralising antibody to prevent HPV infection a recent murine study using an HPV associated tumor model has suggested that in animals with existing disease where cell mediated immunity is required, parenteral immunisation may be a more effective delivery method [37]. The recently approved and widely administered vaccines for HPV, Gardasil® and Cervarix®, are administered intramuscularly, independent of the stage of cycle and the observed protection correlates with circulating levels of IgG [38].

Successful vaccines which protect against infection of the female genital tract

Vaccines are one of the most effective public health and economic advances in medicine. The eradication of smallpox, for example, cost approximately 25 million USD during the campaign, but has since saved approximately 40 million lives, and 275 million USD annually in quarantine and treatment costs [39]. Yet, the vaccine market currently represents only 2% of the worldwide pharmaceutical market [40]. Vaccination to protect against infection and disease in the female reproductive tract represents a key area of innovation and advance for the vaccine field. The successful development and implementation of the HPV vaccine and the HepB vaccine demonstrate that vaccines can be an effective intervention for female reproductive tract health. Vaccination to protect women (and men) against sexually transmitted disease (STD) has incredible potential to save lives, reduce morbidity, decrease global STD expenditures, reduce the spread of HIV/AIDS and contribute to improved reproductive health [41].

Vaccination to prevent infection and cervical cancer: Human Papillomavirus

HPV is the etiological agent for cervical cancer [42]. Cervical cancer is second most common cause of cancer-related deaths in females worldwide. HPV are non-enveloped dsDNA viruses which infect the squamous epithelia. The persistent infection of these squamous epithelia is associated with cervical cancer. Once the infection becomes persistent, the transcription of

two viral oncogenes (E6 and E7) is activated. These proteins inactivate the host cell tumor suppressors p53 and retinoblastoma gene, which ultimately leads to the cervical intraepithelial neoplasia which is the first stage of cervical cancer [43]. The two vaccines clinically in use both prevent the initial infection with HPV (Gardasil®, Merck and Cervarix®, GlaxoSmithKline). Gardasil® has already been demonstrated to protect against neoplasia and induce an immune response detectable for at least 5 years [44-45]. The vaccines are both virus like particle formulations with the viral L1 surface protein. Gardasil® contains 4 L1 protein variants to protect against HPV genotypes 6, 11, 16 and 18. This coverage of the genotypes protects against 70% of the cervical cancers and 90% of female genital infections. Cervarix® is formulated with two of the L1 protein variants; HPV16 and HPV18. The vaccines have now been approved for use in many countries worldwide, however the impact of the vaccination program will take a number of decades to be apparent as the infection and development of cancer is predicted to have as much as a 20 year delay. Recent studies have however provided promising data on the effectiveness of the Gardasil® vaccine. In Victoria Australia there has been a significant decrease in high-grade cervical abnormalities in women aged less than 18 years in the 3 years since the vaccine was introduced [46]. These data suggest that wide-spread introduction of the HPV vaccine in girls, prior to the onset of sexual activity, has the potential to greatly reduce the incidence of cervical cancer. Inclusion of more HPV genotypes in second-generation vaccines should further enhance protection and a vaccine targeting 9 different strains of HPV (MERCK) is currently in Phase II clinical trials. The introduction of the HPV vaccine has demonstrated a number of key advances for this vaccination field; firstly that protection against a major female genital tract infection is possible by vaccination, that the public health and regulatory agencies in several countries have allowed a vaccine program against a major STI to be implemented in teenage girls. Extending vaccination to include males may further reduce the incidence of cervical cancer as well as reducing the incidence of HPV-induced genital lesions in males [47-48].

Reduction of chronic liver disease: Hepatitis B vaccine

Hepatitis B (HepB) is a blood borne and sexually transmitted virus that causes acute and chronic hepatitis. Virus is readily detected in semen and vaginal fluids of infected individuals and it is believed that most adults, who are not intravenous drug users, acquire infection

through sexual contact [49]. There are estimated to be approximately 2-billion infected individuals world-wide, however the major burden of this disease arises from the chronic liver inflammation. There are 8 serologically distinct genotypes which are all protected against by the available vaccines. The hepatitis B vaccine has been in use since the early 1980s, with high risk groups initially targeted for immunisation. It is only in recent years that more universal vaccination programs have been implemented with Australia introducing a universal infant vaccination strategy in 2000. The vaccine is based on the viral surface antigen (envelope protein) and contains an aluminium hydroxide adjuvant (Alum). In infants the HepB vaccine is now commonly administered as part of a multi-component vaccine targeting a number of pathogens. One example is the GlaxoSmithKline Infanrix® vaccine (diphtheria, tetanus, acellular pertussis, poliomyelitis, and *Heamophilus influenzae* type B). The 98% decline in rates of hepatitis infection in children under 13 and 76% decline in infection rates in adults over the 15 years since the vaccine was introduced widely in the USA demonstrates the major public health benefits of an effective and well implemented vaccine program [50-51]. Similar successes have been reported in both rural and urban settings from developed and developing countries worldwide. The vaccine is effective in the majority of the population but requires a booster dose in adults which has some issues for compliance in some settings.

Vaccines in the pipeline

Vaccines to prevent against reproductive tract infection have not yet been successfully developed for HIV, *Chlamydia*, gonococcus, HSV and many of other pathogens. There are a number of reasons for these problems. These issues include; the complex life cycles of the organisms, considerable diversity of the sequence of the surface protein antigens, lack of complete understanding of immunological mechanisms of control and protection against the disease, lack of safe, approved adjuvants that will drive vaccine-induced humoral (Th2) and/or cellular (Th1) mucosal immune response and failure to induce longer term immunity. Furthermore, vaccine trials have been hampered by the limited tools, or efforts, to measure protective mucosal immunity within the female reproductive tract. In many cases the demonstration of antibody and cell-mediated responses in peripheral blood following vaccination has not resulted in protection against infection within the FRT. Furthermore, the infectious agents which commonly infect the FRT are long evolved human pathogens and

many have developed multiple mechanisms to evade host immune defences, such that in the case of *N. gonorrhoeae* even natural infection fails to elicit protective immunity and individuals can be re-infected multiple times [52] while the multiple serovars of *C. trachomatis* mean that infection induces only short-lived, serovar-specific immunity [53].

HIV vaccines

Development of a HIV vaccine is one of the most pressing public health priorities worldwide. Currently 35.5 million people worldwide are infected with HIV and approximately 30 million are estimated to have died from AIDS related causes [54]. Unfortunately whilst there are numerous vaccine trials underway and previously attempted there has not yet been an effective vaccine developed for HIV. There are several comprehensive reviews specific to HIV vaccine development; hence we will not explore this area further in this review [55-56].

Herpes simplex virus 2 vaccines

HSV2 infection is the causative agent for genital herpes, with 20% of adults infected in the USA [57]. The infection is contracted through the skin or mucosal surfaces and establishes within the sensory neurons as a life-long latent infection. The latent virus can be reactivated by physical or emotional stress, fatigue, genital irritation or trauma and can either be asymptomatically shed or form painful, fluid-filled blisters/ulcers on the genitalia and surrounding tissues Neonatal transmission results in serious consequences with high incidences of morbidity and mortality. Th1 immunity with a predominance of IFN-γ has been demonstrated to be required in the human immune response to achieve protective immunity [58], and for this reason vaccine methodologies have focussed on inducing a Th1 response.

There have been numerous trials of HSV vaccines. Subunit vaccines based on the surface glycoproteins have been extensively tested. The trials all induced both antibody and T cell proliferation in phase I trials but no significant protection against infection in phase II has been demonstrated. The potential effectiveness of a vaccine which doesn't prevent infection but reduces clinical presentation has been acknowledged as a more realistic goal for HSV2 vaccination [59]. There have been two separate phase III trials which reduced clinical symptoms (70% efficacy), interestingly this effect was only observed in HSV1 and HSV2 seronegative females [60-61]. Thus, prior infection and gender are factors which influence the efficacy of HSV2 subunit vaccines tested to date. A number of different killed and DNA vaccines have been tested for HSV, in either animal models or human clinical trials, with

little success (reviewed [59]). A novel live attenuated vaccine (GlaxoSmithKline) did not protect against recurrence of activation of the latent virus or shedding in a phase II trial with HSV2 seropositive women [62]. The vaccine did induce a strong cytotoxic immune response and thus has potential as a prophylactic vaccine. A phase II clinical trial is currently underway with a different live attenuated strain (AuRix), and a replication impaired live attenuated vaccine is in pre-clinical development by Avant Immunotherapeutics. Depending on the outcomes of these trials, a live attenuated vaccine which prevents the HSV2 symptoms may be one of the next FRT vaccines to progress to clinical use.

Chlamydia vaccines

Chlamydia trachomatis infection is the most common sexually transmitted bacterial disease worldwide, with over 91 million cases estimated annually (WHO). Despite the availability of a sensitive diagnostic test and also effective antibiotics, the incidence of *C. trachomatis* infections continues to increase [63]. One reason for this is the fact that in many cases, *C. trachomatis* genital infections are asymptomatic; 70-90% in females and 30-50% in males. The long-term sequaele of untreated *C. trachomatis* infections can be serious. In women, chronic infection can lead to scarring of the reproductive tract which can subsequently lead to salpingitis, ectopic pregnancy, pelvic inflammatory disease and infertility. In men, infection can lead to inflammation which can result in prostatitis, epididymitis and orchitis [64]. All these factors lead to the urgent need for an effective vaccine to treat *C.trachomatis* genital tract infections.

Early vaccine studies used whole inactivated *Chlamydia* but these led to enhanced immunopathology in some of the vaccinees [65-66] and as a consequence, this has led researchers to take a more cautious and rational approach to develop safe and effective chlamydial vaccines. Mouse studies suggest that both mucosal IgA as well as Th1 $CD4^+$ T cells play a crucial role in controlling chlamydial infections [67]. Mucosal antibodies can be neutralising and therefore play a role in limiting chlamydial urogenital tract infection [68] but the role of IgA in protective immunity is probably less crucial [69]. By comparison, interferon-gamma producing T cells are considered to be the major effector cells in controlling new chlamydial infections. For example, in the mouse model, mice deficient in interferon-gamma receptor but competent in mucosal IgA production fail to clear a primary chlamydial genital tract infection [70].

Recent vaccine studies are starting to show some promise although most work is still being conducted in the *Chlamydia muridarum* – mouse infection model. Other animal models for chlamydial genital infection, and hence vaccine development, include the guinea pig and non-human primates [71] but very few studies have yet been conducted in these non-mouse models. A key question for the development of an effective vaccine is the choice of antigen. Most work to date has focussed on the chlamydial major outer membrane protein, MOMP. While MOMP has both strong B and T cell epitopes and therefore can provide reasonable protection against live challenge, it shows considerable variability between serovars. This has led recently to an increase in attempts to identify new vaccine candidates using a range of approaches [72] including, (a) 2D gel electrophoresis and immunoblotting [73-74] to identify chlamydial antigens recognised by human antibodies. MOMP antibodies tend to dominate in these assays however. (b) Radio-immunoprecipitation combined with 2D gel electrophoresis. Indeed, this approach confirmed the importance of the *Chlamydia*-secreted protease factor (CPAF) [75] which is currently a strong vaccine candidate. (c) Genome-wide protein expression for detecting both antibody and T cell responses [76]. *Chlamydia*'s small genome makes this approach more feasible and it has led to a new set of potential vaccine antigens. (d) Antigen discovery using T cell lines. This technique has been successfully used to generate T cell lines that were protective in a systemic murine infection model [77]. (e) Immunoproteomics [78] has recently been used by Brunham and colleagues to examine the sequence of peptides presented via MHC class II on antigen presenting cells. This approach identified a range of antigens, including several Pmps. Together, these approaches have added a large number of new potential candidates (such as CPAF, PmpD, PmpG, CopN, IncA, NrdB, Pgp3) to the early targets of MOMP, Omp2 and heat shock protein 60. It is likely that an effective chlamydial vaccine will require a cocktail of antigens to be sufficiently effective. The other critical area for an effective vaccine is the method of delivery and the use of adjuvants. A range of delivery systems have been evaluated, including, immunostimulating complexes [79], detergent/surfactant-based adjuvants [80], live viral vectors [81], *Vibrio cholerae* ghosts [82], liposomes [83] and cytokines. However, there is still the need for more work on adjuvants that stimulate the required T cell response and importantly, induce homing of the effector cells to the site of the natural infection; i.e. the genital tract.

Because of the difficulties in demonstrating reasonable protection against live challenge in addition to safety, human clinical trials for a chlamydial STD vaccine are still progressing

very slowly. An important aspect that is often not considered is the dynamics of vaccination strategies. Gray et al. (2009) [84] used a mathematical modelling approach to show that targeting 100% of one sex (eg. females) is likely to have a greater epidemiological impact than administering the vaccine to 50% of both sexes. They suggested that an effective vaccine could have a significant impact if it could significantly reduce the infectious burden in an individual to a level that made transmission less likely, without necessarily having to be completely sterilising. On a promising note, they suggested that a fully protective vaccine (the holy grail of chlamydial vaccine research) could potentially eradicate *Chlamydia* infection within 20 years.

Gonococcus

N. gonorrhoeae sexually transmitted infection can be asymptomatic in both women and men; however the infection is associated with the development of chronic pelvic pain, PID, tubal factor infertility and ectopic pregnancy. There are approximately 300,000 reported gonococcus cases annually in the USA, with the actual incidence predicted to be double this number [85]. Gonococcus vaccine develop has been severely hindered by several factors; (1) the considerable antigenic variability of the surface antigens, (2) the fact that natural infections do not induce a strong humoral or cytotoxic immune response, although natural infections do eventually resolve (reviewed, [86-87]). This may be due to the innate inflammatory response and it appears that long-term protection against re-infection does not occur [88]. (3) Finally an effective animal model has only recently been developed. In spite of this, there have been vaccine trials using the subunit vaccines based on the gonococcus pilin or porin. A subunit vaccine trial using pilus did induce a local antibody response, however it failed to protect against subsequent infections [89]. Porin and other outer membrane proteins have also been considered and tested as potential vaccine antigens; however there are no published reports of any of these progressing clinical trials (see Zhu *et al*., for a more comprehensive review of this topic [86]).

Vaccination to protect pregnant women against reproductive tract infections: benefits and risks

Reproductive tract infections can result in significant additional impacts in pregnant women; hence we will separately review these considerations here. The medical, social, and legal, risks of immunizing pregnant women against infectious agents for their protection as well as their infant's protection are important issues. Emphasis has been placed on maternal immunisation primarily for protecting newborns and infants. There are several groups of infectious organisms that have potential for affecting the pregnant woman, her foetus, or neonate.

i) **Placental infection resulting in congenital diseases**: Toxoplasmosis, cytomegalovirus, HSV, rubella, syphilis, HIV, parovirus B-19, HepB, malaria, and varicella [90-94].

ii) **Infections in neonates**: *Hemophilus (H.) influenzae* type B, *Streptococcus (S.) pneumonia*, Group B *Streptococcus* (GBS), *Staphylococcus (S.) aureus* [95-96].

iii) **Preterm birth**: *Mycoplasma (M.) hominis*, *Ureaplasma (U.) urealyticum*, *Clostridium*, *Leptospira*, *Trichomonas (T.) vaginalis*, bacterial vaginosis, and bacteroides [97-100].

During pregnancy, the maternal immune system must accept/tolerate the implantation and growth of a semi-allogeneic fetus. As a result of this, changes occur in the maternal immune system during pregnancy. These include a general suppression of Th1 immunity and an increase in Th2 immunity. In addition, there is an influx of Treg cells into the uterus during the first and second trimesters of pregnancy, cells that maintain tolerance to the paternal antigens on developing fetus, and expression of a number of molecules such as IDO and non-classical and MHC molecules (HLA-G, HLA-E) that suppress activation of maternal T cells and NK cells respectively (reviewed [101]). Thus, while the administration of live-attenuated vaccines aimed at developing Th1/CMI responses is not recommended during pregnancy, the pregnant immune system still retains the capacity to respond to killed or inactivated vaccines to produce humoral responses that are equivalent to those in non-pregnant women [102-103].

The potential benefits of immunizing pregnant women

Immunisation programmes in pregnancy are among the most cost beneficial health interventions. They can significantly reduce the occurrence of preventable disease thereby benefitting the pregnant women and infant [104]. Preparations of new vaccines are also required against infectious agents that are known to result in reproductive pathology and congenital

malformation if the infection of the mother occurs during pregnancy. Active campaigns to vaccinate the pregnant women should have the following public health benefits:

i) Eliminating teratogenic and embryopathic congenital infections of the embryo and placenta: i.e. HSV, varicella, CMV, toxoplasmosis, rubella, syphilis, HIV, parovirus B-19, HepB, and malaria [105-107].

ii) Eliminating neonatal infections acquired during perinatal period via maternal transplacental antibodies for which the neonate has not developed antibodies: i.e. GBS, *H. influenzae* type B, *S. pneumoniae* [105-106].

iii) Elimination of intrauterine infections that may contribute to premature birth: i.e. Bacteroides, *M. hominis*, *U. urealyticum*, *Clostridium*, *Leptospira*, *T. vaginalis* (bacterial vaginosis) [108-110].

The risks of immunizing pregnant women

The perceived risks of immunizing pregnant women have resulted in a lack of data to support the actual risks to the women. This is paradoxical given that pregnant women are frequently at higher risk of serious sequelae due to infections.

i) In general live and attenuated vaccines are contraindicated during pregnancy as there is primarily a theoretical risk to the foetus. The quadrivalant HPV vaccine for prevention of infection by HPV strains (Gardasil®) is not recommended for use during pregnancy [111].

ii) Transmission of attenuated virus to the placenta or foetus: There is a risk of live vaccine immunisations during pregnancy resulting in infection of the baby as sub-clinical infections have been documented [112-114].

iii) The risk that the vaccine would result in reproductive effects, congenital malformations, abortion, growth retardation, stillbirth, and an array of neurological effects [115-116].

iv) There have been reports of the ineffectiveness of vaccine for pregnant females, specifically the pneumococcus vaccine [117].

However, in spite of these risks several vaccines have been reported to have been administered to pregnant women with no increase in congenital defects for the foetus and no reported adverse outcomes for the mother; including Rubella (Menavix II), MMR (measles, mumps, rubella MMRII) (reviewed, [103]).

In summary, There is considerable need for studies that include pregnant animals during vaccine development (pre-clinical vaccine studies) and also the inclusion of pregnant women in human clinical trials of new vaccines, given the lack of understanding and evidence relating to the impacts of vaccines on pregnant women, the altered immune status during pregnancy, and the potential impact of the vaccine on the mother, foetus or neonate. In addition, more clinical emphasis needs to be placed on vaccinations for women who are intending to get pregnant prior to conception. Furthermore, recognition by vaccine development companies that it is critical to test vaccines in pregnant women, in fact participation of pregnant women in vaccine trials has been permitted since 1994 (Institute of Medicine, USA) [103]. Whilst there may be additional risks in the inclusion of this cohort, the benefits of identifying successful vaccines for this cohort to reduce incidence of these serious infections may well far outweigh the risks [102]. While it is likely that immune responses to vaccines delivered during pregnancy will likely be different, given the altered immune status [101,118], it is also clear that the pregnant immune system retains the ability to respond to vaccination and produce protective immune responses [119]. Finally, it is clear that regardless of the results of clinical trials in pregnant women, clinical confounders may still exist and thus it is still critical that at the clinic a risk: benefit analysis is discussed and considered for each individual on a case by case basis.

Adjuvants are critical for successful vaccine development

The role of adjuvants in vaccine delivery is to improve the immunogenicity of the vaccine antigen(s), particularly subunit vaccines, and direct the type of adaptive response generated by the vaccination. As adjuvants are responsible for initiating and influencing the type of immune response generated by the vaccination, adjuvant choice is an important consideration when designing a vaccine. Although many varied adjuvants are used in animal models, few are approved for human use.

The classical aluminium salts or alum adjuvant have been in use for almost one hundred years. It was first used in vaccination as early as 1926 to boost the immunogenicity of the diptheria toxoid vaccine. Early vaccines were often fixed or heat killed pathogens, containing LPS and other potent immune stimulators. While these early vaccines usually elicited potent immune responses these were often accompanied by adverse side effects. For this reason, current trends in vaccine development involve the use of purified subunit antigens to avoid adverse reactions, however these are usually less effective at stimulating the innate immune response at a level sufficient to initiate a significant adaptive immune response. To overcome this problem stronger adjuvants are required to stimulate a strong innate immune response that is required to induce a long lasting and specific protective immune response. Mucosal immunisation offers an extra level of challenge for good adjuvant design as the default immune response to mucosal delivery is often tolerance and at least with oral delivery both adjuvant and antigen must survive the gastric pH and digestive processes.

Approval of adjuvants other than Alum for human use did not occur until the late 1990's. MF59, an oil-in-water emulsion adjuvant, is composed of small droplets of the natural oil squalene [120]. MF59 is appropriate for use with soluble and hydrophobic proteins as well as particulate antigens. Virosomes are also small and made up of a phospholipid bilayer containing 70% phosphotidylcholine and 20% synthetic phosphoethanolamine. Hemagglutinin (HA) and neuroaminidase (NA) are intercalated into the phospholipid bilayer and appear to enhance uptake of the virosome and associated vaccine [121]. Virosomal vaccination has been successfully tested in humans as an influenza vaccine without adverse affects [122]. There are many stronger adjuvants that are commonly used in animal models such as cholera toxin (CT) and its subunits and derivatives, CpGs, various cytokines such as IL-12, GM-CSF and complement factors. Immune stimulating complexes (ISCOMs) and bacterial products such as monophosphoryl lipid A (MPL) have undergone extensive testing and are closer to becoming a reality for use in human vaccines. New adjuvants are emerging for clinical use in human vaccines. Montanide, a water-in-oil emulsion and CpG, a TLR9 agonist are undergoing clinical trials and show promise [123-124].

Adjuvants influence the polarity of the immune response against the vaccine antigen. The polarity of the ensuing immune response and subsequent memory developed against the vaccine antigen are important in mediating protection. Many of the currently licensed adjuvants, including Alum (the most commonly used) and MF59, direct the immune response

towards a Th2 polarity [125-126]. Many bacterial and viral diseases require a Th1 cell mediated immune response for pathogen control and clearance, so more adjuvants that can act in this fashion are needed. Mucosal routes of administration show promise for the induction of protective immune responses, however the natural default of mucosal immune responses towards tolerance needs to be overcome through the use of a strong, yet safe and appropriately polarising, adjuvants. Our continuing advances in the understanding of innate immunity through the use of animal models have led to the discovery of many novel innate immune stimulators, which are now being trialled in animal studies and human clinical trials as potential adjuvants for human use.

Novel and emerging methods

Multivalent vaccines against multiple organisms are not a new innovation; however, an exciting advance within the field has been to use successful vaccines in combination with antigens from other infectious agents to elicit immunity against both pathogens. There are two examples of attempts to use the HPV virus like particle vaccine strategy to improve the immune response to antigens from other pathogens; fusion of chlamydial MOMP to the HPV vaccine [127], and fusion of the HSV2 protein to the HPV vaccine [128]. Additionally *Vibrio* cholera ghosts in combination with *Chlamydia* subunit vaccine antigens have been tested in animal models, as a means of harnessing the adjuvant properties of the cholera toxin [129-130]. This approach to 'piggy back' on approved successful vaccines could represent a fast and effective step forward for the field and we look forward to the outcomes from these and similar studies.

The abundance of genome sequencing data is going to positively impact on the vaccine field, in fact genome mining approaches have already been used successfully in the detection of vaccine antigens for *Neisseria meningitidis* [131]. This has also enabled *in silico* approaches to identify vaccine antigens, such as a recent study from our own team, which identified several novel chlamydial antigens [132].

Expert Commentary and five year view

Sexually transmitted infections are responsible for serious morbidities and mortalities and pose a major burden on healthcare systems. The sequelae of STIs disproportionately impact on women. Treatments are available for some infections but these are often expensive, require active testing programs and have not been able to halt the increase in rates of infection. Vaccination has the greatest potential to impact on rates of infection however there are major obstacles to the development of vaccines for STIs. These include; understanding the correlates of protection for the major sexually transmitted pathogens, understanding how cycle-associated changes in female sex hormones impact on the induction of and effectiveness of vaccine-induced immunity, defining the best route(s) of immunisation to target the female (and male) reproductive tracts, selection of the best antigen(s) and adjuvant combinations to elicit protective immunity and defining who and when to vaccinate. Antigen discovery research using animal models has identified potential protective antigens for most important STIs and a greater understanding of innate immunity has led to the development of many novel adjuvants and adjuvant combinations that are currently in human clinical trials. The key to developing vaccines against the major human STIs is the translation of knowledge gained from pre-clinical studies in animal models into human clinical trials. The successful development of vaccines to target HPV infections and cervical cancer provide an example of how this can be achieved and has also increased social acceptance of vaccines for STIs.

Key Issues

- Determining the mechanisms of immune protection against the various sexually transmitted pathogens is essential for vaccine development. Cervicovaginal secretions contain both IgG and IgA, and both subclasses may impact on protection. For example IgA may prevent infection via immune exclusion whilst IgG may provide a key link between innate and adaptive immunity by facilitating antibody dependent cellular cytotoxicity (ADCC) by FcR+ cells such as NK cells. Interferon gamma producing CD4 T cells are essential for protection against intracellular pathogens such as *Chlamydia*, however studies in animal models show that recruitment of memory T cells into the FRT only occurs as a result of infection and may not occur in time to

prevent infection. This may mean that induction of sterilising immunity is not possible for some STIs.

- Is sterilising immunity an essential requirement for all STI vaccines? For infections caused by *C. trachomatis* for example, modelling studies suggest that even partially effective vaccines could have a major impact on the incidence of infection at a population level, as a result of decreasing the magnitude and duration of infection in vaccinated individuals and increasing the critical load required for infection of vaccinated individuals. Such outcomes should be effective from a public health perspective in reducing the incidence and burden of a disease, however acceptance of a vaccine that does not provide sterilising immunity would represent a major paradigm shift.
- What is the best route of immunisation to target immunity to the FRT? Studies in animal models suggest that intranasal immunisation, for example, is particularly effective at eliciting protective immunity against a number of sexually transmitted pathogens. Studies of needle-free mucosal (intranasal, oral, vaginal, transcutaneous) vaccination in humans are required to determine if mucosal immunisation elicits better protection than current intramuscular injection, as is used to deliver the HPV vaccines Gardasil® and Cerverix®.
- Adjuvants other than those currently approved for human use (Alum, MF59 and AS03) are essential for the development of effective vaccines against STIs. These must be non-toxic and safe for human use, facilitate mucosal vaccine delivery and allow for induction of immune responses tailored to specific pathogens, for example induction of mixed Th1/Th2 responses with or without a CD8 CTL response. An increased understanding of PAMP/PRR interactions required for activation of innate immunity should facilitate the development of new adjuvants and several combination adjuvants have shown promise in animal models.
- Vaccine efficacy may be impacted by hormonal impacts on the immune response elicited by vaccines. The data supporting this view comes predominantly from animal models and it remains unclear if this will represent an obstacle for development of vaccines to target human STIs. It will be essential to determine in humans if hormonal status affects either induction of immunity by vaccination or the effectiveness of vaccine-induced protection. The success of vaccines targeting HPV infections and cervical cancer is encouraging.

- The ability to implement vaccination programs to the most suitable target age to ensure immunity is effectively acquired before risk of exposure has been a potential hurdle which was recently overcome in the HPV vaccination program (i.e. prepubescent females have recently been targeted for the HPV vaccines; pre-exposure immunisation). Furthermore, could the effectiveness of STI vaccines at the population level be increased by also vaccinating males?
- Gender specific vaccination approaches are already being trialled (eg for HSV) and this may be necessary to successfully protect both the males and females from infection. Could the state of immune privilege that exists in the male reproductive tract represent a barrier to induction of protective immunity against STIs in males and could immune mechanisms of protection differ between genders?

References

1. Jindal N, Aggarwah A, Gill P, Sacharwai B, Sheevani BB. Community-based study of reproductive tract infections, including sexually transmitted infections, among the rural population of Punjab., India. *India J Comm Med*, 34, 359-361 (2009).
2. Hoare A, Wilson DP, Regan DG, Kaldor J, Law MG. Using mathematical modelling to help explain the differential increase in HIV incidence in New South Wales, Victoria and Queensland: importance of other sexually transmissible infections. *Sex Health.* , 5, 169-187 (2008).
3. Russell MW, Mestecky J. Tolerance and Protection against infection in the genital tract. *Immunological Investigations*, 39, 500-525 (2010).
4. Vallely A, Page A, Dias S *et al.* The prevalence of sexually transmitted infections in papua new Guinea: a systematic review and meta-analysis. *PLoS One*, 5, e15586 (2010).
5. Guy RJ, Kong F, Goller J *et al.* A new national Chlamydia Sentinel Surveillance System in Australia: evaluation of the first stage of implementation. *Commun Dis Intell.*, 319-328 (2010).
6. Blandford JM, Gift TL. The cost-effectiveness of single-dose azithromycin for treatment of incubating syphilis. *Sex Transm Dis*, 30, 502-508 (2003).
7. Boyd MA, Hill AM. Clinical management of treatment-experienced, HIV/AIDS patients in the combination antiretroviral therapy era. *Pharmacoeconomics.* 28, 17-34 (2010).
8. Emmert DH. Treatment of Common Cutaneous Herpes Simplex Virus Infections. *American Family Physician*, 61, 1697-1704 (2000).

9. Tawk HM, Vickery K, Bisset L, Lo SK, Selby W, Cossart YE. The current pattern of hepatitis B virus infection in Australia. J Viral Hepatitis. *J Viral Hepat*, 13, 206-215. (2006).

10. Moodley P, Wilkinson D, Connolly C. *Trichomonas vaginalis* is associated with pelvic inflammatory disease in women infected with human immunodeficiency virus. *Clin Infect Dis.*, 34, 519-522 (2002).

11. Forna F, Gülmezoglu AM. Interventions for treating trichomoniasis in women. . *Cochrane Database Syst Rev.*, CD000218 (2003).

12. Petrin D, Delgaty K, Bhatt R, Garber G. Clinical and microbiological aspects of *Trichomonas vaginalis*. *Clin Microbiol Rev.*, 11, 300-317. (1998).

13. Org GF. Global Health Program. *2011 Bill and Melinda Gates Foundation* (2010).

14. Zhao C, Wang I, Lehrer RI. Widespread expression of beta-defensin hBD-1 in human secretory glands and epithelial cells. *FEBS Lett*, 396(2-3), 319-322 (1996).

15. Wira CR, Fahey JV. The innate immune system: gatekeeper to the female reproductive tract. *Immunology*, 111(1), 13-15 (2004).

16. Aflatoonian R, Fazeli A. Toll-like receptors in female reproductive tract and their menstrual cycle dependent expression. *J Reprod Immunol*, 77(1), 7-13 (2008).

17. Aflatoonian R, Tuckerman E, Elliott SL *et al.* Menstrual cycle-dependent changes of Toll-like receptors in endometrium. *Hum Reprod*, 22(2), 586-593 (2007).

18. Bouman A, Heineman MJ, Faas MM. Sex hormones and the immune response in humans. *Hum Reprod Update*, 11(4), 411-423 (2005).

19. Fish EN. The X-files in immunity: sex-based differences predispose immune responses. *Nat Rev Immunol*, 8(9), 737-744 (2008).

20. Stopinska-Gluszak U, Waligora J, Grzela T *et al.* Effect of estrogen/progesterone hormone replacement therapy on natural killer cell cytotoxicity and

immunoregulatory cytokine release by peripheral blood mononuclear cells of postmenopausal women. *J Reprod Immunol*, 69(1), 65-75 (2006).

21. Kaul R, Pettengell C, Sheth PM *et al.* The genital tract immune milieu: an important determinant of HIV susceptibility and secondary transmission. *J Reprod Immunol*, 77(1), 32-40 (2008).
22. Patton DL, Thwin SS, Meier A, Hooton TM, Stapleton AE, Eschenbach DA. Epithelial cell layer thickness and immune cell populations in the normal human vagina at different stages of the menstrual cycle. *Am J Obstet Gynecol*, 183(4), 967-973 (2000).
23. White HD, Crassi KM, Givan AL *et al.* CD3+ CD8+ CTL activity within the human female reproductive tract: influence of stage of the menstrual cycle and menopause. *J Immunol*, 158(6), 3017-3027 (1997).
24. Yeaman GR, Collins JE, Fanger MW, Wira CR, Lydyard PM. CD8+ T cells in human uterine endometrial lymphoid aggregates: evidence for accumulation of cells by trafficking. *Immunology*, 102(4), 434-440 (2001).
25. Yeaman GR, Guyre PM, Fanger MW *et al.* Unique CD8+ T cell-rich lymphoid aggregates in human uterine endometrium. *J Leukoc Biol*, 61(4), 427-435 (1997).
26. Lu FX, Ma Z, Moser S, Evans TG, Miller CJ. Effects of ovarian steroids on immunoglobulin-secreting cell function in healthy women. *Clin Diagn Lab Immunol*, 10(5), 944-949 (2003).
27. Wira CR, Sullivan DA. Estradiol and progesterone regulation of immunoglobulin A and G and secretory component in cervicovaginal secretions of the rat. *Biol Reprod*, 32(1), 90-95 (1985).
28. Hughes GC, Clark EA. Regulation of dendritic cells by female sex steroids: relevance to immunity and autoimmunity. *Autoimmunity*, 40(6), 470-481 (2007).

29. Kovats S, Carreras E. Regulation of dendritic cell differentiation and function by estrogen receptor ligands. *Cell Immunol*, 252(1-2), 81-90 (2008).

30. Bengtsson AK, Ryan EJ, Giordano D, Magaletti DM, Clark EA. 17beta-estradiol (E2) modulates cytokine and chemokine expression in human monocyte-derived dendritic cells. *Blood*, 104(5), 1404-1410 (2004).

31. Murphy AJ, Guyre PM, Wira CR, Pioli PA. Estradiol regulates expression of estrogen receptor ERalpha46 in human macrophages. *PLoS One*, 4(5), e5539 (2009).

32. Liang J, Sun L, Wang Q, Hou Y. Progesterone regulates mouse dendritic cells differentiation and maturation. *Int Immunopharmacol*, 6(5), 830-838 (2006).

33. Wira CR, Fahey JV. A new strategy to understand how HIV infects women: identification of a window of vulnerability during the menstrual cycle. *AIDS*, 22, 1909–1917 (2008).

34. Nardelli-Haefliger D, Roden R, Balmelli C, Potts A, Schiller J, De Grandi P. Mucosal but not parenteral immunization with purified human papillomavirus type 16 virus-like particles induces neutralizing titers of antibodies throughout the estrous cycle of mice. *J Virol*, 73(11), 9609-9613 (1999).

35. Johansson EL, Wassen L, Holmgren J, Jertborn M, Rudin A. Nasal and vaginal vaccinations have differential effects on antibody responses in vaginal and cervical secretions in humans. *Infect Immun*, 69(12), 7481-7486 (2001).

36. Kozlowski PA, Williams SB, Lynch RM *et al.* Differential induction of mucosal and systemic antibody responses in women after nasal, rectal, or vaginal immunization: influence of the menstrual cycle. *J Immunol*, 169(1), 566-574 (2002).

37. Decrausaz L, Domingos-Pereira S, Duc M *et al.* Parenteral but not mucosal immunization is able to induce regression of human papillomavirus associated genital tumors. *Int J Cancer*, (2011).

38. Stanley M, Lowy DR, Fraser I. Chapter 12: Prophylactic HPV vaccines: Underlying mechanisms. In: *Vaccine: HPV Vaccines and Screening in the Prevention of Cervical Cancer*. Elsievier (Ed. (2006) 106-113.

39. Ulmer JB, Liu MA. Science and society – vaccines: Ethical issues for vaccines and immunization. *Nature Reviews Immunology* 2, 291-296 (2002).

40. GAVI. http://www.who.int/immunization_financing/options/en/briefcase_vacproduction.pdf.

41. Kim JH, Rerks-Ngarm S, Excler JL, Michael NL. HIV vaccines: lessons learned and the way forward. *Curr Opin HIV AIDS*, 4, 428-434 (2010).

42. Walboomers JM, Jacobs MV, Manos MM *et al.* Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol*, 189(1), 12-19 (1999).

43. Werness BA, Levine AJ, Howley PM. Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science*, 248, 76-79 (1990).

44. Villa LL, Costa RL, Petta CA *et al.* High sustained efficacy of a prophylactic quadrivalent human papillomavirus types 6/11/16/18 L1 virus-like particle vaccine through 5 years of follow-up. *Br J Cancer*, 1459-1466 (2006).

45. FUTURE I/II Study Group, J. D, Kjaer SK *et al.* Four year efficacy of prophylactic human papillomavirus quadrivalent vaccine against low grade cervical, vulvar, and vaginal intraepithelial neoplasia and anogenital warts: randomised controlled trial. *BMJ.*, 341, 3493 (2010).

46. Brotherton JM, Fridman M, May CL, Chappell G, Saville AM, Gertig DM. Early effect of the HPV vaccination programme on cervical abnormalities in Victoria, Australia: an ecological study. *Lancet*, 377(9783), 2085-2092 (2011).

47. Kim JJ. Focus on Research: Weighing the Benefits and Costs of HPV Vaccination of Young Men. *N Engl J Med*, 364, 393-395 (2011).

48. Giuliano AR, Palefsky JM, Goldstone S *et al*. Efficacy of Quadrivalent HPV Vaccine against HPV Infection and Disease in Males. *N Engl J Med* 364, 401-411 (2011).

49. Anderson ME, Weese JS. Video observation of hand hygiene practices at a petting zoo and the impact of hand hygiene interventions. *Epidemiol Infect*, 1-9 (2011).

50. CDC. A comprehensive immunization strategy to eliminate transmission of hepatitis B virus in the United states: Recommendations of the advisroty committee on immunization practices (ACIP); Part 1: immunization of infants, children and adolescents. *MMWR*, 54, RR-16 (2005).

51. CDC. A comprehensive immunization strategy to eliminate transmission of hepatitis B virus in the United states: Recommendations of the advisroty committee on immunization practices (ACIP); Part 2: immunization of adults. *MMWR*, 55, RR-16 (2006).

52. Noble RC, Kirk NM, Slagel WA, Vance BJ, Somes GW. Recidivism among patients with gonococcal infection presenting to a venereal disease clinic. *Sex Transm Dis*, 4(2), 39-43 (1977).

53. Beagley KW, Timms P. Chlamydia trachomatis infection: incidence, health costs and prospects for vaccine development. *J Reprod Immunol*, 48(1), 47-68 (2000).

54. ADVERT. http://www.avert.org/worldstats.htm.

55. Kelleher P. HIV immunology: what lessons can we learn from recent vaccine trials? *J HIV Ther*, 15(1), 2-5 (2010).

56. McKinnon LR, Card CM. HIV Vaccine Efficacy Trials: A Brief History, and Options for Going Forward. *AIDS Rev*, 12(4), 209-217 (2010).

57. Fleming DT, McQuillan GM, Johnson RE. Herpes simplex virus type 2 in the United States. *N Engl J Med*, 337, 1105-1111 (1997).

58. Bettahi I, Zhang X, Afifi RE, BenMohamed L. Protective immunity to genital herpes simplex virus type 1 and type 2 provided by self-adjuvanting lipopeptides that drive dendritic cell maturation and elicit a polarized Th1 immune response. *Viral Immunol*, 19, 220-236 (2006).

59. Aurelian L. Herpes simplex Virus Type 2 Vaccines: New ground for optimism? *Clin Diagn Lab Immunol*, 11, 437-115 (2004).

60. Stanberry LR, Bernstein DI, Burke RL, Pachl C, Myers MG. Vaccination with recombinant herpes simplex virus glycoproteins: protection against initial and recurrent genital herpes. *J Infect Dis*, 155, 914-920 (1987).

61. Stanberry LR, Spruance SL, Cunningham AL *et al.* Glycoprotein-D-Adjuvant vaccine to prevent genital herpes. *N Engl J Med*, 347, 1652-1662 (2002).

62. Boursnell ME, Entwisle C, Blakeley D *et al.* A genetically inactivated herpes simplex virus type 2 (HSV-2) vaccine provides effective protection against primary and recurrent HSV-2 disease. *J Infect Dis*, 175, 16-25 (1997).

63. Cochrane M, Armitage CW, O'Meara CP, Beagley KW. Towards a Chlamydia trachomatis vaccine: how close are we? *Future Microbiol*, 5(12), 1833-1856 (2010).

64. Cunningham KA, Beagley KW. Male Genital Tract Chlamydial Infection: Implications for Pathology and Infertility. *Biology of Reproduction*, 79, 180-189 (2008).

65. Peipert JF. Clinical practice. Genital chlamydial infections. *N Engl J Med*, 349(25), 2424-2430 (2003).

66. Grayston JT, Woolridge RL, Wang S. Trachoma vaccine studies on Taiwan. *Ann. NY Acad. Sci.* , 98, 352-367 (1962).

67. Kelly KA. Cellular immunity and Chlamydia genital infection: induction, recruitment, and effector mechanisms. *Int Rev Immunol*, 22(1), 3-41 (2003).

68. Morrison SG, Morrison RP. A predominant role for antibody in acquired immunity to chlamydial genital tract reinfection. *J. Immunol*, 175, 7536-7542 (2005).

69. Morrison SG, Morrison RP. The protective effect of antibody in immunity to murine chlamydial genital tract reinfection is independent of immunoglobulin A. *Infect Immun*, 73, 6183-6186 (2005).

70. Johansson M, Schon K, Ward M, Lycke N. Genital tract infection with Chlamydia trachomatis fails to induce protective immunity in γ interferon receptor-deficient mice despite a strong local immunoglobulin A response. *Infect Immun*, 73(1032-1044) (1997).

71. Hafner L, Beagley K, Timms P. Chlamydia trachomatis infection: host immune responses and potential vaccines. *Mucosal Immunol.*, 1, 116-130 (2008).

72. Rockey DD, Wang J, Lei L, Zhong G. Chlamydia vaccine candidates and tools for chlamydial antigen discovery. *Expert Rev Vaccines*, 8(10), 1365-1377 (2009).

73. Caldwell HD, Kuo CC, Kenny GE. Antigenic analysis of Chlamydiae by two-dimensional immunoelectrophoresis. II. A trachoma-LGV-specific antigen. *J Immunol*, 115, 969-975 (1975).

74. Sanchez-Campillo M, Bini L, Comanducci M *et al.* Identification of immunoreactive proteins of Chlamydia trachomatis by Western blot analysis of a two-dimensional electrophoresis map with patient sera. *Electrophoresis*, 20(11), 2269-2279 (1999).

75. Murphey C, Murthy AK, Meier PA, Neal Guentzel M, Zhong G, Arulanandam BP. The protective efficacy of chlamydial protease-like activity factor vaccination is dependent upon CD4+ T cells. . *Cell. Immunol.* , 242, 110-117 (2006).

76. Stemke-Hale K, Kaltenboeck B, DeGraves FJ *et al.* Screening the whole genome of a pathogen *in vivo* for individual protective antigens. *Vaccine*, 23(23), 3016-3025 (2005).

77. Starnbach MN, Loomis WP, Ovendale P *et al.* An inclusion membrane protein from Chlamydia trachomatis enters the MHC class I pathway and stimulates a CD8+ T cell response. *J Immunol*, 171(9), 4742-4749 (2003).

78. Shaw AC, Gevaert K, Demol H *et al.* Comparative proteome analysis of Chlamydia trachomatis serovar A, D and L2. *Proteomics*, 2(2), 164-186 (2002).

79. Dong-Ji Z, Yang X, Shen C, Lu H, Murdin A, Brunham RC. Priming with *Chlamydia trachomatis* major outer membrane protein (MOMP) DNA followed by MOMP ISCOM boosting enhances protection and is associated with increased immunoglobulin A and Th1 cellular immune responses. *Infect Immun*, 68, 3074-3078 (2000).

80. Pal S, Peterson EM, Rappuoli R, Ratti G, de la Maza LM. Immunization with the *Chlamydia trachomatis* major outer membrane protein, using adjuvants developed for human vaccines, can induce partial protection in a mouse model against a genital challenge. *Vaccine*, 24, 766-775 (2006).

81. He Q, Martinez-Sobrido L, Eko FO *et al.* Live-attenuated influenza viruses as delivery vectors for Chlamydia vaccines. *Immunology*, 122(1), 28-37 (2007).

82. Macmillan L, Ifere GO, He Q *et al.* A recombinant multivalent combination vaccine protects against Chlamydia and genital herpes. *FEMS Immunol Med Microbiol*, 49(1), 46-55 (2007).

83. Hansen J, Jensen KT, Follmann F, Agger EM, Theisen M, Andersen P. Liposome delivery of *Chlamydia muridarum* major outer membrane protein primes a Th1 response that protects against genital chlamydial infection in a mouse model. *J Infect Dis*, 198, 758-767 (2008).

84. Gray RT, Beagley K, Timms P, Wilson DP. Modeling the Impact of Potential Vaccines on Epidemics of Sexually Transmitted Chlamydia trachomatis Infection. *J Infect Dis*, 199, 1680-1688 (2009).

85. Workowski KA, Berman SM. Sexually transmitted diseases treatment guidelines, 2006.AUCenters for Disease Control and Prevention. *SOMMWR Recomm Rep*, 55, 1-11 (2006).

86. Zhu W, Chen C, Thomas CE, Anderson JE, Jerse AE, Sparling PF. Vaccines for gonorrhea:can we rise to thec hallenge? *Frontiers in Microbiology*, 2, 124 (2011).

87. Liu Y, Feinen B, Russell MW. New concepts in immunity to *Neisseria gonorrhoeae*: innate responses and suppression of adaptive immunity favor the pathogen, not the host. *Front Microbiol* 2, 52 (2011).

88. Feinen B, Jerse AE, Gaffen SL, Russell MW. Critical role of Th17 responses in a murine model of Neisseria gonorrhoeae genital infection. *Mucosal Immunol*, 3(3), 312-321 (2010).

89. Boslego JW, Tramont EC, Chung RC *et al.* Efficacy trial of a parenteral gonococcal pilus vaccine in men. *Vaccine*, 9, 154-162 (1991).

90. Yinon Y, Farine D, Yudin MH. Screening, diagnosis, and management of cytomegalovirus infection in pregnancy. *Obstet Gynecol Surv*, 65, 736-743 (2010).

91. Serranti D, Buonsenso D, Valentini PC. Congenital toxoplasmosis treatment. *Eur Rev Med Pharmacol Sci*, 15, 193-198 (2011).

92. Sha BE, C. T, Cohn SE *et al.* Postpartum viral load rebound in HIV-1-infected women treated with highly active antiretroviral therapy: AIDS Clinical Trials Group Protocol A5150. *HIV Clin Trials*, 12, 9-23 (2011).

93. Bardají A, Sigauque B, Sanz S *et al.* Impact of malaria at the end of pregnancy on infant mortality and morbidity. *J Infect Dis*, 203, 691-699 (2011).

94. Hannachi N, Marzouk M, Harrabi I *et al.* Seroprevalence of rubella virus, varicella zoster virus, cytomegalovirus and parvovirus B19 among pregnant women in the Sousse region, Tunisia. *Bull Soc Pathol Exot.*, 104, 62-67 (2011).

95. Vasilcan G, Avasiloaiei A, Moscalu M, Dimitriu AG, Stamatin M. Germ circulation in neonatal units--risk factors for maternal-foetal infection. *Rev Med Chir Soc Med Nat Iasi*, 114, 1028-1034 (2010).

96. Steer PJ, Plumb J. Myth: Group B streptococcal infection in pregnancy: Comprehended and conquered. *Semin Fetal Neonatal Med*, 12 (2011).

97. Challis JR, Lockwood CJ, Myatt L, Norman JE, Strauss JFr, Petraglia F. *Inflammation and pregnancy Reprod Sci*, 16, 206-210 (2009).

98. Pararas MV, Skevaki CL, Kafetzis DA. Preterm birth due to maternal infection: Causative pathogens and modes of prevention. *Eur J Clin Microbiol Infect Dis*, 25, 562-569 (2006).

99. Guaschino S, De Seta F, Piccoli M, Maso G, Alberico S. Aetiology of preterm labour: bacterial vaginosis. *Brit J Obs Gyn*, 113, 46-51 (2006).

100. Kirchner L, Helmer H, Heinze G *et al.* Amnionitis with Ureaplasma urealyticum or other microbes leads to increased morbidity and prolonged hospitalization in very low birth weight infants. *Eur J Obstet Gynecol Reprod Biol*, 134, 44-50 (2007).

101. Wheetman AP. Immunity, thyroid function and pregnancy: molecular mechanisms. *Nat Rev Endocrin*, 6, 311-318 (2010).

102. Al-Safi ZA, Shavell VI, Gonik B. Vaccination in pregnancy. *Women's Health*, 7(109–119) (2011).

103. Fox KA, Theiler R. Vaccination in Pregnancy. *Current Pharmaceutical Biotechnology*, 12, 789-796 (2011).

104. Al-Safi ZA, Shavell VI, Gonik B. Vaccination in pregnancy. *Womens Health* 7, 109-119 (2011).

105. Brent RL. Immunization of pregnant women: reproductive, medical and societal risks. *Vaccine*, 21, 3413-3421 (2003).

106. Brent RL. Risks and benefits of immunizing pregnant women: The risk of doing nothing. *Reproductive Toxicology.*, 21, 383-389 (2006).

107. Gilbert-Barness E. Teratogenic Causes of Malformations. *Annals of Clinical & Laboratory Science*, 40, 99-114 (2010).

108. Zhou X, Brotman RM, Gajer P *et al.* Recent advances in understanding the microbiology of the female reproductive tract and the causes of premature birth. *Infect Dis Obstet Gynecol.* , 737425 (2010).

109. Vrachnis N, Vitoratos N, Iliodromiti Z, Sifakis S, Deligeoroglou E, Creatsas G. Intrauterine inflammation and preterm delivery. *Ann N Y Acad Sci*, 1205, 118-122 (2010).

110. Randis TM. Progress toward improved understanding of infection-related preterm birth. *Clin Perinatol*, 37, 677-688 (2010).

111. Smith-McCune K, Sawaya GF. Update on quadrivalent human papillomavirus vaccination and pregnancy outcomes: is contraception advisable? . *Obstet Gynecol Surv*, 114, 1168-1169 (2009).

112. Desoye M, Gauster G, Wadsack C. Placental transport in pregnancy pathologies. *Am J Clin Nutr*, May, 4 (2011).

113. Pimentel MA. Vaccination during pregnancy. *Acta Med Port*, 23, 837-840 (2010).

114. Badilla X, Morice A, Avila-Aguero ML *et al.* Fetal risk associated with rubella vaccination during pregnancy. *Pediatr Infect Dis J*, 26, 830-835 (2007).

115. Jordan EK, Sever JL. Fetal damage caused by parvoviral infections. *Reprod Toxicol*, 8, 161-168 (1994).

116. Wacholder S, Chen BE, Wilcox A *et al.* Risk of miscarriage with bivalent vaccine against human papillomavirus (HPV) types 16 and 18: pooled analysis of two randomised controlled trials. *BMJ*, 340, 712 (2010).

117. Lopes CR BE, Ching TH, Canuto Jde S, Costa VO, Klering EM . 2009;13(2):104-6. Ineffectiveness for infants of immunization of mothers with pneumococcal capsular polysaccharide vaccine during pregnancy. *Braz J Infect Dis*, 13, 104-106).

118. Witkin SS, Linhares IM, Bongiovanni AM, Herway C, Skupskia D. Unique alterations in infection-induced immune activation during pregnancy. *Brit J Obs Gyn*, 118, 145–153 (2010).

119. Wilkin T, Lee JY, Lensing SY *et al.* Safety and immunogenicity of the quadrivalent human papillomavirus vaccine in HIV-1-infected men. *J Infect Dis*, 202(8), 1246-1253 (2010).

120. O'Hagan DT, Ugozzoli M, Barackman J *et al.* Microparticles in MF59, a potent adjuvant combination for a recombinant protein vaccine against HIV-1. *Vaccine*, 18(17), 1793-1801 (2000).

121. Gluck R. Adjuvant activity of immunopotentiating reconstituted influenza virosomes (IRIVs). *Vaccine*, 17(13-14), 1782-1787 (1999).

122. Gluck U, Gebbers JO, Gluck R. Phase 1 evaluation of intranasal virosomal influenza vaccine with and without *Escherichia coli* heat-labile toxin in adult volunteers. *J Virol*, 73(9), 7780-7786 (1999).

123. Herrera S, Fernandez OL, Vera O *et al.* Phase I safety and immunogenicity trial of *Plasmodium vivax* CS derived long synthetic peptides adjuvanted with montanide ISA 720 or montanide ISA 51. *Am J Trop Med Hyg*, 84(2 Suppl), 12-20 (2011).

124. Jahrsdorfer B, Weiner GJ. CpG oligodeoxynucleotides as immunotherapy in cancer. *Update Cancer Ther*, 3(1), 27-32 (2008).

125. Singh M, Carlson JR, Briones M *et al.* A comparison of biodegradable microparticles and MF59 as systemic adjuvants for recombinant gD from HSV-2. *Vaccine*, 16(19), 1822-1827 (1998).

126. Valensi JP, Carlson JR, Van Nest GA. Systemic cytokine profiles in BALB/c mice immunized with trivalent influenza vaccine containing MF59 oil emulsion and other advanced adjuvants. *J Immunol*, 153(9), 4029-4039 (1994).

127. Xu W, Liu J, Gong W, Chen J, Zhu S, Zhang L. Protective immunity against *Chlamydia trachomatis* genital infection induced by a vaccine based on the major outer membrane multi-epitope human papillomavirus major capsid protein L1. *Vaccine*, 29, 2672-2678 (2011).

128. Diniz MO, Lasaro MO, Ertl HC, Ferreira LC. Immune responses and therapeutic antitumor effects of an experimental DNA vaccine encoding human papillomavirus type 16 oncoproteins genetically fused to herpesvirus glycoprotein D. *Clin Vaccine Immunol*, 17, 1576-1583 (2010).

129. Ekong EE, Okenu DN, Mania-Pramanik J *et al.* A *Vibrio cholerae* ghost-based subunit vaccine induces cross-protective chlamydial immunity that is enhanced by CTA2B, the nontoxic derivative of cholera toxin. *FEMS Immunol Med Microbiol*, 55, 280-291 (2009).

130. Eko FO, Ekong E, He Q, Black CM, Igietseme JU. Induction of immune memory by a multisubunit chlamydial vaccine. *Vaccine*, 29, 1472-1480 (2011).

131. Fisseha M, Chen P, Brandt C, Kijek T, Moran E, Zollinger W. Characterisation of native outer membrane vesicles from lpxL mutant strains of *Neisseria meningitidis* for use in parenteral vaccination. *Infect. Immun.*, 73, 4070-4080 (2005).

132. Barker CJ, Beagley KW, Hafner LM, Timms P. I*n silico* identification and *in vivo* analysis of a novel T-cell antigen from *Chlamydia*, NrdB. *Vaccine*, 26(10), 1285-1296 (2008).