PROJECT FINAL REPORT

Publishable summary

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1 - Executive summary

The overall objective of EuroNeut-41 was to design a vaccine that raises antibodies able to prevent HIV cell fusion by blocking the virus fusion protein gp41 in its pre-fusogenic conformation.

The project has been structured in 3 main pathways or "tracks": a Discovery track, aiming at designing gp41-based antigens (Ag) using an iterative improvement process, and two development tracks (Parenteral and Mucosal) to determine the safety and immunogenicity of the Ags and the optimal administration conditions before selecting the most promising candidate for clinical development.

DISCOVERY

Fifty-one vaccine candidates were designed mainly to mimic fusion intermediate conformations of gp41 and in some instance to increase exposure of the 2F5/4E10 epitopes. Among them, 42 could be produced and characterized for solubility, oligomerization state, thermal stability, epitope exposure and structural properties. The most promising Ags were submitted to a more in-depth biophysical investigation of their structure and conformation stability, and for their capacity to interact with neutralising antibodies (Abs) and peptides.

Another design strategy was developed using gammaretroviruses backbone where 2 domains were substituted with HIV epitopes in the N-terminal part of the ectodomain and the membrane proximal external region (MPER) respectively. Thirteen constructs were produced based on this approach.

In addition, 38 constructs were also created through a collaboration with Harvard university, aiming at mimicking the structure of the MPER on the surface of the viral particles.

LAB-GRADE DEVELOPMENT & EXPLORATORY ACTIVITIES

The sequences of the constructs were first gene-optimized for vector expression and synthesized, representing in total 186 expression clones. From these, 42 immunogens could be successfully produced in E. coli at more than 100 mg each and in different buffers. Alternative expression systems including L. lactis and an acellular production system were evaluated. E.coli was clearly more appropriate for these kinds of proteins.

Fourteen Ags with the appropriate design were formulated in liposomes. Semi-solid (gels) or solid formulations (freeze-dried tablets with controlled release) were also developed.

More than 25 protocols were performed in rabbits to: i) screen all the Ags in Parenteral studies, ii) evaluate optimal route of administration and formulations in Mucosal studies. Induction of binding Abs was measured by ELISA and neutralizing activity by PBMC and TZM-bl assays in all serum and vaginal samples.

Other specific initiatives were conducted such as evaluation of the sublingual route for immunization, investigation of the Principal Immunodominant Domain (PID) of gp41, and direct HIV inhibition with an Ag family which demonstrated in vitro antiviral activities comparable to the antiretroviral drug T20 (Fuzeon).

CLINICAL DEVELOPMENT OF THE PARENTERAL VACCINE CANDIDATE

Vaccine candidate EN41-UGR7C (protein UGR7-C + Alum), offered the best compromise in terms of design strategy, immunogenicity in rabbits and manufacturing feasibility and was selected for clinical trial; the GMP manufacturing process was set-up.

Following a toxicology study, MHRA and Ethical approvals, a Phase 1 clinical trial (EN41CT1.1.2.) was conducted in UK to evaluate the safety and immunogenicity of EN41-UGR7C in Alum (3 IM injections, 3 months of F-U) in 24 healthy female volunteers. The vaccine was safe and well tolerated, induced potent serum IgG & IgA responses waning post last vaccination, and low and intermittent mucosal IgG, but did not induce neutralizing activity neither in serum nor vaginal samples.

In parallel, a questionnaire was submitted to a population of women in Haiti to assess their willingness to participate in a future HIV vaccine trial. A large majority was willing to participate; most of them were from a low socio economic population, hence the importance of the ethical committees in this country;

A challenge study was performed in macaques to test the efficacy of EN41-UGR7C using the same protocol as in Human. After immunization, the animals received 10 weekly repeated challenges with SHIV162P3 by vaginal route. Vaccine was well tolerated however all animals became infected in both vaccine and control groups. Only few animals had developed neutralising Abs.

CLINICAL DEVELOPMENT OF THE MUCOSAL VACCINE CANDIDATE

Vaccine candidate EN41-FPA2 (protein FP-UGR7-MPR-A2, formulated in liposomes with the adjuvant MPLA - a TLR4 agonist) was selected as the "Mucosal Ag" offering the best neutralising response in rabbits. The nasal route and a gel formulation were selected based on a “fine-tuning” study conducted in rabbit and macaque. The GMP Manufacturing process was developed and after a toxicology study, MHRA and Ethical Committee approvals, a Phase 1 clinical trial (EN41CT1.1.1.) was conducted in UK to evaluate the safety and immunogenicity of EN41-FPA2 (3 nasal primes, 2 IM boosts, 6 months of F-U) in 48 healthy female volunteers. Three nasal priming followed by 2 IM boosts with EN41-FPA2: i) were safe and well tolerated, ii) could induce systemic specific IgG and IgA as well as mucosal specific IgG and, at lower level, IgA responses, iii) could induce some HIV inhibitory responses in mucosa detected by TZM-bL assay, iv) nasal priming allowed to accelerate the response after the IM boost and activated specific genes.

A challenge study in NHP was also performed with EN41-FPA2, which was well tolerated. In terms of efficacy, except one animal in the vaccine group who remained uninfected after 10 challenges, all animals became infected in all vaccine or control groups.

Potential next steps were identified for this vaccine candidate such as alternative combinations (with other Ags or other formulations, routes, …). Although discrete, the HIV inhibitory response observed in the vaginal secretions of the human volunteers is encouraging and deserves further studies.

2 - Project context and objectives

2.1 - Epidemiology

At the start of EuroNeut-41 in 2008, the annual AIDS epidemic update published by UNAIDS reported that 33.4 million people were living with the Human Immunodeficiency Virus (HIV) in the world, with 2.7 million newly infected during the year. Despite the implementation of global prevention programmes, the epidemic was growing, heavily concentrated among young people and in Sub-Saharan Africa where 63% of all HIV-infected people were living.

In 2012, an estimated 35.3 (32.2–38.8) million people were living with HIV, an increase from previous years as more people are receiving the life-saving antiretroviral therapy. There were 2.3 (1.9–2.7) million new HIV infections globally, showing a 33% decline in the number of new infections from 3.4 (3.1–3.7) million in 2001. At the same time the number of AIDS deaths is also declining with 1.6 (1.4–1.9) million AIDS deaths in 2012, down from 2.3 (2.1–2.6) million in 2005 (UNAIDS report on the global AIDS epidemic 2013) (1).

In 2011, eight million people received ARTs, an increase of 20% from 2010, but representing only the half of the population that needed treatment. At the end of 2012, 9.7 million people had access to antiretroviral therapy, representing 61% of all who were eligible under WHO guidelines. However, in 2013, this coverage represented only 34% of the 28.6 million people eligible in 2013 (HIV/AIDS Fact sheet N°360, WHO) (2).

Despite historic gains in expanding treatment services, efforts to reach universal treatment access face considerable challenges. In 2012, international investment in HIV programmes reached an estimated US$ 8.9 billion an 8% increase / amounts available 2011. To meet the 2015 resource target, at least US$ 2.9–5.4 billion in additional annual funding needs to be generated (UNAIDS report on the global AIDS epidemic 2013) (1).

2.2 - New prevention technologies

During EuroNeut-41 lifespan, new prevention technologies (NPTs) such as microbicides, pre-exposure prophylaxis (PrEP) and antiretroviral treatment as prevention (TASP) demonstrated efficacy. NPTs together with circumcision, that earlier proved to prevent female to male transmission, could open the possibility of bringing the HIV and AIDS pandemic to an end. Scientists predicted that a combination of NPTs and ART would decrease infections by 90%.

- Microbicides are of particular interest to prevent HIV in women of developing countries who are disproportionately affected by HIV and may be unable to negotiate condom use. CAPRISA 004 phase II/b trial of tenofovir 1% gel used intravaginally provided promising results. The gel, when used once up to 12 hours before and once up to 12 hours after coitus, was 50% effective at preventing HIV infection (Abdool 2010) (3) . Two phase III trials are ongoing (FACTS001, MTN020) (Microbicid Trials Network) (4).
- Pre-Exposure Prophylaxis (PrEP) is a prevention method in which healthy people take a daily pill to reduce their risk of becoming infected. When used consistently, PrEP (iPrEx study in 2010) was shown to be effective in men who have sex with men (Grant 2010) (5) and heterosexually-active men and women (Partners PrEP and TDF2 studies in 2011) (Baeten 2011, Thigpen 2011) (6;7) cutting a person’s risk of acquiring HIV by 44 to

73%, depending on adherence to the daily regimen. In July 2012 the FDA approved the use of Truvada® as PrEP among sexually active adults at risk for HIV infection and in June 2013, CDC published a guidance recommending that PrEP be considered as one of several prevention options for people at very high risk of getting HIV.

- Antiretroviral treatment as prevention (TASP) tested in study HPN052 showed that early ART for HIV-positive partner reduces the risk of transmission by 96% in serodiscordant partnerships. This led to 2012 treatment guidelines changes recommending ART for patients at risk of HIV transmission. This was supported by most clinicians who believe that early initiation of ART can slow the spread of HIV in a community by making patients less infectious to others (Kurth 2012) (8). TASP is associated with early testing that has become steadily simpler to use (blood through a finger-prick or a swab of saliva) and less expensive to deliver thanks to technological advances over the past decade.
- Medical male circumcision reduces the risk of female-to-male sexual transmission of HIV by approximately 60% as shown in 2006 by three randomized controlled trials in Kisumu, Kenya, Rakai District, Uganda, and Orange Farm, South Africa (Auvert 2005, Bailey 2007, Gray 2007) (9-11). In the five years since the Uganda trial was completed, high effectiveness was maintained among the men who were circumcised, with a 73% protective effect against HIV infection (WHO Factsheet July 2012).
  WHO and UNAIDS guidance for voluntary medical male circumcision recommends a surgical procedure that can be performed in 20 to 30 minutes. By the end of 2011, more than 1.3 million voluntary medical male circumcisions had been performed for HIV prevention, with nearly a doubling of the number from 2010 to 2011. A five-year Joint strategic action framework was developed by WHO and UNAIDS to accelerate the scale-up of voluntary medical male circumcision in Eastern and Southern Africa 2012-2016.

2.3 - Risks related to the New Prevention Technologies

Recent studies have highlighted setbacks of NPTs.

- Microbicides were shown efficacious when used consistently. However, in CAPRISA 004 study, the protective effectiveness dropped from 50 to 39% after 2.5 years, likely due to decreasing adherence. In phase III trial VOICE the topical microbicide arm, where women were instructed to use the gel every day, was stopped due to failure in preventing infection. This laborious procedure might have compromised the consistent use of medication.

  Intravaginal rings (IVR) are well tolerated and could help overcome the adherence issue. In 2012, a macaque study demonstrated that microbicides could successfully be delivered by IVR to vaginal fluids and tissues and protect macaques from SHIV infection (12). Tackling the adherence issue was also the aim of two phase III trials ASPIRE and RING launched in 2012. The gel was replaced by a monthly changed silicone vaginal ring, slowly releasing dapivirine ARV over time. Results are expeted in 2016.
- PrEP was successful in various studies. However, the FEM-PrEP trial in a high-risk population of heterosexual young women with no stable relationships, showed no benefit of Truvada® in preventing HIV infection. This failure was again related to poor adherence to treatment. Furthermore, safety concerns are associated with PrEP including drug resistance, mild to severe side effects (nausea, vomiting, diarrhea, headache and dizziness) and a false sense of security that may lead to engage in more risky activities.
- Medical circumcision reduces the risk of female-to-male sexual transmission of HIV but not the other way round. In the Ugandan trial, there appears to have been a 61% relative increase in HIV infection among female partners of HIV-positive circumcised men. This could have been related to higher risk of transmission in couples who resumed intercourse before completed wound healing (Wawer 2009) (13). Another study in Kenya showed that women, informed that circumcision reduces a man's risk of HIV infection, perceived lower HIV risk and were more likely to forego condoms with partners of positive or unknown serostatus (Maughan-Brown 2012) (14).

Each NPT presents the possibility for individuals to help reduce their risk of HIV but none on their own can end the epidemic. A comprehensive HIV prevention package should include the provision of HIV testing, TASP, PrEP, microbicides and counseling services, treatment for sexually transmitted infections, the promotion of safer sex practices, the provision of male and female condoms and promotion of their correct and consistent use. However, NPTs raise concerns related to poor adherence, resistance, side effects and increased risky behaviour, not mentioning the high cost of early and daily treatment. In this context, the development of a vaccine should remain a priority.

2.4 - Vaccines

Neutralising and inhibitory antibodies

EuroNeut-41 initially focused on the development of a HIV vaccine able to induce neutralizing antibodies (NAbs). This was based on a large body of data that showed that monoclonal NAbs could protect against the virus, in monkey studies (Burton 2011, Hessell 2009, Ruprecht 2001) (15-17). Since the beginning of the project, the concept of neutralization has evolved beyond the strict definition of "antibodies that neutralize a virus in a PBMC or TZM-bl assay".

In 2009, the results of RV144 large study using a prime-boost strategy with a recombinant vector and an envelope subunit provided new hope that a clinically useful preventive HIV-1 vaccine can potentially be made (Rerks-Ngarm 2009) (18). An immune-correlates study generated the hypotheses that serum V1/V2 binding Abs may have contributed to protection against HIV-1 infection, whereas high levels of serum Env-specific IgA may have mitigated the effects of the protective Abs (Haynes 2012) (19). This suggested that binding Abs and not only traditional NAbs may also participate to protection. For example, HIV-1 replication was shown to be inhibited in Langerhans and interstitial dendritic cells by neutralizing but also Fc-mediated inhibitory Abs (Peressin 2011) (20). Extra-neutralizing mechanisms, including those dependent on interaction of Abs with Fc receptors such as antibody-dependent cellular cytotoxicity (ADCC), or on interaction with complement seem to be involved (Hope 2011, Hessell 2007) (21;22).

Besides using traditional assays to detect neutralizing Abs, EuroNeut-41 dedicated efforts to develop new techniques for the detection of non-neutralising inhibitory Abs.

Mucosal antibodies

Another aspect was taken into consideration when developing vaccine candidates in EuroNeut-41. Most of HIV-1-infected individuals worldwide are women who generally acquire HIV infection after sexual contact. Blocking HIV mucosal transmission and local spread in the female lower genital tract is therefore critical to prevent infection. EuroNeut-41 aimed at designing a vaccine eliciting Abs able to interfere with viral transmission at the mucosal HIV portal of entry. Strong HIV-blocking IgA in genital secretions are thought to confer natural resistance to HIV in exposed, healthy sexual partners of infected subjects (Rerks-Ngarm 2009, Barassi 2004, Devito 2000) (18;23;24). Therefore, efforts were directed to induce such IgA Abs in the genital tract, assuming that they would block the virus by aggregation, inhibition of adherence to the mucus through steric hindrance or inhibition of transcytosis (Mantis 2007, Bomsel 2011) (25;26). These Abs may exert a number of other activities in addition to neutralization of $CD4^+$ T-cell infection, even in the absence of detectable systemic neutralizing responses.

2.5 - gp41

The HIV-1 envelope glycoproteins gp120 and gp41 are generated from the heavily glycosylated precursor protein gp160. Subsequently, trimers of gp120-41 heterodimers assemble to form the viral spikes (Env). A first vaccine approach based on monomeric gp120 from VaxGen failed to elicit broadly neutralising antibodies and to protect from infection or even disease. EuroNeut-41 candidates are based on gp41.

The current model of HIV infection suggests that binding of gp120 to CD4 and co-receptor triggers a conformational change that releases the grip of gp120 on gp41 . As a result, the fusion peptide at the extremity of gp41 is exposed and penetrates into the host membrane. This is followed by large conformational rearrangements within gp41 during which this protein adopts an energetically more favourable conformation, also known as a 6-helix bundle, consisting in an anti-parallel arrangement of three C-terminal helices and a central trimer of three N-terminal helices. This arrangement makes it possible for the viral envelope to fuse with the plasma membrane. In this model, the pre-fusogenic conformations of gp41 are characterised by the fact that the trimer of N-terminal helices (referred to as heptad repeat 1, N-HR) and the three C-terminal helices (referred to as C-HR) are exposed to solvents. In contrast, N-HR is buried by C-HR into the 6-helix bundle conformation.

The molecular mechanism leading to the 6-helix bundle conformation is not fully understood. Particularly, it is not known whether this 6-helix bundle is formed before or after the fusion. It has been suggested that the 6-helix bundles could help bring the cellular and viral membranes in close proximity and that the subsequent higher order clustering of the 6-helix bundles would facilitate membrane fusion (Golding 2002) (27). Alternatively, it has been suggested that, while the formation of the 6-helix bundle is essential for fusion, it occurs only after the formation of a transmembrane pore (Markosyan 2003) (28).

The objective of EuroNeut-41 was to design a vaccine that raises Abs able to prevent cell-virus fusion by blocking gp41 in its pre-fusogenic conformation by targeting: i) either the N-HR or the C-HR, which are thought to be fully exposed to solvents in the prefusion conformation of gp41; or ii) a region of the C-terminal part of

gp41 that is believed to be exposed on the surface during the fusion process; or iii) a particular epitope recognised by the known 2F5 and 4E10 NAbs.

Some of the regions of gp41 are highly conserved among all HIV subtypes, probably because they play a critical role in virus infection and therefore are buried to deny access to B-lymphocyte receptors, avoiding immune selection pressure. These highly conserved regions were the targets of some of the selected vaccine candidates expected to neutralise not only HIV subtype B strains, but also all other HIV subtypes.

As these Abs target regions exposed at the prefusion stage of gp41, they were expected to bind to the virus only after receptor and co-receptor attachment. However, evidence that Abs might act on virus prior its attachment came from the ability of 5-helix protein, an entry inhibitor that disrupts conformational changes in gp41, to interact with HIV-1 Env in a receptor-independent fashion. These results demonstrated that the C-terminal region of the gp41 ectodomain is an accessible target on HIV-1-infected cells for the development of antiviral therapeutics and neutralizing antibodies (Root 2003) (29). It was assumed that access to the target would be easier for vaccine-induced Abs than for receptors on naïve B-lymphocytes.

EuroNeut-41 is a Research and Development project starting with the design of immunogens up to the evaluation of safety and immunogenicity of two selected vaccine candidates in two phase I trials in UK.

A Phase 1 clinical trial using the mucosal route was performed with EN41-FPA2. This study enrolled 48 subjects. Nasal priming followed by parenteral boosting with an adjuvanted vaccine appeared a promising schedule to achieve both long-lasting systemic responses together with mucosal immunity.

The second Phase 1 trial by the parenteral route was conducted with EN41-UGR7C in 24 participants.

In parallel to these trials in human, two studies in macaque used the same vaccine, dose and regimen. The animals were then challenged vaginally with repeated low-dose of SHIV162P3. These experiments aimed at providing efficacy data, should the monkey model be predictive of human.

A preparatory study for a vaccine trial was carried out in Haiti. If the local population demonstrated interest and if satisfactory safety and immunogenicity data were observed in UK, further clinical development could also be considered in this developing country where a HIV vaccine is most needed.

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3 - Main Scientific & Technical Results/foregrounds

The principal aim of the EUROpean consortium on NEUTralising antibodies using gp41 (EuroNeut-41) was to inhibit HIV entry by eliciting neutralising antibodies.

The glycoprotein gp41 is composed of 345 amino acids (aa) with a molecular mass of 41KDa. It does not contain clearly defined variable regions, and is more conserved than gp120.

gp41 is divided into three major domains: the extracellular region or ectodomain (aa 1 to 171), the transmembrane (TM) domain (aa 172 to 193) and the cytoplasmic tail (aa 194 to 345). The ectodomain contains several distinct functional determinants involved in the fusion of viral and host cell membranes: (i) an N-terminal hydrophobic region that functions as a fusion peptide (FP); (ii) a polar region (PR); (iii) two α-helix repeat regions referred as the N-terminal heptad repeat (NHR) and the C-terminal heptad repeat (CHR); (iv)a disulfide-bridged hydrophilic loop that connects the two heptad repeats, also known as the immunodominant (ID) loop; (v) a tryptophane (Trp)-rich region known as the membrane proximal external region (MPER).

3.1 - DISCOVERY

Two partners involved in Discovery fueled the vaccine candidates pipeline for the first 4 years of the Project.

3.1.1- RKI

RKI approach was based on the fact that it is possible to induce neutralising antibodies against retroviruses including porcine endogenous retroviruses (PERVs), feline leukaemia viruses (FeLV) and the Koala retrovirus (KoRV) by immunisation with their respective transmembrane envelope proteins. By epitope mapping, it was shown that the antisera recognized two epitopes, one located in the N-terminal part of the ectodomain (E1), the other in the MPER (E2).

Importantly, despite the large evolutionary distance between HIV-1 and gammaretroviruses, sequence analyses showed that the E2 epitopes identified in the envelope of PERV, FeLV and KoRV shared up to 3 amino acids with an epitope in gp41 of HIV-1, which is recognised by 4E10 MAb.

Screening experiments were performed in order to detect an E1-like domain in HIV gp41. One peptide was identified which was able to increase the binding of both 2F5 and 4E10 MAbs to a peptide containing the epitopes of 2F5 and 4E10. This peptide corresponded to a domain in gp41 similarly localised as the E1 domain in the envelope of the gammaretroviruses.

Therefore, RKI used hybrid proteins containing a backbone of gammaretroviruses from which the E1 and E2 domains were substituted by the E1 and E2 domains derived from gp41 in order to force the induction of neutralizing antibodies against HIV.

These hybrid proteins, but also the corresponding sets of E1/E2 synthetic peptides were used to immunize guinea pigs, goats, rats and rabbits. A total of 8 combinations of E1/E2 peptides and 3 fusion proteins were tested. The

interaction of 2F5 and 4E10 MAbs with these antigens was checked by Western Blot, ELISA and Biacore, demonstrating that these domains were accessible. Nevertheless, none of these immunogens could elicit HIV neutralising activity.

RKI also worked on the production of a gp41 molecule deleted of the cytoplasmic domain that they successfully expressed in 293 T cell line. Protein dimer could be purified from the supernatant at 800 to 900 ng/mL and was recognized by sera from HIV positive individuals. A few rgp41 antisera generated in rat and goat showed neutralizing activity measured by TZM-bl assay. Mutation in the immunosuppressive domain of the gp41 increased the immune response.

3.1.2 - UGR

UGR was the supplier of new immunogens to the whole EuroNeut-41 Consortium. They designed a series of new HIV-1 vaccine candidates classified in 5 families divided into 6 generations, depending on the targeted epitopes and the design strategies. The main driver for the design was to mimic fusion intermediate conformations of gp41 or to increase exposure of the 2F5/4E10 epitopes. The immunogens were optimized using an iterative process based on biophysical characterization and immunogenicity of each antigen generation. However, two major obstacles delayed this iterative process, one related to purification and refolding of the proteins and the other to the measurement of neutralising activity which required several repeats for confirmation. Although biophysical characterisation results of the generation “n” could be provided on time to design the generation n+1, immunological results were only available for improvement of the generation “n+3”. Design of n+1 and n+2 generations was therefore based on modelling, biophysical characterisation and published information rather than on immunogenicity results.

All candidates were biophysically characterized in terms of solubility, oligomerization state, secondary and tertiary structure, thermal stability and epitope exposure.

3.1.2.1 - H family

The H family consisted in six antigens from two different generations:

1) H1, H3, H4, H5 and H6 (1st EURONEUT-41 generation), and

2) H4A (4th EURONEUT-41 generation).

It was generally accepted that the prefusogenic state of gp41 is trimeric and that the NHR and CHR helices do not interact in its conformation. In addition, the loop region between NHR and CHR of gp41 facilitates the protein to adopt the 6-helix bundle (6HB) conformation.

It was hypothesized that deletion of the loop may create a thermodynamic barrier, which was thought to either diminish or abolish the propensity of these molecules to adopt a 6HB conformation. With this hypothesis, five gp41 mutants were designed in which the helices NHR and CHR, or vice versa, were fused together directly to generate constraints that might prevent the adoption of the fusogenic state and therefore favor an alternative arrangement. This alternative arrangement might be characterized by the exposure of epitopes that are hidden in the helix bundle arrangement.

From the biophysical characterization of these antigens, it was determined that all five mutants were highly alpha-helical proteins and very thermostable.

Two classes of antigens were proposed: 1) N-C constructs; antigens in which the NHR motif is N-terminal (named H1 and H3) to the CHR one and 2) C-N constructs; those where the NHR motif is C-terminal (H4, H5 and H6) with respect to CHR.

Within each class, the constructs differ in the length of the NHR and CHR helical segments. All antigens also contain a His tag at their C-terminus (GGGGSHHHHHH).

Unfortunately, at physiological pH, only H1 was soluble and H4 and H6 were partially soluble in freshly prepared solutions but they underwent slow precipitation during storage.

The N-C constructs were mainly oligomeric when refolded from inclusion bodies, whereas the C-N constructs were mainly trimeric. Only the N-C construct H1 was fully trimeric and soluble at both acid and physiological pH. More concerning, these H constructs did not expose the NHR epitope as suggested by the presence of the characteristic near-UV CD band of the NHR-CHR interaction at 293 nm for all these antigens.

In conclusion, these H constructs probably folded or oligomerized to finally adopt the 6 helix-bundle conformation as the most energetically favorable conformation.

The H4 antigen, in which N-terminal C helices are directly connected to the N-helices, was a promising candidate in terms of structural stability, cooperativity and solubility. Nonetheless, in spite of these good properties, no significant exposure of the target NHR epitopes was detected for this antigen indicating that, even though it exhibited a high helicity, the C helices seemed to fold back onto the NHR trimer, therefore occluding the NHR epitopes.

Inspection of H4 sequence revealed that the connection between the C- and N- helices was not performed at the right register to keep the heptad repeats in the correct orientations for optimal packing in an extended trimeric conformation, i.e., a and d positions in the heptad repeat pointing towards the internal interface. It was possible that, because of this inconsistency between the orientation of the C- and N- helices, the connection region might be unstructured in the H4 construct allowing sufficient flexibility to the C-helices to pack against the NHR trimer. In order to avoid this and favour the propagation of the coiled-coil conformation throughout the entire structure, three residues (DKA) were removed at the connection region, finally generating the H4A candidate.

It was also designed an H4A variant (H4D) including several mutations to stabilize an open trimeric conformation of the CHR region and decrease the propensity of the CHR to pack against the NHR trimer into the 6HB conformation. Unfortunately, H4D could not be produced due to problems of expression yield in E.coli and low solubility.

One of the most striking results of the biophysical characterization was that H4A was less soluble than the parental H4 at physiological pH. Arginine was added to increase solubility and pursue its characterization. It was found that both H4 and H4A were trimeric; the size was slightly more expanded for H4A. Moreover, both antigens were highly alpha-helical; H4A lost some ellipticity at pH 7.4, although the experiment could not be performed in presence of arginine due to incompatibility with this technique. As all the H constructs, H4A was also observed to be highly thermostable at both pH values. Finally, the near-UV CD analysis and the analysis of mixtures with CHR peptides showed that H4A did not expose the NHR hydrophobic pocket.

In conclusion, these results indicated that the deletion of 3 amino acids in the NHR-CHR connection region did not improve the properties of this antigen with respect to H4 or the other family members.

3.1.2.2 -The UGR7 family: remodeling the loop region of gp41 for improved solubility

The UGR7 family consisted of 10 antigens organized into three different generations:

1) S30, Q3 and UGR7 (1st EURONEUT41 generation),

2) UGR7A, UGR7B, UGR7C and UGR7D (2nd EURONEUT-41 generation), and

3) UGR7+, UGR7D+ and UGR7E (4th EURONEUT-41 generation).

a) UGR7

One of the challenges for the rational design of HIV vaccine candidates has been the insolubility of the antigens derived from the full-length ectodomain of gp41 at physiological pH. The importance of designing a soluble antigen is to solve the associated problems such as epitope occlusion, difficulties in production and formulation and samples irreproducibility.

It is well known that recombinant forms of the gp41 ectodomain produced in E. coli form highly insoluble aggregates at neutral pH and that solubilization can be accomplished by either reducing the pH below 4 or by deleting the long loop that connects the NHR and CHR regions. It was further demonstrated that the extracellular ectodomain of purified HIV and simian immunodeficiency virus gp41 forms a mixture of high molecular weight aggregates composed of trimers associated by interactions of the loop connecting the NHR and CHR helices.

On the basis of this background, the first UGR candidate, UGR7, was rationally designed to solubilize the gp41 ectodomain at physiological pH by including seven mutations in the immunodominant (ID) loop region ((L82D, W85E, L91G, I92D, T95P, A96E and W103D).

The mutations were selected to a) increase the net charge in the region, b) reduce the hydrophobicity of surface-exposed residues and c) introduce residues such as Pro and Gly that disrupt β-sheet conformations, very common in aggregates.

The electrostatic potential in the ID loop of the mutant variant UGR7 was found to be significantly modified with respect to that for the parent molecule as determined by the computational analysis carried out with the Delphi algorithm implemented in Discovery Studio 2.0 (Accelrys).

The biophysical analysis of the structure and oligomerization state of the mutants showed that mutations in the loop did not alter the oligomerization state, overall structure and stability of the post-fusion conformation of the gp41 ectodomain.

In conclusion, UGR7 showed an impressive high solubility at pH 7.4. The highest concentration value achieved was above 10 mg/mL. This result confirmed the hypothesis initially proposed by Caffrey and co-workers suggesting that gp41 aggregation at physiological pH occurs at the hydrophobic loop.

To investigate the biophysical basis of the increase in solubility of UGR7 at physiological pH relative to the wild type (WT) gp41 ectodomain, eight UGR7 reversed mutants were designed and produced and the contribution of each mutation to solubility was analysed.

The results suggested that the E85W mutation contributed specially to reduce solubility of the ectodomain. W85 was solvent exposed in the model post-fusion structure of the gp41 ectodomain and could mediate intermolecular interactions. However, removing W85 alone was not enough to make a highly soluble gp41 ectodomain because one mutant that contained all the other 6 mutations except this particular one was soluble. P95T and D103W mutations in isolation were unimportant for solubility. D81L and G91L mutations appeared to reduce only very slightly the solubility. This small effect could be ascribed either to a small change in the isoelectric point due to the D81L mutation and/or to the change in hydrophobicity produced by the substitution of the two Leucine residues.

The following generations of UGR7-based antigens were designed to promote neutralizing epitope exposure.

b) Second generation of the UGR7 family: mutations destabilizing the NHR-CHR interactions

The design strategies for this second generation of antigens tried to disrupt the interactions between the NHR and CHR motifs and increase the structural stability of independent NHR and CHR trimers in order to maximize the exposure of the conserved NHR epitopes. Maintaining the solubility at physiological pH and a conformational uniqueness to avoid unspecific aggregation at the same time were also goals to achieve. In general, standard molecular modeling techniques (homology modeling, energy minimization, molecular dynamics simulations, etc) were used for these purposes, as well as specific algorithms for the evaluation of other protein features such as tendency to aggregate, coiled-coil propensities and optimal packing, electrostatics, etc.

Based on this approach, the second generation of antigens of the UGR7 family included four variants where different sets of mutations were introduced sequentially to:

i) Destabilize the 6HB conformation by

- disruption of specific interactions between NHR and CHR
- disruption of electrostatic complementarity (electrostatic steering).

ii) Stabilize isolated trimeric coiled-coils of CHR and NHR helices.

Biophysical analyses showed that although the set A of mutations maintained the solubility at physiological pH, it was not destabilizing enough to expose the epitopes. In contrast the set B of mutations seemed to favor the epitope exposure but decreased the solubility at pH 7.4. The combination of both set A and C of mutations did not affect the solubility at physiological pH and increased the degree of NHR epitope exposure but only at acidic pH.

Based on these conclusions, the next step was to design mutations in UGR7D to improve its solubility at physiological pH while maintaining the degree of epitope exposure.

c) Third generation of the UGR7 family: optimization of solubility and epitope exposure

Three strategies were used in the design of these new versions of the UGR7D antigen:

i) Further destabilization of the 6HB conformation by disruption of additional interactions involving Q655 at the CHR region that establishes a “ring” of hydrogen bonds with certain helices from a neighboring NHR motif. Introducing positively charged residues at this position was reported to significantly increase sensitivity to neutralizing antibodies by destabilizing the 6HB conformation.

ii) Destabilization of 6HB conformation by elimination of disulfide bonds in the loops. According to Molecular Dynamics (MD) simulations using a UGR7D model structure and its (Q142R) mutant, the disulphide bonds at the immunodominant loops seemed to be restricting somehow the conformational

space available for the C-helices. Elimination of the disulfide bonds (C87S, C93S) led to a better exposure of the NHR region during the MD simulations.

iii) The introduction of the UGR7 mutations at the ID loop resulted in a decrease of the isoelectric point and a lower propensity of these loops to aggregate, resulting in a highly soluble gp41 ectodomain at physiological pH. Nonetheless, the introduction of the mutations to disrupt of the electrostatic steering (replacement of negatively charged residues by positive side chains) brought the isoelectric point of UGR7D very close to physiological pH. As a consequence, this antigen showed very poor solubility at neutral pH.

In conclusion, three new antigens were proposed based on the UGR7 scaffold aimed at improving the conformational behaviour and the solubility of UGR7D.

(1) UGR7E (UGR7D + Q142R, C87S, C93S), where the mutations were designed to drive the equilibrium towards more open conformations and facilitate the CHR packing.

(2) UGR7D+ (UGR7D + D82K, D92R, E96R, D103R), to improve the solubility of UGR7D.

(3) UGR7+ (UGR7 + D82K, D92R, E96R, D103R). This candidate was the reference to control epitope exposure and correct folding.

Biophysical characterization of this third generation of UGR7-based antigens showed that the mutations introduced in UGR7+ did not change its apparent conformation or stability relative to the parent molecule, UGR7, except for a significant decrease in solubility at pH 7.4.

This antigen could therefore be considered a good reference antigen representing a 6HB postfusion conformation of the gp41 ectodomain.

The most outstanding result was the significant increase in solubility of UGR7E and UGR7D+. The mutations included in these antigens increased their isoelectric points above 8, therefore increasing the solubility at pH 7.4. Unfortunately, although the mutations included in UGR7D+ increased its solubility relatively to UGR7D, it underwent precipitation over time and during storage. On the other hand, both UGR7E and UGR7D+ showed a similar NHR epitope exposure at acidic pH, as observed in the studies of binding by near-UV CD. These candidates could be considered as slightly improved candidates compared to UGR7D.

3.1.2.3 - Antigens based on a single-chain trimer of gp41 NHR helices: the covNHR family

The covNHR family consisted in 9 antigens organized in three different generations of HIV-1 vaccine candidates: covNHR2 (2nd EURONEUT-41 generation), covNHR3, covNHR4, covNHR5 (4th EURONEUT-41 generation), covNHR3A, covNHR3B, covNHR3AB, covNHR3AC and covNHR3ABC (6th EURONEUT-41 generation).

The sequences of the CHR and NHR gp41 regions are conserved sites on the envelope, most likely because of their critical role on the viral membrane fusion and, therefore, they are just transiently exposed during this fusion process. Despite this short-lived exposure, the CHR and NHR regions can be accessed by small proteins, peptides and antibodies that bind the prehairpin intermediate and further disrupt fusion by preventing subsequent folding into a six-helical bundle.

The NHR region and more specifically its hydrophobic pocket is a “hot spot” for fusion inhibition and therefore an ideal target to focus on to generate a vaccine-elicited antibody response. Thus, the rational design strategy of the covNHR family consisted of the generation of a soluble single-chain trimer of gp41 NHR helices exposing these NHR epitopes.

a) First generation of covNHR-based antigens

The design of a single-chain trimer of gp41 NHR helices was a challenging project since it implied the reversal of one of the helices so that, instead of a parallel trimeric coiled-coil conformation, the construct would have an anti-parallel central helix.

The actual design was carried out in several stages:

- Reversal of the central helix sequence by homology modeling,
- Engineering of short loops connecting the helices containing N- and C- helix capping motifs,

- Engineering mutations to improve stability and solubility of the NHR covalent trimer.

Two candidates, covNHR1 and covNHR2, where proposed based on those premises. The difference between both antigens is that covNHR2 includes the engineering mutations to improve stability and solubility of the NHR covalent trimer. Both variants were cloned in E.coli but, unfortunately, covNHR1 could not be expressed, possibly due to a low structural stability or to a strong aggregation propensity.

Results of the biophysical characterization of covNHR2 showed that this antigen is soluble at both acidic and physiological pH, folds into a stable, highly alpha-helical structure at both pH values and presents a conformation that mimics a solvent-exposed NHR trimer and is recognized by CHR peptides with high affinity. Nonetheless, covNHR2 appeared to be more expanded or prone to oligomerize at pH 7.4. Therefore, it was decided to design a further generation of antigens in an attempt to optimize these properties at pH 7.4.

b) Second generation of covNHR-based antigens

In this second round of design of covNHR constructs, the main goals were to increase stability and improve structural cooperativity and homogeneity at physiological pH while keeping protein solubility high. Three independent lines of action were explored:

i) Increasing the loop length to reduce conformational strain by adding one additional residue to the loops and engineering new favorable capping interactions. The antigen including these modifications was named covNHR3.

ii) Improving the helix 2 packing. Several changes in the second helix sequence were performed to optimize packing with helices 1 and 3, which were left unaltered. These changes in sequence were strictly limited to a and d positions. These mutations gave rise to the covNHR4 antigen.

iii) Stabilizing the monomeric conformation by reducing the available conformational space. Finally, disulfide bonds were engineered at the N- and C- termini of helix 2 in order to stabilize the monomeric conformation. Four different positions were found to be the most suitable positions to mutate, according to geometrical considerations: I8C-E100C and Q59CQ160C. The resulting antigen was called covNHR5.

In conclusion, the biophysical characterization showed that although covNHR4 and covNHR5 folded in a highly alpha-helical structure and exposed the NHR epitopes, they presented solubility restrictions that dismissed them as possible vaccine candidates. covNHR3 showed a slightly better behavior than covNHR2 regarding solubility and structural cooperativity. It also could be produced in higher amounts. However, this protein showed certain structural instability and propensity to self-associate at physiological pH, which may be problematic during manufacturing process development. In addition, a three-fold pseudo-symmetry of the molecule may divert their immunogenicity from gp41 native-like epitopes, reducing their potential to induce potent neutralizing antibodies.

For this reason, a third generation of proteins on the basis of covNHR3 trying to improve their conformational properties and focusing their ability to recognize gp41 CHR peptides in a native-like arrangement was designed.

c) Third generation of covNHR-based antigens

Modified versions of covNHR were designed to reduce the potential interaction of the two non-native-like faces. To do this, some exposed residues were mutated to eliminate hydrophobic pockets and to create additional stabilizing interactions.

The proposed mutations were grouped in three sets:

A: Mutations removing pocket 1: G33K, V81E and G79E.

B: Mutations removing pocket 2: A19Q, L95E, A93E, and Q135K

C: Mutations removing two solvent-oriented tryptophan side chains: W32S and W80S

Five covNHR antigens were then proposed combining one or more of these sets of mutations:

CovNHR3A: including mutations A

CovNHR3B: including mutations B

CovNHR3AB: including mutations A+B

CovNHR3AC: including mutations A+C

CovNHR3ABC: including all mutations

In conclusion, biophysical characterization of these antigens showed a considerable improvement relative to the parent molecule, covNHR3. The constructs present optimal biophysical properties; they are soluble and mainly monomeric at both pH values, they fold in a stable highly alpha-helical conformation and expose the NHR epitope. All these antigens have also been produced in high amounts and have decreased their tendency to stick in filter media with respect to covNHR3.

Furthermore, taking all the biophysical results into account, covNHR3ABC showed the best overall biophysical properties.

Despite their increased stability, the new Cov-NHR3 constructs failed to improve the systemic and mucosal neutralising responses induced following parenteral immunization, compared with the original Cov-NHR3 antigen.

Because these antigens could interfere with the folding of gp41 during the fusion process, they were tested for their direct antiviral activity. Interestingly, CovNHR3-ABC was able to inhibit quite efficiently the infection by SF162 and QHO strains that belong to tier 1 and tier 2 viruses, respectively.

Based on these results, a patent application was submitted.

3.1.2.4 - Antigens based on the full-length gp41 ectodomain: The FP/PR family

The FP/PR family consisted of four antigens that constituted the third generation:

FP-UGR7-MPR-A2 and FP-UGR7-MPR-B1 include the FP region directly attached to the N-HR region, PR-UGR7-MPR-A and PR-UGR7-MPR-B have the FP region replaced by the PR region. The design of the FP/PR candidates was aimed at promoting the exposure of epitopes at the membrane proximal external region (MPER) of gp41 because these epitopes are recognized by broadly neutralising antibodies and it was assumed that if they were correctly presented to the immune system, they could induce such antibodies.

Many attempts to induce these Abs by presenting the linear MPER sequence into various scaffolds had failed. Although Abs were generated, they could not prevent HIV infection. A higher order structure was likely necessary to elicit such broadly neutralising antibodies.

The vaccine candidate FP-UGR7-MPR-A-2 (EN41-FPA2) was designed to mimic this structure: it is composed of the MPER region displayed in the context of a trimeric gp41 ectodomain arranged in a 6HB. This latter structure could act as a stable scaffold to display the MPER in a trimeric arrangement. Seven mutations were included at the loop linking the N-HR to the C-HR to reduce hydrophobicity, increase net charge and minimize propensity to mediate intermolecular association. In addition, the Polar Region was deleted as it may interact with and occlude the 2F5 and 4E10 epitopes. Finally, the presence of the FP adjacent in space to the MPER region was expected to stabilize the latter in an immunogenic conformation.

Recent data indicated that the viral lipid membrane itself may play a critical role in the neutralizing activity of 2F5 and 4E10: these antibodies reversibly attach to the viral membrane and it is assumed that this poised them to capture the transient gp41 fusion intermediate, preventing the completion of the fusion process. For this reason, FP-UGR7-MPR-A-2 was modified to allow presentation of the MPER region in the context of a lipid environment by adding a few hydrophobic residues at its C-terminus to facilitate incorporation of the protein into liposomes.

The adjuvant MPLA was included in the formulation to increase the immunogenicity of the resulting EN41-FPA2 vaccine candidate. MPLA used in EN41-FPA2 is a synthetic derivative of bacterial lipopolysaccharide (LPS). Both LPS and MPLA are ligands of toll-like receptor 4 (TLR4) on cells of the innate immunity. However, whereas LPS is toxic in human, MPLA displays a low-toxicity. There are several variations of MPLA, some are used as adjuvant in licensed human vaccines. The low toxicity of MPLA adjuvant function is associated with a bias in signaling pathway compared to the toxic LPS. When compared with LPS, MPLA induces strong TRIF-associated but weak MyD88-dependent events needed for proinflammatory patterns. Incorporation of this immunostimulant into liposomes ensured uptake of the vaccine antigens by APCs and immunostimulation of the same cells. This targeted stimulation was likely to minimize the risk of adverse systemic immunological reactions.

3.1.2.5 - Synergistic effect between the NHR and MPER regions: the family of epitope combination

The last family corresponded to the 5th generation of antigens. This consisted in 4 different candidates where a combination of epitopes was targeted: UGR7D-MPER-6, covNHR-MPER-1, covNHR-MPER-2 and H4D-MPER-2.

The rationale was based on the observed synergistic effects between different regions or functional domains of gp41. This synergy was observed in fusion inhibitors, in neutralization by antibodies and also in immunogenic responses to antigens.

As a consequence, a vaccine composition simultaneously eliciting antibodies targeting both NHR and MPER epitopes was assumed to intensify antibody response as well as neutralization potency. Antigens exposing simultaneously both types of epitopes were proposed. However, biophysical characterization evidenced a number of problems related to production yield and solubility at physiological pH, and a relatively low exposure of NHR epitopes relative to the antigen families they derived from.

In conclusion, the design of these antigens combining two epitope regions generated really complex protein constructs, difficult to produce and with certainly non-optimal biophysical properties.

3.1.3 - Collaboration with Dr Charloteaux

Another family of antigens was proposed in collaboration with Dr B. Charloteaux, Belgium, expert in molecular modeling and viral fusion proteins. These antigens were designed to mimic the structure of the MPER on the surface of the viral particles.

From the literature, it was assumed that the MPER should not be presented as a trimer of closely associated helices, a state that might correspond to the latent state. The thermostable 6-HB structure (post-fusion state) was not an option either. However, trimeric constructions could be envisaged as long as the MPER was not involved directly in the formation of the trimer, nor closely interacting with gp41 ectodomain. In addition, the MPER association to the viral membrane and/or its crowded molecular environment during the fusion process was thought to be key factor to elicit antibodies like 2F5 and 4E10 (long CDR3). It was also proposed that the MPER interact with the polar region downstream of the fusion peptide.

Therefore, in the proposed constructions, MPER epitope(s) presentation was optimized by acting on its environment (i.e. position at the interface and/or protein environment) rather than by introducing mutations in the epitope(s) to constrain its structure in a given (imperfect and rigid?) conformation. This was motivated by several reasons:

- 2F5 and 4E10 epitopes are close in gp41 primary sequence and their structure when bound to 2F5 and 4E10 are barely compatible
- the MPER region seems to adapt its structure in function of its environment (neighboring protein regions, membranes and lipidic phases) and this environment vary considerably during the fusion process
- MPER neutralisation is not dominated by any single specificity akin to known MPER-specific monoclonal antibodies. Presented in an appropriate set of conditions, the MPER should adopt an appropriate structure eliciting neutralizing antibodies.

The high hydrophobicity of the MPER (~W155-I171) and the presence of an Arg185 in the middle of the predicted transmembrane domain (TMD) (I173-V194) suggested that these regions might adopt a different topology at different steps of the fusion process. This was hypothetical but this could explain for instance how cytoplasmic epitopes can elicit antibodies. Furthermore, the presence of three glycines (GxxxG motif) in the TMD as well as TMD sequence suggested that it might form oligomers. The MPER and TMD would also be directly involved in the destabilisation of the membranes, and thus in the fusion process.

A total of 38 "Harvard" constructs were proposed and 21 were discarded after being subjected to production feasibility. Most of the 17 constructs tended to mimic the conformation that gp41 would adopt when CHR helices fold back against the NHR trimer.

3.1.4 - Other approaches

3.1.4.1 - MPEX

An additional immunogen MPEX formulated in liposome was proposed: it was composed of two MPER regions in order to ensure that the immune response will be against HIV MPER, whatever the direction of the protein inserted into the liposome. The MPEX16 was successfully produced and tested.

3.1.4.2 - PID

The principal immunodominant domain of gp41 (PID) was exclusively proposed for mucosal administration. The rationale for this restriction was that this Ag is already known to induce non neutralising Abs, therefore, there was no interest to elicit such Abs in blood. However, since the PID is highly immunogenic, it could potentially elicit a good response in the genital mucosa, a site usually refractory to immunogens. Anti-PID antibodies could potentially agglutinate the virus on the surface of the mucosa, preventing its passage through this barrier, complementing the activity of neutralising Abs.

PID antigens were produced by Bioneer after design adjustment at UGR. Rabbits were immunised using intranasal, sub-lingual or intravaginal route with MS2-PID-1 alone in drops or mixed with chitosan for evaluation of the immunogenicity induced in blood, in vaginal mucosa and faeces.

To complete the PID approach, SP proposed a passive immunisation study in macaque, with anti-PID antibody application in the mucosal tract, followed by a vaginal challenge with SHIV (see part 9.4).

3.2 - MANUFACTURING

3.2.1 - Gene optimization

GeneArt was in charge of gene optimisation and synthesis. Eighty four constructs were produced for the first and second generation constructs for E. coli and L. lactis.

- In L. lactis, 4 strategies were used, including standard GeneArt optimisation, codon harmonisation, highly expressed L. lactis genes and translational pausing.
- In E. coli BL21, the influence of intragenic methylation sites on protein expression and solubility was analysed.
- While expression of insoluble proteins was influenced by methylation site content, expression of the soluble proteins was not. Expression of the partially soluble H1 protein inversely correlated with the increase of methylation site content.
- The ratio of soluble/insoluble proteins was not influenced by expression level: a decrease of expression level did not translate into an increase in solubility.

In conclusion, optimisation strategy showed individual effects for certain constructs groups, but expression was not modulated following a general rule.

E. coli was selected for expression of the gp41-derived proteins after winning competition against L. lactis expression system. At GeneArt, efforts were concentrated on codon optimization adapted to E. coli.

In order to obtain a broad data base for the identification of parameters important for E. coli gene expression a new approach was proposed based on the generation of a silent mutation library and data mining. Extensive data mining and correlation analyses were performed with the goal to interpret the obtained sequence and expression data. The results indicated that the influence of the codon choice on protein expression in E. coli is a protein specific effect that needs to be considered in the overall context of the gene. This approach was used successfully to identify specific positions within the open reading frame of the target gene which seemed to have an influence on expression level.

Additional potential parameters were used for advanced correlation studies. By scanning the complete reading frame, a matrix of all possible codon pairs was built and the correlation between their cumulative occurrences in different expression level groups was investigated. One sequence ("gp41-best") was designed to contain the maximum possible number of codon pairs that correlated with high expression within the peer group. Another gene ("gp41-worst") was saturated with codon pairs of lowest correlation to high expressing specimen. Both sequences included about the same number of silent substitutions compared to the standard optimized gp41. The two genes ("best" and "worst") were synthesized and subcloned into the same expression vector. Together with the standard optimized gp41-UGR7 and eight randomly picked specimens from the silent library, three independent inductions were performed and the total expressed gp41 was quantified from the cell lysate. Both rationally designed genes expressed higher than the standard optimized version while "best" showed a slightly better performance as compared to "worst".

In conclusion, this methodology proved to be perfectly suited to identify the “best performing codon sequence” for a given protein.

3.2.2 - Lab grade protein manufacturing

One of the milestones of the first project Period was to select an expression system to manufacture the transmembrane proteins, based on the following:

- Characterisation (solubility, purity) of vaccine candidates
- Process yield
- Evaluation of process cost
- Process scalability

Two expression vectors were evaluated: Lactococcus lactis at Bioneer and E. coli at PX Therapeutics (PXT).

First and 2nd generation antigens were tentatively produced in Lactococcus lactis at Bioneer and E. coli at PXT.

Bioneer used Lactococcus lactis where the protein of interest is secreted into the culture medium, simplifying the purification process. However, neither UGR7 nor H4, selected for their higher solubility, could be secreted. Rather, they accumulated intracellularly. Therefore, it was decided to optimise intracellular production using medium or high copy vectors designed for intracellular expression. This resulted in some production of UGR7 but not H4. Attempts to produce CovNHR1 and CovNHR2 allowed intracellular detection of the latter protein, which accumulated in the membrane fraction. Other approaches included the secretion of the covalent trimers using a highly secreted protein as a fusion partner and the intracellular fusion of the covalent trimers to the SUMO peptide.

After several attempts, the production of proteins in this system was abandoned. It became clear that L. lactis was not adapted for production of gp41-derived proteins.

PXT used E. coli expression system. After small scale feasibility study where different expression conditions were tested, PXT selected the best parameters for 1 L scale production. It was noted that gene optimised constructs were better expressed. The proteins were extracted (key parameters were pH and salt concentration) and then purified in 2 steps, quality controlled and shipped to partners for immunisation, characterisation, formulation and as reagents.

E. coli proved to be better adapted than L. lactis and was selected after one year for further production. Although most of the antigens were produced at PXT who was the expert in this expression system, some of the constructs were produced in E. coli at Bioneer who had to integrate this technology and train people accordingly.

During the Discovery Period, PXT delivered 25 research-grade proteins, corresponding to 100 mg or more for almost each candidate, with a total of 64 batches, adapting the buffer to partners' requirements. In addition to the second, third, fourth and fifth generation of antigens, PXT produced the "mucosal antigens" specifically designed for mucosal application.

- 3rd generation antigens: from the 7 candidates of 3rd generation provided by UGR, only 4 could be produced by PXT thanks to the addition of a booster sequence at the N-terminus. Three to six batches were produced for each of the 4 candidates: FP-UGR7-MPER-A, FP-UGR7-MPER-B, PR-UGR7-MPER-A or PR-UGR7-MPER-B. For FP-H1-MPR-A & B, even if level expression was acceptable, the expected amount was not sufficient for formulation purpose.
- "mucosal antigens" (produced during the same round than the 3rd generation of antigens) : from the 16 candidates designed by SP in collaboration with Harvard, 2 were successfully produced in E.coli by PXT: 5D1C, 4B1C and one by Bioneer, 5B. The others were discarded due to insufficient expression and solubility.

For the successfully produced Ags, specific buffers were adapted during downstream process for liposomal formulation at Polymun.

- 4th generation antigens: 7 out of the 8 candidates provided by UGR could be successfully produced by PXT: UGR7-E, UGR7 D+, UGR7+, H4A, and the CovNHR 3, 4, and 5. Derived from H4A, H4D could not be produced despite several attempts as protein precipitated during concentration step.
- 5th generation antigens: only 3 out of the 12 candidates provided by UGR could be successfully produced by PXT: UGR7 D-MPER-6, CovNHR-MPER-2, and H4D-MPER-2. The issues encountered for the 9 other Ags were mainly low expression level or purification recovery.
- PID: for production, the PID was inserted at the tip of the coat protein of the MS2 bacteriophage to be presented in the assemblies of virus-like particles hopefully mimicking the surface of HIV. Bioneer had

experienced great difficulties in producing the first MS2-PID1 antigen and the purified antigen did not seem to assemble into VLP. Two possible reasons were put forward:

- The linker connecting the MS2 tandem monomers was too long (insertion of a Ser-Ala-Met tri-peptide)
- When the PID loop was inserted, part of the MS2 sequence was deleted, possibly compromising the stability of the protein.

To optimize the folding of the PID-containing MS2 dimers with minimal impact on stability, two strategies were proposed by Bioneer and three by UGR. The production of one of these Ags was completed successfully.

MS2-PID-1 was used in drops or mixed with chitosan to immunise rabbits using intranasal, sub-lingual or intravaginal route for evaluation of the immunogenicity induced in blood and vaginal mucosa. Some neutralising activity could be measured. However, due to higher priorities, this program was put on hold.

3.2.3 - Acellular production at Synthelis

For highly hydrophobic proteins that could not be produced in E. coli, several attempts were made to use an in vitro acellular expression system developed by a new Biotech, Synthelis. One protein containing peptides Eisenhower and Kennedy, the MPER and the membrane spanning domain of gp41, called LIPO-gp41 and another protein binding UGR7 to LIPO-gp41 to increase the overall solubility (UGR7-LIPO), were synthesized and presented on liposomes to mimic the presentation on the virus surface. A third candidate was proposed by RKI. Although two out of three proteins could be produced, the content in endotoxins was too high for further development.

3.2.4 - GMP Process assessment

In order to anticipate technical questions that may arise during the manufacturing of the candidate for the clinical trial, it was decided to work on the development process of a limited number of antigens presenting a good probability to be selected based on available biophysical and immunogenicity data. GeneArt started with GMP production of the plasmid devoid of histidine tag. Then, PXT developed and compared production and purification protocols for each of these candidates and produced small batches in compliance with cGMP standards in order to investigate manufacturing feasibility and accumulate quality control and stability results.

This anticipation allowed to save time when the vaccine candidates were definitively selected.

3.3 - SEMI-SOLID AND SOLID FORMULATION

QUB was in charge of developing quality-controlled semi-solid – gel - and solid – tablets - formulations for immediate or sustained sublingual (SL) or vaginal release of the Ags with or without liposomes.

Gels could be used for vaginal route. However protein mixed in gel could be unstable. For this reason, it was recommended to mix the protein to the gel immediately prior administration.

3.3.1 - Tablets.

Tablets could be used for both SL and vaginal routes. Four types of tablets were prepared: directly compressed or freeze-dried, for immediate or sustained release. In a pilot study, tablets were dye-loaded for distribution study post-vaginal and sublingual (SL) administration in rabbit and SL administration in monkey.

In rabbits, selection of the tablets for vaginal use was based on the size and form. Rod shaped freeze-dried formulation with muco-adhesive properties was the most appropriate, with staining observed after 6 hours. These rods were therefore produced, together with the Ag for immunisation studies.

On the contrary, tablets were not suitable for sublingual use in rabbit as they were immediately expelled.

In monkey, the compressed dye-loaded immediate-release SL tablets seemed to best fit the need.

3.3.2 - HEC gel manufacturing and QCs

The gel was later selected for use in human. QUB provided Polymun with the manufacturing technique of 4% hydroxyl-ethyl-cellulose (HEC) gel for the clinical batch. The gel preservative was benzyl alcohol. QUB developed a method to measure the alcohol content in the gel in order to ensure the good stability of the product. Using this method, the alcohol content in the GMP material was checked at the end of the immunizations and proved to be in line with expectations.

3.3.3 - Biodistribution study

The route of mucosal administration was balanced between nasal and vaginal application. To investigate the outcome of liposomes mixed with gel after vaginal administration, a biodistribution study was performed in mice at QUB using in-vivo imaging. This involved the administration of a fluorescent probe in the dosage form into a live animal. After anesthetizing the animal and using laser based fluorescence excitation, the fluorescence signal was mapped onto different organs. One important phenomenon was tissue auto-fluorescence. The computer used a base scan to compensate for the auto fluorescence and mapping the signals to the different organs. This was very reproducible and easily done in small animals with tight skins like mice and rats.

Data clearly showed that the gel was able to maintain the liposomes at the vaginal site for at least 2.5 hrs. At this timepoint, some fluorescence was visible in the abdomen. This could be due to absorption into the stomach when animals clean themselves or to migration of liposomes through the uterus, up the fallopian tubes and deposition within the peritoneal cavity. In this later case, this could be a source of Pelvic Inflammatory Disease in women.

3.3.4 - Antigen degradation in cervico-vaginal fluids

Another study aiming at supporting decision on the route of mucosal administration was launched: QUB tested the stability of the protein FPA without liposome, in human cervico-vaginal fluid (CVF). In-house produced 2F5-HRP conjugate Ab was used for FPA detection. The protein was found to be stable over 24 h in both diluted CVF and in normal saline.

3.3.5 - Quick release, freeze-dried, solid dosage form

QUB explored for the first time the use of a quick release, freeze-dried, solid dosage form for vaginal immunization. These solid dosage forms were generated to avoid the dose leakage that commonly occurs with vaginal administration of Ag in solution. Mice were immunized vaginally with H4A-loaded quick release rods. However, this method failed to elicit a robust immune response.

Detailed analyses of cytokines, stability of H4A in mouse CVF and state of H4A in the dosage form revealed that all parameters might have contributed to the observed lack of efficacy. These were important factors affecting vaginal immunization that deserve further investigation.

3.4 - LIPOSOMAL FORMULATION

Polymun was responsible for the formulation of insoluble proteins in liposomes. The crossflow injection technique allowed large scale GMP incorporation of protein into liposomes under aseptic conditions. The lipid solution in ethanol was mixed with the protein solubilised in detergent. Vesicles formed while the detergent was diluted. A final sterilisation was performed.

MPLA was added as adjuvant in certain experiments. Of note, it is degraded into up to 14 congeners as pH increases. Liposomes +/- MPLA could be administered either parenterally, or mucosally in drops or mixed into a gel.

3.5 - ASSESSMENT OF ANTI-HIV FUNCTIONAL ANTIBODIES

The functional activity of antigen-induced antibodies was assessed by validated standardised assays measuring neutralising activity in PBMC and TZM-bl.

Moreover, non-neutralising inhibitory activity was determined in macrophage assay. The mechanism of inhibition is distinct from the neutralisation of infectivity occurring via Fab fragments and involves the interaction of the Fc portion of the immunoglobulin with the Fcγ receptors present on macrophages and immature dendritic cells (iDC). Such non-neutralizing inhibitory Abs were suggested to limit mucosal HIV transmission (J Virol. 2006 Jun;80(12):6177-81).

It recently appeared that binding yet non-neutralising Abs can potentially inhibit HIV transmission at the barriers of the sexual mucosa by alternative mechanisms: i) particle cross-linking into large complexes that cannot penetrate the mucosal barriers, ii) trapping viral particles within superficial epithelial barriers and the protective mucus of the female reproductive tract, iii) specific targeting and killing of infected cells through Ab-directed cell cytotoxicity and related mechanisms (Nature Medicine 17, 1195–1197 (2011)).

Based on similar concept, additional techniques were developed in the Consortium and were used for secondary and exploratory endpoints in the clinical trials. They included:

- Measurement of specific IgA in the vaginal secretions. Since these Abs are oligomeric, they may contribute to agglutination of the virus on the mucosal surface.
- Measurement of inhibitory activity by a virus capture assay. It was demonstrated that this assay could not be used as a surrogate method for the investigation of the neutralizing activity of serum Abs. However, on the mucosal surface, these capturing Abs could be able to block the virus and prevent infection. These Abs form immune complexes mainly by targeting the principal immunodominant domain (PID) of gp41 (Virology. 2005 Mar 1;333(1):102-13).
- Measurement of virus agglutination. Antibodies capable of binding multiple virions into aggregated complexes without having to block every available envelope spike may have an important role in mucosal prevention of infection. Size exclusion chromatography (SEC) was combined with dynamic light scattering to enable the accurate measurement of multiple size peaks in a reproducible fashion. Ultra purified virions were incubated with the Abs of interest and allowed to form any metastructure that the interaction allows. Populations were then separated through SEC and an assessment of the ability to form immune complexes was performed in a contiguous fashion. Both the polyclonal HIV-Ig and dimeric 2F5 IgA demonstrated a significant tendency to form multiple aggregation size peaks, while 2F5 IgG and pentameric IgM did not. Antibody isotype had a significant influence on the ability to form aggregates either through steric and flexibility properties or by multiple bindings to a single virion (P11-07, AIDS VACCINE 2008). Various biophysical techniques were developed to detect and characterise such aggregates.
- Measurement of antibody-dependent cell-mediated cytotoxicity (ADCC). A role for ADCC in controlling initial development of HIV-1 infection is supported by a growing number of studies and recently anti-gp41 MAb 2F5 was shown to trigger ADCC of both R5 and X4-tropic HIV-1 envelope subunit coated cells at ng/mL concentration. This expanded the prophylactic potential of gp41-directed Abs (AIDS. 2011 Mar 27;25(6):751-9).
- Inhibition of HIV-1 transfer from DC to CD4+ T lymphocytes. During mucosal HIV transmission, iDCs present in the mucosa are among the first cellular targets of the virus. Anti-gp41 Abs where shown to inhibit infection of these cells via a mechanism involving Fcγ receptor II, probably by mediating endocytosis and degradation of HIV-IgG immune complexes, without inducing the maturation of these cells. These Abs that efficiently inhibit in vitro HIV infection of human iDCs could involve other than neutralizing IgGs and participate in the protection of individuals from HIV-1 infection (Blood. 2006 Jun 1;107(11):4466-74).

3.6 - IMMUNOGENICITY STUDIES IN RABBIT

DVMP was in charge of rabbit immunisations and clinical and biological follow-up of the animals. Non-SPF New Zealand rabbits were immunized with the immunogens according to various protocols either by parenteral route or by vaginal route using tablets or gels with or without adjuvant. The primary goal of these studies was to find the right protocol to elicit an immune response detectable in serum and mucosa.

The strategy was 1) to evaluate all candidates in rabbit by the parenteral route, 2) to explore the best way to induce an immune response in the genital tract of the animals, using various routes and schedules of administration, formulations and adjuvants but focusing on a limited number of antigens.

3.6.1 - Technique

The technique for mucosal immunisation and sampling required numerous preliminary attempts and training before being well controlled. The vaginal mucosa had to be protected from alteration by the device to avoid inflammation and/or systemic immunisation. At the end of each study, careful examination of the genital tract was performed after the sacrifice of the animals for detection of lesions and toxicity. This was supported by pictures.

More than 25 studies were performed to investigate the immunogenicity of more than 60 antigens. Formulation of the antigens included lyophilized tablets or rods, gel or liquid drops, liposomes or chitosan. They were mixed with adjuvants such as Cholera Toxin, IMS, Alum or MPLA.

3.6.2 - Parenteral administration

After parenteral injection, all immunogens elicited a high specific IgG response in serum, measured by ELISA. When injections were repeated, the 4th IM administration did not increase the response, but on the contrary

decreased the titer. It was proposed that repeated injections could cause apoptosis of cells already activated. Therefore a new schedule was proposed, composed of 2 instead of 3 IM priming at D0 and 28 followed by a late boost at D56.

The serum IgA response was generally poor but increased in presence of Alum adjuvant.

No or rare IgG or IgA responses could be detected in lavage. They could only be measurable in Weck-cels where the genital secretions were more concentrated. IgG were generally more abundant than IgA.

Some of the immunogens could also induce a neutralising activity in serum and Weck-cels but only exceptionally in vaginal lavages. Again, this could be due to the high dilution of this latter sample.

Some experiments focused on the comparison of two antigens generations in order to confirm immunogenicity despite design modification to improve solubility. This was the case for RAB10-PAR-4G: CovNHR-3, CovNHR-4, CovNHR-5 were tested in parallel, after adsorption on AlOOH adjuvant. All Ags were able to induce IgG Abs in serum and Weck-Cels and neutralising activity in serum. In particular, all CovNHR Ags immunisations were associated with HIV neutralisation, confirming the immunogenicity profile of CovNHR-2 which they were derived from.

3.6.3 Mucosal administration

Mucosal administration was investigated using various routes and forms of the antigens:

3.6.3.1 Sublingual route

The SL route was investigated using UGR7-A given as drops with CT or in immediate or intermediate release freeze-dried rods with LTB adjuvant. None of these approaches could elicit serum IgG or IgA responses. Mucosal responses were weak and transient even after an IM boost. No reliable neutralising response could be induced suggesting that this route of immunization was not advantageous for the induction of HIV specific immune response in rabbits.

Although the SL route was discarded this route was considered worth further exploring because the vaginal route, that induced the best responses in the genital tract, may be difficult to apply in large populations and because the nasal route may be suspected to bypass the protective blood-brain barrier and allow passage of antigens into the brain, in particular in the presence of strong adjuvant. Consequently, several activities were undertaken to investigate the SL route in non human primate (NHP) and human (see below 9. Immunogenicity studies in monkey).

3.6.3.2 - Nasal route

Nasal route was tested because many data show that nasal administration can induce an immune response in the genital mucosa. Three nasal priming with URG7-A and CT in drops induced IgG – not IgA - responses in serum. The overall neutralising activity induced with drops was poor. Mucosal inhibitory activity was only detected after nasal priming followed by 2 IM boosts.

Nasal prime/IM boost and IM prime/nasal boost regimens were compared. Nasal administrations were performed after mixing the FPA2 Ag with HEC gel, the mucoadhesive selected for the clinical trial. Nasal priming did not induce IgG responses in serum. However, both complete regimens of administration were able to induce specific IgG Abs in serum and neutralising activity in serum and Weck-Cels. Higher titers were measured using nasal prime/IM boost schedule compared to the reverse. Although this could demonstrate an advantage of the former, the difference could also be related to timing of sampling with respect to the last systemic immunisation. MPLA was needed to obtain a response earlier, in all animals, and there was a trend for higher titers with increasing doses of the adjuvant.

3.6.3.3 - Vaginal route

Vaginal administration was selected based on recommendations from experts gathered at the beginning of the project: best vaginal responses are elicited after vaginal immunization. Three vaginal priming with URG7-A and CT in drops induced IgG – not IgA - responses in serum. As with the nasal route, the neutralising activity induced with drops was poor.

Three vaginal primes and 2 vaginal boosts with UGR7 in gel elicited serum IgG, mucosal IgG and IgA and responses were higher when the protein was administered in the presence of CT adjuvant. HIV inhibitory responses were modest in serum and lavages after 3 primes and were not improved by 2 vaginal boosts. This activity was increased when UGR7 was combined with CT. Comparing with the previous study, the gel may show an advantage over the drops for immunogenicity.

To further improve Ag retention on the mucosa, freeze-dried solid formulations were prepared. Vaginal priming with sustained-release rod containing UGR7 induced a serum IgG response in the absence of adjuvant. Mucosal IgA were reliably detected in Weck-cels in the absence of a mucosal IgG response. UGR7 vaginal rods also induced a neutralizing response in serum which was improved by higher dose of UGR7. HIV inhibitory activity was also detected in the vaginal lavages. However, placebo rods alone induced an HIV-inhibitory activity, probably by inflammation of the vagina with release of chemokine/cytokine able to neutralise the virus. It was therefore difficult to distinguish the contribution of the induced immune response in the neutralisation of the virus.

Nasal and vaginal routes were also used with other Ags presented in liposomes and MPLA (Harvard 5D1C, MPEX-16 and Harvard 5B). No responses were observed in serum or mucosa after administration whereas good specific IgG responses were elicited in serum by all immunogens after IM injections. Interestingly, after mucosal priming followed by IM boosts, responses in Weck cels were restricted to IgA but they were borne by IgG and IgA after IM injections in the absence of mucosal prime.

3.6.3.4 - Conclusion

The SL route was not potent in eliciting an immune response but both nasal and vaginal routes proved to be efficient. The restricted IgA profile of the response at the mucosal level, as opposed to the IgG/IgA after IM route only is an example of the qualitative modulation of the immune response by mucosal application. In serum, most of the responses to all antigens were IgG rather than IgA, whatever the route of administration.

3.7 - SELECTION OF THE ANTIGEN FOR THE MUCOSAL TRIAL

The planning was precisely followed allowing the selection of the candidate on the exact expected date of May 3- 4 2010 during the annual meeting in Paris.

The aim was to select the candidate for the mucosal trial out of the three generations of immunogens and all the "mucosal antigens". The elicited immune response was the principal criterion for selection. The second critical criterion was the manufacturing feasibility.

FP-UGR7-MPR-A (FPA) and PR-UGR7-MPR-A (PRA) were quoted equally with respect to the immune response. PRA was more stable according to biophysical studies by UGR. With respect to manufacturing, antigens representative of the 3 families: UGR7 A, FPA and HARVARD 4B1C were evaluated for compatibility with GMP production including fermentation, inclusion bodies preparation for the insoluble candidates, resins for purification, mini scale-up and analytical controls.

The 3 proteins displayed good behaviour in fermentation under standard protocol. UGR7 A did not show a homogeneous trimeric form, FPA showed a high level of expression but inclusion bodies preparation had to be improved and storage at -80°C caused aggregation and Harvard candidate presented degradation products.

Although FPA candidate was not ranked first for manufacturing, PXT believed that issues could be addressed.

Regarding formulation, compared with the other antigens FPB, Harvard 4B1C, Harvard 5D1C, MPEX-16 and Harvard 5B, FPA showed the best total score in terms of buffer usability, concentration, formulation behaviour, drug product parameters, drug product stability. Finally, FPA did not demonstrate any safety issue at DVMP. It was thus selected for further development.

Following this meeting, GMP Process development started with FPA.

The RAB10-FT1 "Fine-Tuning study" performed in rabbit investigated the formulation of FPA for mucosal administration and the benefit of MPLA adjuvant. In addition to liposome-FPA with or without MPLA, Ag-free liposomes with or without MPLA were tested as negative controls.

Although analysis of the inhibitory activity was hampered by non-specific activity detected in some samples prior vaccination, groups immunized with the gel formulation for priming and dispersion formulation for IM boost gave the highest inhibitory activity in both sera and Weck-Cels. This conclusion was backed by ELISA results showing higher titers when FPA was adjuvanted with MPLA and administered in presence of gel. These data supported the selection of a gel formulation for mucosal administration in future studies. They also supported the need for MPLA to increase the binding Abs titers and the number of responders.

Based on RAB10-FT1 data, the Consortium decided to use the HEC gel for further mucosal application of FPA, confirmed the need for MPLA adjuvant and adopted the nasal prime/IM boost schedule for clinical protocol.

This immunogen was renamed EN41-FPA2 when referred to in official documentation for Health Authorities.

3.8 - SELECTION OF THE ANTIGEN FOR THE PARENTERAL TRIAL

The Parenteral Track comprised all the steps leading to the selection of the vaccine candidate for the parenteral trial. The objective was to induce a systemic as well a mucosal response in the genital tract. Due to the limited number of freely available non proprietary GMP-grade adjuvants, aluminium salts were used, AlPO4 and AlOOH, or IMS, a water based nanoparticles combined with a soluble immunostimulant, kindly supplied by Seppic.

All antigens from 1st to 5th generation were tested by intramuscular or a combination of intramuscular and subcutaneous routes in rabbits. Based on immunogenicity data, a short list of potential vaccine candidates was identified. This list was refined to take into account the manufacturing feasibility. The selected immunogens H4, H4A, UGR7 and UGR7C/D were extensively characterized at UGR and compared head to head for immunogenicity.

UGR-7-derived candidates had the best immunogenicity score. UGR7-C was selected as it offered new epitopes due to the more open structure than gp41, it contained part of the principal immunodominant domain (PID) that could contribute to the inhibitory activity at the mucosal level since anti-PID Abs were known to capture the virus, and it induced neutralizing responses in serum as well as the best inhibitory activity measured in vaginal secretions. The ELISA titer was increased with higher amount of protein and adjuvant, justifying the dose selected for the clinical trial. Alum significantly increased the titers compared with Ag alone, justifying the need for this adjuvant.

With respect to manufacturing feasibility UGR7-C showed acceptable expression level and solubility.

FPA2 was the candidate for the mucosal trial and UGR7-C was selected for the parenteral trial. It was tempted to combine both Ags to improve the immune response in rabbit:

Two administration protocols were proposed, one where both Ags were given simultaneously as a cocktail, the other where the same amounts of Ag were given in a prime/boost regimen. This comparative approach was extended to other high priority Ags 4B1C and CovNHR-3 that elicited neutralising Abs in earlier experiments. The goal was to increase the neutralising Abs by focusing the response against neutralising epitopes presented in different scaffolds.

Both strategies failed to increase the neutralising response. Although binding Abs were elicited at the expected levels, there was only rare neutralisation detectable in serum or Weck-Cels. The competition between all immunogens might have been responsible for this counterproductive effect.

3.9 - IMMUNOGENICITY STUDIES IN MONKEY

3.9.1 - Mucosal studies with EN41-FPA2

A decision was made to start monkey studies with the selected Mucosal candidate based on the detection of a neutralising response in sera or of binding activity in mucosal samples of rabbits. A GO was given during the 'Mucosal Track' Annual meeting (Paris, 3-4 May 2010), where results obtained with the FPA antigen were presented. Positive neutralising activity and binding antibodies were measurable in both sera and vaginal samplings of the rabbits.

Studies in non-human primates were launched to confirm immunogenicity of EN41-FPA2 and to measure efficacy of the vaccine.

3.9.1.1 - VAC1018 (& VAC1116)

This study was performed to determine the route of administration of FPA in macaque, a species considered more appropriate than rabbit to predict the results in human. FPA was given by nasal or vaginal route to groups of three animals that were boosted by IM injections. A control group received only the IM doses of FPA to explore, by comparison with the other groups, the benefit of the mucosal priming. FPA was given as a simple suspension.

By ELISA, mucosal priming, either nasal or vaginal, was unable to induce detectable specific IgG responses in serum, whereas 2 IM boosts could elicit IgG Abs peaking at ~ 105 ng/mL in all groups. The serum IgA responses were seen only after IM injections and at lower levels in the mucosally primed animals.

Surprisingly, neutralising activity developed in all animals in both serum and vaginal fluids after 3 nasal primes when no positive responses were measured by ELISA. In serum, this activity was sustained in 2 animals after first IM boost. One animal was positive after the second IM boost. IgG purification experiments confirmed that

this activity was associated to the IgG fraction. In conclusion, nasal route proved to be more efficient and was selected for the clinical trial.

In protocol VAC1116, animals were boosted with 3 nasal administrations of FPA to further investigate the safety of this Ag by this route, in prevision of the clinical trial.

In summary, nasal priming or boosting with FPA was not able to induce or boost specific IgG responses in serum, as measured by ELISA. It could boost serum IgA induced by previous parenteral administration. It was not able to elicit or boost IgA responses in vagina. This was in contrast with neutralising results. Three FPA nasal priming were able to elicit an inhibitory activity in serum (VAC1018) and 3 FPA nasal boosts (VAC1116) could sustain or elicit an activity in vaginal secretions.

3.9.1.2 - VAC1126

This study explored the immunogenicity and efficacy of EN41-FPA2 immunogen, using the same dose and regimen of administration as in the clinical trial CT1.1.1. A repeated vaginal challenge with 0.5 AID50 of SHIV162P3 was performed every week for 10 weeks, starting 10 weeks after the last immunisation.

a) Local and systemic tolerance

- No major local reaction was observed at the vaccine site of administration one day after each of the five immunizations
- Nasal and intramuscular administration of FPA-2 protein formulated with or without adjuvant did not induce major changes in blood leukocyte cells
- No major weight and temperature changes was observed all along the study

b) Immunogenicity

- Serum IgG responses were primed following 3 nasal administrations reaching titres of ~103.5 log10 which were boosted to 105.5log10 following 2 IM boosts. Serum IgA responses mirrored the kinetics of the serum IgG albeit 2 logs lower.
- Vaginal mucosal IgG responses were transient with titers reaching ~103 log10 after three nasal primes and dropping prior to peaking at ~104 after 2 IM immunisations for animals that received vaccine. Vaginal mucosal IgA responses mirrored the kinetics of the vaginal IgG responses albeit at approximately a log10 lower.
- Neutralising responses were low and transient with no difference between groups.
- During the challenge phase, serum and vaginal specific IgG responses were boosted, indicating cross reactivity of Abs directed against the virus and EN41-FPA2, the ELISA coating Ag. All animals (both active and control groups, except one in the active group) reached serum IgG levels of ~106.5log10. Nevertheless, serum IgA were not boosted by the virus whereas vaginal IgA were transiently boosted after challenge.

c) Efficacy

- Macaques in the control group acquired infection after a maximum of 7 challenges. Seven macaques in the EN41-FPA2 immunised group acquired infection after a maximum of 4 challenges and a single macaque (BN100) remained uninfected after the 10 challenges.
- Comparison of the groups for acquisition of infection was investigated using survival curve analysis. The three survival curves of vaccinated and control groups weren’t significantly different from each other ($p=0.8905$; log-rank (Mantel-cox) test).

As animal BN100 in the EN41-FPA2 group remained aviremic after the 10 challenges, the presence of viral RNA and DNA was investigated in its axillary and inguinal lymph nodes. All samples were negative for both SHIV RNA and SHIV DNA, confirming that the animal was not infected by the SHIV162P3 virus.

d) Conclusion

- Except one immunized monkey, all other 23 animals became infected during the challenge phase, without difference between groups.
- EN41-FPA-2 vaccine did not protect against acquisition of infection by SHIV162P3 after repeated low dose intravaginal challenge
- EN41-FPA-2 vaccine did not reduce the viremia levels in monkey after acquisition of infection.

3.9.2 - Parenteral study with EN41-UGR7C: VAC1129

This study investigated the immunogenicity of EN41-UGR7C immunogen, using the same dose and regimen of administration as in the clinical protocol. A repeated vaginal challenge with 0.5 AID50 of SHIV162P3 was performed every week for 10 weeks, starting 10 weeks after the last immunisation.

a) Local and systemic tolerance

No local reaction was observed at the vaccine site of administration on the day after each of the 3 immunizations. No major weight and temperature changes were observed all along the study. IM administration of EN41-UGR7C did not induce major changes in blood leucocytes cells.

b) Immunogenicity

- EN41-UGR7C induced persistent specific serum IgG but more transient specific vaginal IgG. Low specific serum IgA were also induced and only weak and transient specific IgA in the vagina were measured.
- SHIV challenge boosted both serum & vaginal UGR7-C-specific IgG in infected animals, evidencing a cross-reactivity between anti-UGR7-C and anti-SHIV gp41. Vaginal specific IgA were slightly boosted and only transiently.
- Neutralization responses were sporadic and very low after vaccination, with no differences between groups. Some neutralization was detectable in sera only post-infection, with no difference between groups.

c) Efficacy

Ten out of 12 macaques in the control group acquired infection after a maximum of 7 challenges and 11 out of 12 vaccinated animals got infection after 6 challenges. There was no difference between the groups for percentage of infection nor viral load at peak or area under the curve. Two and one animal in the control and vaccinated group remained uninfected after 10 challenges, respectively. The same was true for BN100 who was not infected in VAC1026 and remained uninfected after 10 additional exposures to SHIV162P3.

d) Conclusion

Except one immunized monkey and two controls, all other animals became infected during the challenge phase, without difference between groups.

EN41-UGR7C vaccine did not protect against acquisition of infection by SHIV162P3 after repeated low dose intravaginal challenge.

EN41-UGR7C vaccine did not reduce the viremia levels in monkey after acquisition of infection.

3.9.3 - Investigation of the sub-lingual route of immunisation

In recent years, increasing attention was directed to sub-lingual (SL) route to induce systemic and mucosal immune protection against viral infections. This SL mode of vaccination was therefore investigated in EuroNeut-41, in macaque, through a collaboration with Inserm funded by ANRS (Agence Nationale de Recherche sur le SIDA, France).

Based on literature, it was clear that the immunogen administered SL should remain on the mucosa to increase the probability of eliciting an immune response. Therefore, the UGR7-B was coupled to cholera toxin B subunit (CTB). CTB can bind to GM1 gangliosides present on mucosal surface, working as muco-adhesive.

A pilot study of SL immunisation with CTB-UGR7B conjugate in presence of native cholera toxin (CT) as adjuvant was performed in female cynomolgus macaques. This protocol allowed to induce UGR7B-specific Abs of IgG isotype in all immunized animals and IgA in blood and vaginal secretions in some of them. Three out of four immunized macaques showed neutralizing activity which persisted in one animal.

Two SL booster administrations were given to the macaques and circulating UGR7B-specific Ab-secreting cells (ASCs) were transiently detectable in the blood at day 5 but not at day 7 post-immunisation. UGR7B-specific ASCs were also identified in the spleen. They were mainly of IgG isotype.

The increase of serum UGR7B-specific Abs titers (IgG, IgA) after SL boost indicated that the SL priming with CTB-UGR7B and CT was able to generate a B cell memory response.

Based on knowledge acquired in the pilot study, a second experiment was designed in a larger number of macaques to compare SL and IM routes of immunisation.

a) ELISA

The 3 SL immunizations induced UGR7B-specific Abs of IgG and IgA isotypes in the sera of all immunized macaques. The 2 SL boosts did not amplify the Ab responses but contributed to increase the numbers of responders (IgG and IgA responses: 6/6 responders after the prime-boost versus 3/6 after the priming).

The 3 IM immunizations schedule was as efficient as the combined immunization to induce Ab responses in the serum but did not induce any Ab response in the rectal secretions, and only weak responses in the vaginal secretions. This result indicated that the SL priming is important to induce mucosal Ab responses.

The combined immunization (3SL – 2 IM) was the most efficient: the IM boost increased the intensity of the IgG antibody response in the serum of all the macaques by a factor 40 (mean endpoint titer:11,000 after combined versus 350 after SL immunization). This regimen was the only one to induce notable Ab responses in the rectum in comparison to SL immunization and IM immunization (responders: 4/6 versus 1/6 versus 0/6). In addition, it was more efficient than the SL immunization to induce Ab responses in the vaginal secretions (responders: 6/6 versus 2/6).

b) Neutralisation

No neutralisation was detectable by PBMC assay. Using TZM assay, sera samples obtained two weeks and 3 months after the second boost of the combined immunization gave a weak (IC50%) neutralization (6/6 responders) whereas only 3/6 sera samples of the macaques that received a SL immunization were functional and only 1/6 after IM immunization. No neutralisation activity was detectable in vaginal secretions.

c) Conclusion

The combined SL-IM immunization regimen was the best protocol to induce UGR7B-specific Abs in the blood and rectal and vaginal secretions of the macaques. The functionality of the antibodies produced was weak, nevertheless the combined immunization seemed to be the most efficient to generate neutralizing antibodies.

3.9.4 - Passive immunization with anti-PID antibodies

The principal immunodominant domain of gp41, PID, is able to induce binding Abs in most if not all infected individuals. Although Abs directed against this epitope are non neutralizing, their binding activity could be important to capture the virus on the surface of the mucosa and prevent the infection at the portal of entry.

An immunogen exposing the PID was created and administered with a regimen such as to elicit Abs in the mucosa. To bring the proof-of-concept that such anti-PID Abs could protect against infection, a 2-step passive immunization study was proposed in monkey: first the pharmacokinetics of anti-PID IgA administered by vaginal route was conducted in 2 animals (THE1206 study); second, the protective effect of these Abs was analyzed after SHIV challenge by the same entry (THE1111 study).

a) The immumnogens

Two anti-PID IgG MAbs from Polymun, 3D6 and 4B3, were class-switched to IgA and produced in stably transfected CHO cells. Both 3D6 and 4B3 dIgAs recognized their respective epitope on a recombinant HIV-1

gp140. They were purified at large scale. The secretory component was also produced for assembly with the dIgA to reconstitute the secretory IgA (sIgA). By capture assay, these Abs showed a strong binding capacity to the virus, higher than their IgG counterpart. Several hundred mg of the two anti-PID sIgA formulated in 1.6% HEC gel were manufactured by Polymun to supply CEA for both experiments in macaque.

b) THE1206 Pharmacokinetics study with anti PID IgA antibodies

Two female cynomolgus macaques were involved in this study;

- Vaginal fluid sampling was performed using Weck-Cel spears at 0, 1, 2, 4, 6, 24 hours, 2, 3 and 4 days after administration for quantifying 4B3 and 3D6 antibodies in vaginal weck-cel;
- Blood was drawn at 0, 1, 2, 4, 6, 24 hours, 2, 3 and 4 days after administration for quantifying 4B3 and 3D6 antibodies in serum and to determine blood cell formula on day 0 and day 4.

Surprisingly, the amount of Ab measured by ELISA was already quite low or even undetectable one hour after administration. On the contrary, the antibody concentration was as expected in the syringes, the original container. This might be due to degradation of the protein in the vaginal secretions, despite the addition of protease inhibitor during sampling.

c) THE1111 Efficacy study with anti PID IgA antibodies

Despite these results, a challenge study (THE1111 study) was launched in monkeys passively immunised with these MAbs because the product was already manufactured and its stability over time was not known.

A total of 15 female cynomolgus macaques were distributed into two groups of six and one group of 3 animals, respectively:

- Group 1: 6 animals were treated intravaginally (IVAG) with 2 mL of placebo (HEC) gel;
- Group 2: 6 animals were treated IVAG with 2 mL of HEC gel containing 20 mg/g of each IgA antibody 4B3 and 3D6, for a total of 40 mg of each antibody (80 mg total);
- Group 3: 3 animals were treated IVAG with 2 mL of HEC gel containing 20 mg/g of each IgG antibody: 2F5, 4E10 and 2G12 for a total of 40 mg of each antibody (120 mg total);

One hour later, all animals were challenged IVAG with 10 AID50 SHIV162P3; this timepoint was selected in order to compare with another study using the same anti-PID antibodies but of IgG isotype and where animals were challenged one hour post-IVAG antibody application. In this study, the IgG antibodies were administered at the concentration of 30 mg/g each and at a dose of 30 mg of antibody per mL, that is 60 mg of each anti-PID IgG antibody per animal.

All animals became infected in both anti PID IgA/IgG and control groups, whereas 50% remained uninfected in a historical study where monkeys received 3 neutralising MAbs 2F5, 4E10 and 2G12 (TriMab).

d) Conclusion

Pharmacokinetics studies on anti PID antibodies showed a clear difference between IgA and IgG subtypes after intravaginal application in monkey. Although IgG were recovered more than 64 hours post-administration, IgA could not be detected one hour after application. This may explain the absence of protection of the animals after challenge with 10 AID50 SHIV162P3, despite the fact that IgA showed higher HIV agglutination capacity in vitro, compared with the IgG antibodies.

It was proposed to collaborate with another European Consortium, CHAARM, to reproduce the study since there are plans for several monkey studies. Discussions are ongoing between CEA, Polymun and CHAARM coordinator.

3.10 - MANUFACTURING DEVELOPMENT AND SCALE-UP OF THE PRODUCT

3.10.1 - GMP Manufacturing of EN41-FPA2

Once FPA2 was selected, the plasmid was reconstructed at GeneArt with appropriate GMP documentation. The manufacturing process was developed at two locations, PXT for the protein and Polymun for the formulation consisting of liposome with MPLA. A Quality Agreement was signed between both parties to clarify responsibilities.

At PXT, a Master Cell Bank was produced by transformation of a BLR(DE3) E. coli strain with the plasmid and was thoroughly characterized. The upstream process was progressively scaled-up. The downstream process was optimized and an ”end-to-end” batch was produced, followed by a preclinical batch for the toxicology studies and the clinical batch.

The protein displayed an alpha-helical structure by far-UV CD and a 6-helix bundle conformation by near-UV CD. The DLS analysis showed an oligomeric state for the samples as previously observed for other FPA samples.

At Polymun, the formulation in liposomes of each batch of protein was performed using the Ethanol-Injection method. After sterilizing filtration, the bulks were filled into vials which were stored at 2 – 8 °C until shipment.

Characterisation directed towards the protein formulated in liposome was very limited because of the strong interferences of the liposomes in the far-UV CD and ATR-FTIR techniques. By near-UV CD the spectrum looked very similar to those previously reported for the non liposomal formulated protein, indicating that the overall 6HB conformation of the protein was not affected by the insertion into liposomes.

3.10.2 - Production of HEC gel

The gel proposed by QUB was composed of 4% hydroxyl-ethyl-cellulose (HEC, Natrosol) and 1.1% benzyl-alcohol as preservative in phosphate buffer (PBS).

A GMP batch of gel was produced at Polymun under aseptic conditions for the clinical trial. For preparation, the polymer was mixed with the benzylalcohol for wetting the gel powder. This was followed by mixing with PBS. The gel was then distributed in sterile 3 mL Luer-lock syringes capped with sterile Luer tip caps. Each syringe was filled with 1 g of gel (approximately 1 mL). Two hundred and sixty HEC gel syringes were stored at 2 – 8 °C until shipment to the clinical site.

Polymun released the batch and provided the documentation to be included in the IMPD.

To monitor the preservation of the gel, the alcohol content was measured at QUB after manufacturing and was controlled after one year, i.e. at the end of the proposed shelf life.

In the frame of the toxicology studies conducted at CIT, the protein was mixed with the HEC gel before administration to animals, according to an SOP provided by SP. To check the homogeneity of the protein repartition within the gel after mixing, Polymun performed electrophoresis on the mixture and provided a report to the toxicology CRO confirming homogeneity.

3.10.3 - Production of EN41-UGR7C

When the Consortium agreed to select UGR7-C for further clinical development, the expression vector was produced following GeneArt internal GMP-like conditions and guidelines in order to provide the appropriate documentation. The expression construct together with the GeneArt GLP-source documentation for gene synthesis and sub-cloning were delivered to PXT for GMP manufacturing. All steps of fermentation and purification were established.

The protocol allowed obtaining a recovery >25 mg of product/Liter of fermentation with a purity >99% (RP-HPLC method), mainly (>90%) under trimeric conformation (SEC-HPLC method) and with a concentration (UV method) of 0.45mg/mL. The bulk was immediately sent at 4°C to subcontractor Carbogen Amcis for Fill & Finish within a week to avoid formation of aggregates that may occur during longer storage. The final product consisting in 785 vials containing 0.5 mL each was stored at -20°C at PX’Therapeutics.

3.11 - PRECLINICAL GLP TOXICOLOGY STUDIES

CIT was selected through a tender among 3 Contract Research Organisations (CROs) to perform the toxicology studies and to provide the risk assessment.

3.11.1 - Mucosal vaccine candidate

Three studies were performed on EN41-FPA2: in rabbit, with a regimen that mimicked the clinical protocol; in rat, to complete the safety assessment of the adjuvant and in vitro for genotoxicity.

a) In rabbit: no local reactions were noted at the nasal administration sites. Local acute inflammatory reactions with myofiber degeneration/necrosis and heterophilic infiltrates were observed at the IM injection sites 24 hours after administration, correlating with increased C-reactive protein levels and higher blood heterophilic counts in animals treated by the IM route with EN41-FPA2 or the adjuvant. Although C-reactive protein levels remained slightly higher, no lesions were observed at the IM injection sites 14 days after the last administration. Regarding immunogenicity, results showed specific IgG and IgA responses measured by ELISA in serum after 4 IM or 3 nasal followed by 2 IM administrations and poor IgG response in vaginal secretions. Neutralising activity was only detected sporadically in these SPF animals. This was reminiscent of another study in SPF rabbits where responses were poor compared to non SPF animals.

b) In rat: the adjuvant did not elicit any systemic changes, except higher white blood cell and fibrinogen levels in some females treated by the IM route alone and the lymphoid system stimulation observed in animals treated by the IM or IN/IM routes. These changes were attributed to the reversible local inflammation at the injection site.

c) Genotoxicity studies: two in vitro genotoxicity studies were performed on the Liposome and MPLA adjuvant formulation: under the experimental conditions, the product did not show any mutagenic activity in the bacterial reverse mutation test with Salmonella typhimurium nor in Mammalian cell gene mutation test in L5178Y TK+/- mouse lymphoma cells (MLA), either in the presence or in the absence of a rat metabolizing system.

In monkey, the safety of EN41-FPA2 was also investigated in the context of the non-GLP immunogenicity study VAC1116 (see above) where two groups of 3 adult female cynomolgus macaques received 3 intranasal administrations of FPA mixed in HEC gel at a human dose to mimic the planned clinical trial. The study showed that 3 nasal administrations of EN41-FPA2 in HEC gel was well tolerated with no significant lesion related to vaccine candidate in olfactory bulb and brain sections of the treated animals.

3.11.2 - Parenteral vaccine candidate

The safety of EN41-UGR7C was assessed in rabbit with a regimen mimicking the clinical protocol

EN41-UGR7C was well tolerated systemically and locally. Inflammatory blood biomarkers were transiently increased (fibrinogen and C-Reactive protein) after administration.

Immunogenicity results showed specific IgG and IgA responses measured by ELISA in serum after 4 intramuscular administrations and poor IgG responses and weak to borderline IgA responses in vaginal secretions. As those samples were contaminated with blood, the origin of these antibodies was unclear.

No specific HIV-1 inhibitory activity could be detected in the serum and vaginal secretions collected one day and 14 days after the fourth immunisation despite the presence of HIV-specific IgG and IgA measured by ELISA.

3.12 - CLINICAL DEVELOPMENT

Two tracks were defined that focused on the preparation and conduct of clinical trials to validate in human the data found in Discovery:

- the Parenteral track,
- the Mucosal track.

In both cases, the objective was to investigate the capacity of the selected candidate/formulation/adjuvant/route(s) to induce an immune response in both blood and genital mucosa.

The criteria to move to clinical trial were based on preclinical immunogenicity and toxicology data.

Parenteral route trial could be started if i) functional Abs were raised in blood by the selected Ag and ii) binding Abs were elicited in mucosa

Mucosal route trial, possibly a mucosal/parenteral trial, could be started if binding Abs were detected in mucosa.

3.12.1 - Mucosal clinical trial with EN41-FPA2 (study EN41CT1.1.1.)

The Phase I clinical trial with EN41-FPA2 was started in January 2012 - after MHRA Regional Ethical Committee and local R&D approvals - at Surrey CRC, UK, with PX Therapeutics as the sponsor - and was completed in June 2013 with the LVLS.

This was a First in Human Phase 1, randomised, single-centre, observer-blind clinical trial of safety and immunogenicity of nasal-prime and intramuscular boost immunisation with EN41-FPA2 HIV vaccine in healthy female volunteers.

Study was conducted on 48 volunteers, and dose of EN41-FPA2 vaccine was (for both IN & IM administrations) 200 µg of FPA2 protein + 160 µg MPLA. For Nasal administration, 1 mL of EN41-FPA2 suspension was mixed v/v with HEC gel.

There were 3 Nasal Administrations and 2 IM boosts, all spaced-out of a month, and a 6-month follow-up.

For safety purposes the enrolment was stepwise, in three cohorts receiving increasing doses of the vaccine by nasal route (dose escalation) whereas the IM dose remained constant.

Beside safety, the primary objective of the trial was the evaluation of EN41-FPA2 immunogenicity assessed by measurement of specific serum IgG responses by ELISA. The secondary objective comprised the measurement of specific binding Abs in vaginal secretions and the neutralising activity against HIV in serum and vaginal samples using PBMC and TZM-bl assays.

HIV inhibitory activity was measured using a macrophage assay where the role of Ab Fc is taken into consideration together with Fab since, by binding to Fc receptors on cell surface, virus-Ab complexes can be phagocytosed by macrophages. Ab-dependent cell cytotoxicity in presence of NK cells was also investigated as well as aggregation and virus capture to assess the capacity of Abs to block the virus on the mucosal surface. Inhibition of HIV transfer from DC to CD4+ T lymphocytes was explored since DC are one of the first target cells for the virus in mucosa and they can transport and disseminate the virus to other cells.

All these assays were used for exploratory end-points.

Main results & conclusions:

- Three nasal priming followed by two IM boosts with EN41-FPA2 is safe and well tolerated. There were 3 withdrawals during the study, in Cohort 3, however no subject was withdrawn due to safety concern.
- EN41-FPA2 is immunogenic and is able to induce systemic specific IgG and IgA responses, as well as mucosal specific IgG and, at lower level, IgA responses.
- Immunisation with EN41-FPA2 did not elicit neutralizing activity in blood nor in mucosa measured by PBMC assay (some problems of background inhibitory activity occurred for PBMC assays in soft-cups). However it could induce low inhibitory responses in mucosa detected by TZM-bL assay.
- Regarding more specifically the role of nasal priming, EN41-FPA2 could not elicit a response when given by the nasal route, but there was a trend for earlier responses after IM boost by ELISA in serum and more frequent low inhibitory activity in the mucosa detected by TZM-bL assay. In addition, by transcriptomics, nasal priming clearly activates specific genes compared to placebo group.

3.12.2 - Parenteral clinical trial with EN41-UGR7C (study EN41CT1.1.2.)

The Phase I clinical trial with EN41-UGR7C was started in April 2013 - after MHRA Regional Ethical Committee and local R&D approvals - at the Royal Free Hospital, UK, with PX Therapeutics as the sponsor - and was completed in February 2014 with the LVLS.

This was a First In Human Phase 1, randomised, single-centre, observer-blind, placebo-controlled trial of safety and immunogenicity of EN41-UGR7C HIV vaccine candidate administered intramuscularly in healthy female volunteers

Twenty four volunteers were enrolled in this study and were distributed into 2 groups:

- . Group 1 (15 subjects) receiving the protein (210 µg of UGR7-C) mixed in alum (560 µg)
- . Group 2 (9 subjects) receiving 0.9% sodium chloride.

There were 3 intramuscular administrations at months 0, 1, 4, and a 3-month follow-up duration.

There was no withdrawal during study.

Main results & conclusions:

- Three intramuscular administrations with EN41-UGR7C is safe and well tolerated.
- EN41-UGR7C induced potent serum IgG responses detected after second IM, improved significantly after third IM. Serum binding IgA responses were also induced albeit at lower levels. However serum Abs responses rapidly waned post last vaccination.
- Low level intermittent mucosal IgG responses were seen in the active group.
- There was no neutralizing activity detected neither in serum or vaginal samples, there was however some problems of background inhibitory activity.

3.12.3 - Willingness Questionnaire in Haiti

The efficacy of a HIV vaccine can only be tested in a country with a high incidence of infection. After the phase I in UK where the safety of the vaccine is evaluated, the next development phase could take place in Haiti, a developing country where the incidence of HIV infection is still high.

A collaboration with the US was discussed and accepted by the Scientific Officer of the European Commission. A letter proposing a new phase I trial in Haiti with EuroNeut-41 vaccine candidates was sent by Gheskio and Sanofi Pasteur coordinator to the HIV Vaccine Trials Network (HVTN).

Meanwhile, to determine the interest of people in Haiti to participate to a vaccine clinical trial, it was proposed to administer a generic “willingness to participate” questionnaire to females coming for services at GHESKIO and found to be in good health. The questionnaire was proposed by social workers specifically trained to offer this type of services.

Interestingly, the large majority of the interviewed women are willing to participate to a vaccine trial. Not surprisingly, most are in primary/secondary school and with no or low income. This justifies the importance of having several ethical committee reviews before initiation of a trial in this country.

3.12.4 - Safety and immunogenicity of SL immunisation in Human

A characterization study was performed in human to explore the systemic and mucosal immune response induced by a vaccine after SL administration. For this purpose, a tetravalent HPV subunit vaccine was used by SL or IM route in a group of 12 and 6 healthy female volunteers aged 19-31 years, respectively. The vaccine was given at 0, 4 and 16 weeks.

IM Ags delivery induced or boosted HPV-specific serum IgG and pseudovirus-neutralizing Abs, HPV-specific cervical and vaginal IgG, and elicited circulating IgG and IgA ASCs.

SL Ags induced ~38- fold lower serum and ~2-fold lower cervical/vaginal IgG than IM delivery, and induced or boosted serum pseudovirus neutralizing Abs in only 3/12 subjects.

Neither route reproducibly induced HPV-specific mucosal IgA.

This Characterisation study “Systemic and mucosal immune responses to sublingual (SL) or intramuscular (IM) Human Papilloma Virus (HPV) antigens in healthy female volunteers” was completed and the manuscript was published in PLOs One.

In conclusion, alternative delivery systems and adjuvants should be required to enhance immune responses following SL immunization in Human. The study in NHP (see above) suggested that coupling the immunogen to a mucoadhesive such a CTB could improve the response.