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Human Papillomavirus Type 16 Viral Load is Decreased Following a Therapeutic Vaccination

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Abstract

In the dose-escalation phase of a Phase I clinical trial in which six subjects each were vaccinated with PepCan at the 50, 100, 250, and 500µg per peptide dose, the 50µg dose showed the best histological regression rate. Ten additional subjects were vaccinated at this dose in the final dose phase. As with the dose-escalation phase, no dose-limiting toxicities were observed. Overall, the histological regression rates were 50% at the 50µg dose (7 of 14) and 100µg dose (3 of 6), and

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Conflict Of Interest: Mayumi Nakagawa is one of inventors named in patents and patent applications describing PepCan. Other authors do not have any conflict of interest to disclose.

Ethical Approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the Institutional Review Board, the Food and Drug Administration, and with the 1964 Helsinki declaration including its later amendments.

Informed Consent: Informed consent was obtained from all individual participants included in the study.

45% overall (14 of 31). Of subjects in whom HPV type 16 (HPV 16) was detected at entry, it became undetectable in 3 subjects after vaccination, and the viral loads significantly decreased in 9 subjects in whom HPV 16 infection was detected at entry and exit ($p=0.008$). Immune profiling revealed increased T-helper type 1 cells after vaccinations ($p=0.02$ and $p=0.0004$ after 2 and 4 vaccinations respectively). T-helper type 2 cells initially increased after 2 vaccinations ($p=0.01$), but decreased below the baseline level after 4 vaccinations although not significantly. Pre-vaccination regulatory T-cell levels were significantly lower in histological responders compared to non-responders ($p=0.03$). Feasibility of testing plasma for multiplex cytokine/chemokine analysis and of performing proteomic analysis of PBMCs was examined for potentially identifying biomarkers in the future. While these analyses are feasible to perform, attention needs to be given to how soon the blood samples would be processed after phlebotomy. As sufficient safety of PepCan has been demonstrated, enrollment for the Phase II clinical trial has been opened.

Keywords

HPV; viral load; cervical intraepithelial neoplasia; therapeutic vaccine; T-cells; clinical trial

Introduction

Cervical cancer cases are almost always associated with high-risk HPVs with an annual incidence of 528,000 cases and mortality of 266,000 cases globally [1]. Although effective preventative measures such as Papanicolaou smear screening, HPV-DNA testing, and prophylactic vaccines are available, cervical cancer is still the fourth most common cancer in women worldwide. HPV can also cause anal, oropharyngeal, penile, vaginal, and vulvar cancers, and is estimated to be responsible for 5.2% of the cancer burden [2,3]. Of approximately 200 HPV types described to date, a few dozens are considered to be high-risk or associated with malignancy. HPV type 16 (HPV 16) is the most common, and accounts for approximately half of cervical cancer cases [4]. Therefore, virtually all HPV vaccines include HPV 16 as a source of antigen. Many versions of HPV therapeutic vaccines are in development (recently reviewed in [5]) including a DNA-based vaccine (E6 and E7 genes of HPV types 16 and 18 delivered by electroporation) for which successful Phase II clinical trial results were recently reported [6]. The HPV therapeutic vaccine being developed by our group, called PepCan, contains four current good manufacturing practice-grade synthetic peptides covering the HPV 16 E6 protein and Candin® (Allermed, San Diego, CA), a colorless extract of *Candida albicans*, as a novel adjuvant [7]. The idea to use Candin as a vaccine adjuvant came about from clinical studies that showed that injections of recall antigens such as Candin are effective in regressing common warts [8-13]. Furthermore, *in vitro* studies have shown T-cell proliferative effects of Candin and its ability to induce IL-12 secretion by Langerhans cells [14], the main antigen presenting cells in skin.

While the standard surgical treatments for high-grade squamous intraepithelial lesion (HSIL) are very effective, a non-surgical alternative is needed because of a recently recognized, unintended side effect, namely an increase in the pre-term delivery rate from 4.4% to 8.9% [15]. Furthermore, prophylactic vaccines are not effective once HPV infection is established [16]. Therefore, if an HPV therapeutic vaccine is approved for clinical use, it is likely to

become the first line therapy for women with HSIL who wish to become pregnant. The dose-escalation portion of this single center, single arm, dose-escalation, Phase I clinical trial (NCT00569231) was previously reported [17]. PepCan was administered intradermally every 3 weeks, and a loop electrical excision procedure was performed 12 weeks after the last injection. This manuscript reports the results of the final dose phase, and the feasibility of performing cytokine/chemokine and proteomic analyses for identifying potential biomarkers in the Phase II study (NCT02481414).

Methods

Final Dose Part of Phase I Clinical Trial

The study design, vaccine composition, methods for laboratory testing (peripheral HPV 16-specific T-cell responses, peripheral immune cell phenotype, and HPV-DNA), and statistical analyses were previously described for the dose-escalation portion of the clinical trial [17]. Subjects with biopsy-confirmed HSIL regardless of HPV types detected were eligible. In the final dose phase, ten additional subjects were vaccinated at the 50µg per peptide dose, which was shown to have the highest histological regression rate. This protocol was approved by the Institutional Review Board, and a written informed consent was provided by each subject.

HPV 16 Viral Load

A method described by Mirabello and colleagues was followed [18] to determine the HPV 16 viral load in subjects in whom HPV 16 was detected at entry and at exit using Linear Array HPV Genotyping Test (Roche Molecular Diagnostics, Pleasanton, CA). Thin-Prep samples (Hologic, Marlborough, MA) containing cervical cells were used for both analyses. Briefly, quantitative real time PCR was carried out using iQ SYBR Green PCR Master Mix (BioRad Laboratories, Hercules, CA) with diluted DNA and HPV 16 E6 primers (5'-aaagccactgtgtcctgaaga-3' and 5'-ctgggtttctctacgtgttct-3', [19]) or GAPDH primers (5'-cgagatccctccaaaatcaa-3' and 5'-catgagtccttcacgataccaa-3', [20]). Amplifications were performed using a BioRad CFX 96 Real-Time PCR Detection System (BioRad Laboratories) with an initial denaturation at 95°C for 5 min. This step was followed by 40 cycles of 95°C for 10 sec and 55°C for 20 sec for HPV 16 E6; or 30 cycles of 95°C for 15 sec, 50°C for 30 sec, and 72°C for 20 sec for GAPDH. Data acquisition was performed at 510 nm at 55°C and 72°C respectively. The standard curve for HPV was derived by amplification of a serially diluted quantitative synthetic HPV 16 DNA (American Type Culture Collection, Manassas, VA) in the presence of 100pg of human placental DNA. The standard curve for GAPDH was obtained using serial 10 fold dilution of placental DNA starting with 250ng of DNA. The amount of DNA was converted to cell number by assuming that 6.6pg of DNA is present in a diploid cell [18]. Mean threshold cycle (CT) values of triplicate specimens at each dilution were used to generate standard curves, and mean CT values of sample duplicates were used for calculating quantities. Entry and exit DNA samples from the same subjects were analyzed in the same PCR run. HPV 16 viral loads were determined using E6 copy numbers, and were not meant to be an absolute quantification. The specificity of amplifications was determined through melting curve analyses.

Multiplex Cytokine/Chemokine Analysis

Plasma samples from 19 consecutive blood samples drawn at a research clinic between July 2014 and March 2015 were separated by centrifugation and frozen in a -80°C freezer at 1 hour and 2 hours after blood draw. Plasma samples separated within 1 hour from pre-vaccination blood sample and post-2 vaccination blood samples were available from 19 subjects throughout the study. Pre-vaccination plasma samples only were available from 2 more subjects (n=21). The quantities of IL-1 β , IL-1 receptor agonist (IL-1RA), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17A, eotaxin, basic fibroblast growth factor (FGF), G-CSF, GM-CSF, IFN- γ , IFN- γ induced protein 10 (IP-10), monocyte chemoattractant protein 1 (MCP-1), MIP-1 α , MIP-1 β , platelet-derived growth factor subunit B (PDGF-BB), regulated on activation, normal T-cell expressed and secreted (RANTES), TNF- α , vascular endothelial growth factor (VEGF), IL-2 receptor α (IL-2R α), chemokine (C-X-C motif) ligand 1 (CXCL1), hepatocyte growth factor (HGF), IFN- α 2, LIF, chemokine (C-C motif) ligand 7 (CCL7), macrophage migration inhibitory factor (MIF), chemokine (C-X-C motif) ligand 9 (CXCL9), β -nerve growth factor (β -NGF), stem cell factor (SCF), stem cell growth factor β (SCGF- β), TRAIL, IL-16, and IL-18 in selected plasma samples were determined using a commercially available Bio-Plex kit (Bio-Rad Laboratories) according to the manufacturer's instructions using a MAG-PIX instrument (Bio-Rad Laboratories). Plasma levels of TGF- β 1, TGF- β 2, and TGF- β 3 were determined using a separate Bio-Plex kit as acid treatment was required.

Proteomic Analysis

Blood was drawn from a healthy donor (40ml) and vaccine recipients (60-80ml) in vacutainer tubes containing sodium heparin, and PBMCs were isolated using a ficoll-hypaque gradient method. For proteomic analysis, one vaccine recipient was from the 50 μ g/peptide group, two vaccine recipients were from the 100 μ g/peptide group, and two additional vaccine recipients were from the 250 μ g/peptide group. One subject was a complete responder. Another subject was a partial responder while the remaining three subjects were non-responders.

For the healthy donor, half of the blood sample was processed on the day of the blood draw, and the other half on the following day. For vaccine recipients, all samples were processed on the day of blood draw and as soon as possible. Twenty ml of blood was diluted with 15ml of sterile 0.9% sodium chloride (NaCl) solution in a polystyrene 50ml conical tube, and 15ml of Ficoll-Paque PLUS (GE Healthcare, Fairfield, CT) was gently underlaid below the mixture. The tube was centrifuged (485g) for 25 minutes at room temperature, with the brake turned off. The buffy coat layer was transferred to another tube using a sterile polyethylene transfer pipet, and the tube was filled with NaCl solution to the 50ml mark. The tube was centrifuged at 175g for 20 min. The cells in 10% of the overlaying wash buffer were retained and washed once more in NaCl solution (485g for 10 minutes). PBMCs were frozen in FCS with 10% DMSO, and stored under liquid nitrogen.

Duplicate proteomic analyses were conducted on PBMCs from the healthy donor. PBMC samples from vaccine recipients were analyzed once. The frozen cell pellets were transported on dry ice to the University of Arkansas for Medical Sciences Proteomics Core

Laboratory where mass spectrometry was performed. After thawing, cell pellets (3×10^6) were lysed and digested with trypsin using filter aided sample preparation (FASP) in 4% SDS, 100mM Tris-HCl pH 7.6, 0.1M DTT [21]. DNA was sheared by sonication and lysate clarified by centrifugation. Protein extracts were alkylated with 50mM iodoacetamide in 8M urea, 100mM Tris-HCl pH 8.5. Protein was then digested with sequencing grade porcine trypsin (Promega, Madison, WI) at a 1:100 enzyme to protein ratio in 50mM ammonium bicarbonate. One hundred μ g of each lysate was digested and one fiftieth of the digest was analyzed for each sample by mass spectrometry. Tryptic peptides were separated on reverse phase Jupiter Proteo resin (Phenomenex, Torrance, CA) on a 100×0.075 mm column, using a nanoAcquity UPLC system (Waters Corporation, Milford, MA). Peptides were eluted using a 60 minute gradient from 97:3 to 35:65 buffer A:B ratio. Buffer A = 0.1% formic acid, 0.5% acetonitrile; buffer B = 0.1% formic acid, 75% acetonitrile. Eluted peptides were ionized by electrospray (1.9 kV) followed by MS/MS analysis using collision induced dissociation on an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, San Jose, CA). MS data were acquired using the Fourier transform mass spectrometry analyzer in profile mode at a resolution of 60,000 over a range of 375 to 1500 m/z with lockmass correction (445.1200 m/z). MS/MS data were acquired for the top 15 peaks from each MS scan using the ion trap analyzer in centroid mode and normal mass range with a normalized collision energy of 35.0. Tandem mass spectra were extracted by Thermo MSFile Reader version 2.2. Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using MaxQuant (Max Planck Institute of Biochemistry, Martinsried, Germany; version 1.3.0.5) to search the IPI human protein database (version 3.87, 91520 entries), assuming the digestion enzyme strict trypsin using 1.0% false discovery rate thresholds for both protein and peptide identification. Following an initial re-calibration of peptide masses at 5ppm tolerance, MaxQuant analysis was performed with a fragment ion mass tolerance of 0.5 Da and a parent ion tolerance of 2ppm. Carbamidomethyl of cysteine was specified as a fixed modification. Oxidation of methionine and acetylation of protein N-termini were specified as variable modifications. The MaxQuant label-free quantification algorithm [22] was applied to allow comparison across individual MS runs. Scaffold (version 4.3.2, Proteome Software Inc., Portland, OR) was used to verify MS/MS based peptide and protein identifications. MaxQuant precursor ion intensity data were normalized in Scaffold by summing the top three unique peptide precursors for each protein. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold Local false discovery rate algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides in at least one of the biological samples. Protein probabilities were assigned by the Protein Prophet algorithm [23]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Statistical Analysis

Wilcoxon signed-rank test was used to compare (1) pre-vaccination and post-4 vaccination HPV 16 viral loads in persistently HPV 16-positive subjects, and (2) pre-vaccination and post-2 vaccination plasma cytokine/chemokine levels. Wilcoxon rank-sum test was used to compare pre-vaccination cytokine/chemokine levels between histological responders and

non-responders. Non-parametric repeated measures analysis was used to compare cytokine/chemokine levels between plasma samples separated at 1 hour and 2 hours after phlebotomy.

With the proteomics data, normalized precursor intensities were log-transformed for statistical analysis to stabilize variance. Pearson's correlation coefficient was used to evaluate the consistency of intensities between replicate samples from the healthy donor. For samples from the 5 vaccine recipients at 3 time-points, proteins were tested for change in mean log-intensity from baseline to post-4-vaccinations using a paired version of the moderated *t*-test, as implemented in the R/Bioconductor package *limma* [24]. The package *limma* is widely used in gene expression array analysis and increasingly in proteomic contexts. The moderated *t*-test is an empirical Bayes approach, equivalent to shrinkage of the estimated sample variances towards a pooled estimate, resulting in more stable inference when the sample size is small. We only tested those 294 proteins for which at least two patients had intensity data available at both baseline and post-4-vaccinations, which ensured that variance estimates were specific to each protein. Corrections for multiple comparisons were not performed.

Results

Final Dose Part of Phase I Clinical Trial

The demographic characteristics, HPV types, new immune responses to HPV 16 E6, and histological analyses of subjects who received vaccination in the final dose phase is summarized in Supplementary Table 1. Eight of 10 subjects completed the study. As with the dose-escalation part of the Phase I clinical trial [17], no dose-limiting toxicities were reported, and the most common adverse event (AE) was mild to moderate immediate injection site reactions which were reported with all injections. The AEs for subjects who received the 50µg per peptide dose (6 subjects from the dose-escalation phase and 10 subjects from the final dose phase) are summarized in Supplementary Table 2.

Histological regression rates, virological response rates, and immunological response rates of different dose levels are summarized in Table 1. The final regression rate of the 50µg dose group (n=14) was 50%, which was the same as the 100µg dose (n=6). Although the study is not powered to detect differences in efficacy among different dose levels, there was a trend of lower doses (50 and 100µg) being more effective than the higher doses (250 and 500µg). No statistically significant differences were detected when histological response rates were compared (1) between subjects with entry diagnosis of cervical intraepithelial neoplasia (CIN) 2 versus CIN3, (2) between subjects < 25 years of age versus > 25 years of age, and (3) between subjects who were HPV 16-positive versus those who were not. The number of HPV types detected prior to vaccination ranged from 0 to 6 types (Supplementary Table 1 and Table 1 of [17]). The rate of at least one HPV type becoming undetectable at exit was the highest (85%) at the 50µg dose (Table 1). When the HPV types which became undetectable were grouped into HPV 16, HPV 16-related, other high-risk types, and low-risk types, the rate of undetectability was paradoxically higher for non-HPV 16 types (Supplementary Table 3). Immunological responses to HPV 16 E6, as measured by IFN- γ ELISPOT assay, were similar among the first 3 dose levels in terms of detecting positive response to at least one new E6 region and for the increase in response being statistically

significant (Table 1). The lowest response rate was observed for the 500µg dose level. In 3 of 8 subjects who completed the final dose phase, the increase in CD3 T-cell response to at least one HPV 16 E6 region was statistically significant, as shown in Supplementary Fig. 1. No significant increases to HPV 16 E7 regions were measured.

Immune profiling (Fig. 1) showed statistically significant increases in circulating T-helper type 1 (Th1) cells after 2 ($p=0.02$) and 4 vaccinations ($p=0.0004$). T-helper type 2 (Th2) cells initially increased significantly ($p=0.01$) but decreased to below the baseline level after 4 vaccinations, although not significantly. Regulatory T-cell (Treg) levels were minimally changed. The differences in Treg levels prevaccination ($p=0.03$) and post-2 vaccinations ($p=0.04$) between these two groups were statistically significant (Fig. 1b).

HPV 16 Viral Load

Of 13 subjects in whom HPV 16 DNA was detected prior to vaccination, it became undetectable in 3 subjects and was persistent in 9 subjects. One HPV 16-positive subject did not complete the study (Supplementary Table 1). In the patients with persistent HPV 16 viral loads, a significant decrease (mean of 840 copies per cells to 76.6 copies per cell, $p=0.008$) was observed in 8 of the 9 patients (Fig.2).

Multiplex Cytokine/Chemokine Analysis

The comparisons of cytokine/chemokine plasma concentrations between plasma separated at 1 hour and 2 hours after 19 blood draws revealed statistically significant differences in 15 of 44 analytes (Table 2). Most were decreased over time but some increased. Comparisons of cytokine/chemokine levels between prevaccination and post-2 vaccination revealed a statistically significant decrease only in RANTES (median decrease of 1,703pg/ml, decrease observed in 14 of 19 subjects examined, $p=0.003$). Comparison of prevaccination cytokine levels between histological responders and non-responders showed significantly higher levels among responders compared to non-responders in 8 of 44 analytes examined (Supplementary Table 4).

Proteomic Analysis

Proteomic analysis of PBMCs from a healthy donor was carried out by processing them on the day of and one day after the blood draw (each in duplicate), while PBMC samples from the five selected vaccine recipients were processed from blood samples drawn prior to vaccination, after two vaccinations, and after 4 vaccinations. A total of 548 proteins were present in at least one replicate from the healthy donor. In samples drawn from the healthy donor, normalized precursor intensities from duplicate liquid chromatography-dual mass spectrometry (LC-MS/MS) runs on PBMCs processed on the same day as blood draw were highly correlated ($r=0.85$, Fig. 3), as were data from replicate runs on PBMCs processed on the day after blood draw ($r=0.92$) among the 304 proteins detected in all 4 runs. However, correlation coefficients of four possible comparisons between same day PBMCs and next day PBMCs were substantially lower ($r=0.66$, 0.64, 0.75 and 0.70). Therefore, the timing of PBMCs processing should be kept constant.

A total of 651 proteins were detected in at least one of 15 samples (5 patients $\times$ 3 time-points). A paired moderated *t*-test identified 24 proteins significantly changed in abundance between baseline and after four vaccinations ($p < 0.05$), with 17 decreasing and 7 increasing. The molecular weight, fold change between baseline and post-4 vaccination, *Limma* *p*-value, and biological functions of these 24 significantly changed proteins are summarized in Table 3.

Discussion

As this study was a Phase I clinical trial, the primary end point was safety. No dose-limiting toxicities were reported during the dose-escalation [17] and the final dose phases demonstrating that PepCan is safe. The most common AE was immediate injection site reactions, which occurred with 100% of injections, and were characterized with redness and itching. Other commonly reported AEs were myalgia, nausea, headache and fatigue ([17] and Supplementary Table 2). In addition, we examined histological response, virological response, immunological response (Table 1, Supplementary Fig. 1), and also characterized peripheral immune cells before and after vaccinations (Fig. 1). Although the 50 μ g dose level was chosen for the final dose phase for having had the best histological regression rate (83%) in the dose-escalation phase ($n=6$), the final regression rate of the 50 μ g dose at the study completion ($n=14$) was the same as the 100 μ g dose, suggesting that the initial higher response may have been a statistical aberration due to a small sample size. However, the virological response was still the highest at the 50 μ g dose. The lowest response rates for the highest dose (500 μ g) among histological, virological, and immunological responses are consistent and raises a possibility that immune tolerance may have been induced when a larger amount of antigens were injected.

To our knowledge this is the first clinical trial of an HPV therapeutic vaccine which examined HPV 16 viral loads in addition to HPV-DNA testing. Although 9 subjects had persistent HPV 16 infection, the viral loads were significantly decreased (Fig. 2) suggesting that longer observation may lead to even lower HPV 16 viral load or even reaching levels below the threshold of detection. This rationale justifies the design of the Phase II clinical trial in which the observation period will be extended to 12 months from the time of last vaccination instead of 3 months used in the Phase I clinical trial. The decision to extend the observation period to 12 months was made previously based on results of another clinical trial of peptide-based HPV therapeutic vaccine in which women with HPV 16-positive high-grade vulvar intraepithelial (VIN) lesions were treated [25]. Complete histological regression rate of 25% at 3 months was nearly doubled to 47% at 12 months. No enhancement of regression was recorded by 24 months. Although no data appear to be available for change in HPV 16 viral loads in unvaccinated women with CIN2/3, only 38% decrease in 1 year (from 0.85 copies per cell to 0.53 copies per cell) was reported in unvaccinated women under 30 who had normal cervical cytology [26].

The detectability of HPV 16 after vaccination in those subjects who had HPV 16 at entry may correlate well to histological regression, as the 3 subjects whose HPV 16 infection became undetectable all had no evidence of dysplasia in loop electrical excision procedure samples. On the other hand, 7 of 9 subjects in whom HPV 16 infection persisted were

histological non-responders, one subject was a partial responder, and another subject was a complete responder who had CIN 1 in loop electrical excision procedure sample. In a clinical trial of another HPV therapeutic vaccine in which patients with high-grade vulvar intraepithelial neoplasias were treated with a topical immunomodulator, imiquimod, for 8 weeks and were injected with TA-CIN (fusion protein of HPV 16 E6, E7, and L2 proteins) at weeks 10, 14, and 18 [27], 63% (12 of 19) had complete histological regression at 52 weeks while only 36% (5 of 14) showed clearance of HPV 16 suggesting HPV 16 infection persisted longer beyond regression of vulvar intraepithelial neoplasias in that study.

As our earlier *in vitro* studies [14,7] have demonstrated that *Candida* most commonly induces IL-12 secretion by Langerhans cells, whether HPV peptides are present or not, the Th1 promoting effect after 2 and 4 vaccinations ($p=0.02$, and 0.0004 , respectively; Fig. 1a) is likely to be an adjuvant effect. The adjuvant only arm included in the Phase II clinical trial will further elucidate the role of *Candida* in Th1 promotion. Th2 level curiously increases initially after 2 vaccinations ($p=0.01$), but decreased below the baseline after 4 vaccinations. It may be that the presence of HSIL or the condition that allows progression to HSIL maybe Th2 promoting which requires more than 2 injections to be reversed. While Treg levels were basically unchanged following vaccinations, the Treg levels prior to vaccination and after 2 vaccinations were significantly different between histological regressors and persistors ($p=0.03$ and 0.04 , respectively; Fig. 1b). These results raise a possibility that pre-vaccination Treg level maybe used as a biomarker for histological non-responsiveness to therapeutic vaccination, as no subject with pre-vaccination Treg levels $\geq 0.7\%$ responded.

In order to incorporate additional analyses for identifying potential biomarkers in the Phase II study, selected plasma and PBMC samples were analyzed to assess the feasibility of performing multiplex cytokine/chemokine assay and proteomic analysis, respectively. Attention would need to be paid as to how soon after blood draw samples are processed as 34% (15 of 44) of cytokines/chemokines analyzed had statistically significant differences between values determined between plasma samples separated 1 hour and 2 hours after blood draw (Table 2). Likewise, correlation coefficients of comparisons between same day PBMCs and next day PBMCs were lower compared to those from duplicates from same day as well as the next day, suggesting the importance of being consistent as to how long after the blood draw the samples should be processed.

When changes in the prevaccination and post-2 vaccination cytokine/chemokine levels were compared, only RANTES was significantly decreased. RANTES is also known as chemokine (C-C motif) ligand 5 or CCL5, and it binds to a G protein coupled receptor [28]. It has chemotactic role for T cells [29,30], eosinophils [31], monocytes [29], dendritic cells [32], mast cells [33], and basophils [33], and it can also activate NK cells [34]. Higher plasma RANTES levels in order of stages IV, III, II cervical and breast cancer cases have been reported [35]. For the current study, whether or not this significant decrease can be reproduced in our Phase II clinical trial as well as whether the decrease extends beyond 2 vaccinations would be of interest to determine.

When pre-vaccination cytokine/chemokine levels were correlated with histological response, 8 cytokine/chemokines were significantly elevated in histological responders compared to

non-responders (Supplementary Table 4). As the purpose of these analyses were to establish the feasibility for Phase II, corrections for multiple analyses were not performed. While the paradoxically higher levels of Th2 cytokines (i.e., IL-6 and IL-10) is interesting, one would need to wait for the results of future studies to draw conclusions. In short, the Phase I clinical trial of PepCan has demonstrated its safety, and decrease in HPV 16 viral load in those who were HPV 16-positive at entry. It would be feasible to perform multiplexed cytokine/chemokine analysis of plasma samples and proteomic analysis of PBMC samples, but attention would need to be paid as to when these samples are processed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AE	adverse event
β-NGF	β-nerve growth factor
CCL7	chemokine (C-C motif) ligand 7
CIN	cervical intraepithelial neoplasia
CT	threshold cycle
CXCL1	chemokine (C-X-C motif) ligand 1
CXCL9	chemokine (C-X-C motif) ligand 9
FASP	filter aided sample preparation
FGF	basic fibroblast growth factor
HGF	hepatocyte growth factor
HPV 16	HPV type 16
HSIL	high-grade squamous intraepithelial lesion

IL-1RA	IL-1 receptor agonist
IL-2Rα	IL-2 receptor α
IP-10	IFN- γ induced protein 10
LC-MS/MS	liquid chromatography-dual mass spectrometry
MCP-1	monocyte chemotactic protein 1
MIF	macrophage migration inhibitory factor
NaCl	sodium chloride
PDGF-BB	platelet-derived growth factor subunit B
RANTES	regulated on activation, normal T-cell expressed and secreted
SCF	stem cell factor
SCGF-β	stem cell growth factor β
VEGF	vascular endothelial growth factor

Fig. 1.

a, circulating immune cells before, after 2, and after 4 vaccinations in all vaccine recipients who completed the study (n=31). **b**, circulating immune cells in responders (●) and non-responders (■). Percentages of CD4 cells positive for CD4 and Tbet are shown for Th1 cells, positive for CD4 and GATA3 are shown for Th2 cells, and positive for CD4, CD25, and FoxP3 for Tregs. The bars represent standard error of means. Data for the subjects from the dose-escalation phase (n=23) was previously described [17].

Fig. 2.
HPV 16 viral loads for subjects who were positive for HPV 16 at entry and exit (n=9).

Fig. 3.

Scatterplot matrix of protein intensities in PBMCs from a blood sample drawn from a healthy donor. Half of the sample was ficolled immediately on the day of the blood draw, and the other half was processed the following day. Each portion was analyzed in duplicate. The data shown are signal intensities of the peptide precursors representing specific proteins. Signal intensities were normalized to the overall signal intensity across all proteins within a run, and were log-transformed (base 10) to correct for the right-skewed distribution. The panels with dots show the six pairwise scatterplots of signal intensity of the 4 runs. Each point represents one of the 304 proteins detected in all 4 runs. The scatterplots were overlaid with lowess smoothers to aid visualization. The panels with numbers show the Pearson correlation coefficient for each pair of runs.

Table 1

Summary of histological, virological, and immunological responses to PepCan.

Characteristics	Dose (n)	50µg (14)	100µg (6)	250µg (6)	500µg (5)	All (31)
Histological regression, % (n)						
Responders		50 (7)	50 (3)	33 (2)	40 (2)	45 (14)
Virological response, ^a % (n)						
		85 (11 of 13) ^b	50 (3)	50 (3)	40 (2)	63 (19 of 30) ^b
Immunological response, % (n)						
New response to HPV 16 E6		64 (9)	67 (4)	83 (5)	20 (1)	61 (19)
Significant response to HPV 16 E6		43 (6)	50 (3)	50 (3)	20 (1)	42 (13)

^aAt least one HPV type detected at entry becoming undetectable at exit.

^bOne subject had no HPV detected at entry in the 50 µg group.

Difference between cytokine/chemokine measurements from plasma samples separated at 1 hour and 2 hours after phlebotomy.

Table 2

Cytokine	Minimum (pg/ml)	Median (pg/ml)	Mean (pg/ml)	Maximum (pg/ml)	P-value
IL-13	-6.2	-0.6	-1.2	0.4	0.05
IL-17A	-78.3	-5.3	-14.3	11.0	0.03
FGFbasic	-46.8	-5.2	-9.7	7.4	0.04
GSCF	-88.0	-7.4	-20.3	8.8	0.02
MIP1a	-5.5	-0.3	-1.0	0.5	0.01
RANTES	-1,992	1,410	1,712	6,880	0.004
HGF	-25.7	6.5	6.5	30.4	<0.00001
LIF	-52.3	-3.8	-10.8	4.5	0.006
MIF	-76.9	112	318	2,788	0.007
SCGF	-4,622	-2,106	-2,137	175	<0.00001
TRAIL	-122	-11.9	-19.4	3.5	0.00003
IL-16	-50.5	137	228	886	0.00001
PDGFBB	-55.3	30.2	50.9	270	0.03
TGF-β1	-3,037	1,037	1,090	3,252	0.02
TGF-β2	-211	123	90.8	321	0.05

Table 3

Proteins significantly changed in quantity between baseline and 4 vaccinations.

	Accession Number	MW (kDa)	Fold Change	Limma <i>p</i> value	Biological Process
<i>Cellular Metabolism</i>					
trifunctional protein subunit α	IP100031522	83	4.43	0.0095	fatty acid oxidation
acetyl-CoA acetyltransferase 1	IP100030363	45	4.53	0.0279	fatty acid oxidation
voltage-dependent ion-selective channel protein 3	IP100031804	31	0.38	0.0347	mitochondrial porin, cilium inhibition
facilitated glucose transporter 3	IP100003909	54	0.24	0.0158	glucose transport
fructose biphosphate aldolase	IP100418262	48	0.28	0.0305	glycolysis
<i>Oxidative damage prevention</i>					
peroxiredoxin-6	IP100220301	25	0.33	0.0131	peroxidase, phospholipase
<i>Proteases</i>					
heparanase-1	IP100410297	61	0.34	0.024	cellular transmigration
cathepsin G	IP100028064	29	0.39	0.0311	pathogen killing, chemokine release, receptor activation
<i>Protease inhibitors</i>					
leucocyte elastase inhibitor	IP100027444	43	0.23	0.0163	intracellular protease inhibition
α -2-macroglobulin	IP100478003	163	4.29	0.0355	Clears serum proteases, cytokine activity modulator
<i>Nuclear Proteins</i>					
histone 1 $\times$	IP100021924	22	3.24	0.0484	nucleolar chromatin packaging
lamin-B2	IP100009771	70	65.69	0.0441	nuclear lamina, nuclear flexibility
matrin-3	IP100017297	95	0.32	0.0309	nuclear matrix
14-3-3 η	IP100216319	28	0.39	0.0232	class switch recombination
<i>Actin-binding proteins</i>					
stomatin	IP100219682	32	0.46	0.0458	ion channel regulator
integrin-linked protein kinase	IP100013219	51	0.36	0.0179	cytoskeletal adaptor
coronin-1C, isoform i3	IP100867509	59	0.23	0.0163	cell motility, calcium signaling
<i>Cell adhesion</i>					
inter- α inhibitor heavy chain H2	IP100305461	107	10.04	0.0216	hyaluronic acid modification

	Accession Number	MW (kDa)	Fold Change	Limma <i>p</i> value	Biological Process
P-selectin	IP100295539	91	0.32	0.0204	endothelial adhesion
<i>Immunomodulation</i>					
cyclophilin A	IP100419585	18	0.42	0.0463	suppresses inflammatory cytokines
<i>Other</i>					
dermcidin	IP100027547	11	3.85	0.0144	cutaneous antimicrobial defense
clusterin	IP100291262	52	0.34	0.0214	multifunctional
multimerin-1	IP100012269	138	0.58	0.0410	thrombus regulation
putative uncharacterized protein	IP100550731	26	0.21	0.0061	unknown