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Authors

Gonzalez, Paula
Hildesheim, Allan
Herrero, Rolando
et al.

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Rationale and design of a Long Term Follow-up study of women who did and did not receive HPV 16/18 vaccination in Guanacaste, Costa Rica

Paula Gonzalez[1,2], **Allan Hildesheim**[3], **Rolando Herrero**[2], **Hormuzd Katki**[3], **Sholom Wacholder**[3], **Carolina Porras**[1], **Mahboobeh Safaeian**[3], **Silvia Jimenez**[2], **Teresa M. Darragh**[4], **Bernal Cortes**[1], **Brian Befano**[5], **Mark Schiffman**[3], **Loreto Carvajal**[1], **Joel Palefsky**[4], **John Schiller**[6], **Rebeca Ocampo**[1], **John Schussler**[5], **Douglas Lowy**[6], **Diego Guillen**[1], **Mark H. Stoler**[7], **Wim Quint**[8], **Jorge Morales**[1], **Carlos Avila**[1], **Ana Cecilia Rodriguez**[1], and **Aimée R. Kreimer**[3] **for the Costa Rica HPV Vaccine Trial (CVT) Group**

[1]Proyecto Epidemiológico Guanacaste, Fundación INCIENSA, Guanacaste, Costa Rica [2]International Agency for Research on Cancer, Lyon, France [3]Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, MD, USA [4]University of California, San Francisco (UCSF), California, USA [5]Information Management Services (IMS), Calverton, Maryland [6]Center for Cancer Research, National Cancer Institute, Bethesda, MD, USA [7]Department of Pathology, University of Virginia, Charlottesville, VA, USA [8]DDL Diagnostic Laboratory, Rijswijk, the Netherlands

Keywords

Human papillomavirus; vaccines; methods; long term follow-up

Introduction

Cervical cancer affects more than 500,000 women per year worldwide (1). Persistent infection with carcinogenic HPV is the necessary cause of cervical cancer (2), and also causes a subset of cancers of the anus, vulva, vagina, penis, and oropharynx (2), comprising

Corresponding author: Paula Gonzalez, Proyecto Epidemiológico Guanacaste, Fundación INCIENSA, Frente aeropuerto Daniel Oduber, Solarium 8C, Liberia, Guanacaste, Costa Rica, pgonzalez@proyectoguanacaste.org.

Conflict of Interest and Role of the Funding Source.

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approximately 70,000 additional cases of HPV-associated cancers per year (3). HPV 16 and 18 are responsible for 70% of cervical cancers (4) and for most cases of HPV-driven cancers at the other anatomical sites (5;6). HPV prophylactic vaccines have the potential to dramatically reduce the burden of HPV-associated disease if incorporated into cervical cancer prevention programs, especially in developing countries.

Two HPV vaccines are approved in most countries: the bivalent (Cervarix®, GlaxoSmithKline Biologicals) and quadrivalent (Gardasil™, Merck and Co, Inc.) vaccines, which confer near complete protection against HPV-16/18 infection and disease in women naïve to these types prior to vaccination (7;8). The quadrivalent vaccine additionally protects against HPV 6 and 11, which cause most genital warts (8). Recently the US Food and Droug Administration (FDA) approved a new nonavalent vaccine produced with technology similar to the quadrivalent vaccine but directed against nine HPV types (HPV 6, 11, 16, 18, 31, 33, 45, 52 and 58).

Data from the Costa Rica Vaccine Trial (CVT)(9), our community-based vaccine efficacy study, confirmed that the bivalent vaccine is highly efficacious against HPV-16/18 persistent infections and resultant CIN2+ among women unexposed to HPV at the time of initial vaccination, and observed partial cross-protection against HPV 31, 33 and 45 comparable to published estimates (10–12). CVT was initiated in 2004 and enrolled 7,466 women aged 18 to 25 years. Women were randomized to receive the HPV or control (Hepatitis-A) vaccine, and were followed for 4 years with high participation rates (9;10;13). Novel findings from our trial included that: 1) the vaccine does not treat existing infections (14); 2) fewer than 3 doses of the vaccine protect as well as the full 3-dose series for 4 years (15); 3) antibodies levels achieved following two doses (0 and 6 months) of the HPV-vaccine are high and only slightly lower than those observed after three doses (one dose antibodies levels were lower than those of two and three doses, but higher than natural infection levels, and remained stably elevated over four years)(16); 4) the vaccine protects against HPV-16/18 infections at the anus and oral region (17;18); 5) vaccine impact declines with increasing age at vaccination (10); 6) vaccination induces cross-neutralizing potential in sera of vaccinated individuals (19); 7) modest levels of antibodies generated by natural HPV infection provide partial protection against re-infection (20); and, 8) vaccination of young adult women leads to a modest decrease in the number of women who require treatment for HPV-associated cervical disease in the initial years following vaccination (21). As promised in the informed consent, at the end of CVT, participants were unblinded to their vaccine status and cross-over vaccination was offered.

At the completion of CVT in 2010, the Long Term Follow-Up Study (LTFU) was implemented, to extend follow-up of CVT participants in the HPV-arm of CVT to 10 years and enroll a new, screening-only, control group in order to provide necessary data that will allow for continued investigation into the risks and benefits of the prophylactic HPV-vaccine.

The goals of this paper are to 1) report the rationale for the LTFU study to extend the follow-up of CVT participants and the inclusion of a new unvaccinated control group

(UCG), 2) describe the design and methods of the LTFU study, 3) present data from the enrollment phase of the LTFU study and 4) evaluate the validity of the UCG.

Rationale for LTFU

The LTFU study was designed in order to evaluate 1) the 10-year impact of HPV-16/18 vaccination of young adult women; 2) determinants of the immune response to HPV and the vaccine and markers of long-term protection; and 3) the natural history of HPV and cervical disease in a vaccinated population, including behavior of other oncogenic HPV types in the absence of HPV-16/18 infections ("disease unmasking").

HPV arm—To evaluate the long-term efficacy of the HPV-vaccine, the follow-up period of CVT women originally vaccinated with the HPV-16/18 vaccine was extended by 6 years with screening at 6, 8, and 10 years after initial HPV vaccination.

Control-arm—Women in the original CVT control-arm, regardless of whether they accepted cross-over vaccination, were followed for 2 additional years, to monitor vaccine safety post-crossover and maximize detection of persistent infections and lesions resultant from HPV exposure that occurred before cross-over to the HPV vaccination. Of these women, roughly 600 accepting cross-over are being followed for the full 6 years as part of a special group providing additional samples for immunogenicity studies.

UCG—To account for the loss of the randomized original control-arm (due to cross-over), a new control group (n=2,827) was enrolled from the same geographic areas and birth cohorts as the original CVT women. Women in this group will be followed for 6 years in LTFU via screening only, to provide a contemporaneous referent group for rates of HPV acquisition, clearance, and disease progression in unvaccinated women.

Ethical justification for the UCG—Women asked to enroll in the UCG are over 20 years old, thus far older than the ideal age for vaccination (9 or 10 to 13 years according to WHO (22)). 50% were older than 26, the maximum age generally recommended for catch-up vaccination (23), and thus vaccination was not standard of care. Among sexually experienced women (97% of those recruited), HPV vaccination is not effective at treating established infection (14), whereas screening programs followed by treatment are highly effective. Participants received high quality cervical cytology screening, due to the extensive quality assurance measures in place in the study (9), HPV testing is used for deciding follow-up among screen-positive women and state-of-the-art treatment is provided when necessary.

Vaccination of adolescents has not yet been incorporated into the Costa Rican national health care system vaccination program, and implementation of catch-up vaccination of young adult women appears highly unlikely to be considered by national authorities. Women may obtain the vaccine outside of the study if they choose; such information will be documented and used in the analytic phase of the study.

Materials and Methods

Brief review of CVT- the randomized, blinded phase

CVT was a community-based, double-blind, randomized controlled phase III trial of the bivalent vaccine, provided by GSK for the trial under a clinical trial agreement with NCI. Between 2004 and 2005, 7,466 women were enrolled and randomized in a 1:1 ratio to receive either Cervarix or Hepatitis-A control vaccine in a three dose schedule at 0, 1 and 6 months.

Women residing in the provinces of Guanacaste and Puntarenas, Costa Rica, identified via a population census specifically conducted for the study, were invited to attend a study clinic. After explanation of study aims and procedures, those willing signed the informed consent. A risk factor interview was administered and a medical history, physical exam and urine pregnancy test were conducted to evaluate their eligibility. Eligibility and exclusion criteria have been published (9). At enrollment and follow up visits, a pelvic examination was performed on sexually-experienced women with collection of cervical cells for liquid-base cytology and HPV-DNA testing, and blood was drawn. Women were followed annually for 4 years, or every 6 months if they had minor cytologic abnormalities (i.e. atypical squamous cells of unknown significance (ASC-US)/HPV-positive and Low-grade squamous intraepithelial lesion (LSIL)). Women with evidence of cytologic high-grade disease (i.e. high-grade squamous intraepithelial lesion (HSIL)/Cancer, atypical squamous cells, cannot exclude HSIL (ASC-H) or atypical glandular cells (AGC)) or with persistent minor abnormalities were referred to colposcopy for evaluation and treatment, when needed.

Participation was 30.5% among invited women and 59.1% among eligible women; compliance with blood and cervical specimen collections was nearly 100%(9). Retention rates were high: only approximately 5% of participants discontinued the study over the four-year study period.

To evaluate vaccine efficacy at non-cervical sites, at the final study visit, oral, vulvar and anal samples were requested (the latter two among sexually-active women only) for HPV-DNA detection.

Post-close out from CVT: Disease ascertainment and Crossover vaccination

With the purpose of detecting as much disease as possible a new colposcopy referral algorithm that considered type-specific high-risk HPV results was implemented after CVT closeout. Women with a history of persistent HPV-16/18 infection were referred for colposcopic evaluation. Women with incident HPV-16/18 or with persistent oncogenic HPV other than 16/18 and those with minor cytological abnormalities at the last CVT visit were referred to accelerate screening every six months if they agreed to participate in the LTFU study. If not they were sent for colposcopic evaluation and treatment if needed.

After CVT participants were informed about their vaccine status, they were offered the study vaccine (HPV or Hepatitis-A) that they did not receive at enrollment into CVT as well as Hepatitis-B vaccine; a new informed consent was obtained. Participants who received Hepatitis-A vaccine at enrollment in CVT (control-arm) were offered HPV vaccination (i.e.

Cervarix®) following a negative urine pregnancy test before each vaccination and Hepatitis-B vaccine (i.e. Twinrix®). Participants who had received the bivalent HPV-vaccine at enrollment in CVT (HPV-arm) were offered Hepatitis-A and B vaccines (i.e. either Twinrix®, Havrix® or Engerix-B® depending on whether the participant was eligible to receive both vaccines or just one of them); no pregnancy test was administered. The medical history was reviewed to confirm that there were no contraindications for vaccination. A total of 2,699 women (77.5% of eligible CVT participants in the control-arm) received at least one dose of the HPV-vaccine, 2,752 (79.6% of eligible CVT participants in the HPV-arm) received at least one dose of Hepatitis-A vaccine and 4,726 (68.1% of eligible CVT participants in both arms) received at least one dose of Hepatitis-B vaccine during crossover.

The first 600 women from the CVT control-arm who received HPV-vaccine during this crossover phase were invited to participate in an immunogenicity subcohort (ISC) designed to collect additional blood samples at vaccination visits, and one month after the final vaccine dose in order to study the immune response to the vaccine.

Initiation of LTFU: Regulatory supervision

The primary IRB reviewing and following the LTFU study was the Costa Rica IRB; the NCI IRB also approved it. An external advisory body ("Working Group") that includes experts from Costa Rica and worldwide was established during CVT to provide scientific support and direction; this group continues to oversee the LTFU study.

Organization of the study

After the crossover phase ended, the Puntarenas clinic was closed to save resources. The LTFU study is being conducted using study clinics located in some of the major districts of Guanacaste. The staff at each clinic includes a clinician, an interviewer, a field work supervisor, a driver and a janitor. The headquarters in Liberia coordinates appointments using a data-management system developed for CVT and modified for LTFU; the headquarters also houses the fully equipped biospecimen repository (24), document center, and teams of study physicians, data entry, information technology and quality control, as well as processing laboratories for cervical sample aliquotting, cytology slide production, blood processing, cryopreservation, histology, and HPV testing by hybrid capture 2 (HC2). Participant records and specimens are centralized at the Liberia headquarters and transported daily to and from the clinics in study vehicles. Samples are stored at our biorepository in Liberia until they are sent to the collaborating international laboratories or to the NCI biorepository for long-term storage. Cytology and histology interpretation occurs in San José.

Enrollment of participants into LTFU

CVT Participants—Women in the HPV-arm of CVT (excluding those from some areas of Puntarenas, and those who withdrew from CVT prior to the four-year visit) were invited to participate in LTFU during their final CVT study visit. Those willing to participate signed the inform consent. The four-year CVT visit was defined as the baseline visit for the LTFU study and was used to define LTFU study-visit windows.

UCG—In order to identify women for the UCG, a new census of women ages 20 to 30 was conducted in 2008. During the census, all households were visited by study staff members to obtain the name, date of birth, ID number, exact address, contact person information, and telephone number of potential participants. The total number of women in the census was 22,240. Intensive checks were carried out to ensure CVT participants were not included in the pool of potential participants.

All women in the census were randomly assigned a personal identification number in the database, and a random sample of 3,000 women frequency-matched to CVT participants by year of birth and geographic location was selected. To replenish the sample pool once 50% of the UCG was enrolled, another random sample of 2,000 women was chosen from the census restricting the selection to the women within the age groups and geographic regions not already covered by the enrolled control women. The enrollment goal was 3,000 women, similar in size to the original control-arm of CVT.

The enrollment visit of the UCG occurred contemporaneously with the final CVT study visit. Outreach workers visited potential enrollees at their homes to deliver the invitation to participate in the study with an appointment date to the nearest of our clinics and, a copy of the informed consent. On the day of the clinic visit, potentially eligible women had an extensive discussion of the informed consent document with a trained interviewer; clinicians were always available to answer questions. Women who decided not to participate or who were deemed ineligible were offered a physical exam and a cytology with colposcopic evaluation and treatment of women screening abnormal as needed, at no cost to them. All women were offered transportation in the study vehicles or reimbursement of travel expenses; however they were not paid for participation in the study. After signing the informed consent, a computerized interview on risk factors was administered by a trained interviewer. The questionnaire elicited information on education, marital status, income, household facilities, menstrual history, sexual, reproductive and contraceptive history, and smoking. Among UCG women cervical screening history was also queried and if cervical treatment was reported histologic specimens were recovered for diagnosis confirmation.

The study visit continued with a complete medical history and physical exam including a pelvic exam among sexually-experienced women to assess final eligibility. Eligibility criteria included birth date between July 1978 and November 1987, residency in Guanacaste Province or selected areas of Puntarenas during 2004–2005, being able to speak/understand Spanish and, apparent mental competency. Women were excluded if they had a history of cervical cancer, a history of hysterectomy, any important medical condition that precluded participation, or prior HPV vaccination. Participation was delayed if a woman was pregnant or less than three months postpartum.

To assess the comparability of the UCG with CVT participants in terms of their risk of exposure to HPV, an additional questionnaire about lifetime HPV-risk factors was administered to all women (CVT and UCG participants) during the LTFU enrollment visit, so that the responses were queried at the same time and in the same way, to avoid potential recall bias.

Clinical procedures and specimen collection at LTFU enrollment for all participants

A pelvic exam with collection of anal and cervical samples was conducted among sexually experienced women. To avoid sample contamination from the cervix, anal samples were collected first, using a dry swab that was inserted 3–4 cm in the anus, rotated once, and then removed and rinsed in 1mL PreservCyt® (PC) solution. The swab was left in the vial and the specimen was frozen in liquid nitrogen (LN) vapor phase at the clinic.

After anal sampling, the vaginal speculum was placed and cervical secretions were collected with two polyvinyl acetate-based Merocel sponges (Medtronic Xomed, Inc) by gently placing each sponge on the cervical os for 30 seconds. The sponges were placed into separate empty 10 mL tubes and frozen in LN immediately. Cervical cells for cytology and HPV testing were collected with a Cervex brush® (Rovers Medical Devices B.V. ®) by firmly rotating the brush 5 times around the cervical os. In women with ectopy, the Cervex brush was also used to sample the squamo-columnar junction. The brush was vigorously rinsed in 20mL of PC and stored in coolers at about 20 degrees Celsius. An additional Dacron swab was used to obtain more cells, by rotating it 360 degrees in the cervical os, and placing it in PC. These cells were immediately frozen in LN. Participants received treatment when cervico-vaginal infections were detected.

At the lab, three 0.5 mL aliquots were extracted from the 20mL PC vial following PCR-safe procedures for HPV DNA genotyping, after which a cytology slide was prepared and the residual volume was used for HC2 testing. Blood was collected to obtain serum, plasma and buffy coat from all participants. Aliquots of whole blood collected in Citric Acid-Dextrose (ACD) preservation medium were placed in vials with ascorbic acid and metaphosphoric acid buffers, for folic acid and ascorbic acid preservation respectively. As a benefit to UCG participants, a CBC was performed; if any measure out of normal range was detected, the participant was referred to the social health care system. Among a 10% random sample of UCG women, an additional 40 mL of blood sample was collected in heparinized tubes, for cryopreservation of lymphocytes as described (9). Oral samples for HPV testing were collected using 15 mL of Scope® mouthwash and were sent to our laboratory in Liberia where they were centrifuged. The pellet was washed, re-suspended in PBS and frozen as previously described (17).

Of note, when recruitment of the UCG commenced, anal and oral sample collection were not included in the LTFU protocol, although they were being collected from CVT women as part of the last CVT visit. At the point where ~50% of the UCG was enrolled, a protocol amendment was approved to collect anal and oral cells.

Initial intensive cervical disease detection among UCG women

As part of CVT, women were actively screened and treated when necessary during the 4 years of follow-up, and they additionally were evaluated by a rigorous colposcopy referral algorithm after their close out from CVT. However women in the UCG had presumably received only cytology-based screening as part of the regular health care system (or no screening at all). Thus, we designed a strict colposcopy-referral algorithm to identify and

treat prevalent disease in women in the UCG so that they would be more comparable to women in the original control-arm of CVT in terms of future incident disease.

For the UCG, this colposcopy-referral algorithm consisted of an initial co-testing with cytology and HC2. If both tests were negative, women were scheduled for 2-year follow-up visits. Women with cytological evidence of high-grade disease were referred to colposcopy for evaluation and treatment as necessary. HC2-positive women and women with minor cytological abnormalities had a second round of "accelerated screening" with co-testing 6 months after their first visit; if both HC2 and cytology were negative (ASC-US/HPV-negative is considered normal), they reverted to biennial follow-up; if either test was positive, they were referred to colposcopy. Unsatisfactory cytology or insufficient HC2 results were considered equivalent to a positive result for the purpose of clinical management.

Follow-up visit and management of cervical cytological abnormalities

At the time of this writing LTFU is ongoing. Follow-up screening visits are scheduled to occur every 2 years with cytology with ASC-US triage by HC2; women with minor abnormalities are followed every 6 months with cytology and HC2 tests. If both tests are normal, women return to regular screening every 2 years. If the cytology is abnormal they are referred to colposcopy for evaluation and treatment as necessary. HPV-positive participants with normal cytology are invited to a second accelerated screening visit with co-testing in 6 months. If both are normal, women return to the 2-year screening schedule, while if either of the tests is positive, they are referred to colposcopy.

At all follow-up visits, a questionnaire collecting risk factors information between current and previous study visits is administered. A pelvic exam is performed among sexually experienced women, and cervical samples are collected and handled in the same way as the enrollment visit except that during follow-up, HPV testing by HC2 is restricted to women attending the accelerated screening visits, women attending colposcopy, and women with an ASC-US cytology result.

Anal cells were collected among all sexually experienced women at all visits up to the 2-year visit prior to cervical sample collection. For the 2-year visit, the procedure for anal sampling was modified to allow for anal cytology preparation for research purposes only. Specifically, the Dacron swab is saturated with water before sample collection, then introduced up to 7 cm into the anal canal (until it stops against the wall of the rectum), and then rotated against the walls of the anal canal for at least 30 seconds while removing the swab, which is then vigorously washed in a vial with 20 mL of and then discarded; the PC vials are stored in coolers at about 20 degrees Celsius.

At each follow-up visits, blood for serum and plasma is collected from all women. Among women in the ISC, saliva and oral sponges for immune studies are collected at each visit and, at biennial visits, a mouthwash sample and an additional 40mL blood sample for cryopreservation of lymphocytes are also collected. For saliva collection, women are asked to accumulate saliva in the mouth for 30 seconds and spit in a cryovial using a straw; then, one polyvinyl acetate-based Mero-cell sponge is gently placed on the oral mucosa of the

right cheek for 15 seconds and repeated with the other face of the sponge for 15 additional seconds; the procedure is carried out again with a second sponge on the left cheek. The sponges are placed into an empty tube and frozen in LN immediately.

HPV-DNA detection and genotyping

Cervical, anal and oral samples are sent to DDL Diagnostic Laboratory in the Netherlands for broad spectrum PCR-based HPV DNA testing. Briefly, DNA extraction is done by using the MagNA Pure LC Isolation station (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) and the Total Nucleic Acid Isolation Kit (Roche Diagnostics GmbH, Roche Molecular Biochemicals, Mannheim Germany), as described by the manufacturer. Extracted DNA is tested using the SPF10 PCR primer system and a DNA enzyme immunoassay detection of amplimers (DEIA) followed by genotyping using the LiPA25 version 1 line detection system as described. LiPA25 detects 25 HPV genotypes, including carcinogenic (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 or 73) and non-carcinogenic (6, 11, 34, 40, 42, 43, 44, 53, 54, 66, 70, and 74) types. To ensure that HPV16 and HPV18 infections are not missed, all specimens positive for HPV DNA using SPF10 DEIA but negative for HPV16 or HPV18 by LiPA25 are also tested using HPV16 or 18 type-specific primers (25;26). Testing is conducted with staff blinded to previous PCR results from the same woman, as well as HC2 and cytology results.

Cervical and anal cytology

Liquid-based cervical and anal cytology slides are prepared with a ThinPrep 2000 processor to obtain thin layer samples that are stained with a modified Pap stain at the Liberia laboratory. Extensive quality control measures are in place including relative humidity control which can affect specimen quality (9). Samples are interpreted using Bethesda System criteria at a local laboratory with repeat screening by two cytotechnologists and final adjudication by the cytopathologist (MA).

Clinical management of the study participants is based on the Costa Rica cervical cytopathology interpretation. Compared to CVT where all cervical slides read as abnormal in Costa Rica and a 10% sample of the slides read as negative in Costa Rica were re-screened and re-interpreted in the United States (9), during LTFU a system is in place to continually evaluate the quality of the cytology staining only by a US expert cytotechnologist (CE). This was decided and approved by the local IRB because the agreement over the four years of CVT follow-up was good (kappa=0.68 (95% CI: 0,66–0,69) (Supplemental table 1). Additional analyses also showed that only 0.56% of the cytology slides that were read as normal in Costa Rica and reinterpreted in the US (N=3,685) corresponded to histologically confirmed disease (CIN2 or worse) that would had not been detected without the review, and to detect them, 3.15% of those 3,685 women had to go through unnecessary colposcopy visits.

The cytopathologist received extensive training on anal cytology interpretation before starting to read these samples with retraining every year. Anal slides are read from a selected group of women selected based on known risk factors for HPV related anal disease are interpreted in CR and reinterpreted in the US by an expert on anal cytopathology (TD). Anal

cytologic results are used only for research purposes unless HSIL including ASC-H is detected in which case women undergo anoscopy and treatment as necessary.

Monitoring of adverse events and pregnancies

As part of the continued evaluation of vaccine safety during LTFU, we continue to document serious adverse events (any untoward medical condition occurring to any study participant including those from both CVT and UCG), independent of their possible relationship with vaccination. However, with authorization from the local IRB, we have excluded from documentation serious adverse events that were very frequent during CVT and deemed clearly not related to vaccination, including: Cesarean section due to previous C-section, cephalopelvic disproportion, arrested active labor, fetal macrosomy; C-section due to pre-existing conditions of the mother; dengue fever; cholelithiasis; urinary tract infections; infections of a surgical wound; abscess; sepsis postpartum (i.e., endometritis); postpartum anemia; peripartum bleeding; and traffic accidents. We also maintained a toll free number for participants to report adverse events.

Pregnancies reported to any member of the study team are documented and followed until resolution, the outcome is documented, including characteristics of the delivery and babies. All congenital abnormalities of a baby are reported. Based on a request from the IRB, at the 4-year CVT visit, information on medical events in the categories of congenital malformations, endocrine and metabolic conditions, autoimmune diseases, hearing and visual problems, learning disabilities, mental retardation and death occurring to children of participants who were born from pregnancies initiated within one year of vaccination were queried.

Specimen handling, data management and quality control

Standard operating procedures (SOPs) for the labeling, transporting, storing, processing and shipping of specimens developed for the CVT were revised and adapted if necessary for their use during the LTFU study. Cold chain for samples is assured by using coolers and small vapor-phase liquid nitrogen shippers, both with thermometers and SOPs to manage deviations. Samples are temporarily stored at the local biorepository and tracked using the NCI biospecimen inventory system; BSI-II (Information Management Services (IMS), MD). Samples are sent as needed to laboratories in Costa Rica and/or shipped to collaborating laboratories outside Costa Rica and to the long-term repository in the US under temperature controlled conditions.

The CVT data-management system was modified for use in the LTFU study by IMS, in collaboration with Costa Rica computer experts. Data-entry staff key all case report forms; for LTFU, double keying was eliminated due to the very low error rate reported during the CVT (0.0032%). Extensive data cleaning is carried out locally and logical edits are conducted periodically at IMS. The same safety and back-up protocols for data protection established for CVT are followed for LTFU (9).

The quality system established for CVT was maintained (9). Since LTFU is an epidemiological cohort study and not a clinical trial, external monitoring as done during CVT was no longer necessary; internal monitoring by quality assurance staff was

implemented, with review of all informed consent forms, eligibility criteria, serious adverse events and pregnancies as well as full chart review in a random sample of 25% of the visits.

Statistical methods

In this article we present the participation rates, compliance with study procedures and LTFU baseline characteristics of women included in the LTFU study in the three arms: the CVT HPV-arm, original control-arm and UCG.

Since we previously documented balance on CVT enrollment characteristics between the HPV and original control arms (9;10), in this manuscript we compare the UCG and the original control-arm at LTFU enrollment, working under the transitive law that if they are the same, the characteristics of the HPV-arm and UCG should be balanced.

While there was no expectation that the two groups would be identical, maximizing the similarity in risk for HPV acquisition over the long-term follow-up would enable residual differences to be statistically controlled by covariate adjustment. To compare the two groups in terms of risk of HPV infection, and to quantitate the magnitude of observed differences, three approaches were implemented: 1) compare LTFU baseline characteristics and reported sexual behaviors; 2) evaluate similarity in predicted risk of cervical carcinogenic HPV infection at different time points based on a model developed using reported characteristics; and 3) calculate vaccine efficacy (VE) four years after vaccination using the two control groups and holding the HPV-arm constant.

In studies with large sample size, small difference between groups can quickly lead to significant p-values, which can lead to false claims of meaningful differences. To avoid this pitfall, we do not provide p-values for the first approach, and instead describe differences that may be important between the original control-arm and UCG (27).

For the second approach, risk estimates were generated in the original control-arm based on covariates measured at their CVT enrollment visit. A logistic regression GEE model (Proc GENMOD in SAS) was fit using an unstructured correlation matrix to account for correlation between outcomes within a woman. Cervical carcinogenic HPV infection at any study visit after enrollment during the main trial was the outcome; this was assessed for women reporting having initiated sexual activity only (so they would have some risk of HPV acquisition). The following covariates were included in the model based on their prior association with HPV infection: age, years since sexual debut, marital status (married, widowed/divorced/separated, single), number of lifetime sexual partners (1, 2–3, 4–5, 6+), number of pregnancies (0, 1+), and visit age. Next, we applied the risk estimates generated by the model to women in the LTFU study (both original control-arm and UCG), to predict their 2-year risk of having cervical carcinogenic HPV infection at that time based on their reported characteristics at the first LTFU visit. After assigning each woman a risk estimate, we calculated the mean and interquartile range (IQR), as well as splaying out the risk by decile.

For the third approach, the prevalence of any oncogenic cervical HPV infection among all participants measured one-time 4-years post-vaccination was expressed as the number of

infected women per 100 women (stratified by HPV-arm, original control-arm, and UCG); asymptotic confidence intervals (95%CI) around the prevalence were estimated. The complement of the ratios of the prevalence for the HPV and control-arms comprised the VE estimates. Exact confidence intervals for vaccine efficacy were calculated based on the binomial distribution of the number of events in the HPV-arm among the total number of events in the HPV and each of the control-arms (28;29). This analysis was repeated using HPV-16/18 infections and oncogenic cervical HPV infection excluding types with evidence of vaccine protection (i.e. 16/18/31/33/45) as endpoints.

Results

Participation rates and compliance with study procedures

CVT—Out of the 7,466 women enrolled in the CVT, 1,417 were not eligible to participate in the LTFU study because they reside in Puntarenas or withdrew from CVT before the 4th year visit, resulting in 6,049 women eligible to participate in the LTFU study (Figure 1). Of these 486 were not recruited, resulting in 5,563 CVT women enrolled in the LTFU study (92% of eligible women). Participation in the LTFU was similar among the two CVT-arms (2,792 from the HPV-arm and 2,771 from the control-arm).

UCG—Out of the 5,000 women selected for contact from the census, after excluding 80 duplicates and 246 non eligible women, 4,674 were eligible to participate in the LTFU study. Of these, 1,839 were not recruited, resulting in 2,836 (61% of eligible) women enrolled in the study; this compares to 59% of eligible women recruited into CVT from the initial census prior to CVT (9).

Compliance with data and specimen collection and laboratory testing was extremely high for women coming from CVT and those joining the UCG (over 90%), except for anal samples that were collected from over 70% of women (Table 1).

As described, women in the UCG went through a strict colposcopy referral algorithm to quantitate and treat existing prevalent disease. This process could comprise up to one 6-month re-screening visit and one or more colposcopy visits. Once a woman was returned to the regular 2-year screening visit, or high-grade histologically confirmed disease was detected, the process was considered complete. 1,002 women from the UCG required this intensive process due to either HC2 positivity or cytological abnormalities at the enrollment visit. Out of these, 723 (72.2%) completed the process before attending the 2-year visit, 282 (20.2%) attended some of these visits but not the full process before the 2-year visit and 77 (7.7%) did not attend any of these visits before the 2-year visit; these proportions resemble the proportion of women in CVT who did not comply with accelerated screening or colposcopy visits during the trial.

Compliance with the first biennial follow-up visit was 92% for women in the HPV-arm, 93% for women in the original control-arm, and 89% for women in UCG.

Characteristics of participating women and comparison of original control-arm and UCG

Women in the original-group and the UCG were similar with respect to age at baseline, area of residence, age at first sexual intercourse, number of lifetime sexual partners, and number of sexual partners in the last month (Table 2). They were also similar with respect to HPV positivity and prevalence of cytological abnormalities. Compared to women in the original control-arm, women in the UCG attained lower levels of education (17.8 vs 26.2% attending University, respectively), were more likely to be married (71.3 vs 64.2%, respectively), and had more pregnancies (16.7 vs 27.1% nulliparous, respectively).

In the evaluation of the similarity in predicted risk using a model based on reported characteristics at baseline, a strong overlap of predicted future HPV infection risks was observed between the two control groups. Specifically, the mean predicted risk of cervical carcinogenic HPV detection at the 2-year visit was 26.7% (IQR 18.6% to 33.6%) for the original control-arm and 25.5% (IQR 17.4 to 31.1%) for the UCG. When risk was further stratified according to deciles, the full distribution also overlapped (Figure 2). VE against one-time detection of cervical carcinogenic HPV infections 4 years after vaccination (i.e. LTFU baseline visit) using the original control-arm was 21.8% (95%CI 12.5 to 30.2%) and using the UCG was 23.2% (95%CI 14.1 to 31.2%); these similarities in VE were driven by the comparable underlying attack rates in the control arms (25.1% and 25.5%, respectively).

VE against one-time detection of cervical HPV-16/18 infection was 74.7% (95%CI 66.0 to 81.4%) and 78.0% (95%CI 70.6 to 83.8%), respectively, demonstrating that VE was similar when either the original control-arm or the UCG were used as referent group (Table 3).

Prevalence of HPV and of cytologic abnormalities by age at LTFU baseline visit for each arm is presented in supplemental tables 2 and 3.

Prevalent disease detection in the UCG during the intensive screening process

Of the 2,836 women enrolled in the UCG, 27 and 92 women were diagnosed with CIN2 and CIN3+, respectively; 20 women who reported cervical treatment prior to study entry had a diagnosis of CIN2+. As a reference, during the combined enrollment and follow-up phase of CVT (i.e. 4 years of cumulative data from screening during CVT), 78 and 105 women from the original control-arm (n=2,729) were diagnosed with a CIN2 and CIN3+, respectively.

Discussion

To our knowledge, the CVT is the only clinical trial of an HPV-vaccine in the public domain, and is one of the two population-based studies initiated (30) before the registry of the current vaccines. CVT confirmed findings from the pharmaceutical-sponsored trials, and more importantly, provided valuable insights about critical public health issues including vaccine efficacy of fewer than three doses and at non-cervical anatomical sites in women.

There are several long term follow-up vaccine studies ongoing. The original FUTURE and PATRICIA studies in Finland (30;31) will be monitored using passive follow-up through the cancer registry for 10 and 15 years, with an active update every 4 and 5 years, for FUTURE and PATRICIA, respectively. Several small studies with active follow-up recently reported

end-of-study results (or are close to completion): HPV013/025 (n=220 women who received the bivalent HPV-vaccine in 2004–2005), will follow women annually for 10 years for immunogenicity and safety (32); HPV023 (n=433 women who received either the bivalent or placebo vaccines) recently reported 10 years of duration of protection and the placebo-arm was offered the HPV-vaccine (33;34); and NCT00316706 (n=563 adolescents randomized to receive the bivalent HPV-vaccine or placebo) will be followed annually for 10 years for immunogenicity and safety (35). Finally, the IARC randomized clinical trial in India, which aims to investigate the efficacy of 2 vs 3 doses, was initiated in 2009 with planned initial follow-up for 5 years and continued surveillance for 20 years.

Our LTFU study is unique in its combination of a considerable sample size with an active follow-up design, including the extensive collection of biological samples for evaluation of immunogenicity and efficacy. LTFU will provide valuable information that cannot be obtained in the passive, registry-based studies or the small studies with active follow-up currently ongoing, such as the investigation of longer-term vaccine efficacy against cervical high grade disease, long term efficacy and immunogenicity by number of doses, and HPV and cervical precancer natural history in a vaccinated population, including the effect of eliminating HPV16 and 18 in rates of infection and disease caused by other HPV types. Further, LTFU is the only one including a new unvaccinated control group, which is essential for providing underlying rates of infection and disease. Since women in the UCG were not randomized, we view the LTFU as an epidemiological cohort study and no longer an RCT, as CVT was. In the present analysis, differences were observed in education, marital status and number of pregnancies between the new and original control groups. Beyond these covariates, the groups were comparable for all other risk factors and predicted future risk of HPV infection, indicating that the observed differences likely do not impact HPV exposure and, if necessary, small differences can be accounted for using statistical adjustment. Yet, the impact of the intensive screening that women in HPV-arm went through as part of CVT, compared to the minimal or absent screening of women in the UCG, could have resulted in increased detection of CIN2 lesions likely to regress in the HPV-arm as well as decreased detection of CIN3+ lesions over the time span of LTFU, due to truncation by treatment of CIN2 lesions that were going to progress. To account for this, women in the UCG went through an aggressive colposcopy algorithm at LTFU enrollment to try to quantitate and treat prevalent disease and homogenize detection of future disease with that of the original control-arm. While the results herein show considerable detection of prevalent disease in the UCG, it was not possible to quantitate the actual differences in disease during the 4 years of the RCT between the 2 control groups. Reassuringly, use of either control group provides similar VE estimates against viral outcomes, which suggests that VE against incident disease should also be similar. In total, these findings highlight the internal validity of the UCG and support its use for future VE evaluations.

Our follow-up is currently planned to 10 years; this may be inadequate to fully evaluate our main aims related to HPV-vaccination, including, quantitating the duration of vaccine efficacy for the three-dose regimen, as that will likely exceed 10 years. However, our focus on fewer than three doses could be the first sign of waning protection. Additionally, the impact of vaccination on disease related to other HPV types which are slower to progress than HPV-16/18 may not be revealed for more than a decade.

The LTFU is a comprehensive study which hopefully will provide answers to important questions, not answered by original vaccine trials, about the long term effects of prophylactic HPV-vaccines including those related to efficacy of less than three doses and the etiology of HPV-related cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CVT	Costa Rica vaccine trial
LTFU	Long term follow up study
UCG	New unvaccinated control group, followed by screening only
ISC	Immunogenicity sub-cohort
LN	Liquid nitrogen
PC	PreservCyt® solution
HC2	Hybrid capture 2
ACD	Anticoagulant Citrate Dextrose Solution
DEIA	DNA enzyme immunoassay
LiPA	Line probe assay
SOP	Standard operating procedure
IQR	Interquartile range
VE	Vaccine efficacy

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Names and Affiliations of investigators in the Costa Rica Vaccine Trial (CVT) group are as follows

Proyecto Epidemiológico Guanacaste, Fundación INCIENSA, San José, Costa Rica—Mario Alfaro (cytopathologist), Manuel Barrantes (field supervisor), M. Concepción Bratti (co-investigator), Fernando Cárdenas (general field supervisor), Bernal Cortés (specimen and repository manager), Albert Espinoza (head, coding and data entry), Yenory Estrada (pharmacist), Paula González (co-investigator), Diego Guillén (pathologist), Roland Herrero (co-principal investigator), Silvia E. Jiménez (trial coordinator), Jorge Morales (colposcopist), Luis Villegas (colposcopist), Lidia Ana Morera (head study nurse), Elmer Pérez (field supervisor), Carolina Porras (co-investigator), Ana Cecilia Rodríguez (co-investigator), Libia Rivas (clinical coordinator).

University of Costa Rica, San José, Costa Rica—Enrique Freer (director, HPV diagnostics laboratory), José Bonilla (head, HPV immunology laboratory), Alfanso García-Piñeres (immunologist), Sandra Silva (head microbiologist, HPV diagnostics laboratory), Ivannia Atmella (microbiologist, immunology laboratory), Margarita Ramírez (microbiologist, immunology laboratory).

United States National Cancer Institute, Bethesda, MD, USA—Allan Hildesheim (co-principal investigator & NCI co-project officer), Hormuzd Katki (stastitician), Aimée R. Kreimer (co-investigator), Douglas R. Lowy (HPV virologist), Nora Macklin (trial coordinator), Mark Schiffman (medical monitor & NCI co-project officer), John T. Schiller (HPV virologist), Mark Sherman (QC pathologist), Diane Solomon (medical monitor & QC pathologist), Sholom Wacholder (statistician).

SAIC, NCI-Frederick, Frederick, MD, UDA—Ligia Pinto (head, HPV immunology laboratory), Troy Kemp (immunologist).

Women’s and Infants’ Hospital, Providence, RI, USA—Claire Eklund (QC cytology), Martha Hutchinson (QC cytology).

Georgetown University, Washington, DC, USA—Mary Sidawy (histopathologist),

DDL Diagnostic Laboratory, Netherlands—Wim Quint (virologist, HPV DNA testing), Leen-Jan van Doorn (HPV DNA testing).

Highlights (for review)

- A vaccine trial to evaluate HPV-16/18 vaccine was conducted in Costa Rica.
- At the end of the trial a Long-term Follow-up study was initiated to evaluate 10-years impact of HPV vaccination.
- Due to cross-over vaccination at the end of the trial and the consequent loss of the original control-arm, a new unvaccinated control-group was included for the long-term follow-up phase.
- We present study methods, enrollment data and compliance with study procedures.
- We show the validity of using the new unvaccinated control-group for vaccine efficacy evaluations.

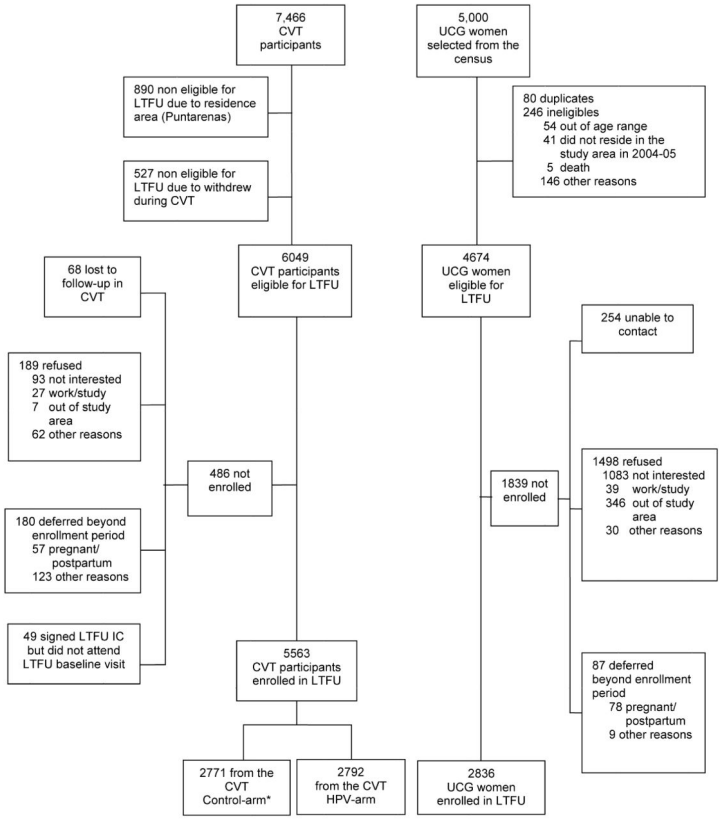

Figure 1. CONSORT diagram indicating participation in the Costa Rica Vaccine Trial Long-term follow up study

* 65 women from CVT control-arm receive the HPV vaccine at crossover before LTFU baseline visit

** 57 women from CVT and 3 from UCG did not have enrollment cervical sample or HPV result available

CVT: Costa Rica Vaccine Trial

LTFU: Long Term Follow Up study

UCG: Unvaccinated Control Group

IC: Informed Consent

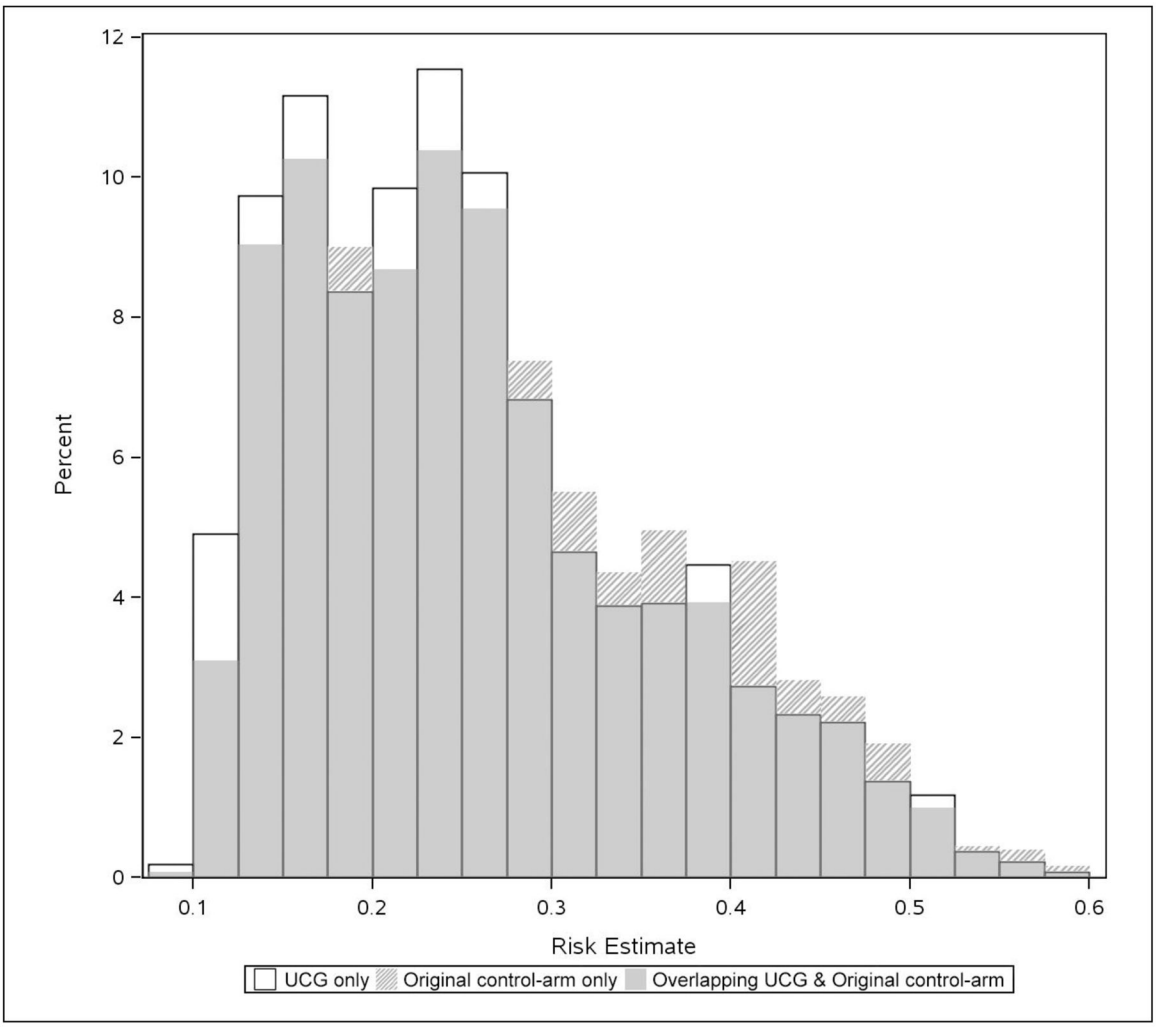

Figure 2.

Overlay of the distribution of future predicted risk of HPV infection (in year two) by deciles of the population in the original control-arm and the new unvaccinated control group, based on reported risk factors at enrolment into the long-term follow-up study.

Table 1

Compliance with interview and specimen collection during the Costa Rica Vaccine Trial Long term follow-up study (LTFU) baseline visit

	LTFU baseline visit for CVT women			UCG enrollment visit		
	Eligible[§]	Collected/tested		Eligible	Collected/tested	
		N	%		N	%
Data						
Interview	5563	5534	99.5	2836	2831	99.8
Screening interview[*]	5563	5549	99.8	2836	2814	99.2
Pelvic Exam	5260	5252	99.9	2764	2763	99.9
Samples						
Cervical secretions	5260	5251	99.8	2765	2765	100.0
PreservCyt	5260	5254	99.9	2765	2765	100.0
Cervical cells for RNA preservation	5260	5254	99.9	2765	2765	100.0
EDTA preserved blood	5517	5467	99.1	2836	2818	99.4
Zinc free serum separation blood	5562	5522	99.3	2836	2820	99.4
ACD preserved blood	5562	5494	98.8	2836	2816	99.3
Heparin preserved 40mL blood[ǂ]	N/A	N/A	N/A	293	272	92.8
Mouth wash[¤]	5563	5171	93.0	1297	1244	95.9
Anal sample[¤]	5260	3765	71.6	1273	988	77.6
Laboratory results						
Cytology results	5263	5254	99.8	2766	2765	99.9
HC2 HPV results	5263	5254	99.8	2766	2762	99.9
Cervical PCR HPV	5254	5253	99.9	2765	2763	99.9

CVT: Costa Rica Vaccine Trial

UCG: Unvaccinated Control Group

ACD: Acid Citrate Dextrose anticoagulant solution

[*] Interview designed to compare original control-arm and the UCG at LTFU baseline

[§] Number of visits completed where the sample should be collected

[¤] Collection of oral and anal samples among new control participants started 29 June 2010 when 1539 women had already been enrolled

¥ Collected only among the immunogenicity sub-cohort

‡ Collected only among the immunogenicity sub-cohort and a 10% random sample

Table 2

Descriptive characteristics of the original control-arm and the new unvaccinated control group (UCG).

Characteristic	Original control-arm N (%)	UCG N (%)
Age at baseline		
<25	976 (36.1%)	857 (30.2%)
25–26	632 (23.4%)	692 (24.4%)
27–28	643 (23.8%)	646 (22.8%)
29+	451 (16.7%)	638 (22.5%)
Median (IQR)	26 (24–28)	26 (24–28)
Years of Education		
Primary-5th or less	225 (8.3%)	437 (15.4%)
Primary-6th	515 (19.0%)	682 (24.1%)
Secondary 1st–3rd	487 (18.0%)	608 (21.5%)
Secondary 4th or more	763 (28.2%)	595 (21.0%)
University	707 (26.2%)	505 (17.8%)
Marital Status		
Single	809 (29.9%)	661 (23.3%)
Married	1735 (64.2%)	2021 (71.3%)
Widowed/Divorced	152 (5.6%)	145 (5.1%)
Number of Pregnancies		
0	732 (27.1%)	473 (16.7%)
1	898 (33.2%)	813 (28.7%)
2	689 (25.5%)	816 (28.8%)
3	264 (9.8%)	467 (16.5%)
4+	119 (4.4%)	264 (9.3%)
Median(IQR)	2 (1–2)	2 (1–3)
BMI		
Low weight	66 (3.0%)	104 (3.7%)
Normal weight	899 (41.2%)	1157 (40.9%)
Over weight	685 (31.5%)	850 (30.1%)
Obese	525 (24.1%)	716 (25.3%)
Age at first sexual intercourse		
Virgin	143 (5.3%)	70 (2.5%)
14 or younger	291 (10.8%)	444 (15.7%)
15	375 (13.9%)	419 (14.8%)
16	331 (12.3%)	381 (13.5%)
17	412 (15.3%)	420 (14.8%)
18	403 (14.9%)	401 (14.2%)
19 or older	717 (26.5%)	672 (23.7%)
Median (IQR)	17 (15–19)	17 (15–18)
Number of lifetime sexual partners		

Characteristic	Original control-arm N (%)	UCG N (%)
0	143 (5.3%)	70 (2.5%)
1	796 (29.5%)	800 (28.2%)
2	563 (20.8%)	661 (23.3%)
3	424 (15.7%)	482 (17.0%)
4+	765 (28.3%)	780 (27.5%)
Median (IQR)	2 (1–4)	2 (1–4)
Number of sexual partners in the last 12 months		
0	224 (8.3%)	158 (5.6%)
1	2172 (80.4%)	2356 (83.2%)
2+	295 (10.9%)	290 (10.2%)
Median (IQR)	1 (1–1)	1 (1–1)
Frequency of Condom Use		
Never	885 (34.6%)	1056 (38.2%)
Rarely	429 (16.8%)	426 (15.4%)
Sometimes	457 (17.9%)	450 (16.3%)
Usually	268 (10.5%)	261 (9.5%)
Always	514 (20.1%)	549 (19.9%)
Oral Contraceptive Use		
Never	341 (13.3%)	452 (16.4%)
Former	1161 (45.4%)	1251 (45.3%)
Current	1052 (41.1%)	1040 (37.6%)
HPV positivity		
Any HPV	1114 (43.5%)	1138 (41.2%)
HPV 16	141 (5.5%)	195 (7.1%)
HPV18	70 (2.7%)	60 (2.2%)
Other oncogenic HPV	545 (21.3%)	576 (20.9%)
Non oncogenic HPV	638 (24.9%)	612 (22.2%)
Cytology result		
Normal	2123 (86.4%)	2347 (88.4%)
LSIL	259 (10.5%)	225 (8.5%)
HSIL	75 (3.1%)	82 (3.1%)

* Evaluated among monogamous women only

IQR: Interquartile range BMI: Body mass index

LSIL: Low grade cervical intraepithelial lesion (includes also atypical squamous cells - unknown significance (ASC-US)/HPV-positive)

HSIL: High grade cervical intraepithelial lesion (includes ASC-ruled out HSIL and typical glandular cells (AGC))

Table 3

Efficacy against one-time prevalent cervical HPV infection four years after vaccination, using the original control-arm and the new unvaccinated control group (UCG), in intention to treatment cohort.

Outcome	Arm	# Women	# Events	Rate per 100 Women (95%CI)	Vaccine efficacy 95%CI
HPV16 or 18 cervical HPV infection	HPV-arm	2788	53	1.9 (1.4 to 2.5)	--
	Original control-arm	2702	203	7.5 (6.6 to 8.6)	74.7 (66.0 to 81.4)
	UCG	2833	245	8.7 (7.7 to 9.7)	78.0 (70.6 to 83.8)
Any oncogenic cervical HPV infection	HPV-arm	2788	546	19.6 (18.2 to 21.1)	--
	Original control-arm	2702	677	25.1 (23.5 to 26.7)	21.8 (12.5 to 30.2)
	UCG	2833	722	25.5 (23.9 to 27.1)	23.2 (14.1 to 31.2)
Oncogenic cervical HPV infection excluding type 16/18/31/33/45	HPV-arm	2788	458	16.4 (15.1 to 17.9)	--
	Original control-arm	2702	455	16.8 (15.5 to 18.3)	2.4 (−11.1 to 14.3)
	UCG	2833	468	16.5 (15.2 to 17.9)	0.6 (−13.1 to 12.6)