Smokers at Higher Risk for Undetected Antibody for Oncogenic Human Papillomavirus Type 16 Infection

Dorothy J. Wiley,[1] Edward Wiesmeier,[2,4] Emmanuel Masongsong,[1] Karen H. Gylys,[1] Laura A. Koutsky,[5] Daron G. Ferris,[6] Eliav Barr,[7] Jian Yu Rao,[3] and The Proof of Principle Study Investigative Group

[1]Division of Primary Care, School of Nursing; Departments of [2]Obstetrics and Gynecology and [3]Pathology and Laboratory Medicine, University of California at Los Angeles School of Medicine; [4]Arthur Ashe Student Health Center, University of California at Los Angeles, Los Angeles, California; [5]Department of Epidemiology, School of Public Health, University of Washington, Seattle, Washington; [6]Departments of Family Medicine and Obstetrics and Gynecology, Medical College of Georgia, Augusta, Georgia; and [7]Department of Biologics Clinical Research, Merck Research Laboratories, West Point, Pennsylvania

Abstract

Objective: **To determine the association between tobacco smoking and serologic evidence of human papillomavirus type 16 (HPV16)–specific antibodies among HPV16 DNA–positive women.**
Design, Setting, and Participants: **Baseline health history, physical examination, and laboratory data for 205 HPV16 DNA–positive women with no prior cytologic evidence of squamous intraepithelial lesions who were enrolled subsequently in a randomized clinical trial.**
Main Outcome Measure: **HPV16-L1 antibody (anti-HPV16 antibody) detected from serum using RIA or ELISA.**
Results: **Eighty-seven percent (179 of 205) of women tested positive for HPV16 DNA using cervicovaginal swabs or lavage specimens, and 26 women showed similar results using swab specimens of external genitalia alone. HPV16-infected women who reported increasingly greater levels of daily cigarette smoking were less likely to test positive for anti-HPV16 antibodies than nonsmoking women (P = 0.02). Smokers were twice as likely as nonsmokers to test negative for anti-HPV16 antibodies, even after controlling for the effects of other covariates in the analyses (adjusted odds ratio, 0.5; 95% confidence limits, 0.2-0.9). Although Papanicolaou test findings and smoking characteristics were poorly correlated (r^2 = 0.01), women who showed atypical cells of unknown significance or squamous intraepithelial lesion were twice as likely to test anti-HPV16 antibody positive as women who showed normal Papanicolaou tests (adjusted odds ratio, 2.0; 95% confidence limits, 1.1-3.7).**
Conclusion: **These data suggest that smoking may influence the long-term risk for cancer by perturbing early immune responses to the virus and may increase the likelihood of persistent infection. Patient education messages should alert women to this additional risk of smoking. A clinical trial of smoking cessation should be explored as a therapeutic intervention for primary HPV16 infection.** (Cancer Epidemiol Biomarkers Prev 2006;15(5):915–20)

Introduction

Human papillomavirus (HPV) may be the most common sexually transmitted infection; nearly 18% of the female population, ages 12 to 59 years, shows serologic evidence of HPV16 infection (1-5). More important, half of all cervical cancers worldwide are attributed to HPV16 (6-8). Although the estimated treatment cost for HPV infection in the United States totals nearly US$4 billion annually, the overall cost of human suffering worldwide is far greater and makes our understanding of these infections an important priority (2-4).

It is likely that the relationship between tobacco smoking (smoking) and HPV-related cancers is complex. Nonetheless, epidemiologic data suggest that smoking is positively associated with abnormal cervical cytology (9-12), cervical intraepithelial neoplasia grade 3, and cervical cancer (11, 13). Smoking may affect a number of pathways leading to cancer; generally, smoking is associated with metaplasia, neoangiogenesis and proliferation in epithelium and with overexpression of p53 and Ki-67 in dysplasias (14, 15). In addition, smoking has been associated with the formation of chemically stable DNA adducts in cervical epithelium that may induce genetic instability (14, 16-18). Last, tobacco byproducts may modulate inflammation and local immunity and may perturb apoptotic pathways (19-21). Several laboratories have reported lymphocytosis, particularly of $CD4^+$ T cells, among smokers (22-25).

Because HPV-related cancers are ultimately due to the host's inability to clear viral infection, immune factors are likely to be very important in the establishment of persistence. Dissecting the role that smoking plays at each phase of infection and carcinogenesis could better inform our interventions and prevention strategies. Cross-sectional data from 205 women, ages 16 to 23 years, who tested positive for HPV16 DNA at their first study visit were analyzed to determine whether smoking was associated with detection of HPV16-L1 antibody in serum specimens (anti-HPV16 antibody).

Materials and Methods

Setting and Study Group. A proof-of-principle study was undertaken to test whether an HPV16-L1 virus-like particle vaccine would prevent HPV16 infection. The overall design and interim results from this study (heretofore referred to as the Proof-of-Principle Study) have been described previously (26). Briefly, a total of 2,392 women, ages 16 to 23 years, who reported fewer than five sex partners during their lifetime and no prior occurrences of low-grade or high-grade squamous intraepithelial lesion (SIL) volunteered to participate in a phase II b trial (26). After written informed consent was obtained, women

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Requests for reprints: Dorothy J. Wiley, School of Nursing, University of California at Los Angeles, Los Angeles, CA 90095-6919. Phone: 310-825-0803; Fax: 310-206-0606. E-mail: dwiley@ucla.edu

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underwent a complete physical examination that included Papanicolaou (Pap) testing, collection of genital tract specimens for virus testing, and venipuncture for serum antibodies. Study sites were located at 16 centers throughout the United States. Biological specimens were collected using standard procedures as follows. Venous blood was separated into cells and serum within 30 minutes of collection and cryopreserved at −20°C until testing. Cervical cell specimens were collected into preservative solution using Papette brooms and cytobrushes for Pap testing; residual specimens were cryopreserved for future testing. Dacron swabs of the endocervix and ectocervix, and external genitalia were collected into separate tubes containing transport media and were cryopreserved at −20°C until PCR-based testing for HPV16 DNA. Pap tests were evaluated initially by cytotechnicians and reviewed by board-certified cytopathologists available to local centers. Subsequently, they were reviewed by a central panel of nationally recognized, board-certified pathologists who were blinded to other diagnoses (27). Pap test cellular changes were evaluated as atypical cells of unknown significance (ASCUS), low-grade or high-grade SIL, atypical glandular cells of unknown significance, squamous cell carcinoma, or adenocarcinoma (27).

Standardized questionnaires were used to collect information on the participant's number of male of sex partners, contraceptive method used, and alcohol and tobacco consumption. Additionally, self-report for family history of anogenital cancers and personal and family medical illnesses were recorded as part of the initial study visit. Genital swab specimens tested HPV16 DNA positive for 205 of 2,392 (8.6%) enrolled women, and data gathered from these women during the initial visit comprised our analyses.

Study End Point. The end point of interest was detection of HPV16-L1 antibodies using a type-specific competitive RIA (28) and an ELISA (29). Briefly, for RIA, a limited amount of HPV16 virus-like particles were fixed to test wells, and equal amounts of immune sera and monoclonal antibodies were incubated together and later radioactively labeled for quantitation (28). Results from individual specimens were compared with a standard curve, and specimens measuring ≥6 milli-Merck units/mL, an arbitrary scale, were considered positive (28). Additionally, at this first study visit, capture ELISA was done to quantify anti-HPV16 antibodies in serum. Briefly, HPV16-L1 virus-like particles were expressed in yeast and fixed to plates using monoclonal antibodies (29). Isotype-specific titers in serum specimens were determined by end point dilutions of 1:300 using anti-human IgG coupled to horseradish peroxidase (29). Women who tested positive using either RIA or ELISA were classified as ''anti-HPV16 antibody positive.'' All subjects were tested by both RIA and ELISA at the initial study visit, and our data suggest that the assays agreed moderately well (i.e., $\kappa = 0.41$).[8,9]

Exposure of Interest. For these analyses, women who reported smoking tobacco products within 3 months of the first study visit (smokers) were compared with nonsmokers. Nonsmokers included women who had never smoked and women who reported smoking cessation ≥3 months before the first study visit. To evaluate the effect of daily tobacco use, women who reported 1 to 5 cigarettes daily, women who smoked 6 to 10 cigarettes, and women who smoked ≥11 cigarettes daily were compared with women who reported no daily use. Thus, we evaluated whether dose and recent smoking cessation were associated with detection of HPV16 antibodies and with cervical cytologic abnormalities.

Other Covariates of Interest. To evaluate confounding in these data, the relations between age, race/ethnicity, historical evidence of external genital warts and other sexually transmitted infections, number of male sex partners, use of barrier contraceptive methods or not, time since first intercourse, anti-HPV16 antibody status, and cytologic abnormalities were summarized. Cervical cytology was evaluated using (cervical) cells collected by cytobrush and Papette broom, and specimens were preserved and analyzed using Thin Prep technology and Pap staining. Some experts suggest that the interpretive variability for Pap test is high, especially across less severe classifications of abnormality (30). High-grade SIL was uncommon in this study group; thus, cytologic abnormalities were evaluated in two ways: women were classified as having ''ASCUS or more severe'' and as ''low-grade SIL or more severe'' (12, 30). Women diagnosed with atypical glandular cells of unknown significance were treated as high-grade SILs for these analyses.

In addition, age was analyzed as a continuous variable, and the mean age of the study group served as the comparison group. Women who reported being Caucasian or of mixed ancestry that included Caucasoid were classified together; African American, Asian, and Hispanic were classified accordingly, and all other racial/ethnic designations were grouped together as ''other race.'' Women who reported having ever been diagnosed or treated for external genital warts were compared with women who had not. Women who reported syphilis, gonorrhea, pelvic inflammatory disease, chlamydia, or genital herpes infections (heretofore referred to as ''other STIs'') were compared with those who had not. Women who reported two to five male sex partners were compared with subjects who reported only one. Time since first intercourse was estimated from the data as the difference between the woman's estimated age (from the data) and the age (in years) when the subject reported having had intercourse for the first time with a male partner. Women who reported using cervical caps, male condoms, or diaphragm contraceptive methods were collectively classified as using ''barrier contraceptives'' and were compared with women who reported other forms of contraception (i.e., oral, implantable, or injectable contraceptives; intrauterine devices, spermicides, or ''abstinence'').

Analyses. Tabular and descriptive statistics were used to contrast the odds for Pap test abnormalities and the presence of HPV16-specific antibodies for smokers and nonsmokers and other covariates of interest. Additionally, a maximum likelihood logistic regression model was developed to determine whether the risk for testing positive for HPV16 antibodies was affected by the covariates of interest (Proc Logistic, SAS; ref. 31). The final model estimated the odds of testing positive for HPV16-specific antibodies as a function of smoking status. Additionally, to control for potential confounding and to examine effect modification, we adjusted for Pap test, abnormalities, race/ethnicity, self-report for history of external genital warts, number of sex partners, age, use of barrier contraceptive methods, and time since first intercourse.

Deviance statistics, using a 0.05 level of significance, were used to evaluate a series of multiplicative models; no statistical interaction terms were found to be superior in comparison to the additive model included herein (31). Global model fit was evaluated using Hosmer-Lemeshow statistic (31). Last, two multivariate models were explored to determine the effect of Pap test findings; first, women showing ASCUS or SIL were compared with women with no identifiable lesion. Second, the risk for testing positive for anti-HPV16 antibody for women with high- or low-grade SIL was compared with otherwise similar women. In each model, we controlled for the effects of other covariates. The estimates from both models largely agreed. However, the model that compared women with ASCUS or SIL to those with no identifiable lesions showed better global fit statistics than the model that evaluated SIL alone, and those findings are

[8] Unpublished data.
[9] Eliav Barr, February 5, 2006, personal communication.

reported herein (i.e., Hosmer-Lemeshow statistic: X^2 = 8.8, 8 degrees of freedom, P = 0.4 versus X^2 = 11.9, 8 degrees of freedom, P = 0.2, respectively).

Results

Descriptive Statistics. Of the 205 women who were positive for HPV16 DNA at the initial visit, 71% (145 of 205) reported no tobacco use; 18% (37 of 205) reported smoking fewer than one or up to five cigarettes daily; and 7% (15 of 205) and 4% (8 of 205) reported smoking 6 to 10 or ≥11 cigarettes daily. Half of the women who tested HPV16 DNA positive also tested anti-HPV16 antibody positive at the initial study visit (53%, 108 of 205). Altogether, smokers were half as likely as nonsmokers to test positive for antibodies [odds ratio (OR), 0.5; 95% confidence limits (95% CL), 0.3-0.9]. Our univariate statistics suggest that there is a dose-response relationship between smoking and anti-HPV16 antibody positivity (P = 0.02; Table 1). For these analyses, nonsmokers and prior smokers who reported cessation for >3 months before the first study visit served as the reference group. Women who reported they had stopped smoking within 3 months of the baseline visit, current smokers that smoked up to five cigarettes daily, and those who smoked 6 to 20 cigarettes daily were 2, 1.4, and 3.3 times less likely than the referent group to test positive for anti-HPV16 antibody, respectively (Table 1).

For both cytologically normal and ASCUS/SIL-affected women, nonsmokers were about twice more likely than smokers to test positive for anti-HPV16 antibody (i.e., for no identifiable lesions and ASCUS/SIL: OR, 1.8; 95% CL, 0.8-4.0 and OR, 2.4; 95% CL, 1.0-5.9, respectively). Self-report for smoking tobacco products and Pap test findings were poorly correlated in these data (r^2 = 0.01). Most women in this study group reported themselves to be Caucasian, 9% were African American, 8% were Hispanic, 6% were of Asian ancestry, and 3% reported other racial heritage (Table 1). The mean and median number of years between first intercourse and the first study visit were 3.2 and 3 years, respectively. Most participants were young adults at the time of enrollment (i.e., the mean and median age was 20.1 and 20 years, respectively).

Women in this study sampled varied when compared with women in the overall vaccine trial who tested HPV-DNA negative. For example, women who tested positive for HPV16 DNA were more likely to report having had multiple male sex partners before enrollment in the study (Table 1). Only we found no statistical association between the number of lifetime male partners reported by women and either race (P = 0.5) or smoking (P = 0.6). Thirty-nine percent (80 of 205) of women reported using a barrier contraceptive method: diaphragm, condoms alone, or in combination with another contraceptive method; additionally, HPV16-infected smokers were more likely to report using barrier contraception than were nonsmokers (crude OR, 1.7; 95% CL, 1.0-3.1). Although 39% (81 of 205) of women reported using oral contraceptives only, contraceptive implants were reported by 10% (23 of 205), and 11% (24 of 205) reported being (sexually) abstinent at the time of their first study visit. Only 12% (25 of 205) of women

Table 1. Univariate and multivariate analyses of associations between behavioral and demographic risk factors and testing anti-HPV16 antibody positive for 205 women who tested HPV16 DNA positive using PCR at the first study visit

Characteristic	No. (% of column within category)	No. testing anti-HPV16 antibody positive (row %)	Unadjusted OR (95% CL)	Adjusted OR (95% CL)*,†
Cigarette smoking†				
Nonsmokers				
Never smoker and women with ≥ 3 mo smoking cessation	131 (64)	70 (53)	1‡	1
Smokers				
Stopped smoking < 3 mo	14 (7)	5 (36)	0.5 (0.2, 2.2)‡	0.5 (0.3, 0.9)
<1 up to 5 cigarettes daily	37 (18)	16 (43)	0.7 (0.3, 3.8)‡	
6-20 cigarettes daily	23 (11)	6 (26)	0.3 (0.1, 1.1)‡	
Pap test				
No intraepithelial lesions	118 (58)	47 (40)	1	1
ASCUS or SIL	87 (42)	50 (57)	2.0 (1.2, 3.6)	2.0 (1.1, 3.7)
Self-report for history of external genital warts				
No	188 (92)	86 (46)	1	1
Yes	17 (8)	11 (65)	2.0 (0.8, 6.1)	2.5 (0.8, 7.6)
Race/ethnicity				
Caucasian	160 (78)	72 (45)	1	1
African American	20 (10)	13 (65)	0.7 (0.3, 1.3)	1.5 (0.7, 3.2)
Asian	5 (2)	1 (20)		
Hispanic	14 (7)	8 (57)		
Other	6 (3)	3 (50)		
Lifetime number of male intercourse partners				
1	19 (9)	7 (37)	1	1
2	28 (14)	17 (61)	1.6 (0.6, 4.3)	1.2 (0.4, 3.5)
3	46 (22)	22 (48)		
4	59 (29)	29 (49)		
5	53 (26)	22 (42)		
Barrier contraceptive methods				
No	125 (61)	59 (47)	1	1
Yes	80 (39)	38 (48)	1.0 (0.6, 1.8)	1.1 (0.6, 1.9)
History of ''other STIs''				
No	182 (89)	84 (46)	1	1
Yes	23 (11)	13 (57)	1.5 (0.6, 3.6)	1.0 (0.4, 2.7)

*Estimates are adjusted for the effect of smoking, history of external genital warts, history of STIs (i.e., chlamydia, pelvic inflammatory disease, syphilis, gonorrhea, and genital herpes), age, number of years since first intercourse, barrier contraceptive methods, race/ethnicity (White versus other), lifetime number of male sex partners.

†Hosmer-Lemeshow model fit statistic: X^2 = 5.1, 8 degrees of freedom, P = 0.8.

‡Cochran-Armitage trend test Z = −2.3, P = 0.02.

reported having ever been diagnosed with other STIs before the first study visit.

Multivariate Analyses. After we controlled for the effects of other covariates, the model showed that women who smoked were twice less likely to test positive for HPV16 antibodies than were nonsmokers (adjusted OR, 0.5; 95% CL, 0.3-0.9; Table 1). Conversely, the independent association between cytologic abnormalities and anti-HPV16 antibodies in serum was positive. Women who showed ASCUS or SIL remained twice more likely than women showing no identifiable lesions to test positive for anti-HPV16 antibodies (adjusted OR, 2.0; 95% CL, 1.1-3.7; Table 1).

Relations between anti-HPV16 antibody in serum and other covariates were mixed. For example, these data suggested the prior history of external genital warts was positively associated with anti-HPV16 antibody in serum (adjusted OR, 2.5; 95% CL, 0.8-7.6; Table 1). Although we noted differences in the race, lifetime number of male sex partners, and reports of prior STIs in this sample, our adjusted analyses suggested that these characteristics were not associated with detection of anti-HPV16 antibodies in serum (Table 1). When we held the number of years since first intercourse and other covariates constant in our analyses, the risk for testing positive for anti-HPV16 antibodies decreased 10% for every added year of chronological age (i.e., adjusted OR, 0.9; 95% CL, 0.7-1.1). Conversely, for each additional year since first sexual intercourse, the risk of testing positive for anti-HPV16 antibodies increased by 18% (adjusted OR, 1.2; 95% CL, 1.0-1.4).

Discussion

These findings are important because they suggest that, among young women with HPV16 infection, smoking cigarettes may impair the development of an HPV16-specific humoral immune response. It remains to be seen whether HPV16 DNA positive smokers are at higher risk for persistent HPV16 infection and high-grade lesions than similar nonsmokers. Furthermore, these data do suggest a causal pathway between smoking and cancer that may be malleable to behavioral interventions.

The natural history of HPV16 humoral immunity suggests that antibody responses to genital HPV16 infection are delayed, and minimally, these data suggest that smoking may delay normally acquired immunity further yet. Onda et al. have shown that, on average, HPV16-infected women develop cervical HPV16-specific IgA and serologic evidence of IgG about 11 months following primary infection; the median time to detection of HPV16-IgA in serum is 19 months (32). However, measurable antibody at the site of infection may be short-lived. Among HPV16 DNA–positive women who had serum IgG antibodies to HPV16, Onda et al. found that HPV16 IgA antibodies persisted a median of 12 months in cervical secretions and nearly 14 months in serum after incident infection (32). However, more than half of women showed persistent serum IgG for >36 months following incident infection (32). No studies have reported how tobacco exposure specifically influences these trends, but our analyses suggest that, at the very least, the time to antibody detection may be delayed in both serum and at the cervix, and it remains to be seen whether these antibody-negative women ever develop detectable antibody over time.

It is unclear whether tobacco metabolites act alone or in concert with HPV16 viral proteins to affect the function of human immune cells. These data cannot delineate those relations more clearly. However, it is unlikely that these findings are due to systematic differences in exposure to the virus. Even after we adjusted for the effects of age, number of years since first intercourse, lifetime number of male sex partners, history of external genital warts or other STIs, use of barrier contraceptives, race/ethnicity, and concomitant Pap test abnormalities, the relationship between smoking and detection of anti-HPV16 antibody in serum persisted.

Others have shown that persistently HPV16-infected women are at high risk for developing high-grade cervical intraepithelial neoplasias and cervical cancer (33-37). Our analyses do not clarify how smoking may influence HPV16-specific acquired cellular immunity during early HPV16 infection. Nonetheless, our data do support the notion that detectable antibody may be the result of cellular disruption that comes of cytologic lesions. Specifically, after controlling for the effects of other covariates, women with ASCUS or SIL were twice as likely to test positive for antibody as were women showing no intraepithelial lesion on Pap test. An association between cellular disruption and development of immune responses has been reported in animal models and may suggest a pathway for acquiring cellular immune responses to this virus (38, 39).

These analyses may be limited. For example, investigating antibody responses alone may be less significant than understanding how smoking-related molecular events contribute to cytologic abnormality and malignancy. Associations detected using cross-sectional data may not be supported in longitudinal analyses; in addition, others have found that HPV-specific antibody in serum is delayed (32, 40), and our cross-sectional analyses cannot distinguish between females that may eventually respond and those who have failed. Women that enrolled in this study are, on average, well educated and Caucasian; thus, our findings may not be generalizable to other populations. Last, many of our polychotomous variables are self-report data; thus, the direction of residual bias cannot be determined, even if there is nondifferential misclassification (41).

Oncogenic HPV infections and the related intraepithelial lesions and cancers are important domestic and international public health problems. Smoking remains a significant public health problem for women throughout the world (42-48). These findings support the need for enhanced public health messages that convey yet another smoking-related health risk to women. Additionally, we believe primary prevention as a sole strategy may be a challenge for some years to come; sufficient herd immunity using efficacious vaccines may take some time to achieve. Thus, it may be important to test whether short-term or long-term behavioral smoking cessation interventions might influence immune responses and the long-term risk for persistent oncogenic HPV infection. Together, primary and secondary prevention will likely reduce the risk for cervical cancer in developed and developing countries alike.

The Proof-of-Principle Study Investigative Group

Roles	Names
Principal Investigator	Laura A. Koutsky, University of Washington, Seattle, WA.
Site Investigators	Kevin Ault, University of Iowa, Iowa City, IA; Karl Beutner, Dow Pharmaceutical Sciences, Vallejo and Davis, CA; Darron Brown, Indiana University, Indianapolis, IN; Henry Buck, University of Kansas, Lawrence, KS; Robert Edwards, University of Pittsburgh, Pittsburgh, PA; Daron Ferris, Medical College of Georgia, Augusta, GA;

Roles	Names
	Stanley Gall, University of Louisville, Louisville, KY; Laura A. Koutsky and Leslie Miller, University of Washington, Seattle, WA; Christine M. Peterson, University of Virginia, Charlottesville, VA; Yolanda Wade, Rutgers University, New Brunswick, NJ; Dorothy Wiley and Edward Wiesmeier, University of California-Los Angeles, Los Angeles, CA; Cosette Wheeler, University of New Mexico, Albuquerque, NM; Pauline Wood and David Whitaker, University of Rhode Island, Kingston, RI; Franklyn Judson, Denver Public Health Department, Denver, CO; Archana Chatterjee, Creighton University, Omaha, NE; Anna Giuliano, University of Arizona, Tucson, AZ.
Pathology Panel	Robert Kurman, Johns Hopkins University, Baltimore, MD; Mark Stoler, University of Virginia, Charlottesville, VA; Brigitte Ronnett, Johns Hopkins University, Baltimore, MD; Alex Ferenczy, McGill University, Montreal PQ, Canada.
Cytology Laboratories	Raheela Ashfaq, University of Texas-Southwestern Medical Center, Dallas, TX; Suzanne Selvaggi, Loyola University Medical Center, Chicago, IL; Women's and Infants' Hospital, Pittsburgh, PA; Nancy Kiviat; University of Washington, Seattle, WA; Vito Santarsieri, DIANON New City, NY.
Central Pathology Laboratory	Douglas Baker, Cassandra Fletcher, Covance Central Laboratory Services, Indianapolis, IN; Douglas King, Michael Glant, Carol Eisenhut, Diagnostic Cytology Laboratories, Indianapolis, IN.
Central PCR and Serology Facility	Kathrin U. Jansen (Project and Laboratory Head); Frank Taddeo; Anthony DiCello; Weili Li, Judith Smith, Rhonda Heffelfinger-Wenner; Dawn Campbell, Rocio Marchese, Joanne Erick; Merck Research Laboratories, West Point, PA.
Vaccine R&D Facility	Ann Lee (Project Head); Michael Kosinski; Hugh George, Victor Goetz, Peter De Phillips, Yang Wang, David Volkin, Li Shi, PK Tsai, Robert Sitrin Merck Research Laboratories, West Point, PA.
Clinical Coordinating Center	Eliav Barr (Clinical Monitor); Frances Alvarez (Lead Coordinator); Lisa Chiacchierini (Lead Statistician); Michael Dallas (Unblinded Statistician); Myrna Buiser; Margaret Swope; Suzanne Schild; Annemarie Thornton; Gretchen Suhr; Margaret Nelson; Paula Smith; Cheryl Lightfoot; Daniel Johnson; Ken Fujimori; Christine Kirk; Patricia Krout; Dylan Pugliese; Liping Zhang, Merck Research Laboratories, Blue Bell, PA.

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