

Distribution of Human Papillomavirus Types 16 and 18 Variants in Squamous Cell Carcinomas and Adenocarcinomas of the Cervix

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ABSTRACT

The distributions of human papillomavirus (HPV) types detected in cervical adenocarcinomas and squamous cell tumors differ. However, whether the distributions of intratypic HPV variants seen in these two histological forms of cervical disease differ is unknown. Our objective was to compare the distribution of HPV intratypic variants observed in squamous cell carcinomas (SCC) and cervical tumors of glandular origin (e.g., adenocarcinomas; AC) for two HPV types commonly observed in cervical tumors, HPV16 and HPV18. Participants in a multicenter case-control study of AC and SCC conducted in the eastern United States were studied. A total of 85 HPV16 and/or HPV18 positive individuals (31 diagnosed with AC, 43 diagnosed with SCC, and 11 population controls) were included. For HPV16-positive individuals, both the noncoding long control region and the E6 open reading frame were sequenced, and classified into phylogenetic-based lineage groups (European, Asian-American, African1, and African2). For HPV18-positive individuals, the long control region only was sequenced and classified into known intratypic lineages (European, Asian-American, and African). The distribution of these different intratypic lineages among AC cases, SCC cases, and population controls was compared using standard methods. Non-European HPV16 and/or HPV18 intratypic variants were observed in 42% of ACs compared with 16% of SCCs and 18% of population controls ($P = 0.04$). Intratypic variants from the Asian-American lineage of HPV16 accounted for the differences seen between histological groups. The differences observed between AC and SCC cases were strongest for HPV16, and persisted in analysis restricted to Caucasian women, suggesting that the effect cannot be explained by differences in the ethnic make-up of AC versus SCC cases. Cervical AC and SCC differ not only with respect to the distribution of HPV types detected but also with respect to intratypic variants observed. Non-European HPV16 and/or HPV18 variants are commonly seen in AC. A possible hormonal mechanism is suggested to explain the observed findings.

INTRODUCTION

More than 50 types of HPV³ are known to infect the female genital tract and a subset of ~12 of these are known to have oncogenic potential. In fact, there is strong support for the involvement of HPV

infection in the pathogenesis of nearly all cervical cancers (1). This strong link between HPV and cancer is observed for both squamous (*i.e.*, SCC) and glandular (*e.g.*, adenocarcinomas and adenosquamous carcinomas) forms of the disease. However, while a HPV infection appears to be required for the development of both SCC and AC, the distribution of HPV types seen in these two forms of the disease differ. HPV16 is the type most frequently involved in the development of SCC of the cervix (50% of squamous tumors are HPV16 positive), whereas both HPV16 and HPV18 play a prominent role in the development of AC of the cervix (2, 3). Furthermore, studies have suggested that cofactors associated with the development of SCC and AC are distinct. In addition to HPV infection, factors such as cigarette smoking and multigravidity are associated with increased risk of SCC, whereas these same exposures are associated with decreased risk of AC, pointing to differences in the biological mechanisms through which these different tumor types arise (3, 4).

It was suggested recently that intratypic variants of HPV (variants are defined as viruses that differ from other viruses of the same type by <2% of the nucleotide sequence in specific regions of the viral genome) might confer differential risk of cervical disease (5–11). Studies that have evaluated HPV16 variants and risk of SCC of the cervix and their precursor high-grade lesions have observed an increased risk of disease associated with non-European variants of HPV16 (5–9). More limited data also support a similar trend for HPV18 and SCC of the cervix (10, 11). Much less is known about the involvement of HPV variants in the development of cervical tumors of histological types other than SCC (9, 10). In one study that included 16 HPV16- or HPV18-positive AC and 19 HPV16- or HPV18-positive SCC, the presence of non-European variants was observed more commonly among AC (75%) than SCC (47%) (10). In a second report of six HPV16-positive AC cases, all six of the cases were found to be infected with a non-European form of HPV16 (9), suggesting a predilection of AC for non-European variants of HPV16.

In the present study, we evaluated the distribution of HPV16 and HPV18 variants in a group of cases diagnosed with AC of the cervix, SCC of the cervix, and population controls who participated in a multicenter case-control study of these tumors in the eastern United States (12). We report differences in the distribution of HPV variants for these histologically distinct forms of the disease, which may provide insight into the different mechanisms through which these different tumor types arise.

MATERIALS AND METHODS

The National Cancer Institute sponsored multicenter case-control study of cervical adenocarcinomas, adenosquamous carcinomas, and other tumors of glandular origin has been described previously (12). Briefly, we identified incident *in situ* and invasive cervical cancers of glandular origin (including adenocarcinomas, adenosquamous carcinomas, and other rare forms of glandular tumors referred henceforth as AC) from six medical centers in the

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³ The abbreviations used are: HPV, human papillomavirus; SCC, squamous cell carcinoma; AC, adenocarcinoma, adenosquamous carcinoma, or other rare forms of glandular tumors; LCR, long control region; OR, odds ratio; CI, confidence interval; ORF, open reading frame; GRE, glucocorticoid responsive element.

Northeastern/Mid-Atlantic corridor of the United States over a 51-month period between 1992 and 1996. Women diagnosed between 1992 and June 1994 (the date the study began) were retrospectively ascertained, whereas those diagnosed between July 1994 and March 1996 were ascertained prospectively. Pathologic material from 88% of participating cases was reviewed by an expert panel comprised of three pathologists and confirmed the original diagnosis. The original diagnosis was used for the remaining 12% of cases. One hundred thirty-eight AC cases (68% of those eligible) agreed to participate and had biological specimens collected for HPV testing.

In addition to AC cases, a second case group was recruited, consisting of a comparable number of women diagnosed with *in situ* or invasive SCC. SCC cases were matched to AC cases on age, hospital, diagnosis date, and stage (preinvasive *versus* invasive). One hundred thirty-six SCC cases (53% of those eligible) agreed to participate and had biological specimens collected for HPV evaluation.

A final group of age, ethnicity, and telephone exchange matched controls (matched to AC cases at a 2:1 ratio) with an intact uterus and no known cervical disease was recruited using a modified random digit dialing method. Two hundred fifty-five (54% of those determined via a telephone screening to be eligible) agreed to participate and provided specimens required for HPV testing.

All of the participants were administered an in-person interview by a trained interviewer. The interview elicited information on sociodemographic characteristics, sexual and reproductive behavior, cigarette smoking, medical history, and other factors postulated to be associated with the development of either SCC or AC. Published results from this study (3, 4, 12, 13) have identified the following factors as being associated with AC: oral contraceptives (for *in situ* disease only; OR, 6.0 for >6 years of use *versus* never), smoking (OR, 0.6 for current *versus* never smokers), hormone replacement therapy (OR, 2.7 for ever *versus* never use of unopposed estrogens), and gravidity (OR, 0.4 for gravid *versus* nulligravid women). Factors found to be associated with SCC include smoking (OR, 1.6 for current *versus* never smokers) and multigravidity (OR, 2.3 for 5+ pregnancies *versus* 1–2).

Participating subjects contributed cervical cells for HPV DNA testing. Specimens were collected using dacron swabs, placed into 1.0 ml of STM buffer (Digene Corp., Gaithersburg, MD), and stored frozen until testing. Details of specimen collection have been reported previously (12, 14). In brief, participants were asked to provide one self-administered cervicovaginal specimen. In addition, two clinician-administered specimens were collected at the time of pelvic examination. The two clinician-administered specimens were collected from the ectocervix and the endocervix, with the exception of cases whose cervix had been surgically removed. For these patients the two clinician-administered specimens were collected from the vaginal cuff.

All of the specimens were tested for the presence of one of 27 HPV types (including HPV types 16 and 18) using a MY09/MY11 L1 consensus primer-based reverse-line blot PCR detection method, as described previously (14). Because each participant provided up to three specimens (one self-administered and two clinician-administered) for HPV testing, a woman was considered HPV-positive for a given HPV type if any of her specimens were positive for that type. As expected, HPV was confirmed to be an important risk factor for both tumor types in our population (3). The ORs associated with HPV infection by any type were 24 (95% CI, 9.0–65) for AC and 15 (95% CI, 6.7–32) for SCC whose specimens were collected before treatment. ORs associated with HPV16 and HPV18 infection were 48 (95% CI, 14–160) and 105 (95% CI, 23–490), respectively, for AC, and 30 (95% CI, 12–77) and 20 (95% CI, 4.6–84), respectively, for SCC sampled before treatment (3). Thirty-six glandular cases, 45 squamous cases, and 18 controls were found to be positive for HPV16 and/or HPV18, and were targeted for HPV variant testing.

Viral variant classification was determined by sequencing the LCR of HPV16 and HPV18, and the E6 ORF for HPV16. The LCRs of HPV16 and HPV18 were amplified by nested PCR. The HPV16 E6 ORF was amplified as described previously (15). The LCR PCR products were purified using the Quickstep PCR kit (Edge Biosystems, Gaithersburg, MD), whereas the HPV16 E6 PCR products were isolated after gel electrophoresis using the Qiagen Gel extraction kit (Qiagen, Valencia, CA). Isolated PCR products were sequenced on both strands in the Albert Einstein College of Medicine sequencing core facility. Assignment of variants into lineages was performed based on previous studies correlating the geographic origin of HPV variants and the topography of the resulting phylogenetic tree (16–18). Accordingly, HPV16-positive spec-

imens were classified into European, African1, African2, or Asian-American lineages. HPV18-positive specimens were classified into European, African, or Asian-Amerindian lineages. In analyses comparing European variants to non-European variants, the African1, African2, and Asian-American lineages of HPV16 were combined into the non-European group. For HPV18, the African lineage was combined with the European lineage and compared against the Asian-Amerindian lineage. Similar findings (data not shown) were observed in analyses that excluded women infected with the African lineage of HPV18, and compared women infected with the Asian-Amerindian lineage of HPV18 against those infected with the European lineage.

We were successful in obtaining variant data for 66 HPV16-positive subjects, 16 HPV18-positive subjects, and 3 subjects positive for both HPV16 and HPV18. These 85 individuals comprise the analytical study group [31 AC cases (5 *in situ* and 26 invasive), 43 SCC cases (16 *in situ* and 27 invasive), and 11 population controls]. Restriction of the study to individuals positive for HPV16 or HPV18 allowed us to evaluate the effect of HPV variants on disease progression without concern for possible confounding by factors that might be associated with risk of infection.

The distribution of HPV16 and/or HPV18 variants between case groups and of cervical cancer risk factors between variant groups was compared using Fisher's exact test (19). ORs and 95% CIs were also computed. Medians were compared using the Wilcoxon test (20). We evaluated the distribution of HPV16 and/or HPV18 variants combined and separately. Analysis restricted to Caucasian women was performed to confirm that results were not confounded by ethnicity (also known as population stratification). Analyses restricted to invasive cancer cases were also performed but are not presented because they yielded results similar to those reported herein.

RESULTS

More than 80% of AC cases (81%), SCC cases (81%), and controls (82%) were Caucasian. Approximately 10% of all three of the study groups were of African descent, whereas the remaining subjects were of Hispanic, Asian, or Native American descent.

Table 1 presents the distribution of HPV16 and HPV18 variants observed in our study and their lineage classification. The European variants of both HPV16 (78%) and HPV18 (47%) were the most frequently detected isolates in our population. For HPV16-positive specimens, the Asian-American variants were detected in 13% of the specimens, and the African1 and African2 variants were observed in 4% and 3% of the specimens, respectively. For HPV18, the Asian/Amerindian variants were detected in 37% of specimens tested, whereas African variants were observed in 16% of specimens.

Fig. 1 (A–C) presents the distribution of non-European variants of HPV16 and/or HPV18 among AC cases, SCC cases, and controls. A significant association was observed between the presence of non-European variants of HPV16 and/or HPV18 and disease status ($P = 0.04$). This association was even stronger in analysis restricted to Caucasian women ($P = 0.009$). Overall, 42% of AC cases were infected with non-European variants of HPV16 and/or HPV18, compared with 16% of SCC cases and 18% of population controls. The effects observed were significant for HPV16 ($P = 0.02$) but not for HPV18 ($P = 1.00$). Interestingly, the increased prevalence of non-European HPV16 infections seen among individuals diagnosed with AC was restricted primarily to infections with variants from the Asian-American lineage (Table 2). Of HPV16 positive AC cases, 38% were infected with Asian-American variants of the virus, compared with 3% of SCC cases and none of the population controls ($P = 0.002$). ORs and 95% CIs comparing the distribution of variants between study groups are also shown in Table 2.

Because AC cases comprise a diverse group of tumors of glandular origin, we examined the distribution of non-European variants of HPV16 and/or HPV18 in different subsets of AC cases (Table 3). The prevalence of non-European variants of HPV16 and/or HPV18 was observed to be 40% or higher for adenocarcinomas of endocervical or

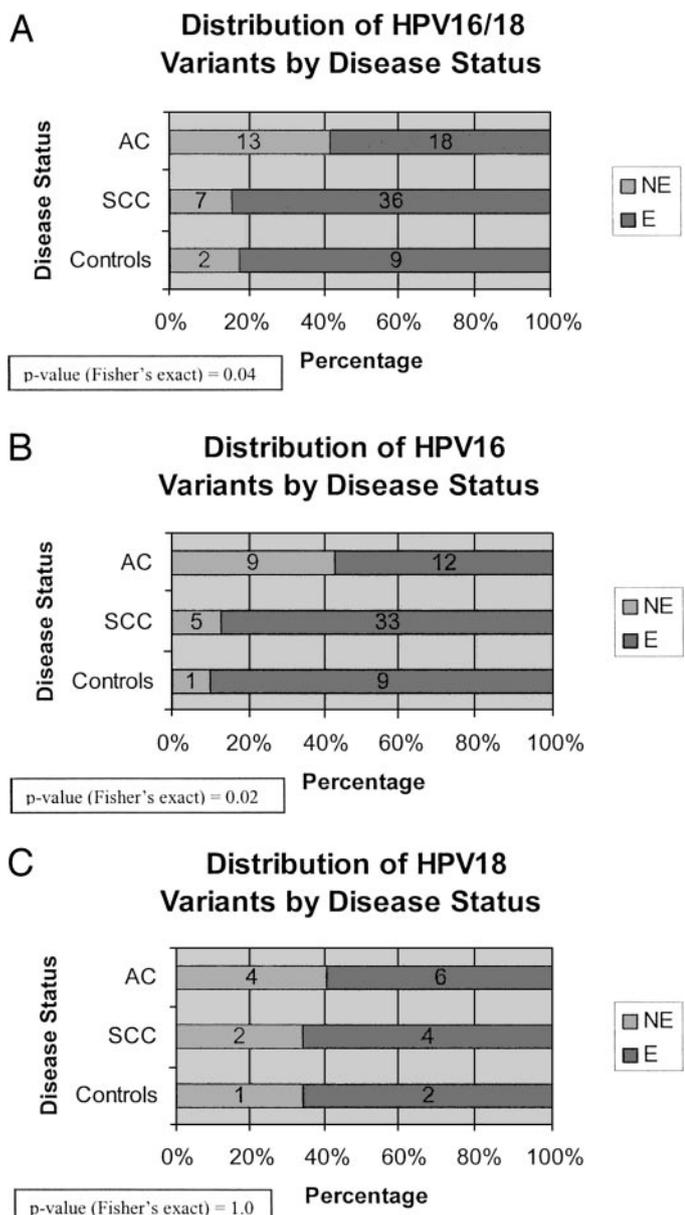


Fig. 1. NE = non-European variants; E = European variants (HPV18 variants of African lineage included with European variants—see “Materials and Methods.”). Three individuals were infected with both HPV16 and HPV18.

biological mechanisms that explain the different distribution of HPV variants seen among these two distinct histological subtypes of cervical tumors. We observed an expected difference in the ethnic make-up of individuals infected with the non-European *versus* the European variants of HPV16 and HPV18 (presence of non-European variants is known to correlate with non-Caucasian ethnicity). No other significant differences were noted between the two groups, although

a nearly significant difference was seen between individuals infected with the European *versus* non-European variants with respect to number of pregnancies ($P = 0.06$).

To evaluate the possibility that tumors containing non-European variants of HPV16 and/or HPV18 are more aggressive and occur at younger ages than those containing European variants of these viruses, we examined the age distribution of cases occurring in women infected with European and non-European variants of HPV16 or HPV18. *In situ* and invasive cancers were examined separately, as were AC and SCC cases. No significant differences were noted in the median age for any of the groups evaluated. The median age for *in situ* cases infected with the non-European variant of HPV16 was 37 ($n = 3$; range = 30–61) compared with 30 ($n = 15$; range = 21–61) for those infected with European variants of HPV16 ($P = 0.27$). For invasive cancers, the median age for cases infected with non-European variants of HPV16 was 38 ($n = 9$; range = 25–49) compared with 43 ($n = 29$; range = 22–68) for those infected with European variants of HPV16 ($P = 0.16$). Similar patterns were observed when analysis was stratified by histological type and when HPV18 variants were examined (data not shown).

We also evaluated variation at individual nucleotide positions other than the positions that define specific variant lineage groups. No significant differences were noted between study groups. Of note, we did not observe evidence of a difference in the distribution of E6 350 (55% of AC cases, 36% of SCC cases, and 50% of controls were infected with HPV16 containing a G rather than T at nucleotide position 350; $P = 0.39$) or LCR 7521 variants (67% of AC cases, 61% of SCC cases, and 90% of controls were infected with HPV16 containing an A rather than G at nucleotide position 7521; $P = 0.23$). Finally, among individuals infected with a European variant of HPV16, no significant differences between groups were noted when the number (0, 1, 2+) of nucleotide positions varying from the prototype virus were examined ($P = 0.15$ for HPV16 LCR and $P = 0.27$ for HPV16 E6 regions).

DISCUSSION

It is well documented that the distribution of HPV types differs between AC and SCC of the cervix (2, 3). Whereas HPV16 accounts for the majority of SCC, both HPV16 and HPV18 are prominent in AC. We now report that the distribution of HPV16 variants also differs for these two histological subgroups of cervical cancer. Specifically, non-European variants of HPV16 were more commonly seen in AC than SCC. This effect persisted in analysis restricted to Caucasian women. Interestingly, in our population, the Asian-American variant of HPV16 appears to be largely responsible for the excess of non-European variants seen in AC cases.

These findings are particularly relevant given that we and others have demonstrated that AC and SCC differ considerably with respect to their nonviral risk factor profile (3, 4, 12, 13, 21, 22). Most notably, factors linked to AC but not SCC are similar to those seen for endometrial adenocarcinomas and point to an important hormonal involvement in the pathogenesis of cervical glandular cancers. Also, it

Table 2 Distribution and risk associated with HPV16 lineages

Lineage Group	% Ctrls ($n = 10$)	% SCC ^a ($n = 37$)	% AC ($n = 21$)	OR [SCC vs. Ctrl] (95% CI)	OR [AC vs. Ctrl] (95% CI)	OR [AC vs. SCC] (95% CI)
European	90%	89%	57%	1.0	1.0	1.0
African	10%	8%	5%	0.82 (.08, 8.8)	0.75 (.04, 14)	0.92 (.09, 9.7)
Asian-American	0%	3%	38%	Inf.	Inf.	22 (2.5, 200)
P^b	0.002	1.0		0.04	0.001	
	(Overall)	(SCC vs. Ctrls)		(AC vs. Ctrls)	(AC vs. SCC)	

^a Exact lineage could not be determined for 1 squamous case.

^b P s from Fisher's exact test.

Table 3 Distribution of HPV16 and/or HPV18 non-European variants by histological subgroups of AC cases

Histological Group	N	% non-European
Adenocarcinoma <i>in situ</i>	4	25%
Adenocarcinoma, endocervical/endometrioid/NOS	14	50%
Adenocarcinoma, other ^a	7	43%
Adenosquamous carcinoma	5	40%
Adenoid basal carcinoma	1	0%

^a Includes 1 clear cell tumor, 1 serous tumor, and 5 well-differentiated villoglandular tumors.

has been observed that normal endocervical columnar cells (site often associated with AC) invariably express estrogen and progesterone receptors, whereas basal cells in the squamous epithelium (site associated with SCC) are negative for progesterone receptors and only infrequently positive for estrogen receptors (23–25). In this context, it is interesting to note that the HPV genome contains a number of GREs and estrogen-responsive elements that may play an important role in the regulation of viral gene expression, and that at least two GREs exist (at positions 7477–7491 and 7643–7657) in the LCR region of the HPV16 genome that we sequenced as part of the present work (26, 27). Sequence variability in these two regions is seen between the different variant lineages of HPV16 observed in our study. In particular, all of the Asian-American variants have sequence differences within the 7477–7491 GRE. The functional significance of these differences remains to be elucidated.

In addition to potential differences in the hormone responsiveness of different HPV intratypic variants, functional differences in either the oncogenic potential of different variants and/or differences in their ability to circumvent the natural immune surveillance of viral infections of the host are possible (28–32), although it is unclear whether such differences would be expected to be differential by cell type (SCC *versus* AC). More *in vitro* work will be required to elucidate functional differences between intratypic variants of HPVs and to determine the molecular basis for their propensity to induce different histological types of cervical cancer.

The lack of an excess of non-European variants of HPV16 and/or HPV18 among cases diagnosed with SCC relative to our population controls is puzzling but might be population specific (5–11). Published studies conducted in Latin America, Japan, and the United

States have reported increases in the prevalence of HPV16 non-European variants among cases diagnosed with squamous cancers and their high-grade precursor lesions compared with HPV16-infected controls. It should also be noted that the number of HPV16- and/or HPV18-positive population controls in our study was small ($n = 11$), and resulting estimates of HPV variant distribution in this group are unreliable.

Limitations of the present study should be noted. First, the sample size of our study was modest, despite its multicenter nature. However, given the rarity of the disease being evaluated, this study comprises the largest study to date to evaluate the association between HPV variants and risk of cervical adenocarcinomas. Second, the response rates to our study (68% for AC cases, 53% for SCC cases, and 54% for controls) were less than ideal, given the widespread difficulties in obtaining good response rates in United States based epidemiological studies. Whereas the possible biases associated with nonresponse are difficult to assess, there is no *a priori* reason to expect that nonparticipants are more or less likely to be infected with specific HPV variants. Third, our study design involved both retrospective and prospective ascertainment. Therefore, participants recruited retrospectively were often sampled for HPV testing after surgical removal of the affected tissue. This resulted in an overall rate of HPV positivity among cases that was lower than expected given that HPV is a necessary cause of cervical cancer. We cannot rule out the possibility that this has biased the distribution of HPV variants seen in our study. For any such bias to affect our overall findings, however, the bias would have had to be differential between AC and SCC cases.

In summary, we report differences in the distribution of HPV16 intratypic variants by histology. Non-European variants (particularly the Asian-American variants of HPV16) were more commonly seen among AC cases than SCC cases. This finding, along with the known differences in cofactors associated with the development of AC *versus* SCC, suggests different etiologies for these two histological subgroups of cervical cancer. A possible hormonal mechanism for the differences observed is suggested. Although difficult to conduct given the rarity with which AC of the cervix occur, larger studies are needed to validate results reported herein.

Table 4 Prevalence and risk associated with of non-European HPV16 and/or HPV18 variants by selected factors

Risk factor ^a	European (%) <i>n</i> = 60	Non-European (%) <i>n</i> = 25	OR (95% CI)	<i>P</i>
Ethnicity				0.001
Caucasian	90%	55%	1.0	
African-American	5%	27%	9.5 (2.1, 43)	
Hispanic	3%	9%	4.8 (0.61, 37)	
Other	2%	9%	9.5 (0.80, 110)	
Duration of cigarette smoking				1.00
Never	31%	30%	1.0	
≤10 years	21%	20%	0.97 (0.22, 4.1)	
10–20 years	22%	25%	1.1 (0.29, 4.5)	
>20 years	26%	25%	0.99 (0.25, 3.9)	
Duration of oral contraceptive use				0.13
Never	17%	10%	1.0	
<2 years	25%	45%	3.0 (0.53, 17)	
2–6 years	20%	30%	2.5 (0.41, 15)	
>6 years	38%	15%	0.65 (0.09, 4.5)	
Number of pregnancies				0.06
None	21%	5%	1.0	
1–2	32%	60%	7.8 (0.90, 67)	
3	13%	20%	6.5 (0.61, 69)	
4+	34%	15%	1.9 (0.17, 20)	
Hormone replacement therapy use				1.00
Never	85%	90%	1.0	
Unopposed estrogens	10%	5%	0.49 (0.06, 4.4)	
Other HRT	5%	5%	0.98 (0.10, 10)	

^a Three individuals missing information on duration of cigarette smoking, number of pregnancies, and hormone replacement therapy use; five individuals missing information on duration of oral contraceptive use.

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