Hybrid Capture 2 Test Results After an Initial Equivocal RLU/CO Value Are Dependent on Age

Camille T. Elkins, MD,1 Christiaan E. de Vries, MD,1 Julie Stephens, MS,2 and Adrian A. Suarez, MD1

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Abstract

The effect of age on Hybrid Capture 2 (HC2) tests initially falling within the equivocal range has not been determined. We identified 359 cervicovaginal liquid cytology specimens with initial equivocal values. First and second retest relative light units/cutoff (RLU/CO) values were compared for women of 3 different age groups (15-29, 30-49, and ≥50 years). The proportion of first retests with an RLU/CO of less than 1 increased with age (P < .001). Of the 56 second retests performed, only 4 had an RLU/CO of 1 or more. The proportion of “positive” HC2 results following the current HC2 algorithm decreased with increasing age (P < .001), showing that HC2 test results after an initial equivocal value are dependent on age. Follow-up demonstrated cervical intraepithelial neoplasia grade 2 or worse (CIN2+) in 6 (5.9%) women 15 to 29 years old and in 5 (6.3%) women 30 to 49 years old. No CIN2+ was found on follow-up of 34 of 57 women 50 years and older. These results likely reflect human papillomavirus infection prevalence and question the use of identical cutoff values regardless of age for detection of CIN2+. Persistent cervical infection with high-risk human papillomavirus (HPV) types causes cervical cancer and its precursor lesions.1 HPV DNA testing, which has greater sensitivity but lower specificity for cervical intraepithelial neoplasia grade 2 or worse (CIN2+) compared with cytology, was first incorporated in the American Cancer Society (ACS) cervical cancer screening guidelines in 2002.2 According to the most recent guidelines by the ACS, the American Society for Colposcopy and Cervical Pathology, and the American Society for Clinical Pathology, HPV DNA cotesting is recommended in women 30 to 65 years old, allowing for an increased screening interval of 5 years.3 However, the performance characteristics vary among different commercially available HPV DNA tests, and excessive analytic sensitivity is currently deemed a significant concern due to poorer specificity.4

The Hybrid Capture 2 (HC2) system (Qiagen, Valencia, CA) is a widely used Food and Drug Administration–approved test for detection of HPV DNA by probe hybridization and chemiluminescence. A liquid cervicovaginal specimen’s signal is measured in relative light units (RLU) and compared with the average signal of positive controls provided by the manufacturer. According to manufacturer recommendations, the RLU/cutoff (RLU/CO) ratio value is considered positive when greater than or equal to 1, corresponding to a concentration of 1 pg/mL.5 However, an initial RLU/CO value between 1 and less than 2.5 is considered equivocal and prompts a confirmatory retest. If a first retest is negative, then the result of a second retest is considered the final result.5 HC2 performance at different cutoff values has been addressed by several studies. Some point out that higher HC2 cutoff values (up to RLU/CO ≥10) would result in beneficial reductions in positive HC2
results not associated with high-grade cervical intraepithelial neoplasia while maintaining acceptable sensitivities. Others have warned about the potentially missed clinically significant lesions.

We are interested in age as a major determinant of HPV infection prevalence and, consequently, of HC2 performance. In a recent study of women 50 years and older, we found no cases of CIN2+ upon follow-up of specimens with an initial RLU/CO between 1 and less than 10 undergoing HC2 testing at our institution. We now set out to determine the characteristics of the HC2 test strictly within the equivocal RLU/CO range in a wide age range, with the detection of CIN2+ as the end point. We examined 359 consecutive cervicovaginal liquid cytology specimens with initial equivocal-range RLU/CO values (1 to <2.5) spanning 3 age groups (15-29, 30-49, and ≥50 years). All RLU/CO values were analyzed, and all available histologic and cytologic follow-up was retrospectively obtained and reviewed.

Materials and Methods

Case Material

We retrospectively identified 359 consecutive cervicovaginal liquid cytology specimens with initial equivocal HC2 HPV tests. Specimens included both in-house material and specimens referred to our laboratory from other institutions solely for HC2 testing. These HC2 tests included reflex HPV testing on atypical squamous cells of undetermined significance (ASCUS) interpretations as well as physicians’ requests for testing regardless of cytologic diagnosis. Subsequent cytologic and histologic specimens were reviewed when available. This study was conducted under appropriate institutional review board approval (OSU IRB 2002H0089) from The Ohio State University.

HPV Testing

HC2 testing was performed according to the manufacturer’s instructions. Most specimens were collected using the SurePath system (BD, Franklin Lakes, NJ). A minor proportion of specimens was collected using the ThinPrep system (Hologic, Marlborough, MA). All initial equivocal HC2 (RLU/CO 1 to <2.5) results were included in the study.

Statistical Analysis

Summary statistics were calculated by age group (15-29, 30-49, and ≥50 years) and test performed (initial, first retest, and second retest). Data were described by age group and test performed using line graphs. The dichotomized RLU/CO result at each test was compared between the age groups with either a χ² or Fisher exact test. Low-grade squamous intraepithelial lesion (LSIL)/cervical intraepithelial neoplasia grade 1 (CIN1) or CIN2+ in the follow-up was compared between the age groups with χ² tests. Figure 1 was produced using Partek software (Partek, St Louis, MO). All other analyses were performed using SAS version 9.2 (SAS Institute, Cary, NC).
**Results**

**Initial RLU/CO Ratios by Age**

We identified 359 consecutive “equivocal” HC2 test results at our institution from 349 patients ranging in age from 15 to 74 years. The tests were divided according to the patients’ age: 15 to 29 years (n = 163), 30 to 49 years (n = 139), and 50 years or older (n = 57).

**Retesting With Equivocal Initial RLU/CO Ratios**

Cases with equivocal results (RLU/CO 1 to <2.5) were retested in accordance with HC2 manufacturer guidelines. Figure 1 shows RLU/CO values for all tests and retests. Twelve (7.4%) specimens from women aged 15 to 29 years, 28 (20.1%) specimens from women aged 30 to 49 years, and 16 (28.1%) specimens from women 50 years and older had a first retest less than 1. This difference between the age groups’ first retest RLU/CO values was statistically significant ($P < .001$) based on a $\chi^2$ test. Initial equivocal tests that had a first retest value of less than 1 underwent a second retest. Of these 56 second retests, only 4 had an RLU/CO of 1 or higher. The proportion of values of second retests less than 1 was not statistically significant ($P = .426$) between the age groups based on a Fisher exact test. Overall, the 359 initial equivocal tests resulted in 307 (85.5%) final positive reports. The distribution of final positive results was statistically significant ($P < .001$) between the age groups based on a $\chi^2$ test, with a decrease in the proportion of positive results as age increases.

**Follow-up**

**Ages 15 to 29 Years**

We identified 163 tests in the 15- to 29-year-old age group. Of these, corresponding index Papanicolaou (Pap) smear tests were found for 142 cases. The initial cytologic interpretations were as follows: 16 negative for intraepithelial lesion or malignancy (NILM; 11.3%), 125 ASCUS (88.0%), and 1 LSIL (<1%). Follow-up was available for 102 cases, ranging from 2 weeks to 34 months (average, 16.6 months): only cytologic follow-up for 44 cases, only histologic follow-up for 14 cases, and both cytologic and histologic follow-up for 44 cases. A low-grade lesion (LSIL/CIN1) was found in 36 cases (35.3%). There were 6 (5.9%) follow-up diagnoses of CIN2+. No cases with initial equivocal HC2 and an NILM diagnosis had a follow-up of CIN2+.

**Ages 30 to 49 Years**

We identified 139 tests in the 30- to 49-year-old age group. Ninety-nine corresponding index Pap smear tests were found. The initial cytologic interpretations were as follows: 27 NILM (20.1%), 70 ASCUS (52.4%), and 1 LSIL (<1%). Follow-up was available for 80 cases, ranging from 1 to 37 months (average, 19.2 months): cytologic follow-up only for 33 cases, histologic follow-up only for 3 cases, and both cytologic and histologic follow-up for 44 cases. A low-grade lesion (LSIL/CIN1) was found in 25 cases (31.3%). Five cases (6.3%) had follow-up diagnoses of CIN2+. No cases with initial equivocal HC2 and an NILM diagnosis had a follow-up of CIN2+.

**Age 50 Years and Older**

We identified 57 tests for women 50 years and older. There were 42 corresponding index Pap smear tests found. The initial cytologic interpretations were as follows: 21 NILM (50.0%), 20 ASCUS (47.6%), and 1 LSIL (2.4%). Follow-up was available for 34 cases, ranging from 1 to 32 months (average, 16.4 months). Only cytologic follow-up was available for 18 cases, no cases had only histologic follow-up, and 16 cases had both cytologic and histologic follow-up. A low-grade lesion (LSIL/CIN1) was found in 8 cases (23.5%). No CIN2+ lesions were found on follow-up regardless of cytologic diagnosis.

**Discussion**

HC2 tests with initial RLU/CO values within the equivocal range were evaluated to ascertain the influence of age on final HC2 results and on detection of CIN2+. A positive HC2 was reported in 307 of the 359 HC2 tests.
(85.5%), mostly due to a first retest with an RLU/CO value of 1 or more. However, a significant increase (P < .001) in the proportion of first retests with RLU/CO values less than 1 was detected among specimens from 349 women as age increased. Furthermore, in the vast majority of cases (52/56, 93%), first retest RLU/CO values less than 1 were followed by second retest RLU/CO values less than 1, ultimately leading to negative HC2 test reports. The likelihood of specimens with initial equivocal RLU/CO values leading to final positive HC2 reports decreased with age, from 93.3% in women 15 to 29 years old to 71.9% in women 50 years and older (P < .001). Although not proportional, these findings likely reflect the overall HPV infection prevalence curve. However, population-based studies specific to our geographic area are lacking. Given an initial equivocal RLU/CO, the current HC2 manufacturer-recommended interpretative algorithm led to detection of CIN2+ only in women younger than 50 years. Hence, in keeping with our previous work,18 we consider the proportion of final HC2 positive reports inappropriately high in women 50 years and older because not a single CIN2+ was detected.

Test performance is affected by the prevalence of the condition to be detected in a given population. HPV infection prevalence, which is the product of acquisition of new infections and persistence of infections, significantly varies with age in many populations.16 A large cross-sectional polymerase chain reaction (PCR)–based study demonstrated different age-specific curves along with prevalences that varied more than 10-fold among 15 different areas of 4 continents. Areas of Italy, the Netherlands, Spain, Argentina, and others showed an HPV prevalence peak for those younger than 25 or 35 years, followed by a steady decline. However, a second generally smaller peak was detected in women 55 years and older in areas of Chile, Colombia, Mexico, and Costa Rica.16,17 Castle et al17 documented that type-specific persistence of HPV infection is a main contributor to the second peak in the Costa Rican population-based study. US studies, with a variety of HPV detection and sampling methods, have shown that HPV prevalence peaks in young women relatively shortly after the initiation of intercourse and is then followed by steady declines.14,15,19,20 Wheeler et al19 recently reported prevalence peaks in women 20 years and younger for HPV-16, HPV-18, and any carcinogenic HPV among 47,617 women in a New Mexico statewide surveillance program. The National Health and Nutrition Examination Survey detected a peak HPV prevalence of 44.8% among women 20 to 24 years old in a US nationally representative sample using self-collected vaginal swab specimens analyzed with PCR.15 The prevalence in this study dropped to 19.6% in women 50 to 59 years old.15 Similarly, the Addressing the Need for Advanced HPV Diagnostics (ATHENA) study showed peak prevalences of HPV-16 and HPV-18 infection in women 21 to 24 years old; these were followed by a steady decrease to less than 1% in women 50 years and older.20 The reports by Datta et al14 and Castle et al13 are particularly relevant to our current study because they used the HC2 system. Datta et al14 reported on 9,657 women who received routine cervical screening at 26 clinics in Boston, Baltimore, Denver, New Orleans, Seattle, and Los Angeles. Overall HC2 positivity was 23%, with a peak of 35% in women 14 to 19 years old that decreased to 6% in women 50 to 65 years old. Data from Kaiser Permanente Northern California (n = 580,289) reported by Castle et al13 showed HC2 positivity peaking at 10.82% and then decreasing to 3.67% in women 30 to 34 and 60 to 64 years old, respectively. Of note, a slight rise in HC2 positivity was detected in women 70 years and older by Castle et al,13 and a slight increase in pooled high-risk HPV types excluding HPV-16 and HPV-18 was reported in women 70 years and older by the ATHENA group.20 So, the existence and magnitude of an increase in HPV infection prevalence later in life in areas of the United States that, like ours, lack population-based studies is an open question. What is clear from the data we are presenting here is that such a phenomenon, if at all present in our population, does not translate into detection of CIN2+ given initial equivocal HC2 RLU/CO values in women 50 years and older.

The HC2 RLU/CO level could potentially be adjusted for age to improve the performance of the test. Several investigators have evaluated HC2 with cutoff levels above the manufacturer-recommended RLU/CO of 1 or more. On the basis of their systematic review of the PubMed database up to August 2010, Rebolj et al10 concluded that HC2 cutoff levels between an RLU/CO of 2 or more and 10 or more would result in at least 90% sensitivity for CIN2+ compared with an RLU/CO of 1 or more. This conforms to recent guidelines for HPV detection in a primary screening setting issued by an international panel.21 For example, Ronco et al7 compared conventional cytology with HC2 in a large randomized controlled trial. The relative sensitivity for CIN2+ was 1.92, and the relative positive predictive value was 0.8 among women 35 to 60 years old with the usual RLU/CO of 1 or more. However, the authors conclude that an RLU/CO of 2 or more is preferable in women of this age group because the relative positive predictive value increased to 0.99, whereas the relative sensitivity decreased only to 1.81.7 Similar recommendations were issued from A Randomized Trial In Screening To Improve Cytology (ARTISTIC) in England.8 Jarboe et al22 reported the detection of CIN2+ to be significantly lower in women with ASCUS (3.2% vs 17.3%) when the RLU/CO was 10 or less. Similarly, the ASCUS-LSIL Triage Study (ALTS) reported that among women with ASCUS, increasing the HC2 cutoff to 10 pg/mL or more would produce only a slight decrease...
in sensitivity but warned that this might be unacceptable if maximal detection of cervical intraepithelial neoplasia grade 3 or worse (CIN3+) is paramount.12 Of note, with the exception of women 29 years and older with LSIL, it appears from these same ALTS data that the reduction in sensitivity for CIN3+ with this higher threshold may depend on age. The larger reductions in sensitivity were seen in younger age groups (18-22 years), whereas in women 29 years and older, the reduction was only 3%.12 In addition, Sargent et al8 have reported that confirmation of HC2 results with a second molecular test was better with an HC2 cutoff level of an RLU/CO of 2 or more compared with an RLU/CO of 1 or more and that the correlation improvement, given the higher threshold, was even better for their group of older women (35-64 years).

The current study included specimens collected in SurePath and ThinPrep media. These media use different fixatives: ethanol and methanol based, respectively. However, this is unlikely to have influenced our results as similar HC2 performance has been demonstrated by Siddiqi et al23 in women with ASCUS and by Qureshi et al24 in women with LSIL. Our current retrospective study is limited by cases lacking follow-up and by the unselected nature of our patients, including index specimens with different cytologic diagnoses. In addition, the specimens were derived from an unknown mix of clinical settings that we presume to at least partially represent opportunistic screening or follow-up of previous abnormal findings. Furthermore, our perspective is somewhat limited by the lack of population-based data specific for our geographic region. However, we have documented that CIN2+ is distinctively rare in women 50 years and older with initial RLU/CO values within the equivocal range and that age is a major determinant of these HC2 test final results. Hence, further larger studies should evaluate the possibility of age-adjusted HC2 interpretation guidelines in primary screening as well as other clinical scenarios.

From the 1Department of Pathology and 2Center of Biostatistics, The Ohio State University College of Medicine, Columbus, OH.

Address reprint requests to Dr Suarez: Dept of Pathology, The Ohio State University Medical Center, 410 Doan Hall, 410 W 10th Ave, Columbus, OH 43210; e-mail: Adrian.Suarez@osumc.edu.

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