**The PapilloCheck® Assay for the Detection of High Grade Cervical Intraepithelial Neoplasia**

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Running title: PapilloCheck® assay for CIN2+ detection

**Abstract**

Human papillomavirus (HPV) testing is used in primary cervical screening, as an adjunct to cervical cytology for the management of low grade abnormal cytology, and in a test of cure. PapilloCheck® (Greiner Bio-One) is a PCR-based DNA microarray system that can individually identify 24 HPV types, including the 13 high risk (HR) types identified by Hybrid Capture-2 (HC2). Here we compare PapilloCheck® with HC2 for the detection of high grade cervical intraepithelial neoplasia (CIN2+) in a total of 8610 cervical cytology samples from the ARTISTIC population-based cervical screening study. We performed a retrospective analysis of 3518 cytology samples from Round 1 ARTISTIC enriched for underlying CIN2+ (n=723), and a prospective analysis of 5092 samples from Round 3 ARTISTIC. Discrepant results were tested using the Roche Reverse Line Blot (RLB) or Linear Array (LA) assay. The relative sensitivity and specificity of HR PapilloCheck® compared with HC2 for the detection of CIN2+ in women aged over 30 years were 0.94 (95% CI 0.91,0.97) and 1.05 (95% CI 1.04,1.05), respectively. HC2 missed 44/672 (7%), whilst HR PapilloCheck® missed 74/672 (11%) CIN2+ lesions. 36% of HC2-positive normal cytology samples were HR HPV negative both by PapilloCheck® and RLB/LA, indicating that the use of HR PapilloCheck® rather than HC2 in population-based primary screening would reduce the number of additional tests required (eg reflex cytology) in women where underlying CIN2+ is extremely unlikely. HR PapilloCheck® could be a suitable HPV detection assay for use in the cervical screening setting.

**Introduction**

Organised cervical screening by cytology has been effective at reducing cervical cancer incidence and mortality in countries where resources permit (1). The established role of persistent high risk human papillomavirus (HPV) infection in cervical carcinogenesis (2-4) has led to the use of HPV testing alongside cytology to triage minor cytological abnormalities, in post-treatment follow up and, more recently, as the primary screen of cervical samples (5). The rationale for this lies in the added sensitivity and high negative predictive value of HPV testing compared with cervical cytology (6). Until recently, the only HPV test approved for use in cervical screening programmes was the Hybrid Capture-2 (HC2) assay (Qiagen), which employs an RNA cocktail probe to induce a chemiluminescent reaction upon HPV DNA binding with any of 13 high risk types. HC2 generally has good clinical utility for the detection of high grade premalignant disease of the cervix (cervical intraepithelial neoplasia (CIN)2 or 3) (7, 8), but there have been concerns about its low specificity and positive predictive value (PPV). Several reports have indicated that it erroneously detects low risk HPV types (9-11) and may even cross react with non-HPV DNA (12). This is seen more in older women, where PCR fails to detect high risk (HR) HPV in as many as 50% of HC2 positive samples (13). The net result of this is that women may be inaccurately labelled as ‘at risk’, leading to anxiety and over investigation. An HPV test that offered improved specificity would reduce the number of unnecessary tests performed for women at low risk of clinically significant premalignant disease of the cervix.

PapilloCheck® (Greiner Bio-One) is a PCR-based DNA microarray system that identifies 24 HPV types individually, including the 13 HR types detected by HC2. The assay involves amplification of the viral E1 and hybridisation on a chip spotted with DNA probes for the 24 different HPV types. The aim of this study was to compare the clinical performance of HC2 and PapilloCheck® for the detection of underlying CIN2 or worse (CIN2+) using cervical samples from the ARTISTIC study (6), a randomised population-based trial of 24,510 women undergoing routine cervical screening in the UK National Health Service Cervical Screening programme (NHSCSP). We analysed a total of 8610 samples comprising 3518 archival samples enriched for underlying CIN2+ and 5092 prospectively tested samples using HC2 and PapilloCheck®. HPV positive samples or discrepant results were additionally tested using the Roche prototype Reverse Line Blot assay or the Roche Linear Array assay.

**Methods**

*Study participants and cervical cytology specimens*

The ARTISTIC trial methods and design have been reported elsewhere (6).Women in Greater Manchester attending their general practitioner or a sexual health clinic for routine cervical screening in the NHS programme between 2001 and 2003 were invited to take part in the ARTISTIC study. After giving written informed consent, cervical cytology samples were collected in PreservCyt liquid based cytology (LBC) solution and slides were prepared using the ThinPrep T3000 processor (Hologic) at the Manchester Cytology Centre. Slides were examined by cytoscreeners and graded according to the BSCC classification.Women were randomly allocated in a ratio of 3:1 to have the HPV result revealed and acted upon, or concealed and further management based on cytology alone. Management of women with abnormal cytology was identical in both arms, following national guidance for the English Cervical Screening Programme (14). In the second screening round (three years later), colposcopy referral was after two and not three borderline cytology results. Women in the revealed arm with negative cytology who tested HPV positive were invited for repeat HPV testing at 12 months and if still positive could choose between immediate colposcopy or a repeat HPV test at 24 months followed by colposcopy if still positive. The study was extended to a third round of screening, where women on both randomised arms were managed solely on the basis of cytology, according to national guidelines. Manchester became one of six Sentinel Sites for HPV triage in England, where HPV triage of low grade cytological abnormalities was undertaken. Therefore, women with borderline and mild dyskaryosis cytology who tested HPV positive were referred to colposcopy, whilst those who were negative were returned to routine recall.

*Samples selected retrospectively*

At the time of testing in ARTISTIC, 4ml of the residual LBC sample were pelleted, resuspended in phosphate buffered saline (PBS) and stored at -70oC. Stored aliquots from 3518 ARTISTIC LBC specimens were selected for testing by the PapilloCheck® assay on the basis of their cytology, histology and HC2 result: HC2 positive normal cytology samples from round 1 (n=2164, including 32 CIN2+), abnormal cytology collected between July 2001 and December 2001 (n=836, including 155 CIN2+), and lastly 518 cytology samples taken within 30 months preceding CIN2+ diagnosis before August 2008.

*Samples selected prospectively*

A total of 5092 liquid based cytology specimens routinely collected between August 2008 and September 2009 were tested prospectively for HPV DNA using HC2 and PapilloCheck® assays. For women with repeat cytology within this period, only the first sample was tested. Women who had previous CIN2+ in the ARTISTIC study period were included (n=321).

*HPV testing*

*Hybrid Capture 2 Assay*

All ARTISTIC LBC specimens were prospectively tested for HR HPV using HC2. Briefly, a 4ml LBC aliquot was denatured and the single-stranded HPV DNA was hybridised to a specific probe containing cRNA sequences for 13 HR HPV genotypes (HPV 16, HPV 18, HPV 31, HPV 33, HPV 35, HPV 39, HPV 45, HPV 51, HPV 52, HPV 56, HPV 58, HPV 59 and HPV 68), according to the manufacturer’s instructions. Positive results were expressed as relative light units (RLU) compared to the result for a high risk HPV positive control known to have 1 pg/ml of HPV DNA, which is equivalent to 100,000 HPV copies/ml or 5,000 HPV copies per assay. An RLU/cutoff ratio of ≥1 was used as the threshold for an HPV positive result.

*PapilloCheck® Assay*

The PapilloCheck® assay was conducted according to the manufacturer’s instructions except that an automated rather than the manual PapilloCheck® Extraction kit for DNA extraction was used. Extraction was carried out using the Nuclisens easyMAG system (bioMerieux). A 50ul aliquot of the stored cell pellet was extracted and purified total nucleic acid was eluted in 100ul of extraction buffer. Briefly, amplification and detection was carried out using a 5μl aliquot of extracted DNA added to 20μl of the PapilloCheck® amplification mix combined with 1 unit of HotStar Taq DNA polymerase (Qiagen) and 0.005 units of Uracil-N-Glycosylase (Fermentas). DNA amplification was performed on a GeneAmp 9700 (Applied Biosystems). Following amplification, the PCR products were kept in the dark at -20˚C prior to hybridisation. Scanning and analysis was carried out using the Greiner Bio-One CheckScanner™ and the CheckReport™ Software version 2.2.5. For the purposes of this analysis, the same 13 genotypes as detected by HC2 were considered high risk (as defined by the IARC Monograph working group – ref 15). Results were expressed as HR PapilloCheck® positive or negative. The commercial “PapilloCheck® high-risk” assay now available detects 14 HR types – those as for HC2 plus HPV66. Thus the addition of HPV type 66 has also been assessed separately and all other types detected by PapilloCheck® (6, 11, 40, 42, 43, 44, 53, 70, 73, 82) some of which are considered “probably high risk” have been referred hereafter as “low risk” types.

*Roche prototype Reverse Line Blot and Roche Linear Array assays*

Either one of these Roche assays (collectively referred to as “RLB” hereafter) were performed on all LBC samples that tested positive for HPV by HC2 or PapilloCheck®. These genotyping assays amplify 37 HPV types simultaneously, including the 13 HC2 target types, using PGMY09-PGMY11 (PGMY09/11) L1 consensus primer PCR. The assays were carried out according to the manufacturer’s instructions except that an automated rather than the manual AmpliLute Liquid Extraction Kit (Roche) for DNA extraction was used. DNA was extracted from a 50μl aliquot of the stored cell pellet using the automated Roche MagNA Pure LCextraction system in conjunction with the Total Nucleic Acid Extraction kit (Roche). The purified total nucleic acid was eluted with a low-salt buffer to a final volume of 100ul. This automated system was validated and certified by Roche Molecular Diagnostics using HPV test panels before any testing was carried out on clinical material.

*Statistical analysis*

The clinical performance of PapilloCheck® relative to HC2 was determined according to its ability to detect underlying CIN2+ by calculating relative sensitivities and specificities with 95% confidence intervals. A power calculation indicated that a study with 2100 negative results (HPV negative or colposcopy/histology showing less than CIN2), and 400 positive results (underlying CIN2+), would have more than 90% power to demonstrate relative sensitivity of PapilloCheck® to HC2 to detect CIN2+ of at least 95% (assuming a proportion of discordant pairs of 0.08) and a sensitivity of HC2 of 95%. Relative sensitivity was calculated as the ratio of positive results (underlying CIN2+) by PapilloCheck® to positive results by HC2. The retrospectively selected samples were enriched for underlying CIN2+ to facilitate comparisons of relative sensitivity and 723 (93%) of the total of 776 CIN2+ diagnosed within the study were retrieved. The majority of samples in the prospective study had normal cytology (n=4856 negative results), which facilitated comparisons of relative specificity for CIN2+ detection. CIN2+ histology following negative cytology was rare unless following a referral based on a positive HC2 test result in the revealed arm of the trial, therefore calculations of sensitivity were only based on those CIN2+ that were diagnosed following referral with abnormal cytology (n=672). Relative specificity was calculated using cases either not referred to colposcopy or negative at colposcopy, as the ratio of negative results by PapilloCheck® to negative results by HC2. 95% Confidence Intervals (95% CI) for relative sensitivity and relative specificity were calculated (16) to determine differences between the two assays. Meijer at al (17) proposed that a new test should not have a relative sensitivity of less than 90% of HC2 in women aged over 30 years and thus results are presented separately for women aged under and over 30 years. Direct comparisons between the assays were made using McNemar’s Test for paired samples. PapilloCheck® was also compared to HC2 with a RLU/cutoff ratio of ≥2 because previous work indicated that this threshold has better clinical utility (13).

**Results**

Prospective unselected samples (2008-2009)

Table 1 shows a summary of all test results from the two assays. Of the samples tested prospectively, 4856/5092 (95.4%) had normal cytology, reflecting the success of earlier rounds of screening at detecting and treating prevalent premalignant disease of the cervix. The proportion of all cytology samples that tested positive for HR HPV was 12.1% (617/5092) by HC2 and 7.9% (403/5092) by PapilloCheck®. The proportion of abnormal cytology samples that tested positive for HR HPV was 69.1% (163/236) by HC2 and 54.2% (128/236) by PapilloCheck®. There was overall agreement of 94.5% between the tests when all cytology samples were included. By the end of histological follow-up (October 2009) 18 underlying CIN2+ lesions were detected. 17/18 were HR HPV positive by HC2 and 16/18 were HR HPV positive by PapilloCheck®. The three discordant CIN2+ samples showed one case missed by HC2 that was positive for HPV31 and one case missed by PapilloCheck® that was positive for HPV18; the remaining extra case picked up by HC2 was found positive for HPV70 (see footnote, Table 1).

Retrospectively selected samples

In contrast to the prospectively tested samples, those collected in July-Dec 2001 were taken at a time when LBC had just been introduced and cytological abnormalities were being overcalled (the age-adjusted cytological abnormality rate in LBC samples taken at entry into the ARTISTIC trial declined by 40% over the first two years of the trial (6)). The proportion of borderline and mild (LSIL) cytology positive by both assays is hence lower for these samples (31% in 2001 vs 51% in 2008-09).

2164 samples with normal cytology and testing positive by HC2 from round 1 of ARTISTIC were retrospectively selected. Approximately half of these tested negative with PapilloCheck®. 1621 were in the revealed arm, where around 65% of those who had repeatedly positive HC2 tests were referred to colposcopy based only on their HC2 result (and not cytology). CIN2+ was detected in 31 women (6). PapilloCheck® high-risk was positive in 26/31 (84%) of these cases. Of the 5 CIN2+ lesions ‘missed’ by PapilloCheck®, HPV16 or HPV18 was detected by RLB in 4 samples (footnote table 1).

Sensitivity

Table 2 shows the sensitivity of each assay for detecting CIN2+ in women with abnormal cytology, stratified by age and cytology result. Sensitivity was consistently higher at younger ages and for more severe cytology, and HC2 was consistently more sensitive than HR PapilloCheck®; overall the sensitivity for detecting CIN2+ was 93.5% for HC2 and 89.0% for HR PapilloCheck®, giving PapilloCheck® a relative sensitivity of 0.95 (95% CI: 0.93 – 0.97, p<0.0001). HR PapilloCheck® missed a total of 34 CIN2+ lesions that were positive by HC2 and HC2 missed 4 CIN2+ lesions that were positive by PapilloCheck®. The proportion testing negative by both HC2 and PapilloCheck® was 6.0% (40/672) overall. This proportion was much higher for CIN2+ than for CIN3+, and was higher in the first 6 months of the trial (CIN2+ 21.3% (17/80); CIN3+ 2.7% (2/75)) than later (CIN2 6.8% (16/234); CIN3+ 1.8% (5/283)).

Specificity

Table 3 shows the specificity of the tests based on women without CIN2+ diagnoses by age and cytology in the prospectively collected samples. Overall, PapilloCheck® was significantly more specific than HC2 (92.4% vs 88.2% respectively; p<0.0001), giving a relative specificity of 1.05 (95% CI: 1.04-1.06). The specificity decreased with increasing severity of cytological abnormality and remained higher for PapilloCheck®.

Further typing

Adding HPV type 66 to the HR PapilloCheck® test would have a minimal effect on the results. It increased the overall HR prevalence in the prospective samples from 7.9% to 8.2%. One additional CIN2 case would be picked up by including type 66, but this would hardly alter the sensitivity (89.1% vs 89.0%) or specificity (92.1% vs 92.4%) of the test. Also adding the ‘probable HR’ types 53, 73 and 82 would pick up an additional 8 CIN2+ cases (HPV53: CIN3, HPV73: 2 CIN2, HPV82: 3 CIN2 and 2 CIN3) increasing the overall sensitivity to detect CIN2+ to 90.3% (with a relative sensitivity compared to HC2 of 0.97 (95%CI:0.95-0.98)) and a sensitivity to detect CIN3+ of 93.3% (relative sensitivity of 0.96 (95%CI: 0.94-0.99)). An additional 1% of women (51/5074) would be referred and the specificity would decrease marginally to 91.4% (relative specificity of 1.04 (95%CI: 1.03-1.04)).

Raising the HC2 RLU/cutoff ratio to ≥2 slightly decreased the sensitivity to detect CIN2+ from 93.5% to 91.7%, which was still significantly higher than PapilloCheck® (89.0%), with a sensitivity of 0.97 (95% CI: 0.95-0.99, McNemar’s p-value=0.006) relative to HC2. Sensitivity to detect CIN3+ was also decreased to 95.5% from 96.7%, again with relative sensitivity of 0.97 (95% CI: 0.94-0.99, McNemar’s p-value=0.02). The specificity for the detection of CIN2+ was increased from 88.2% to 90.4% but was still significantly lower than for PapilloCheck® (92.4%) with a relative specificity of 1.02 (95% CI: 1.02-1.03, McNemar’s p-value<0.0001). The sensitivities and specificities for the two assays became similar when the HC2 RLU/cutoff ratio was increased to ≥5.

Table 4 shows good agreement between PapilloCheck® and Roche Line Blot (or Linear Array) for HC2 positive samples collected prospectively (n=602) and selected retrospectively (n=2544). The assays agreed in 82% of women with normal cytology and 91% of those with abnormal cytology (all ages). The ‘false positive’ rate of HC2 in normal cytology samples negative by both RLB and PapilloCheck® was estimated to be at least 20% (188/937) in younger women and 45% (749/1665) in women aged over 30. Of the 3146 samples that tested positive by HC2, 1283 (40.8%) were negative by PapilloCheck® and 1008 (32.0%) were negative for HR HPV by RLB. RLB detected LR types in 25% of samples with normal cytology and 62% of samples with abnormal cytology. The most common types detected were HPV53 (5.8% samples), HPV70 (5.3% samples) and HPV66 (4.9% samples)(footnote 2). Of the 275 samples that tested positive for HR HPV by RLB, HPV16 was detected in 65 (53.5%) samples, HPV52 in 51 (18.6%) samples, HPV18 in 49 (17.8%) samples, HPV45 in 27 (9.8%) samples and HPV59 in 26 (9.5%) samples (footnote 1).

Of the 5,092 samples tested prospectively, 2.8% (n=143) tested HC2+ but no HPV, including low risk types, were detected with either PapilloCheck® or RLB. Of the 617 samples that tested HC2+, only 393 (63.7%) had one or more of the 13 HR types detected by PapilloCheck® or RLB. Of the remaining 224 where no HR types were detected, 36.2% (n=81) had low risk types detected: 28.1% (51/177) in women aged over 30 with normal cytology and 63.8% (30/47) in women aged under 30 or with abnormal cytology. The prevalence of low risk types only by PapilloCheck® was very low among the 5,092 samples (3.6%). 44% (81/183) of these samples with low risk HPV were positive by HC2, but the proportion was larger for type 53 (20/25), type 66 (11/13), type 70 (15/20) and type 82 (9/20) though the numbers were small.

**Discussion**

In this study, a total of 8610 cervical cytology samples from the ARTISTIC trial were tested for HR HPV using the PapilloCheck® assay. The higher specificity of PapilloCheck® substantially reduced the proportion of women testing HR HPV positive, from 12.1% (617/5092) by HC2 to 7.9% (403/5092) in the prospective unselected samples. The majority of these samples had normal cytology (179/214). Thus, if PapilloCheck® high-risk were used instead of HC2 for primary HPV screening, the number of women requiring additional tests (eg reflex cytology) would be reduced by about a third in women where the risk of underlying CIN2+ is less than 5%. Prevalent high-grade lesions were detected and treated in the first screening round of the ARTISTIC Trial following the introduction of LBC and retraining of cytology readers (6). The much lower CIN2+ rate in the prospective study of women screened in 2008-2009 is therefore more relevant to future screening practice in the UK than the prevalence in previous rounds. Overall, the specificity of the PapilloCheck® assay was 92.4% compared to 88.2% with HC2, giving a relative specificity of 1.05 (95% CI: 1.04-1.06). The specificity of the PapilloCheck® assay was significantly higher than HC2 for women with borderline or mild cytology, implying that PapilloCheck® could also be used as a triage test following cytology.

HC2 is a highly sensitive test for detecting CIN2+, but the relatively low specificity leads to high referral rates, particularly in populations with higher HPV prevalence such as young women or those with abnormal cytology. HR HPV DNA was not detected by PapilloCheck® or RLB in 32.0% (1008/3146) of the HC2+ samples (Table 4), which either contained no detectable HPV or only low risk types which may cause CIN2 or CIN3 but are unlikely to cause invasive cancer and should not be picked up by a screening test. Similar rates of detection of HR types by Linear Array (69%) were found in a study conducted by Oh et al. (18). They also found low risk types 53, 66 and 70 in a further 18% of their HC2+ samples and suggest some cross-reaction with HC2 and these types is possible.

Table 4 shows much higher CIN2+ rates among HC2+ women who were also positive by PapilloCheck® (1.9% (27/1412) in normal cytology and 32.2% (145/451) in abnormal cytology) compared to those who were negative by PapilloCheck® (0.4% (5/1190) in normal cytology and 6.5% (6/93) in abnormal cytology). The relative sensitivity for CIN3+ was 95% (95% CI: 93%-98%) in all women where HC2 missed 11/358 (3.1%) CIN3+ cases and PapilloCheck® missed 27/358 (7.5%) CIN3+ cases. Eleven of these 27 cases missed by PapilloCheck® were positive for HPV18 or HPV45 by RLB, another 6 were positive for other HR types, leaving 11 that had either low risk types (n=6 all HC2+) or no detectable HPV infection (n=5 all HC2-). Meijer (17) states that an acceptable alternative test to HC2 should have a relative sensitivity for CIN2+ of no less than 90% in women aged over 30 years. PapilloCheck® fulfils this criterion with a relative sensitivity of 94% (95% CI: 91%-97%) in this age group (Table 2). PapilloCheck® is therefore a suitable alternative to HC2 for primary cervical screening, alongside Abbott M2000, Roche Cobas, Hologic Cervista and Gen-Probe APTIMA HR HPV tests (19).

This is the largest study to date that has compared the clinical performance of PapilloCheck® against an established HPV test or genotyping method. Previous studies have included an analysis of baseline samples from women participating in the POBASCAM trial, where 1,665 cervical cytology specimens, including 192 from women with CIN2+ were tested with both PapilloCheck® and the GP5+/6+ PCR-enzyme immunoassay. They found that the two HPV testing methods performed similarly in terms of CIN2+ detection (20). A population-based study of 878 cervical cytology specimens, in which there were 32 underlying CIN2+ lesions, also found good correlation between PapilloCheck®, HC2 and the the GP5+/6+ PCR-enzyme immunoassay (21). Another study tested a colposcopy-referral population of 239 women of whom 93 had CIN2+ using PapilloCheck® and the linear array assay, and found a high overall concordance rate between the two assays (22).

We conducted parallel retrospective and prospective analyses of ARTISTIC (6) cervical cytology samples in this study. Using ARTISTIC samples had the benefit that the samples were well characterised in terms of clinical follow up data. The advantage of the retrospective analysis was that we were able to enrich our sample set for underlying CIN2+ lesions, which in turn gave sufficient power to the study to allow a meaningful comparison of HC2 versus PapilloCheck® for the detection of CIN2+. Agreement between PapilloCheck® and the Roche line blot assays remained good despite the samples having been processed, frozen and stored for up to six years before they were tested with PapilloCheck®. In the prospective analysis we were able to compare the performance of the two tests contemporaneously under the same conditions using fresh cervical cytology material, but there were only 18 underlying CIN2+ lesions. A prospective study sufficiently large to detect small differences in the performance characteristics of these tests would be extremely expensive and is unlikely to be performed.

Greiner Bio-One has now developed a microarray-based test kit using the same technology as the approved PapilloCheck® which detects the 14 carcinogenic types (Greiner Bio-One, REF 505060). This is important because any HPV test used in primary cervical screening must detect high risk HPV rather than low risk types or a mixture of the two and we have shown that PapilloCheck® (considering these 14 HR types) is significantly more specific than HC2 with only a small decrease in sensitivity. Compared with the HC2 test, which using the Qiagen QiaSymphony and Rapid Capture is capable of processing 88 samples in 9 hours, the PapilloCheck® method employed in this study required 1.5 days to process 80 samples. Greiner Bio–One, which manufactures PapilloCheck®, has now developed an automated platform which is expected to launch in early 2016, and which will be capable of a throughput and ergonomic performance comparable with HC2. We and others have shown that PapilloCheck® also compares well with other PCR based systems such as the Roche Linear Array, and this genotyping capability will have clinical utility for stratifying HPV positive women, especially as vaccinated cohorts come through to cervical screening. In conclusion, this large study has confirmed that the PapilloCheck® high-risk would be a suitable HPV assay in primary screening, providing sufficient sensitivity and improved specificity relative to HC2.

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**Disclosure of interests**

The authors report no conflicts of interest.

**Contribution to authorship**

EC and HK devised the study, analysed the data and wrote the manuscript. AB and AS performed the laboratory investigations and collated the data. HK, CG and JP provided access to the ARTISTIC study samples and clinical data. CG performed the statistical analyses. All authors contributed to data interpretation and reviewed the final manuscript.

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**Table 1: Overall Hybrid Capture 2 (HC2) and PapilloCheck® (PC) HPV test results for retrospectively and prospective collected samples (n=8610)**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **n** | **HC2+** | | | | | | **HC2-** | | | | | | **% Agreement** | **Total CIN2+** |
| **HR PC+** | | | **HR PC-** | | | **HR PC+** | | | **HR PC-** | | |
| **n** | **%** | **CIN2+** | **n** | **%** | **CIN2+** | **n** | **%** | **CIN2+** | **n** | **%** | **CIN2+** |
| **Prospective unselected samples (2008-09)** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| **Normal cytology4** | 4856 | 242 | 5.0 | 1 | 212 | 4.4 | 0 | 33 | 0.7 | 0 | 4369 | 90.0 | 0 | 95.0% | x |
| **Borderline cytology** | 118 | 36 | 30.5 | 0 | 20 | 16.9 | 1 | 1 | 0.8 | 1 | 61 | 51.7 | 0 | 82.2% | 2 (1.7%) |
| **Mild cytology** | 91 | 70 | 76.9 | 6 | 12 | 13.2 | 0 | 0 |  |  | 9 | 9.9 | 0 | 86.8% | 6 (6.6%) |
| **Moderate+ cytology** | 27 | 21 | 77.8 | 8 | 4 | 14.8 | 1 | 0 |  |  | 2 | 7.4 | 0 | 85.2% | 9 (33.3%) |
| **All cytology** | 5092 | 369 | 7.3 | 15 | 2481 | 4.9 | 2 | 342 | 0.7 | 1 | 4441 | 87.2 | 0 | 94.5% | 18 (0.4%) |
| **Retrospectively selected samples** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| **Normal cytology concealed arm (2001-2004)4** | 543 | 301 | 55.4 | 1 | 242 | 44.6 | 0 |  |  |  |  |  |  |  | x |
| **Normal cytology revealed arm (2001-2004)5** | 1621 | 878 | 54.2 | 26 | 743 | 45.8 | 53 |  |  |  |  |  |  |  | 31 (1.9%) |
| **Abnormal cytology (Jul-Dec 2001)** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| **Borderline cytology** | 482 | 109 | 22.6 | 19 | 26 | 5.4 | 1 | 11 | 2.3 | 0 | 336 | 69.7 | 12 | 92.3% | 32 (6.6%) |
| **Mild cytology** | 228 | 113 | 49.6 | 23 | 31 | 13.6 | 0 | 3 | 1.3 | 0 | 81 | 35.5 | 3 | 85.1% | 26 (11.4%) |
| **Moderate+ cytology** | 126 | 104 | 82.5 | 89 | 8 | 6.4 | 3 | 2 | 1.6 | 1 | 12 | 9.5 | 4 | 92.0% | 97 (77.0%) |
| **Additional CIN2+ samples (2002-07)** | 518 |  | 88.8 | 460 |  | 6.2 | 32 |  | 0.4 | 2 |  | 4.6 | 24 | 93.4% | 518 |
| **All CIN2+ Samples** | 723 |  | 87.6 | 633 |  | 5.9 | 43 |  | 0.6 | 4 |  | 5.9 | 43 | 93.5% | 723 |

1 24(10.1%) were high risk positive by RLB, 214 were HR negative by RLB and 10 were inhibitory by RLB

2 21 (63.6%) were high risk positive by RLB (19 with same HR types as PapilloCheck®), 12 were HR negative by RLB, and 1 was inhibitory (no result by RLB)

3 4/5 of these cytological normal, HC2+, HR-PC- samples were positive for HR types by RLB (1 HPV16, 2 HPV18, 1 HPV16/18)

4 These were not referred for colposcopy based on their HPV status. Any referrals, and hence CIN2+ diagnoses occurred due to additional follow-up as a result of an abnormal screening history.

5 Women who were positive by HC2 on 2 or 3 consecutive occasions were offered colposcopy.

**Table 2: Sensitivity of HC2 and PapilloCheck® HPV tests in 672 abnormal cytology samples preceding a diagnosis of CIN2+ and CIN3+**

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Age at test** | **Cytology** | **Histology** |  | **HC2+** | | **HC2-** | | **HC2 sensitivity (%)** | **HR PC**  **sensitivity (%)** | **Relative sensitivity**  **(95% CI)** |
| **n** | **HR PC+** | **HR PC-** | **HR PC+** | **HR PC-** |
| **20-29** | **All abnormal** | **CIN2+** | 330 | 303 | 12 | 0 | 15 | 95.5% | 91.8% | 0.96 (0.94, 0.98) |
|  | **Borderline** |  | 60 | 49 | 1 | 0 | 10 | 83.3% | 81.7% | 0.98 (0.94, 1.02) |
|  | **Mild** |  | 98 | 90 | 6 | 0 | 2 | 98.0% | 91.8% | 0.94 (0.89, 0.99) |
|  | **Moderate+** |  | 172 | 164 | 5 | 0 | 3 | 98.3% | 93.3% | 0.97 (0.95, 1.00) |
| **30-60** | **All abnormal** | **CIN2+** | 342 | 291 | 22 | 4 | 25 | 91.5% | 86.3% | 0.94 (0.91, 0.97) |
|  | **Borderline** |  | 65 | 44 | 5 | 1 | 15 | 75.4% | 69.2% | 0.92 (0.83, 1.02) |
|  | **Mild** |  | 78 | 71 | 3 | 0 | 4 | 94.9% | 91.0% | 0.96 (0.92, 1.00) |
|  | **Moderate+** |  | 199 | 176 | 14 | 3 | 6 | 95.5% | 89.9% | 0.94 (0.90, 0.98) |
| **All women** | **All abnormal** | **CIN2+** | 672 | 594 | 341 | 42 | 403 | 93.5% | 89.0% | 0.95 (0.93, 0.97) |
| **20-29** | **All abnormal** | **CIN3+** | 172 | 162 | 7 | 0 | 3 | 98.3% | 94.2% | 0.96 (0.93, 0.99) |
|  | **Borderline** |  | 27 | 25 | 1 | 0 | 1 | 96.3% | 92.6% | 0.96 (0.89, 1.04) |
|  | **Mild** |  | 46 | 43 | 2 | 0 | 1 | 97.8% | 93.5% | 0.96 (0.90, 1.02) |
|  | **Moderate+** |  | 99 | 94 | 4 | 0 | 1 | 99.6% | 94.9% | 0.96 (0.92, 1.00) |
| **30-60** | **All abnormal** | **CIN3+** | 186 | 165 | 13 | 4 | 4 | 95.7% | 90.9% | 0.95 (0.91, 1.00) |
|  | **Borderline** |  | 25 | 19 | 2 | 1 | 3 | 84.0% | 80.0% | 0.95 (0.81, 1.12) |
|  | **Mild** |  | 24 | 24 | 0 | 0 | 0 | 100.0% | 100.0% | 1.00 |
|  | **Moderate+** |  | 137 | 122 | 11 | 3 | 1 | 97.1% | 91.2% | 0.94 (0.89, 1.00) |
| **All women** | **All abnormal** | **CIN3+** | 358 | 327 | 20 | 4 | 7 | 96.7% | 92.5% | 0.95 (0.93, 0.98) |

1 5/14 (36%) CIN2 and 14/20 (70%) CIN3+ were HR-RLB+. Those negative for HR types were mostly positive for LR types (14/15). The five CIN2 were positive for types 16, 18(n=2), 45 and 58 and the 14 CIN3+ were positive for 18(n=6), 31, 45 (n=5), 51 and 68.

2 3/4 (75%) of these were HR-RLB+ with the same types as the PapilloCheck® result (HPV 16, 31 and 45).

3 15/33 of the CIN2 cases were tested with RLB and all were found HR negative. 2/7 CIN3+ cases were positive for HR types by RLB (both for type 16).

**Table 3: Specificity of Hybrid Capture 2 (HC2) and PapilloCheck® (PC among women without CIN2+ histology**

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Age at test** | **Cytology** |  | **HC2+** | | **HC2-** | | **HC2 specificity (%)** | **HR PC specificity (%)** | **Relative specificity**  **(95% CI)** |
| **n** | **HR PC+** | **HR PC-** | **HR PC+** | **HR PC-** |
| **Prospectively collected samples** | |  |  |  |  |  |  |  |  |
| **20-29** | **All women** | 397 | 72 | 27 | 2 | 296 | 75.1 | 81.4 | 1.08 (1.05, 1.12) |
|  | **Negative** | 359 | 49 | 19 | 2 | 289 | 81.1 | 85.8 | 1.06 (1.03, 1.09) |
|  | **Abnormal** | 38 | 23 | 8 | 0 | 7 | 18.4 | 39.5 | 2.14 (1.25, 3.68) |
| **30+** | **All women** | 4677 | 282 | 219 | 31 | 4145 | 89.3 | 93.3 | 1.05 (1.04, 1.05) |
|  | **Negative** | 4496 | 192 | 193 | 31 | 4080 | 91.4 | 95.0 | 1.04 (1.03, 1.05) |
|  | **Abnormal** | 181 | 90 | 26 | 0 | 65 | 35.9 | 50.3 | 1.40 (1.23, 1.59) |
| **All women** | **All women** | 5074 | 354 | 246 | 33 | 4441 | 88.2 | 92.4 | 1.05 (1.04, 1.06) |
|  | **Negative** | 4855 | 241 | 212 | 33 | 4369 | 90.7 | 94.4 | 1.04 (1.03, 1.05) |
|  | **Borderline** | 116 | 36 | 19 | 0 | 61 | 52.6 | 69.0 | 1.31 (1.16, 1.48) |
|  | **Mild** | 85 | 64 | 12 | 0 | 9 | 10.6 | 24.7 | 2.33 (1.42, 3.82) |
|  | **Moderate+1** | 18 | 13 | 3 | 0 | 2 | 11.1 | 27.8 | 2.50 (0.85, 7.31) |

1 CIN2+ was underestimated in these prospectively collected samples due to incomplete histological follow-up. This will most likely to affect the moderate+ cytology group.

**Table 4: PapilloCheck® and Roche Line Blot HPV test results in HC2 positive samples, prospectively collected (n=602) and retrospectively selected (n=2544) samples combined**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Age at test** | **Cytology** |  |  | **HR PC+** | | | | | | **HR PC-** | | | | | | **Agreement** | **Total HR RLB -** | **Total HR PC -** |
| **n** | **CIN2+** | **HR RLB+** | | | **HR RLB-** | | | **HR RLB+1** | | | **HR RLB-2** | | |
|  |  |  |  | **n** | **%** | **CIN2+** | **n** | **%** | **CIN2+** | **n** | **%** | **CIN2+** | **n** | **%** | **CIN2+** |  |  |  |
| **20-29** | **Normal** | 937 | 19 | 586 | 62.5 | 17 | 63 | 6.7 | 1 | 100 | 10.7 | 1 | 188 | 20.1 | 0 | 82.6% | 26.8% | 30.7% |
|  | **Abnormal** | 264 | 84 | 222 | 84.1 | 82 | 9 | 3.4 | 1 | 10 | 3.8 | 1 | 23 | 8.7 | 0 | 92.8% | 12.1% | 12.5% |
| **30+** | **Normal** | 1665 | 13 | 619 | 37.2 | 9 | 144 | 8.7 | 0 | 153 | 9.2 | 3 | 749 | 45.0 | 1 | 82.2% | 45.0% | 55.6% |
|  | **Abnormal** | 280 | 67 | 202 | 72.1 | 61 | 18 | 6.4 | 1 | 12 | 4.3 | 3 | 48 | 17.1 | 2 | 89.2% | 23.6% | 21.4% |
| **All women3** | **Normal** | 2602 | 32 | 1205 | 46.3 | 26 | 207 | 8.0 | 1 | 253 | 9.7 | 4 | 937 | 36.0 | 1 | 82.3% | 44.0% | 45.7% |
| **Abnormal** | 544 | 151 | 424 | 77.9 | 143 | 27 | 5.0 | 2 | 22 | 4.0 | 4 | 71 | 13.0 | 2 | 91.0% | 18.0% | 17.1% |

1 Among 275 samples that were HR PC- and HR RLB+, the most common HR types detected by RLB were: HPV16 (n=65, 23.5%), HPV52 (n=51, 18.6%), HPV18 (n=49, 17.8%), HPV45 (n=27, 9.8%) and HPV59 (n=26, 9.5%)

2 Among 1008 samples negative by both tests, 25.0% (235/937) with normal cytology and 62.0% (44/71) with abnormal cytology were positive for LR types by RLB, the most common types being HPV53 (5.8%), HPV70 (5.3%) and HPV66 (4.9%)

3 26/3172 HC2+ samples shown in table 1 were inhibited or insufficient when tested by RLB and are not included here