Title: The value of a transformation zone component in anal cytology to detect high grade squamous intraepithelial lesions (HSIL)

Running title: Value of anal transformation zone cells

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**Precis:** In contrast to cervical cytology, the presence of a transformation zone (TZ) component may be an important indicator of sample quality for anal cytology. We found that negative anal cytology specimens without a TZ component were more likely to be falsely negative than those with an adequate TZ component.
Abstract:

**Background:** In a cytology-based screening program intended to prevent anal cancer, the anal transformation zone (TZ) should be adequately sampled, as it is the site most susceptible to development of the cancer precursor, high-grade squamous intraepithelial lesion (HSIL). An adequate TZ component is defined as comprising at least 10 rectal columnar or squamous metaplastic cells. We examined whether the presence of a TZ component in anal cytology correlated with detection of histological HSIL.

**Methods:** In a natural history study of anal human papillomavirus infection in homosexual men, all participants had liquid-based cytology and high-resolution anoscopy (HRA) +/- biopsy at each visit. We compared True Negative cytology (negative cytology with non-HSIL biopsy or negative HRA), False Negative cytology (negative cytology with HSIL biopsy) and True Positive cytology (abnormal cytology with HSIL biopsy) with respect to the presence or absence of a TZ component.

**Results:** Of 617 participants, baseline results included 155 True Positives, 191 True Negatives and 31 False Negatives. Absence of an adequate TZ component was significantly higher for False Negative (32.3%) than for either True Positive (11.0%, p=0.0034) or True Negative (13.1%, p=0.0089) results.

**Conclusion:** Markedly more False Negatives lacked a TZ component than either True Positives or True Negatives. TZ cells may be an important indicator of sample quality for anal cytology as, unlike cervical sampling, the anal canal is not visualised during cytology sampling.
Key words: anal cytology, high-grade squamous intraepithelial abnormality (HSIL), human papillomavirus, transformation zone.
Background:

Cervical cytology-based screening programs have been responsible for marked reductions in cervical cancer incidence and mortality in many countries. As the incidence of anal carcinoma (most of which are also human papillomavirus (HPV)-associated tumours) rises in many parts of the world [1], screening programs have been proposed for high-risk populations, in order to detect the anal cancer precursor, high grade squamous intraepithelial lesion (HSIL).[2, 3] Anal programs are often based on the classic cervical screening triad of cytology, colposcopy and biopsy followed by treatment. [4]

In an anal cytology screening program, the swab or other collection device is usually introduced without direct visualization of the anal canal; in contrast to cervical cytological sampling in which the cervix is visualised during sampling. In both anal and cervical cytology collection, the aim is to sample the transformation zone (TZ), the presumed site of development of HSIL. The Bethesda System (TBS) 2001 [5] (and the recently updated TBS 2014 [6]) does not require the presence of a TZ component in order to classify a cervical Pap test as satisfactory, because there is no clear correlation between the presence of a TZ component and the subsequent histological detection of cervical HSIL. However the correlation between the presence of a TZ component (defined as rectal columnar and/or squamous metaplastic cells) and histological HSIL has not been formally investigated in anal cytology.

Our aim was to investigate the relationship between the presence of a TZ component in anal cytology and the accurate detection of histologically confirmed HSIL.
Methods:

The Study of the Prevention of Anal Cancer (SPANC) is an ongoing, longitudinal natural history study exploring the epidemiology of anal HPV infection and related squamous epithelial lesions among a mainly community-recruited cohort of homosexual men in Sydney, Australia. Ethics approval for SPANC was granted by the Human Research Ethics Committee at the St Vincent’s Hospital, 390 Victoria Street, Darlinghurst, NSW (HREC/09/SVH/168, Protocol Version 1.0 dated 20/04/2010, approved on April 22, 2010). All participants gave written informed consent.

A detailed description of the design of SPANC has been published elsewhere.[7] In brief, the study recruited 617 homosexual men aged ≥ 35 years, 220 (35.7%) HIV positive and 397 (64.3%) HIV negative, between September 2010 and August 2015. Follow-up is estimated to be completed by August 2018. Each participant has 5 clinic visits over a 3 year period. At each visit men undergo a digital anorectal exam and anal ThinPrep (TP) (Bedford, MA, USA) test for anal cytology and HPV genotyping, using Linear Array HPV Genotyping Test (Roche Diagnostics). This is followed by HRA, with biopsy of any lesions suspected of being HPV-related.

All cytological and histological specimens are referred to a specialist anogenital unit within Douglass Hanly Moir Pathology, a large private general pathology laboratory in Sydney. A total of 5 experienced cytotechnologists have shared all cytological screening and 3 experienced anogenital pathologists (JMR, CB, AF) have shared final reporting of all anal TP slides over the course of the study. The pathologists report cytology and histology independently and without knowledge of concurrent or
previous results. Anal cytological reporting utilises the Bethesda system (TBS) 2001, including the definition of a satisfactory anal sample as having a minimum of approximately 2000 nucleated squamous cells. [8] As well as assessing cellularity and identifying abnormal cells, the cytologist screening the TP slide also determines whether the slide has an adequate TZ component. An adequate TZ component in anal cytology is defined (to replicate the cervical criteria) as at least 10 well-preserved rectal columnar or squamous metaplastic cells.

Anal histology specimens are reported by the same three pathologists using Lower Anogenital Squamous Terminology (LAST). [9] LAST recognises two morphological manifestations of HPV infection – HSIL - a neoplastic lesion; and low-grade squamous intraepithelial lesion (LSIL), which reflects a productive viral infection rather than a true cancer precursor and is therefore not considered to represent a positive histology result in this analysis. If more than one area was biopsied, the highest grade of histological abnormality was used in our analysis.

Because the HRA occurred immediately after the collection of the cytological sample, we were able to determine if the cytological report was an accurate representation of the HRA findings at the same visit. We used the following definitions to classify cytology tests: A True Negative is a negative cytology result with no concurrent HSIL biopsy, including cases where no biopsy was taken because of negative HRA. A True Positive is an abnormal cytology result (atypical squamous cells of undetermined significance (ASC-US) +) with concurrent HSIL biopsy. A False Negative is a negative cytology result with concurrent HSIL biopsy.
Using these definitions, we classified the negative cytology results into False Negative and True Negative. For those with HSIL biopsy, the concurrent cytology result was classified into False Negative and True Positive. We then compared False Negative, True Negative and True Positive samples with respect to the presence or absence of an adequate TZ component. We repeated the analysis for metaplastic squamous cells and for rectal columnar cells separately.

This analysis is based on results from the baseline visit.

Results:

617 men attended for a baseline visit. 61 men (including 10 with concurrent HSIL biopsies) had unsatisfactory cytology samples and were excluded from this analysis. 556 men (90.1%) attending the baseline visit had satisfactory anal cytology and form the population for this study.

**False Negatives vs True Positives:**

186 of the 556 men (33.5%) had HSIL on baseline biopsy. (Table 1) Among these men, 155 (83.3%) had True Positive cytology and 31 (16.7%) had False Negative cytology. Overall, 88 (47.3%) had < 10 rectal columnar cells, 42 (22.6%) had < 10 metaplastic cells and 27 (14.5%) samples lacked an adequate TZ component. A False Negative cytology sample was more likely to lack TZ cells (10/31 = 32.3%) than a True Positive cytology sample (17/155 = 11.0%) (OR=3.87, 95% CI 1.56-9.57, p=0.0034). When the TZ component was split into rectal columnar cells and metaplastic cells, this finding remained consistent for metaplastic cells but not for rectal columnar cells, as reported previously.[10]
Stratifying the 155 True Positive cytology samples according to the severity of the abnormality seen on the slide, those with more severe abnormality (ASC-H/HSIL) were less likely to lack an adequate TZ component (8/111 = 7.2%) than those with a less severe cytological abnormality (ASC-US/LSIL) (9/44 = 20.5%, p=0.017).

**False Negatives vs True Negatives:**

There were 222 men (39.9% of total) with negative cytology. (Table 2) 191 of these (86.0%) were then classified as having True Negative cytology results and 31 (14.0%) as False Negative cytology results. Among men with negative cytology, 90 (40.5%) had < 10 rectal columnar cells, 69 (31.1%) had < 10 metaplastic cells and 35 (15.8%) negative samples lacked an adequate TZ component. A False Negative cytology sample was more likely to lack TZ cells (10/31 = 32.3%) than a True Negative cytology sample (25/191 = 13.1%) (OR=3.16, 95% CI, 1.33-1.79, p=0.0089) and consequently negative cytology samples with absent TZ cells were more likely to be False Negative than those with TZ cells present. Although overall presence of TZ cells was significantly associated with the likelihood of a false negative cytology result, the association with the individual components of TZ (rectal columnar cells and metaplastic cells) was not significant.

Apart from the False Negative, True Negative and True Positive samples, there were 179 abnormal cytology results (including 25 HSIL cytopredictions) associated with non-HSIL biopsies or negative HRA. These could possibly be considered ‘False Positive’ cytology and will form part of a separate study.
Discussion:

In a natural history study of HPV-related anal lesions in homosexual men, we have shown that the presence of a TZ component on an anal cytology slide positively correlates with detection of histological HSIL. More False Negative cytology slides lacked TZ component than either True Positive slides or True Negative slides. Negative anal cytology slides with absent TZ cells were more likely to be False Negative than those slides with adequate TZ cells. Our overall performance indicators of anal cytology [10] are comparable to similar studies. [11-15] This suggests that the presence or absence of TZ component is an indicator of cytology sample quality and affirms TBS 2014 [6] recommendation to include the presence or absence of a TZ component in anal cytology reports.

When we examined the two types of TZ cells separately (rectal columnar and squamous metaplastic), we showed that the presence of ≥10 metaplastic cells positively correlates with a slide being True Positive but not True Negative. The presence of ≥10 rectal columnar cells does not individually correlate with either True Positive or True Negative cytology samples. This was reported in our previous study, where we observed a higher sensitivity in the presence of ≥10 metaplastic cells but not in the presence of ≥10 rectal columnar cells.[10] From this we can postulate that squamous metaplastic cells more closely correlate with sampling of the TZ as they are present at the TZ whereas the presence of rectal columnar cells indicates only that the swab has gone past the TZ.

These findings are in contrast to the evidence surrounding absence of a TZ component on cervical Papanicolaou (Pap) slides, which has been recently summarised in the [Type text]
publication of the 2014 Bethesda System for Reporting Cervical Cytology.[6]

Although cross-sectional studies have shown that abnormalities are more common in cervical Paps with, rather than without a TZ component [16-18], longitudinal studies have not shown a higher risk of HSIL after negative Paps with and without a TZ component.[19-24] Retrospective case-control studies have also shown no association between absence of a TZ component and false negative results. [25, 26] Subsequently, most guidelines recommend that, while the presence or absence of TZ component should always be recorded, there is no requirement for an early repeat cervical Pap test after a negative result which lacks a TZ component. [27-29]

This is the first study to specifically examine the importance of a TZ component in anal cytology. Other studies examining overall performance of anal cytology conclude that the absence of a TZ component does not reduce the ability of cytology to predict abnormality. [30-35] However only Palefsky et al [32] used an endpoint of histological diagnosis to compare the accuracy of cytology slides with and without sampling of the TZ. Palefsky et al however only used rectal columnar cells in his analysis, rather than squamous metaplastic and/or rectal columnar cells, as defined by TBS.

We postulate that the difference in importance of a TZ component between anal and cervical cytology may reflect the difference in collection methodology. Cervical cytological sampling requires visualisation of the cervix prior to use of the collection device. This visualisation of the appropriate area is likely to render other ‘surrogate’ indicators of appropriate sampling, such as presence of TZ cells, less important. In contrast, anal cytological sampling is usually conducted without visualisation. As the collector cannot visually confirm sampling of the TZ, the presence (or absence) of a
TZ component in the sample, is the only indicator of whether the area most likely to harbour HPV-related abnormalities has been sampled or not.

Anoscopic-guided cytological sampling of the anal canal has been proposed as a screening method. [35] Vajdic et al found that these samples were more likely to be unsatisfactory due to low cellularity, less likely to be abnormal and more likely to lack a TZ component than blind samples taken from the same men. [35] This is likely to be because the anoscope actually covers much of the area which needs to be sampled.

As SPANC is a longitudinal study, we will have the ability in the future to investigate how results of serial anal cytology, as occurs in a screening program, correlate with histological outcomes. In particular, we will explore whether our current findings with respect to the importance of a TZ component, based on cross-sectional data, remain true with longitudinal follow-up.

Longitudinal data will also be important in determining the relevance of so-called ‘False Positive’ cytology reports. Our 25 HSIL cytopredictions associated with non-HSIL biopsies or negative HRAs may not truly be falsely positive cytology, but reflect instead false negative HRA. It is widely accepted that HRA-guided biopsy is more likely to miss an HSIL lesion than is cervical colposcopy. [4] HRA is technically more difficult and performance of HRA varies with training and experience of the operator.[11, 36, 37] With further follow-up, the significance of these unconfirmed HSIL cytopredictions will become clearer.

If anal screening programs are commenced, HPV testing may also form part of the screening paradigm. In the cervical literature, there is evidence that high-risk HPV
DNA positive test rates are independent of the presence or absence of a TZ component [38, 39]. We plan to further investigate this association between TZ and HPV detection in the anal canal in the SPANC study.

In summary, in contrast to cervical cytology, the presence of a TZ component may be an important indicator of sample quality for screening anal cytology. Negative cytology specimens without a TZ component are more likely to be falsely negative than those with an adequate TZ component. Therefore the presence or absence of a TZ component may be one of a number of factors which needs to be considered in development of a risk stratification paradigm which will underpin the design of an anal screening program in this population.
References:


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39. Zhao C, Austin RM. Human papillomavirus DNA detection in ThinPrep Pap test vials is independent of cytologic sampling of the transformation zone.

*Gynecol Oncol* 2007, **107**:231-235.
Table 1: Presence and absence of rectal columnar cells, metaplastic cells and transformation zone component for the 186 cytology samples with concurrent HSIL histology by cytology classification

<table>
<thead>
<tr>
<th>Cytology Classification</th>
<th>False Negative (n=31)</th>
<th>True Positive (n=155)</th>
<th>All Positive (n=186)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rectal columnar cells &lt;10</td>
<td>16</td>
<td>72</td>
<td>88</td>
<td>0.599</td>
</tr>
<tr>
<td>Rectal columnar cells ≥10</td>
<td>15</td>
<td>83</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>Metaplastic cells &lt;10</td>
<td>13</td>
<td>29</td>
<td>42</td>
<td>0.005</td>
</tr>
<tr>
<td>Metaplastic cells ≥10</td>
<td>18</td>
<td>126</td>
<td>144</td>
<td></td>
</tr>
<tr>
<td>TZ component absent</td>
<td>10</td>
<td>17</td>
<td>27</td>
<td>0.002</td>
</tr>
<tr>
<td>TZ component present</td>
<td>21</td>
<td>138</td>
<td>159</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Presence and absence of rectal columnar cells, metaplastic cells and transformation zone component for the 222 negative samples by cytology classification

<table>
<thead>
<tr>
<th>Cytology Classification</th>
<th>False Negative (n=31)</th>
<th>True Negative (n=191)</th>
<th>All Negative (n=222)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rectal columnar cells &lt;10</td>
<td>16</td>
<td>74</td>
<td>90</td>
<td>0.176</td>
</tr>
<tr>
<td>Rectal columnar cells ≥10</td>
<td>15</td>
<td>117</td>
<td>132</td>
<td></td>
</tr>
<tr>
<td>Metaplastic cells &lt;10</td>
<td>13</td>
<td>56</td>
<td>69</td>
<td>0.159</td>
</tr>
<tr>
<td>Metaplastic cells ≥10</td>
<td>18</td>
<td>135</td>
<td>153</td>
<td></td>
</tr>
<tr>
<td>TZ component absent</td>
<td>10</td>
<td>25</td>
<td>35</td>
<td>0.007</td>
</tr>
<tr>
<td>TZ component present</td>
<td>21</td>
<td>166</td>
<td>187</td>
<td></td>
</tr>
</tbody>
</table>
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