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**Human Papillomavirus type distribution in Vulval
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3 **1 Human Papillomavirus type distribution in Vulval Intraepithelial Neoplasia**
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6 **2 determined using PapilloCheck DNA microarray**
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10 4 Dean Bryant, Nirmala Rai, Gareth Rowlands, Sam Hibbitts, Joanne Jones, Amanda
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3 **20 Abstract**
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5
6 21 Vulval intraepithelial neoplasia is a precursor of vulval carcinoma, and is frequently
7
8 22 associated with Human Papillomavirus (HPV) infection. Estimates of HPV
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10 23 prevalence in vulval intraepithelial neoplasia vary widely in the UK. The objective of
11
12 24 this study was to assess HPV infection in a sample of women with vulval
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14 25 intraepithelial neoplasia, confirmed histologically, and determine the proportion of
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16 26 disease associated with HPV types targeted by prophylactic HPV vaccines. HPV
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18 27 infection was assessed in biopsies from 59 patients using the Greiner Bio-One
19
20 28 PapilloCheck[®] DNA chip assay. Valid results were obtained for 54 cases. HPV
21
22 29 infection was present in 43 of the 54 cases (79.6%: 95% CI 67.1-88.2%). The most
23
24 30 common HPV types were HPV 16 (33/54: 61.1%), HPV 33 (8/54: 14.8%), HPV 6
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26 31 (5/54: 9.3 %) and HPV 42 (3/54: 5.6%). The mean age of HPV positive women was
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28 32 significantly less than the mean age of HPV negative women.

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30 33 This is the largest UK series of vulval intraepithelial neoplasia in which HPV type has
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32 34 been investigated, and 34/54 (63.0 %, 95% CI: 49.6-78.6 %) cases were associated
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34 35 with HPV 16/18, which are targeted by current prophylactic HPV vaccines.
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37 **37 Key words**

38 HPV, Human papillomavirus, VIN, Vulval intraepithelial neoplasia, Papillocheck
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3 39 **INTRODUCTION**
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5 40 Vulval intraepithelial neoplasia is frequently a painful, distressing condition, and a
6
7 41 precursor lesion of invasive vulval carcinoma. In recent decades the incidence of
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9 42 vulval intraepithelial neoplasia has increased, while the mean age of patients has
10
11 43 decreased. The incidence of vulval cancer rose by 17% between 1979 and 2001
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13 44 [MacLean, 2004], with 996 new cases registered in England in 2000 [MacLean,
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15 45 2006].
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19 46 Human Papillomavirus (HPV) positivity in vulval intraepithelial neoplasia lesions is
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21 47 reported in international meta-analyses as 84% with most cases attributable to HPV
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23 48 type 16 [De Vuyst et al., 2009]. The most likely explanation for the increased
24
25 49 incidence of vulval intraepithelial neoplasia is the substantial rise in HPV infections
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27 50 of the lower genital tract [Peto et al., 2004]; the lifetime risk of HPV infection being
28
29 51 estimated at up to 79% [Syrjanen et al., 1990].
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32
33 52 The aim of this investigation was to identify HPV types present in vulval
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35 53 intraepithelial neoplasia, confirmed histologically, using the Greiner Bio-One
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37 54 PapilloCheck[®] DNA chip assay and determine the proportion of vulval intraepithelial
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39 55 neoplasia potentially preventable by HPV prophylactic vaccination.
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57 MATERIALS AND METHODS

58 Study population

59 The study population comprised women attending a specialist vulval intraepithelial
60 neoplasia clinic at the University Hospital of Wales, Cardiff, UK between 2003 and
61 2009, with vulval intraepithelial neoplasia confirmed histologically, who gave written
62 informed consent for a biopsy to be taken for research purposes. The study was
63 approved by South Wales LREC (SMKW/EL/03/5178). 59 cases were investigated,
64 with an age range of 22-82 years, and a mean age of 45.8 years. There were six cases
65 of vulval intraepithelial neoplasia grade 1, two of vulval intraepithelial neoplasia
66 grade 2, and fifty one cases of vulval intraepithelial neoplasia grade 3.

68 Sample collection and processing.

69 Separate biopsies were taken for histology and research use. The research biopsy was
70 immediately placed in liquid based cytology medium (which fixed cells and preserved
71 DNA). DNA was extracted from the research biopsy using the Qiagen QIAamp kit
72 (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions.

74 Pathology review

75 A recent review of vulval intraepithelial neoplasia histopathology recommended
76 replacement of the vulval intraepithelial neoplasia 1/2/3 subdivisions with two
77 categories reflecting the putative aetiology of the disease: usual vulval intraepithelial
78 neoplasia (HPV-associated); and differentiated vulval intraepithelial neoplasia
79 (associated with lichen sclerosis) [Sideri et al., 2005]. However, the existence of
80 differentiated vulval intraepithelial neoplasia is disputed, and in practice this diagnosis
81 is rarely made [McCluggage, 2009]. The histopathology of lesions in this study is

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3 82 reported using both the vulval intraepithelial neoplasia 1/2/3 and usual/differentiated
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5 83 classification. Pathology review was undertaken blind to HPV status by an
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8 84 experienced Consultant Histopathologist with a special interest in gynaecological
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10 85 pathology (GR).
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15 87 **PapilloCheck[®] assay**

17 88 HPV infection was identified using the Greiner Bio-One Papillocheck[®] assay (Greiner
18 89 Bio-One GmbH, Frickenhausen, Germany), which enables simultaneous detection and
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20 90 typing of 24 different HPV types in a single reaction (HPV 16, 18, 31, 33, 35, 39, 45,
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22 91 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, 82, 6, 11, 40, 42, 43 & 44) . The assay uses
23
24 92 multiplex PCR with fluorescent primers to amplify a 350bp fragment of the E1 gene.
25
26 93 The PCR product is then hybridised to a PapilloCheck[®] DNA array, comprising 28
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28 94 probes, each in 5 replicate spots. To identify false negatives the assay includes
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30 95 amplification of the human ADAT1 gene. Hybridization is assessed using the
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32 96 CheckScanner[™] array reader. Amplification, hybridization, detection and
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34 97 interpretation were performed according to the manufacturer's instructions. A valid
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36 98 result was defined as one which passed all of the PapilloCheck[®] internal controls
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38 99 (including DNA adequacy, sample inhibition, and hybridisation).
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46 100 Previous studies have confirmed the analytical [Dalstein et al., 2009; Jones et al.,
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48 101 2009] and clinical validity [Halfon et al., 2009; Hesselink et al., 2009] of this assay in
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50 102 large sample sets as compared to GP5+/6+, HC2 and Roche Linear Array. The assay
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52 103 is certified within the European Union as an *in vitro* diagnostic for qualitative
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54 104 detection of HPV in clinical specimens.
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3 107 **RESULTS**

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5 108 **HPV type distribution**

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8 109 HPV type distribution, stratified by histology, is shown in Table I. Valid results were
9
10 110 obtained for 54 of the 59 cases. HPV infection was present in 43/54 cases (79.6%:
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12 111 95% CI 67.1-88.2%). HPV 16 and/or 18 were present in 34/54 cases (63.0 %, 95%
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14 112 CI: 49.6-78.6%). HPV 16 and/or 18 were the only types present in 26/54 cases (48%,
15
16 113 95% CI: 35.4-61.2%).

17
18
19 114 High Risk (HR) HPV types were present in 38 cases and Low Risk (LR) types in 9
20
21 115 cases; 4 cases contained both HR and LR types. Multiple HPV types were present in
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23 116 10 cases; in these cases the distribution of types was: one vulval intraepithelial
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25 117 neoplasia grade 1 sample contained HPV 18, 42 and 44/55; all other cases with
26
27 118 multiple types were vulval intraepithelial neoplasia 3 and the combinations of types
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29 119 were: 3 cases with HPV 16 and 33; 2 cases with HPV 16 and 6; and one case each of
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31 120 (16,18), (33, 42), (16, 31, 33), (16, 51). In the 5 cases where only LR types were
32
33 121 present, 3 were HPV 6 (histology: two cases of vulval intraepithelial neoplasia grade
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35 122 3, and one vulval intraepithelial neoplasia grade 3 with well-differentiated carcinoma
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37 123 and viral warts), 1 was HPV 40 (vulval intraepithelial neoplasia 1) and 1 contained
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39 124 HPV 42 (vulval intraepithelial neoplasia 1).
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48 126 **HPV and age**

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50 127 HPV positive cases were aged 22-81 with a mean of 43.4 years. HPV negative cases
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52 128 were aged 33-82 years with a mean of 54.9 years. The means of these groups were
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54 129 significantly different (t test $p = 0.019$).
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60 131 **HPV and histology**

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3 132 There was no significant correlation between HPV positivity and grade of lesion. Of
4
5 133 the HPV positive cases, 40/43 (93.0%) showed vulval intraepithelial neoplasia grade 2
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8 134 or worse. This was slightly lower in HPV negative lesions, where 9/11 (81.8%)
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10 135 showed vulval intraepithelial neoplasia grade 2 or worse, but this difference was not
11
12 136 significant (Fisher's $P = 0.266$).

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14
15 137 There was no significant correlation between HPV type and histological grade;
16
17 138 however it was noticeable that all of the HPV 16 positive cases were associated with
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19 139 vulval intraepithelial neoplasia 2 or worse, while a high risk type (HPV 18) was
20
21 140 identified in only 1/5 vulval intraepithelial neoplasia grade 1 cases with valid HPV
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23 141 results.

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27 142 Slides were available for pathology review for 54/59 cases. 52/54 cases were "Usual
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29 143 vulval intraepithelial neoplasia"; 2/54 cases were "Probable Differentiated vulval
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31 144 intraepithelial neoplasia". Both cases of probable Differentiated vulval intraepithelial
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33 145 neoplasia were in HPV negative patients: one an 82 year old woman with vulval
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35 146 intraepithelial neoplasia 3, the other a 61 year old woman with vulval intraepithelial
36
37 147 neoplasia 1 (but with a history of vulval cancer). Among the cases with valid HPV
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39 148 results, 42/49 cases of usual vulval intraepithelial neoplasia were HPV positive
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41 149 (85.7% 95%CI: 73.3-92.9%).
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3 150 **DISCUSSION**
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5 151 This is the largest UK series of vulval intraepithelial neoplasia in which HPV type has
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7
8 152 been determined. The main limitation of this study was the use of a self selected
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10 153 sample, i.e. women attending a specialist vulval intraepithelial neoplasia clinic. This
11
12 154 might bias the sample towards women with persistent or recurrent disease, which
13
14 155 might possibly result in some over-representation of more persistent types (HPV 16).
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16 156 A further limitation was the use of separate biopsies for pathology and HPV analysis;
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18 157 in practice both biopsies were immediately adjacent and cases were only included if
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20 158 both biopsies were part of the same macroscopically visible lesion. It is however
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22 159 theoretically possible that the research biopsy may not be representative of the
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24 160 diagnostic biopsy.
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31 162 Several studies have investigated HPV prevalence in vulval intraepithelial neoplasia
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33 163 in the UK and estimate HPV positivity at 37.5 to 100%, with the proportion of HPV
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35 164 positive cases containing HPV 16 varying from 37.5 to 93.3% [Abdel-Hady et al.,
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37 165 2001; Baldwin et al., 2003; Daayana et al., 2010; Fiander et al., 2006; Gasco et al.,
38
39 166 2002; Todd et al., 2002; Tristram and Fiander, 2005; Woo et al., 2007]. The sample
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41 167 size in these studies ranged from 8-29 cases. The sample size for the current study is
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43 168 larger, and the findings are more consistent with the overall results of a recent
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45 169 international meta-analysis, in which 84.0% of vulval intraepithelial neoplasia cases
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47 170 tested HPV positive (67.5% were HPV 16, 7.7% were HPV 33, and 4.6% were HPV
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49 171 18) [De Vuyst et al., 2009]. The corresponding proportions in the current study were
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51 172 overall HPV positivity of 79.6% (61.1 % were positive for HPV 16, 14.8% for HPV
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53 173 33, and 3.7% for HPV 18). These are also similar to a separate meta-analysis which
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3 174 indicated an overall HPV positivity of 80.4% for vulval intraepithelial neoplasia 2/3
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5 175 (HPV 16 present in 71.2%, HPV33 in 7.7% and HPV 18 in 5.5% [Smith et al., 2009]).
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8 176 Patients who tested positive for HPV infection were significantly younger (by over 11
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10 177 years on average) than those testing negative. This is consistent with HPV associated
11
12 178 disease being more common in younger women and vulval intraepithelial neoplasia
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14 179 associated with lichen sclerosis being more common in older patients [Bonvicini et
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16 180 al., 2005].
17
18 181 The finding that 85.7% of usual vulval intraepithelial neoplasia were HPV positive is
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20 182 in accord with data previously reviewed [van de Nieuwenhof et al., 2008]. The 7 HPV
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22 183 negative usual vulval intraepithelial neoplasia patients represent an interesting group;
23
24 184 it is not clear whether these patients are accounted for by mis-diagnosis of
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26 185 differentiated vulval intraepithelial neoplasia (possibly due to sampling error), or
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28 186 whether there is a subset of usual vulval intraepithelial neoplasia that is truly HPV
29
30 187 negative.
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34 189 Three cases showing vulval intraepithelial neoplasia grade 2 or worse were associated
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36 190 with a low risk HPV type alone (HPV6). One of these cases showed vulval
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38 191 intraepithelial neoplasia grade 3 with well differentiated squamous cell carcinoma and
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40 192 viral warts; it is possible that the warts were associated with the HPV 6 but the vulval
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42 193 intraepithelial neoplasia and carcinoma were not. This apparent association between
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44 194 high grade vulval intraepithelial neoplasia and LR HPV types runs contrary to the
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46 195 suggestion that LR types cause only warts and low grade lesions. However an
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48 196 association between LR HPV types and high grade disease was observed in the De
49
50 197 Vuyst meta analysis [De Vuyst et al., 2009] which reported approximately 5% of
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52 198 vulval intraepithelial neoplasia 2/3 as linked to HPV 6.
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6 200 HPV associated vulval intraepithelial neoplasia is most common in women in their
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8 201 30's and 40's [Hart, 2001]. The incidence of VIN is approximately 5 per 100,000
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10 202 women per year and is increasing [Joura, 2002]. HPV infection is endemic in the UK
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12 203 with almost 30% of women in the 20-25 year age range now infected with a high risk
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14 204 anogenital HPV type [Hibbitts et al., 2008]. Hence, in the short term, the number of
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16 205 women affected by vulval intraepithelial neoplasia is likely to continue increasing.
17
18 206 HPV vaccination has been shown to be 100% effective in prevention of vulval
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20 207 epithelial neoplasia 2/3 associated with HPV 16/18 in a per protocol population [Joura
21
22 208 et al., 2007]. In the UK vaccination of 12-13 year old girls against HPV 16/18
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24 209 infection began in 2008. Hence it is likely to be 20 to 30 years before the effects of
25
26 210 this intervention become apparent in reduced incidence of vulval intraepithelial
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28 211 neoplasia. Ultimately however, as HPV 16/18 were present in 63.0% of vulval
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30 212 intraepithelial neoplasia, our data suggest that greater than half of vulval
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32 213 intraepithelial neoplasia cases could be potentially prevented by vaccination.
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Histology	HPV Type								
	16	18	31	33	51	6	40	42	44/55*
VIN 1	0	1	0	0	0	0	1	2	1
VIN 2	1	0	0	0	0	1	0	0	0
VIN 3	32	1	1	8	1	4	0	1	0
Total	33	2	1	8	1	5	1	3	1
%	61.1	3.7	1.9	14.8	1.9	9.3	1.9	5.6	1.9

Table I. HPV type distribution within histological grade.

There were 10 cases showing multiple infection, hence the number of types identified is greater than the number of cases. Percentages indicate the proportion of cases that tested positive for a given type.

*the HPV 44 probe cross-hybridizes to HPV 55, hence these types are reported together.