Vulvo-vaginal epithelial tumors in mares: A preliminary investigation on epithelial-mesenchymal transition and tumor-immune microenvironment

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ABSTRACT

Vulvo-vaginal epithelial tumors are uncommon in mares and data on the epithelial to mesenchymal transition (EMT) and the tumor-immune microenvironment (TIME) are still lacking. This is a study investigating the equus caballus papillomavirus type 2 (EcPV2) infection state as well as EMT process and the tumor microenvironment in vulvo-vaginal pre-neoplastic/ benign (8/22) or malignant (14/22) epithelial lesions in mares. To do this, histopathological, immunohistochemical, transcriptomic, *in situ* hybridization, and correlation analyses were carried out. Immunohistochemistry quantification showed that cytoplasmic E-cadherin and β-catenin expression as well as nuclear β-catenin expression were features of malignant lesions, while benign/pre-neoplastic lesions were mainly characterized by membranous E-cadherin and β-catenin expression. Despite this, there were no differences between benign and malignant equine vulvo-vaginal lesions in the expression of downstream genes involved in the canonical and non-canonical wnt/β-catenin pathways. In addition, malignant lesions were characterized by a lower number of cells with cytoplasmic cytokeratin expression as well as a slightly higher cytoplasmic vimentin immunolabeling. The TIME of malignant lesions was characterized by more numerous $CD204^+$ M2-polarized macrophages. Altogether, our results support the hypothesis that some actors in TIME, such as $CD204^+$ M2-polarized macrophages may favor the EMT process in equine vulvo-vaginal malignant lesions providing new insights for future investigations in the field of equine EcPV2-induced genital neoplastic lesions.

Keywords: epithelial to mesenchymal transition, equus caballus papillomavirus type 2, genital carcinomas, horse, immunohistochemistry, real-time PCR, tumor-immune microenvironment

Squamous cell carcinoma (SCC) represents the most common malignant cutaneous tumor in horses, accounting for 7-37% of all skin lesions. It can develop at any site on the skin and mucosa, although non-pigmented skin and muco-cutaneous junctions, such as eyelids and external genitalia of both male and female horses, are the preferential sites.[40,46,55]

Growing evidence has suggested that equus caballus papillomavirus type 2 (EcPV2) is likely the etiological cause of equine SCCs, including penile and preputial SCC,[40,46] as well as equine vulvo-vaginal carcinoma.[35,38,46,55] In the last decade, numerous studies have investigated the prevalence and the possible role of EcPV2 in inducing penile and preputial epithelial preneoplastic lesions and both benign and malignant tumors.[46] However, the role of EcPV2 in the pathogenesis of genital lesions in mares remain elusive.[55,35,38,16,43] Similar to the equine male counterpart, vulvo-vaginal carcinoma, represent a malignant tumor that can occur with a de novo onset or arise from precursor lesions, like plaques and papillomas.[52,53]

Recently, different equine SCCs have been reported to undergo the epithelial to mesenchymal transition (EMT) process. The transition from an epithelial to mesenchymal phenotype plays a pivotal role in processes like physiological embryogenesis and fibrosis.[33,50] The reactivation of this process has been proposed as a pivotal driver of tumor progression, invasiveness, and cancer metastasis.[10,27] More specifically, EMT consists of a multistep process, characterized by the activation of the main transcription factors (TWIST, ZEB, SNAIL1, and SLUG), which subsequently leads to the loss of epithelial marker expression (E-cadherin, β-catenin, and cytokeratin), promoting the acquisition of a

mesenchymal phenotype (N-cadherin and vimentin expression), finally enabling invasiveness during cancer progression.[11]

In the last decades, a research field that has provided new insights on tumor progression and potential therapeutic targets, is the tumor-immune microenvironment (TIME). It is becoming more evident that tumors develop in a heterogenous and dynamic ecosystem, wherein the interactions between different players (i.e. stromal cells, endothelial cells, immune cells) orchestrate tumor reprogramming and drive cancer progression, representing an interesting network from which new potential therapeutic targets can be identified.[19,51] Immune cells represent a particularly interesting component of this microenvironment playing a potentially critical role when employed for both prognostic or therapeutic purposes, as demonstrated by the recent successful application of immunotherapeutic protocols.[5] Although data about the immune microenvironment characterization has been reported in equine SCC,[34] there is still a lack of these information for equine vulvo-vaginal epithelial preneoplastic and neoplastic lesions.

EMT and TIME are two different and parallel processes. Indeed, in the last few years, a direct and synergistic interaction among the two has been highlighted in different types of cancer.[13–15,17,47] In particular, EMT seems to enhance immune evasion by increasing the number of intratumoral myeloid-derived suppressor cells and the expression of PD-L1 in several types of cancer. As a result, the signaling pathways stimulated by molecules involved in tumor immune suppression could lead to the induction of EMT.[47]

It is fairly well known that naturally occurring tumors in domestic animals can provide precious opportunities to study cancer *in vivo*. To date, several studies investigated the EMT process in different equine tumors,[2,4,45,30] but there is still a lack of data regarding equine vulvar SCC. As a matter of fact, most information on the biology and features of preneoplastic and neoplastic epithelial lesions in mares are borrowed from the available studies on penile and preputial epithelial lesions in male horses. Therefore, this study aims

at providing insights on the biology of vulvo-vaginal epithelial tumors in mares, focusing in particular on the characterization of EMT and TIME, and on their possible interaction in supporting tumor progression

MATERIALS AND METHODS

Case selection

Cases were retrospectively selected from the archives of the veterinary pathology section of the Department of Veterinary Medicine of the University of Perugia and the Institute of Pathology of the University of Veterinary Medicine of Hannover based on the following inclusion criteria: 1. histological diagnosis of epithelial hyperplasia/dysplasia, papilloma, carcinoma *in situ*, and SCC; 2. confirmed vulvar or vaginal localization of the lesions; 3. availability of greater than 0.5 cm^2 formalin-fixed, paraffin-embedded (FFPE) tumor tissue per section. When available, surgical margins without neoplastic tissue were selected together with neoplastic tissue for histological and immunohistochemical comparisons.

All samples were used for histopathological and immunohistochemical evaluations. For viral DNA testing, *in situ* hybridization, and gene expression assessment, only 19 samples (8 benign/pre-neoplastic lesions and 11 SCC) were evaluated due to samples availability restrictions. Three SCC cases were excluded due to scarce FFPE material availability and poor genomic quality. In addition, 2-3 cm in diameter samples were collected at the slaughterhouse from 6 macroscopically healthy vulvar and vaginal mucosa. These were used as healthy control group (CTRL) for immunohistochemical analysis of EMT, *in situ* hybridization, and gene expression analysis.

Histological evaluations

Slides from the 22 cases were blindly evaluated by two board-certified veterinary pathologists (IP and MC). Diagnoses were reassessed and different histological characteristics were described. A differentiation score was also assigned to malignant tumors (0=undifferentiated, when it was not possible to see signs of squamous differentiation; 1=poorly differentiated, when rare signs of squamous differentiation were present; 2=moderately differentiated, when signs of squamous differentiation are present and occasional keratin pearls are seen; 3=well differentiated, when squamous differentiation is maintained throughout the tumor). Mitotic count (MC) was evaluated in 2,37 mm^2;[32] and the presence/absence of vascular invasion, ulceration, and necrosis were recorded. The presence and extent of koilocytosis, hypergranulosis, hyperkeratosis, (0=absent; 1=mild, when focally present; 2=moderate, when multifocal areas where observed, but less than 50%; 3=prominent, when >50% of the epithelium was affected) stroma (0=absent; 1=fine fibrovascular stroma; 2=thin to thick bundles of fibrovascular stroma; 3=thick to desmoplastic stroma), and inflammation (0=absent; 1=mild, when focal area or small multifocal infiltrates <10 inflammatory cells where observed; 2=moderate, when multifocal infiltrates of <50 cells where observed; 3=severe, when multifocal aggregates >50 inflammatory cells to diffuse infiltration of inflammatory cells were observed) were also assessed. The different inflammatory cell populations were evaluated semiquantitatively both within the neoplastic tissue and, when possible, in the extratumoral tissue (0=absent; 1=mild; 2= moderate; 3=severe, as assessed for inflammation). The distribution of inflammatory cells and their localization (predominantly intratumoral, predominantly extratumoral, or both) was evaluated, together with the severity in each compartment (0=absent; 1=mild; 2=moderate; 3=severe, as assessed for inflammation). The tumors were also classified into immune-infiltrated, immune-altered, and immune-deserted.[24] In case of non-concordance among the two pathologists, data were re-discussed and a final agreement was reached.

148 **DNA Extraction and EcPV2 detection**

149 The presence of the *L1, E2, E6,* and *E7* genes of EcPV2 was tested in 19 samples and
150 healthy mucosa. DNA was extracted from two, 5 μm thick sections as previously described[6]
151 and quantified by QUBIT 3 (ThermoFisher Scientific, Waltham, MA, USA). 100 ng of DNA
152 were used for viral gene detection and for assessing DNA amplifiability by *beta-2-*
153 *microglobulin* (*B2M*) gene amplification. Real-time polymerase chain reaction (PCR) was
154 performed as previously described,[44] using 200 nM of the probe, 100 nM of each primer and
155 Taq DNA Polymerase MasterMix (Biorad Laboratories, Berkeley, CA, USA) with the
156 following thermal profile: 95 °C for 10 minutes, then 39 cycles of 95 °C for 15 seconds, and
157 60 °C for 60 seconds. Oligonucleotide sequences of primers and probes are reported
158 Supplemental Table S1, The CFX96 Real-Time System (Biorad Laboratories, Berkeley, CA,
159 USA) was used, setting a Cq of 38 as cut-off for virus positivity.

160

161 **RNA Extraction and viral and host gene expression**

162 Total RNA was extracted from 19 samples and healthy mucosa using five FFPE sections (5
163 μm thickness) using RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Invitrogen,
164 ThermoFisher Scientific, Waltham, MA, USA) according to manufacturer's instructions.
165 SuperScript IV VILO Master Mix (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA)
166 was used to reverse-transcribe 250 ng of total RNA from each sample that was diluted 1:10
167 for real-time PCR. Gene expression of *L1*, *E2, E6*, and *E7* viral genes was evaluated using
168 probes and primers reported in Supplemental Table S1 following the protocol used for viral
169 DNA (previous section). The same protocol was applied to directly test the RNA to exclude
170 possible contaminations by EcPV2 genomic DNA. For host relative gene expression the
171 following genes were tested: *C-X-C motif chemokine ligand 8* (*CXCL8*), interleukins (*IL2,*
172 *IL17A, IL23A, IL12A, IL12B, IL10*), *transforming growth factor-beta* (*TGFB*), *interferon*
173 *gamma* (*IFNG*), *AP-1 transcription factor subunit* (*FOSL1*), *lymphoid enhancer binding factor*

1 (*LEF1*), *catenin beta 1* (*CTNNB1*), *hypoxia inducible factor 1 subunit alpha* (*HIF1A*), *von Hippel-Lindau tumor suppressor* (*VHL*), and egl-9 family hypoxia inducible factors (*EGLN1, EGLN2, EGLN3*). Primer sequences are reported in Supplemental Table S2. The Power SYBR Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) was used, applying the following thermal profile in the CFX96 Real-Time System (Biorad Laboratories, Berkeley, CA, USA): 95 °C for 3 minutes, then 50 cycles of 95 °C for 15 seconds and 60 °C for 30 seconds and finally the melting curve going up in the range 58–95 °C with an increment of 0.01 °C/second. *B2M* was utilized as reference.[45] The primer pairs first used in this study are represented in the table by accession numbers and were designed including an intron or spanning an exon-exon junction through Primer3web tool v. 4.1.0 (https://primer3.ut.ee). Technical replicates were included in the reaction, collecting fluorescence data at the end of the extension step of each cycle and to construct the melting curve. Relative expression was calculated through the $2^{-\Delta\Delta Cq}$ method. For samples with no detectable amplification, a Cq of 42 was chosen as the detection threshold.

In Situ **Hybridization**

The in-situ hybridization (ISH) used in this study was the RNAscope ISH Technology (Advanced Cell Diagnostics, Hayward, CA). The presence of *EcPV2 E6/E7* oncogenes were investigated directly on neoplastic FFPE samples. The assay was optimized and performed according to manufacturer's instructions. The tissue samples were cut at 4 µm thickness, placed on SuperFrost Plus (Fisher Scientific) glass slides and deparaffinized in a series of xylene and 100% ethanol steps. On each section, the protease was added and pre-treated through heating in the dry oven HybEZ for 1h at 60°C. The RNA-specific sequence was identified through the binding with the target probe. The following process was the hybridization of the probe with signal-amplifying molecules in six successive steps. For each step, the amplifying solution, up to the complete coverage of the slide, was added and

subsequently incubated in the dry oven HybEZ for either 30 minutes for *AMP-1*, *AMP-3*, and *AMP-5*, and 15 minutes for *AMP-2*, *AMP-4*, and *AMP-6*. Between each step, the slide was washed with a 1x wash buffer for 2 minutes at room temperature. The detection of the amplified signal was performed using the chromogenic substrate (Fast RED, abcam, Cambridge, UK). Slides were then counterstained with Gill 's hematoxylin for 2 minutes at room temperature. In this study, we used RNAscope 2.5 HD Kit (Advanced Cell Diagnostics, Hayward, CA, USA), according to their prescribed procedure. As positive control, a sample of SCC positive for *EcPV2* DNA and RNA, confirmed by histology and molecular biology, was used. The slides were evaluated with a Zeiss Axio Scope A1 microscope (Zeiss, Jena, Germany) at increasing magnifications (10x, 20x, and 40x) and were considered positive if red punctuate dots in epithelial neoplastic cells were detected.

Immunohistochemistry

Immunohistochemistry was carried out as previously described.[2,34] Briefly, immunolabeling was performed with standard protocols on serial sections, using antibodies anti-pan-cytokeratin AE1/AE3, E-cadherin, β-catenin, N-cadherin, vimentin, ZEB-1, TWIST-1, and HIF-1α for EMT and anti-CD3, CD20, FoxP3, MUM1, IBA1, CD204, and MPO for characterizing immune infiltrate. Details about protocols and positive controls are summarized in Supplemental Table S3. Avidin–biotin complex (ABC) peroxidase kit (Vector Labs) was used after secondary antibody application. The immunolabeling was revealed with 3,3´-diaminobenzidine tetrahydrochloride (DAB) system (Vector Labs) for pan-cytokeratin AE1/AE3, E-cadherin, β-catenin, N-cadherin, vimentin, ZEB-1, TWIST-1, and HIF-1α and with 3-amino-9-ethilcarbazole (AEC Single Solution, Abcam) for CD3, CD20, FoxP3, MUM1, IBA1, CD204, and myeloperoxidase (MPO). Counterstaining was performed with Meyer's hematoxylin. Negative controls were run by omitting the primary antibody and incubating sections with TBS.

Healthy vulvar tissues obtained from mares at the slaughterhouse (control group) were used to asses baseline expression of EMT-related markers in a normal vulvar epithelium. Positive cells were evaluated manually by counting 10 evenly distributed fields within the tumor periphery/invasive front at 400x magnification (field of view size: 0.0305 mm^2) using a Nikon Eclipse E800 microscope (Nikon Corporation, Japan) with a Nikon PLAN APO lens and equipped with a Camera DIGITAL SIGHT DS-Fi1 (Nikon Corporation, Tokyo, Japan). Pictures were acquired with a DS camera control unit DS-L2 (Nikon Corporation, Japan) and stored in a USB device. For control samples, 10 high-power fields (400×) evenly distributed within the mucosal epithelial layer were analyzed.

Statistical Analysis

Descriptive statistics were used to describe basic features of the data and values are expressed as medians (Mdn) and interquartile range (IQR). Parametric and non-parametric tests were used to test hypotheses. Mann-Whitney U tests were performed to assess differences among groups. Correlation analysis was performed using the Spearman's test (ρ). Statistical tests were performed with IBM SPSS (version 21). After normality check of gene expression data through the Shapiro–Wilk, the non-parametric Kruskal–Wallis test was used to assess differences between CTRL, benign/pre-neoplastic lesions (BPL), and malignant epithelial tumors (MET) groups. The post-hoc Dunn's multiple comparison test was applied and a p-value (*p*) threshold was set at 0.05 for the statistical significance. Data analysis was performed with GraphPad Prism 5.04 (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

Case selection, histological characterization, and correlations among variables

Twenty-two cases of epithelial vulvo-vaginal lesions were retrospectively retrieved. The median age of both BPL and MET bearing mares was 18 years. All the tumors collected were primary lesions at their first presentation, from non-neutered mares. No staging information nor follow-up information were available for the selected cases.

From the 22 retrospectively selected cases, 14/22 (64%) were diagnosed as METs (13 SCC and 1 adenosquamous carcinoma), 1/22 (4%) was an *in situ* carcinoma, 4/22 (18%) were papillomas, and 3/22 (14%) were classified as epithelial hyperplasia/dysplasia. *In situ* carcinoma, papillomas, and epithelial hyperplasia/dysplasia were grouped together as BPLs. Representative images of the BPL and MET groups are shown in Figures 1a-d. Eight out of fourteen cases (57%) of malignant tumors were graded as well differentiated, 3/14 (21%) as moderately differentiated, and 4/14 (29%) as poorly differentiated. No case was classified as undifferentiated. The median MC of all cases was 29.75 mitoses/2,37 mm^2 (IQR=14.12-43.62), and was 17.5 mitoses/2,37 mm^2 (IQR=5.0-30.0) in the BPL group and 37.5 mitoses/2,37 mm^2 (IQR=24.62-44.37) in MET. The MC was not significantly different between the two groups, but was higher in cases where ulceration was present ($p<0.05$). Vascular invasion was observed in 3/22 cases (14% of cases), which were all SCCs. Data are summarized in Supplemental Table S4.

Further analyzing 22 cases of vulvo-vaginal lesions, the amount of koilocytosis showed a strong inverse correlation with malignancy ($p<0.001$; $\rho=-0.709$), similarly to hypergranulosis, which had a moderately strong inverse correlation ($p<0.05$; $\rho=-0.524$). The presence and extent of hyperkeratosis had a strong correlation with the degree of differentiation ($p<0.01$; $\rho=0.790$). The severity of intratumoral inflammation was also strongly associated with the amount of stroma ($p<0.001$, $\rho=0.659$) and with the overall inflammation of the specimens ($p<0.001$, $\rho=0.699$). On the other hand, the severity of extratumoral inflammation had a strong positive correlation with the degree of differentiation ($p<0.001$, $\rho=0.895$) and with the

overall degree of inflammation ($p<0.001$, $\rho=0.818$). The macroscopically normal vulvar mucosae sampled at the slaughterhouse did not show any histopathological alterations.

Vulvo-vaginal equine MET invariably express EcVP-2 oncogenes, whereas in BLP lesions, viral replication was not confirmed in all cases

MET and BPL groups were investigated for the presence of EcPV2 DNA. All samples were positive for *EcPV2-L1* viral DNA. Sixteen out of 19 (84%) cases were positive for *EcPV2-E2* viral DNA. The 3 negative samples included 2 METs and 1 BPL. All cases were positive for *EcPV2-E6* and *E7* viral DNA (Supplementall Table S5).

Next, MET and BPL groups were investigated in order to verify EcPV2 oncogenes *L1, E2, E6,* and *E7* expression. The *L1* gene was expressed ($L1^+$) in 17/19 (89%) samples, with 2 BPLs being negative for *L1* expression ($L1^-$). *E2* expression was detected in 13/19 (68%) samples; 3 METs and 3 BPLs were negative. On the other hand, 17/19 (89%) samples were positive for *E6* as well as for *E7*, while 2 BPL samples were negative. 16/19 (84%) samples expressed both *E6* and *E7* oncogenes. Interestingly, all METs expressed both *E6* and *E7* mRNAs whereas expression was detected in only 5/8 (62%) BPLs (Supplemental Table S5). Healthy mucosa samples (CTRL group) were negative for EcPV2-*L1* viral DNA detection.

Viral gene expression was investigated in 19 tumors using *in situ* hybridization. *E6/E7* oncogenes expression was detected in 10/19 (53%) samples. 8/11 (73%) METs were positive, whereas only 2/8 (25%) BPLs were positive. Dots were observed within the cytoplasm of epithelial cells, both in MET and BPL samples (Figs. 1e-f; Supplemental Table S6). The *in situ* hybridization for *E6/E7* oncogenes was negative in the CTRL group.

Intermediate filaments rearrangements, "cadherin switching" and activation of the wnt/β-catenin pathways are feature of the EMT process in equine vulvo-vaginal MET

In order to verify if EMT is a feature of equine vulvo-vaginal malignant neoplasia, expression of the main EMT markers was investigated in tumor cells. Representative pictures of cytokeratin and vimentin immunolabelings in equine vulvo-vaginal MET can be found in Figures 2a-b. Quantification of cells immunolabeled for intermediate filaments such as cytokeratins (pancytokeratin AE3/AE1) revealed a significantly ($p \leq 0.001$) lower number of cells expressing cytoplasmic cytokeratin in the invasive front of METs when compared to BPL and CTRL (Fig. 3a). On the other hand, the number of cells showing cytoplasmic vimentin immunolabeling in the invasive front of METs was slightly higher compared to BPL and CTRL (Fig. 3b).

Focusing on adhesion molecules expression, E-cadherin quantification revealed a significantly lower overall number of cells expressing E-cadherin in the invasive front of METs when compared to BPL ($p \leq 0.01$) and CTRL ($p \leq 0.001$). METs had a significantly lower number of cells with a membranous E-cadherin immunolabeling than BPL ($p \leq 0.01$) and CTRL ($p \leq 0.0001$), whereas the number of cells with aberrant cytoplasmic E-cadherin expression was higher in METs and BPLs compared to the CTRL (Figs. 3c-e). On the other hand, quantification of N-cadherin-expressing cells revealed that the number of cells immunolabeled for N-cadherin was significantly higher in METs compared to BPL ($p<0.05$).. In particular, the number of cells with membranous or cytoplasmic N-cadherin immunolabeling was higher in METs compared to BPL and the CTRL (Figs. 3f-h). Representative images of E- and N-cadherin immunolabelings in equine vulvo-vaginal MET can be found in Figures 2c-d. Despite evidence of intermediate filaments rearrangement and cadherin switching, quantification of the immunolabeling for transcription factors involved in the EMT process showed a lack of expression of ZEB-1 in all samples from all

groups. On the other hand, TWIST-1 was detected in only four samples from the MET group, and was mainly nuclear (Supplemental Figure S1). Additional transcription factors involved in the EMT process, such as β-catenin and HIF-1α, were subsequently investigated.

Representative pictures of β-catenin and HIF-1α immunolabelings in equine vulvo-vaginal MET can be found in Figures 4a-b. The number of cells expressing β-catenin in the invasive front of METs was significantly lower than in BPL ($p \leq 0.01$) and CTRL ($p \leq 0.001$). The number of cells with a membranous β-catenin expression was significantly lower in METs compared to BPL ($p \leq 0.01$) and CTRL ($p \leq 0.001$), whereas the number of cells expressing nuclear β-catenin was significantly higher in METs than in BPL ($p \leq 0.01$). Interestingly, the number of cells with a cytoplasmic β-catenin expression was higher in both METs and BPLs compared to the normal mucosa (Figs. 5a-d).

HIF-1α expression was evaluated based on previous findings that reported the involvement of HIF-1α in the EMT process in equine tumors.[2] Vulvo-vaginal METs displayed a significantly ($p \leq 0.01$) higher number of cells expressing HIF-1α compared to normal mucosa. Interestingly, BPLs had the highest number of cells with a cytoplasmic HIF-1α expression. On the other hand, the number of cells expressing nuclear HIF-1α was significantly higher in METs than in BPLs ($p < 0.05$) or CTRL ($p \leq 0.001$; Figs. 5e-g). Taken together, these findings were suggestive of an EMT process in vulvo-vaginal METs. Identification of β-catenin nuclear translocation and the HIF-1α nuclear and cytoplasmic expression in the malignant and non-malignant lesions, respectively, prompted us to further investigate the expression of selected downstream pathway-related genes.

Investigation of down-stream genes of non-canonical wnt/Ca2+ and canonical wnt/β-catenin pathways showed that *FOSL-1* ($p < 0.05$) and *LEF-1* ($p \leq 0.01$) were significantly more expressed in METs compared to CTRL group, while only *LEF-1* was also significantly more expressed in BPLs compared to CTRL ($p < 0.05$). *CTNNB1* gene expression displayed no differences among the groups. Subsequently, investigation of the gene expression of

enzymes involved in the cytoplasmic degradation of HIF-1α, namely *EGNL1, EGNL2, EGNL3,* and *VHL* revealed a significantly higher expression of *EGNL3* ($p<0.05$) and *VHL* ($p<0.05$) in the BPL group compared to CTRL lesions. Interestingly, *HIF-1A* gene expression was significantly higher in both MET ($p<0.05$) and BPL ($p\leq0.01$) groups compared to CTRL (Fig. 6).

Spearman's correlation test analysis of the epithelial marker expression revealed that there was moderately strong positive correlations between the number of cytokeratin expressing cells and E-cadherin ($p\leq0.001$; $\rho=0.793$) and β-catenin ($p\leq0.01$; $\rho=0.673$) immunolabelled cells as well as a there was a moderate positive correlation ($p<0.05$; $\rho=0.535$) between vimentin and N-cadherin expression. Furthermore, moderate negative correlations ($p\leq0.01$; $\rho=-0.641$) was also detected between the number of β-catenin and vimentin immunolabelled cells as well as numbers of HIF-1α and E-cadherin immunolabelled cells ($p<0.05$; $\rho=-0.520$). Finally, the number of vimentin immunolabelled cells showed a moderate negative correlation ($p\leq0.01$; $\rho=-0.641$) with β-catenin immunolabelled cells.

Sub-cellular protein expression was also investigated for correlations. Membranous β-catenin had a strong positive and moderate negative correlation with membranous ($p\leq0.001$; $\rho=0.840$) and cytoplasmic ($p<0.05$; $\rho=-0.479$) E-cadherin immunolabelling, respectively. On the other hand, membranous E-cadherin immunodetection showed a moderately strong negative correlation with nuclear β-catenin ($p<0.05$; $\rho=-0.569$). In addition, cytokeratin immunolabelling showed moderately strong positive correlations with membranous E-cadherin ($p\leq0.001$; $\rho=0.785$) and membranous β-catenin ($p\leq0.001$; $\rho=0.717$), whereas nuclear β-catenin immunodetection showed a moderate negative correlation ($p\leq0.01$; $\rho=-0.647$). On the other hand, there was a moderate negative correlation between the number of vimentin immunolabelled cells and membranous E-cadherin ($p<0.05$; $\rho=-0.515$). In addition, there was a moderate negative correlation ($p\leq0.01$; $\rho=0.604$) between membranous E-cadherin and nuclear HIF-1α immunolabelling. There was also a moderate

negative correlation between the number of N-cadherin immunolabeled cells ($p<0.05$; $\rho=-0.506$) and cytoplasmic HIF-1α immunolabelling, whereas there was a moderate positive correlation ($p\leq0.01$; $\rho=0.601$) with nuclear HIF-1α. Furthermore, nuclear HIF-1α also showed a moderate positive correlation ($p<0.05$; $\rho=0.044$) with the number of vimentin immunolabelled cells as well as with nuclear β-catenin immunodetection ($p\leq0.01$; $\rho=0.604$). On the transcriptome level, there was a moderately strong direct correlation ($p\leq0.001$; $\rho=0.785$) between *LEF1* and *CTNNB1* gene expression.

When analyzing previously investigated features, like mitoses and differentiation of tumor cells, we found that the overall number of vimentin immunolabelled cells showed a moderately strong negative correlation with the tumor differentiation ($p<0.05$; $\rho=-0.637$). There was a strong negative correlation ($p\leq0.01$; $\rho=-0.855$) between *TGFB* gene expression and tumor differentiation. On the other hand, there was a moderately strong positive correlation ($p\leq0.001$; $\rho=0.708$) between *FOSL1* and the number of mitoses. Furthermore, the extent of koilocytosis showed a moderately strong positive correlation with overall numbers of cells expressing either cytokeratin ($p\leq0.01$; $\rho=0.668$) or E-cadherin ($p<0.05$; $\rho=0.496$), while it was negatively correlated with overall numbers of vimentin immunolabelled cells ($p<0.05$; $\rho=-0.547$). In addition, the overall number of HIF-1α-positive cells was associated with tumors where vascular invasion was observed ($p<0.05$).

In conclusion, our last analysis showed a moderately strong negative correlation among the overall number of E-cadherin immunolabelled cells and the extent of tumor inflammation ($p<0.05$; $\rho=-0.495$) and the severity of tumoral inflammation ($p<0.05$; $\rho=-0.499$; $p\leq0.01$; $\rho=-0.683$), as well as, the overall number of β-catenin immunolabelled cells showed a moderately strong negative correlation with the severity of intra-tumoral inflammation ($p\leq0.01$; $\rho=-0.606$).

Vulvo-vaginal equine papillomavirus-induced carcinomas show a CD204-rich intratumoral immune environment, associated with higher mitotic count and the presence of ulceration

Once we demonstrated that equine vulvar METs undergo the EMT process, we wanted to characterize the tumor microenvironment by investigating the tumor-associated immune infiltrate. Immune infiltrates in both intra/peri-tumoral tissue as well as extratumoral tissue was evaluated in all the cases belonging to MET and BPL groups. Extratumoral tissue was available in 18/22 cases. Some markers could not be investigated due to the lack of tissue caused by serial recuts.

All METs were characterized by a mild to moderate infiltration of intratumoral inflammatory cells, whereas in the BPL group, the intratumoral inflammation varied from absent to moderate. The classification based on T cell infiltration revealed that 5/14 (36%) METs were immune deserted, 8/14 (57%) showed an immune-altered profile of infiltration, and only one (7%) was infiltrated (Figs. 7a and 8a).

Comparing the quantity of intratumoral and extratumoral positive cells, results showed that CD3-, MUM1- (Figs. 7d and 8c) and MPO-positive cells (Figs. 7g and 8h) were higher within the intratumoral compartment ($p<0.001$; $p=0.001$and $p<0.05$, respectively). Instead, CD20 was higher in the extratumoral tissues, whereas few B cells were observed within the neoplastic tissue (Figs. 7b and 8b; $p<0.001$; Supplemental Figure S2). No statistically significant differences were observed for FOXP3 (Figs. 7c and 8d) and IBA1 (Figs. 7e and 8e)

When comparing the expression of the immune markers in the BPL and MET groups, the expression of intratumoral CD204+ cells was significantly higher in the MET group when compared to the BPL group ($p=0.001$; Figs. 7f and 8f-g). No association was observed with the other markers in both intra- and extratumoral locations. Moreover, the number of intratumoral $CD204^+$ cells was the only variable that showed a moderate positive correlation

with the number of mitoses ($p<0.05$; $\rho=0.507$). Intratumoral CD204+cells were also associated with the presence of ulceration ($p<0.05$).

The number of intratumoral CD3+cells showed a moderately strong positive correlation with the number of intratumoral CD20+cells ($p<0.05$; $\rho=0.554$). It showed a negative association with intratumoral MUM1 ($p=0.01$; $\rho=-0.538$). Intratumoral MUM1 was, in turn, associated with intratumoral CD20 expression ($p<0.05$; $\rho=0.507$). Also, extratumoral CD3 showed a strong positive correlation with extratumoral CD20 expression ($p<0.01$; $\rho=0.701$). Extratumoral CD204+ cells were instead moderately positively associated with extratumoral CD20 and extratumoral CD3 expression ($p<0.05$; $\rho=0.613$ and $p<0.05$; $\rho=-0.521$, respectively). A very strong positive correlation was observed between extratumoral MUM1 and extratumoral FoxP3 ($p<0.001$, $\rho=0.828$), whereas a moderately strong positive correlation was present between extratumoral MUM1 and both extratumoral CD20 ($p<0.05$, $\rho=0.602$), and extratumoral MPO. extratumoral MPO was also positively correlated with extratumoral CD3 ($p<0.05$, $\rho=0.594$). Representative images of different immune markers in METs and BPLs are reported in Supplemental Figure S3.

Gene expression analysis revealed that *IL17A* expression was significantly higher ($p<0.001$) in METs when compared to both BPL and CTRL. Moreover, *CXCL8* ($p<0.01$), *IFNG* ($p<0.05$), *IL12A* ($p<0.05$), and *IL23* ($p<0.05$) expression was significantly higher in METs when compared to CTRL. Furthermore, a significant upregulation of *IFNG* ($p<0.05$), *IL10* ($p<0.01$), *IL12A* ($p<0.05$), *IL23* ($p<0.05$), and *TGFB* ($p<0.05$) as well as a downregulation of *IL2* ($p<0.05$) was identified in BPL samples compared to CTRL (Figure 9).The correlation of immune cell markers with RNA expression was observed only for the expression of MPO, which was positively correlated with the expression of *IL12B* ($p<0.001$; $\rho=0.921$) and *IL17A* ($p<0.05$; $\rho=0.560$). The protein expression of CD204 was instead inversely correlated with the expression of *IL10* ($p<0.05$; $\rho=-0.515$). Also IBA1 expression was positively correlated with the expression of *IL12B* ($p<0.05$; $\rho=0.604$). Gene expression, comparing three groups

(MET, BPL and CTRL), revealed a significant higher expression of *CXCL8* ($p<0.01$), *IFNG* ($p<0.05$), *IL12A* ($p<0.05$), and *IL23* ($p<0.05$) when comparing METs to CTRL.

Intratumoral CD204$^+$cells are associated with the activation of EMT, particularly with the process of cadherin switching and nuclear expression of HIF-1α

The expression of intratumoral CD204 was negatively associated with the expression of different molecules involved in the EMT process, namely membranous E-cadherin ($p<0.01$; $\rho=-0.592$) membranous β-catenin ($p<0.01$; $\rho=-0.620$), and cytokeratin ($p<0.01$; $\rho=-0.587$). On the other hand, the expression of intratumoral CD204 was positively associated with the membranous expression of N-cadherin ($p<0.05$; $\rho=-0.457$) and with nuclear expression of HIF-1α ($p<0.05$; $\rho=-0.537$).

DISCUSSION

Vulvo-vaginal epithelial tumors are uncommon in mares and data on these tumors are still lacking. The aim of this study is to characterize vulvo-vaginal epithelial pre-neoplastic and neoplastic lesions, focusing in particular on the EMT and on the TIME. EMT is a highly coordinated sequential biological process in which epithelial cells lose epithelial biomarker expression and assume a metastable phenotype characterized by mesenchymal biomarker expression.[3,11,50] This process has been investigated in different equine tumors,[2,4,45] but never in genital epithelial pre-neoplastic and neoplastic lesions of mares.

The current study revealed an overall lower number of cells expressing epithelial markers at the invasive front of METs together with a higher number of cells expressing mesenchymal markers, suggesting an EMT process. Cytoplasmic E-cadherin and β-catenin as well as nuclear β-catenin expression were features of METs, while BPLs were mainly characterized by membranous E-cadherin and β-catenin expression. It is noteworthy to

consider that membranous β-catenin localization was positively and negatively correlated with E-cadherin expression on the membrane and in the cytoplasm, respectively, as well as nuclear β-catenin was negatively correlated with membranous E-cadherin expression. Since subcellular translocations of E-cadherin and β-catenin are known to favor the induction of the EMT process,[3,41] we postulate from our results that one of the triggers from a benign to a malignant transformation in equine vulvo-vaginal tumors might be represented by the loss of membranous E-cadherin and β-catenin expression as well as by β-catenin nuclear translocation. Nevertheless, our results showed that the expression of downstream genes involved in the canonical and non-canonical wnt/β-catenin pathways were not significantly different between benign and malignant equine vulvo-vaginal tumors. However, future studies are warranted to investigate the gene expression of these two pathways more in depth.

Epithelial cells undergoing the EMT process gradually lose cell–cell adhesion molecules, modulate their polarity, and rearrange their cytoskeleton, which becomes dynamic and flexible following the replacement of cytokeratin by vimentin.[27,39] Similarly in our study, cytoplasmic cytokeratin expression was correlated with membranous E-cadherin and β-catenin as well as it was negatively correlated with nuclear β-catenin expression. Furthermore, cytoplasmic vimentin expression was negatively correlated to E-cadherin membranous localization. These findings suggest that E-cadherin and β-catenin translocations influence, or are influencedby, the intermediate filament rearrangements, favoring the EMT process in malignant equine vulvo-vaginal tumors and, thus conferring migratory and invasive features, typical of malignant tumors.[3,27,39] In our study, METs also showed another hallmark of EMT known as “cadherin switching” [54] compared to BPL or control tissues.

Another distinctive feature of METs detected in this study was the higher number of cells with nuclear HIF-1α expression compare to BPL or control tissues. The EMT process is

reported to be triggered not only by wnt/β-catenin pathways activation or by other transcription factors like TWIST-1 or ZEB, but also by the activation of the HIF-1α pathway, which confers increased tumor cells invasion in different human cancers.[9,20,48] Based on the fact that membranous E-cadherin was inversely correlated with nuclear HIF-1α, we postulate that loss of membranous E-cadherin and nuclear HIF-1α expression are part of the malignant transformation of equine vulvo-vaginal neoplasia. In addition, the positive correlation of nuclear HIF-1α with N-cadherin and vimentin expression as well as with nuclear β-catenin leads us to hypothesize that nuclear expression of HIF-1α is associated with the mesenchymal-like state of epithelial tumor cells undergoing EMT in equine vulvo-vaginal malignant neoplasia. All together these findings corroborate the hypothesis that the EMT process is happening in malignant equine vulvo-vaginal tumors, which isfurther supported by the fact that similar findings have been described in other equine neoplasia undergoing the EMT process.[2,4,30]

In humans, the occurrence of EMT and its role in tumor progression have been studied in a number of cancers, including several gynecological cancers.[8,37,44,56] Human vulvar SCCs are characterized by an aggressive nature and by an infiltrative invasion pattern, which is favored by the EMT process in the neoplastic cells.[18] Human vulvar squamous cell carcinomas undergoing EMT are characterized by a loss of E-cadherin expression, and vimentin and nuclear β-catenin expression.[37] Interestingly, in human vulvar SCCs, the occurrence of EMT is associated with a negative human papillomavirus status[37] and is likely to be associated with p53 mutations.[25,31,37] The majority of the malignant equine vulvo-vaginal tumors investigated in this study were found to express *E6/E7* oncogenes. This leads us to speculate that despite a similar expression pattern of EMT markers, the equine counterpart might have a different pathogenesis than the human ones. However, the status of p53 mutations in these tumors should be evaluated in future studies.

Equine vulvo-vaginal METs were characterized by a higher infiltration of inflammatory cells, when compared to BPLs. In human medicine, a pro-inflammatory microenvironment in vulvar high-grade squamous intraepithelial lesion is predictive of vaccine-induced immune infiltration and associated with a better clinical response. On the other hand, cold lesions did not develop such a pro-inflammatory environment post-vaccination.[1] Benign, suspected pre-neoplastic lesions in mares are usually papillomas, with intraepithelial carcinoma being less common.[35,40] For this reason, a comparison with the human counterpart should not be taken as straightforward. Nevertheless, it could be postulated that the mild inflammatory infiltrate observed in equine BPLs could be a predisposing factor for the development of carcinomas and a potential histologic criteria to be evaluated to predict responses to a possible future vaccination.

In the MET group, the classification based on tumor T cell infiltration showed results similar to what reported in humans.[23] Unlike humans, where HPV-negative lesions represent the majority of cases[22], all cases of our study group resulted to be EcPV2-positive. Unfortunately, follow-up data were not available in our case, but we could hypothesize that a different T cell infiltration patterns could reflect different prognosis or susceptibility to immunotherapy.[24]

The presence of a statistically significant increased number of different pro-inflammatory cellular populations composed of intratumoral infiltration of lymphocytes, neutrophils, and mature plasma cells was observed. Interestingly, the intratumoral infiltration of B cells ($CD20^+$) cells was lower when compared to the extratumoral tissue. These results are similar to what has been reported in equine penile SCCs[34] and may represent the inflammatory response in cases of equine genital EcPV2-induced malignant tumors.

In our study, the number of MPO^+ cells was significantly associated with *IL17A* expression. *IL17A* expression by MPO^+ tumor-associated neutrophils has been postulated to promote

557 EMT processes through JAK2/STAT3 signaling in human gastric cancer.[28] This finding
558 might suggest a possible role of MPO^+ tumor-associated neutrophils in promoting the EMT
559 process in equine vulvo-vaginal METs.

560 Data obtained by the evaluation of CD204 expression showed some of the most interesting
561 results of this study. First, the expression of this marker was higher in METs when compared
562 to BPLs. CD204 is recognized as a possible marker for pro-tumoral M2-polarized
563 macrophages in different cancers, both in humans and animals.[6,21,36,42,49] This leads us to
564 postulate that MET infiltration by $CD204^+$ M2-polarized macrophages could be associated
565 with immunoescape processes leading to an immunosuppressed tumor. However, the lack
566 of significantly higher *IL10* and *TGFB* gene expression in equine vulvo-vaginal METs of our
567 study does not seem to support this hypothesis. On the other hand, the pro-tumoral role of
568 $CD204^+$ M2-polarized macrophages population seems to be supported by the association
569 with a higher mitotic count and the with presence of ulceration, both variables commonly
570 associated with malignancy and poor prognosis in cancer. Interestingly, intratumoral CD204
571 expression was associated also with the expression of different key molecules for the EMT
572 process. As a matter of fact, intratumoral CD204 expression was inversely correlated with
573 membranous E-cadherin and β-catenin expression as well as cytokeratin, and was instead
574 positively associated with N-cadherin membranous expression and HIF-1α nuclear
575 expression. Taken together, these results seem to indicate an association of intratumoral
576 $CD204^+$ cells with the progression of the EMT process in equine vulvo-vaginal METs. This
577 hypothesis is supported by results in other studies that found a correlation among tumor-
578 associated macrophages and EMT.[7,12,26,29] In order to further clarify this aspect, future
579 studies should gather insights on $CD204^+$ M2-polarized macrophages in horses and explore
580 other possible immunosuppressive pathways mediated by this cellular population.

The authors recognize that this study presents some limitations. First, the limited number of cases does not allow to draw any firm conclusion due to small sample size. Unfortunately, this is due to the rare occurrence/report of the disease. Second, there was a lack of follow up and staging data. This did not allow us to associate any of our pathological finding to an actual clinical outcome. In addition, the lack of information about the presence of metastasis, and the lack of eventual samples from metastatic sites, limited the investigations on the EMT process. Third, some data on the transcriptome level did not fully support our findings on IHC quantifications, i.e. gene expression of the wnt/βcatenin pathway-related genes or *IL10* and *TGFB*. This might be due to the fact that the RNA was extracted from FFPE material and this represent a limitation because of an inferior quality of RNA due to the formalin fixation of the samples. However, a future multicentric study should include more institutions and possibly include the presence of clinical data in the inclusion criteria as well as the collection of fresh material for transcritptome analysis.

In conclusion, this is a study investigating the EcPV2 infection state as well as EMT and the tumor microenvironment in vulvo-vaginal BPL or MET lesions in mares. Despite a similar EMT-related protein expression pattern, human and equine vulvo-vaginal cancers share some differences like the negative papillomavirus infection status in the human counterpart that undergoes in EMT. However, our study showed that changes like loss of membranous E-cadherin and β-catenin expression, and increased N-cadherin and vimentin expression as well as nuclear β-catenin and HIF-1α expression are hallmarks of malignant transformation in equine vulvo-vaginal epithelial tumors. In addition, our results seem to support the hypothesis that some actors in TIME, such as M2-polarized macrophages expressing CD204, may favor EMT, providing new insights for future investigations in the field of equine EcPV2-induced genital neoplastic lesions.

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AUTHOR CONTRIBUTIONS

The study was designed by ER, WB, and AG.

Pathology evaluation was performed by FA, LdP, IP, BP, LM, MC, CP, VM and MP

Immunolabelling was conducted and analyzed by FA, LdP, IP, BP, MC, VM and MP

PCR was performed and analyzed by KC, SM, FF, ER and CGdC

Data analysis and interpretation were performed by FA, LdP, IP, BP, MC, CB, KC, SM, LM, FF, CGdC and ER

Figures were prepared by FA, LdP, and IP

The original draft was written by FA, LdP, IP, and ER

The manuscript was reviewed, edited, and approved by all authors.

Funding was acquired by ER

The project was supervised by LM, ER, AG and WB

DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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DATA AVAILABILITY

All the paper related material can be accessible per request to the corresponding author.

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FIGURE LEGENDS:

Figure 1: Equine vulvo-vaginal epithelial neoplasia. Vulvo-vaginal mucosa, horse. (a-d) Hematoxylin and eosin. (a) Equine vulvo-vaginal mucosa. Overview of a sample belonging to the benign/pre-neoplastic lesions (BPL) group. (b) Equine vulvo-vaginal mucosa. Higher magnification of (a). Moderate basal cell proliferation with mild to moderate, multifocal to coalescing, lymphoplasmacytic inflammation. (c) Equine vulvo-vaginal mucosa. Overview of a sample belonging to the malignant epithelial tumors (MET) group. (d) Equine vulvo-vaginal mucosa. Higher magnification of (c). Diffuse desmoplasia. High numbers of mitotic figures. Squamous differentiation and multifocal “keratin pearls”. (e-f) *In situ* hybridization for *E6/E7* oncogenes. (e) Vulvar papilloma. The labelling is represented by magenta dots which appear to be predominantly located in the cytoplasm of basal keratinocytes. (f) Squamous cell carcinoma. Dots are disseminated in the cytoplasm of neoplastic cells.

Figure 2: Equine vulvo-vaginal epithelial neoplasia. Vulvo-vaginal mucosa, horse. Immunohistochemistry for cytokeratin AE1/AE3 (a), vimentin (b), E-cadherin (c), and N-

802 cadherin (d) in equine vulvo-vaginal malignant epithelial tumors (MET). (a) There are
803 moderate numbers of cytokeratin AE1/AE3 immunolabelled tumor cells. There are a few
804 tumor cells that do not show cytokeratin AE1/AE3 cytoplasmic immunolabelling
805 (arrowheads). (b) There are occasional vimentin immunolabelled tumor cells with a weak
806 cytoplasmic labeling (arrowhead). (c) On the left, there are moderate to high numbers of E-
807 cadherin immunolabelled tumor cells with a membranous staining. From the middle to the
808 right, there are high numbers of cells that do not show membranous E-cadherin
809 immunolabelling. (d) There are moderate numbers of membranous N-cadherin
810 immunolabelled tumor cells (arrowhead).

811

812 **Figure 3:** Frequency of cytokeratin AE1/AE3, vimentin, E-cadherin, and N-cadherin
813 expression and sub-cellular localization in equine vulvo-vaginal benign/pre-neoplastic
814 neoplastic lesions (BPL), malignant epithelial tumors (MET), and healthy mucosa (CTRL).
815 (a) Quantitative analysis. Comparison of numbers of cytokeratin AE1/AE3 immunolabelled
816 tumor cells across BPL, MET, and control groups (Mann-Whitney-U; ***: $p \leq 0.001$). (b)
817 Quantitative analysis. Comparison of numbers of vimentin immunolabelled tumor cells
818 across BPL, MET, and CTRL groups. (c) Quantitative analysis. Comparison of numbers of
819 E-cadherin immunolabelled tumor cells across BPL, MET, and CTRL groups (Mann-
820 Whitney-U; **: $p \leq 0.01$; ***: $p \leq 0.001$). (d) Quantitative analysis. Comparison of numbers
821 of tumor cells with membranous E-cadherin immunolabelling across BPL, MET, and CTRL
822 groups (Mann-Whitney-U; **: $p \leq 0.01$; ****: $p \leq 0.0001$). (e) Quantitative analysis.
823 Comparison of numbers of tumor cells with cytoplasmic E-cadherin immunolabelling across
824 BPL, MET, and CTRL groups (Mann-Whitney-U). (f) Quantitative analysis. Comparison of
825 numbers of N-cadherin immunolabelled tumor cells across BPL, MET, and CTRL groups
826 (Mann-Whitney-U; *: $p<0.05$). (g) Quantitative analysis. Comparison of numbers of tumor

cells with membranous N-cadherin immunolabelling across BPL, MET, and CTRL groups. (h) Quantitative analysis. Comparison of numbers of tumor cells with cytoplasmic N-cadherin immunolabelling across BPL, MET, and CTRL groups (Mann-Whitney-U). Data are shown as box and whisker plots. The bounds of the box plot indicate the 25th and 75th percentiles, the bar indicates medians, and the whiskers indicate minima and maxima. Membr= membranous; cyto= cytoplasmic.

Figure 4: Equine vulvo-vaginal malignant epithelial neoplasia. Vulvo-vaginal mucosa, horse. Immunohistochemistry for β-catenin (a) and HIF-1α (b). (a) There are moderate numbers of β-catenin immunolabelled tumor cells. A few tumor cells show weak nuclear immunolabelling (arrowhead), whereas occasional tumor cells have cytoplasmic labeling (asterisk). (b) There are high numbers of HIF-1α immunolabelled tumor cells with frequent nuclear staining.

Figure 5: Frequency of β-catenin and HIF-1α expression and sub-cellular localization in equine vulvo-vaginal benign/pre-neoplastic neoplastic lesions (BPL), malignant epithelial tumors (MET), and healthy mucosa (CTRL). (a) Quantitative analysis. Comparison of numbers of β-catenin immunolabelled tumor cells across BPL, MET, and CTRL groups (Mann-Whitney-U; **: $p \leq 0.01$; ***: $p \leq 0.001$). (b) Quantitative analysis. Comparison of numbers of tumor cells with membranous β-catenin immunolabelling across BPL, MET, and CTRL groups (Mann-Whitney-U; **: $p \leq 0.01$; ***: $p \leq 0.001$). (c) Quantitative analysis. Comparison of numbers of tumor cells with cytoplasmic β-catenin immunolabelling across BPL, MET, and CTRL groups (Mann-Whitney-U). (d) Quantitative analysis. Comparison of numbers of tumor cells with nuclear β-catenin immunolabelling across BPL, MET, and CTRL groups (Mann-Whitney-U; **: $p \leq 0.01$). (e) Quantitative analysis. Comparison of numbers

of HIF-1α immunolabelled tumor cells across BPL, MET, and CTRL groups (Mann-Whitney-U; **: $p \leq 0.01$). (f) Quantitative analysis. Comparison of numbers of tumor cells with cytoplasmic HIF-1α immunolabelling across BPL, MET, and CTRL groups (Mann-Whitney-U). (g) Quantitative analysis. Comparison of numbers of tumor cells with nuclear HIF-1α immunolabelling across BPL, MET, and CTRL groups (Mann-Whitney-U; *: $p<0.05$; ***: $p \leq 0.001$). Data are shown as box and whisker plots. The bounds of the box plot indicate the 25^{th} and 75^{th} percentiles, the bar indicates medians, and the whiskers indicate minima and maxima. Membr= membranous; cyto= cytoplasmic; nucl= nuclear.

Figure 6: Gene expression levels of HIF-1α and wnt/β-catenin pathways-related genes in equine vulvar lesions samples. Differences (malignant epithelial tumors (MET) vs control (CTRL), benign/pre-neoplastic neoplastic lesions (BPL) vs CTRL, MET vs BPL) were evaluated using the Kruskal–Wallis test and applying the post-doc Dunn's multiple comparison. The asterisks indicate the statistical significance: * $p < 0.05$, and ** $p < 0.01$.

Figure 7: Number of intratumoral, extratumoral, and overall positive immune cells in benign preneoplastic lesions (BPL) and malignant epithelial tumors (MET). (a) Quantitative analysis. Comparison of the number of CD3 immunolabelled immune cells (Mann-Whitney-U) (b) Quantitative analysis. Comparison of the number of CD20 immunolabelled immune cells (Mann-Whitney-U) (c) Quantitative analysis. Comparison of the number of FOXP3 immunolabelled immune cells (Mann-Whitney-U) (d) Quantitative analysis. Comparison of number of MUM1 immunolabelled immune cells (Mann-Whitney-U) (e) Quantitative analysis. Comparison of the number of IBA1 immunolabelled immune cells (Mann-Whitney-U) (f) Quantitative analysis. Comparison of the number of CD204 immunolabelled immune cells (Mann-Whitney-U ***: $p \leq 0.001$) (g) Quantitative analysis. Comparison of the number

of myeloperoxidase (MPO) immunolabelled immune cells (Mann-Whitney-U). Data are shown as box and whisker plots. The bounds of the box plot indicate the 25^{th} and 75^{th} percentiles, the bar indicates medians, and the whiskers indicate minima and maxima. Intra= intratumoral; extra= extratumoral; over= overall.

Figure 8: Equine vulvo-vaginal epithelial neoplasia. Vulvo-vaginal mucosa, horse. Immunohistochemistry for CD3(a), CD20(b), MUM-1 (c), Foxp3 (d), IBA1 (e), CD204(f, g), and myeloperoxidase (MPO) (h). (a) Equine vulvo-vaginal squamous cell papilloma. In immune-altered tumors, $CD3^+$ lymphocytes were present in peritumoral stroma and usually did not infiltrate among neoplastic cells. (b) Equine poorly differentiated vulvo-vaginal squamous cell carcinoma. $CD20^+$ B cells were more often localized in the extratumoral tissues, whereas only few cells were observed in direct contact with tumor cells. (c) Equine well-differentiated vulvo-vaginal squamous cell carcinoma. The number of $MUM1^+$ cells was higher in areas in direct contact with the tumor and occasional positive cells could also be seen among neoplastic cells. (d) Equine well-differentiated vulvo-vaginal squamous cell carcinoma. The number of $Foxp3^+$ cells did not show differences among intra and extratumoral tissues. $Foxp3^+$ cells were often seen infiltrating among tumor cells. (e) Equine poorly differentiated vulvo-vaginal squamous cell carcinoma. $IBA1^+$ cells were often scattered among tumor cells. (f) Equine vulvo-vaginal papilloma. In BPL, $CD204^+$ cells were often limited to extratumoral stroma and did not infiltrate among neoplastic cells. (g) Equine well differentiated vulvo-vaginal squamous cell carcinoma. Intratumoral $CD204^+$ cells were often intermingled with neoplastic cells. Occasionally, $CD204^+$ cells showed phagocytosis of neoplastic cells (arrow). (h) Equine vulvo-vaginal papilloma. MPO^+ cells (mostly neutrophils) were observed predominantly near areas of ulceration.

Figure 9: Gene expression levels of immune infiltrate-related targets in equine vulvar lesions samples. Differences (malignant epithelial tumors (MET) vs Control (CTRL), benign/pre-neoplastic neoplastic lesions (BPL) vs CTRL, MET vs BPL) were evaluated using the Kruskal–Wallis test and applying the post-doc Dunn's multiple comparison. The asterisks indicate the statistical significance: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Supplemental Figure S1: Equine vulvo-vaginal epithelial neoplasia. Immunohistochemistry of TWIST-1 in equine vulvo-vaginal malignant epithelial tumors (MET). (a) There are occasional TWIST-1 immunolabelled tumor cells showing a weak nuclear immunolabelling (arrowhead). (b) Quantitative analysis. Comparison of numbers of TWIST-1 immunolabelled tumor cells across benign/pre-neoplastic neoplastic lesions (BPL), MET, and control (CTRL) groups (Mann-Whitney-U). (C) Quantitative analysis. Comparison of numbers of tumor cells with cytoplasmic TWIST-1 immunolabelling across BPL, MET, and control groups. (d) Quantitative analysis. Comparison of numbers of tumor cells with nuclear TWIST-1 immunolabelling across BPL, MET, and control groups (Mann-Whitney-U). Data are shown as box and whisker plots. The bounds of the box plot indicate the 25^{th} and 75^{th} percentiles, the bar indicates medians, and the whiskers indicate minima and maxima. Cyto= cytoplasmic; nucl= nuclear.

Supplemental Figure S2: Number of intratumoral, extratumoral, and overall positive immune cells in all groups. (a) Quantitative analysis. Comparison of number of CD3 immunolabelled immune cells (Mann-Whitney-U) (b) Quantitative analysis. Comparison of number of CD20 immunolabelled immune cells (Mann-Whitney-U) (c) Quantitative analysis. Comparison of number of FOXP3 immunolabelled immune cells (Mann-Whitney-U) (d) Quantitative analysis. Comparison of number of MUM1 immunolabelled immune cells

(Mann-Whitney-U) (e) Quantitative analysis. Comparison of numbers of IBA1 immunolabelled immune cells (Mann-Whitney-U) (f) Quantitative analysis. Comparison of numbers of CD204 immunolabelled immune cells (Mann-Whitney-U) (g) Quantitative analysis. Comparison of numbers of myeloperoxidase (MPO) immunolabelled immune cells (Mann-Whitney-U). Data are shown as box and whisker plots. The bounds of the box plot indicate the 25^{th} and 75^{th} percentiles, the bar indicates medians, and the whiskers indicate minima and maxima.

Supplemental Figure S3: Immunohistochemical expression of CD3, CD20, FOXP3, MUM1, IBA1, CD204, and myeloperoxidase (MPO). (Column a) Immunolabeling of the selected markers on a vulvo-vaginal papilloma. (Column b) Immunolabeling of the selected markers on a vulvo-vaginal squamous cell carcinoma.