Towards a tailored therapeutic approach for vulvar cancer patients

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DISCUSSION AND FUTURE PERSPECTIVES

The overall survival rates of vulvar squamous cell carcinoma (VSCC) have not improved over time (chapter 1), and recurrence rates are as high as 40% ten years after primary treatment.[1] Current treatment causes impressive morbidity due to loss of function to adjacent vital structures such as the bladder, anus and/or clitoris, lymphedema, sexual and psychological dysfunction, and wound healing disorders.[2, 3] Because elderly women are especially affected by this disease[4], treatment decisions must be individualized with respect to comorbidity, low complication rates, and optimal treatment.[5] The scope of this thesis was to examine better tools for individual risk assessments of VSCC patients. In addition, we performed an in-depth analysis of the tumor microenvironment (TME) as a first step towards a new therapeutic approach for VSCC, called immunotherapy.

TOWARDS IDENTIFICATION OF PATIENTS AT RISK FOR ADVERSE CLINICAL OUTCOME

One of the main clinical challenges in the treatment of VSCC is the high recurrence rate. The most frequently described risk factors for recurrent disease are the presence of precursor lesions[6-11], and a minimal peripheral surgical margin (MPSM) of less than 8mm.[6-16] Based on small cohorts with inconsistent measurements and outcomes[14, 17-20], a prominent international guideline recommends a re-excision when the MPSM is less than 8mm after primary surgery.[14] Increasing evidence shows that there is a limited role for the MPSM to prevent local recurrences, suggesting that the MPSM should not be used as a determining factor for adjuvant treatment.[21, 22] Only patients with tumor-positive margin were associated with a higher chance of developing recurrent disease[22], implicating that it could be sufficient to remove a tumor with histologically confirmed tumor-free margin, irrespective of the MPSM. It is clear that the prognostic role of MPSM for recurrent disease warrants clarity. This starts with a consensus guideline on how to uniformly measure and report on the MPSM. Based on an online survey amongst 57 pathologists, an international consensus guideline was developed in which we defined the MPSM as the minimum distance from the peripheral edge of the invasive tumor nests towards the inked peripheral surgical margin in a straight line through the tissue and measured in millimeters (**chapter 2**). This definition should promote reproducibility of the measurement in future studies and establish the prognostic role of the MPSM in local recurrences.

Other tumor characteristics such as tumor size, presence of lymph vascular space invasion (LVSI), and involved lymph nodes were also found to be predictive for clinical outcome[9, 23, 24], although the interobserver variability of LVSI is also substantial in other squamous cell carcinomas.[25]

Another pathological measurement, critical in adjuvant treatment decisions, is the depth of invasion (DoI). Methods for measuring the DoI have been published[26], but again the

level of reproducibility remains surprisingly low.[27, 28] The consequence of an inaccurately measured DoI over 1mm is extensive, because a DoI of more than 1mm results in an uni- or bilateral inguinofemoral lymphadenectomy via separate groin incisions, or staging of the lymph nodes with the sentinel lymph node procedure.[3, 29] The use of DoI, as currently determined, should thus be questioned. Hence the need to search for predictive and more accurate measurements that can replace the rather inconsistent measurements of MPSM, DoI, and LVSI. It would require a prospective trial with a standardized and detailed pathology instruction form, to elucidate the clinical importance of these and other clinicopathological measurements. The outcomes of such a trial might enable clinicians to provide guidance in (adjuvant) therapeutic decisions and obtain a fine balance between radicality and overtreatment.

A second clinical outcome measurement is the overall survival, which is currently estimated based on the FIGO stage of disease consisting of clinicopathological variables such as involvement of the lymph nodes, DoI, and size of the tumor.[30] Significant differences in clinical outcome were observed in patients within the same histological tumor stage, demonstrating the need for prognostic refinement in VSCC.[31, 32] The addition of genomic profiling to the other prognosticators has led to a more accurate estimation of the risk of death or recurrent disease in many cancer types.[33-35] In VSCC, three molecular subtypes were proposed based on the presence of HPV and *TP53* mutations showing distinct clinical outcome.[36] Because current clinical practice mostly relies on immunohistochemistry (IHC), it is valuable to find surrogate IHC markers for HPV and *TP53* mutations. A 'block-type' p16-IHC staining has already been shown to be a good surrogate marker for the presence of integrated HPV.[37] We used p53-IHC patterns to predict mutations in the *TP53* gene and assessed the interobserver variability of the patterns. We found an impressive sensitivity of 100%, specificity of 95%, and accuracy of 0.92 between p53-IHC and *TP53* mutational analysis in VSCC, indicating that p53-IHC patterns are excellent surrogate markers for *TP53* mutations (**chapter 3**). Some diagnostic challenges may arise, as only two trained pathologists scored p53-IHC patterns with optimized laboratory protocols. An online virtual collection and training tool for p16-IHC and p53-IHC interpretation in VSCC could help pathologists to become familiar with these staining patterns. The interpretation of p53-IHC should always be reviewed together with p16-IHC and morphological features such as precursor lesions of the same case. In case of doubt or difficulties with the interpretation of IHC in a clinical setting where it has direct therapeutic consequences, cases should be analyzed with next-generation sequence (NGS) to confirm the mutational status of the tumor.

We assessed the prognostic value of p16-IHC and p53-IHC in a large retrospective cohort of 413 VSCC. All tumors were first scored based on p16-IHC and categorized as either HPV-associated or HPV-independent VSCC. In the latter group, p53-IHC was scored as either wildtype or mutant expression. This resulted in three molecular subtypes: HPV-associated (HPVpos VSCC), HPV-

independent and p53 wildtype (HPVneg/p53wt VSCC), or HPV-independent and p53 mutant VSCC (HPVneg/p53mut VSCC). The molecular subtypes had an important prognostic value for overall survival and recurrence-free period. The molecular subtype displayed higher prognostic value for recurrence-free period than the FIGO stage of disease (**chapter 4**). On multivariate analysis, we observed a difference in recurrence-free period by molecular subtypes but not for overall survival. The reverse was true for FIGO stage which correlated with overall survival but not with recurrence-free period. This might be explained by the design of our study, where we pooled both local and locoregional recurrences, while it is generally accepted that recurrences in lymph nodes are a strong predictor for worse overall survival.[38, 39] Due to our limited sample size of involved lymph nodes, we were not able to separate these different types of recurrences. When local and locoregional recurrences are analyzed separately, it would not be surprising if the molecular subtype forms an independent prognosticator for both local recurrences and overall survival.

In many cancers, a coordinated and dense infiltration with immune cells is prognostic for longer survival and associated with a favorable response to therapy.[40-42] In colorectal cancer, the extend of the immune infiltrate is superior in predicting clinical outcome compared to conventional tumor staging.[43] Limited data in the literature described the presence of M2 macrophages, regulatory T cells (Tregs) and $CD8^+$ T cells in VSCC (**chapter 5**). In **chapter 6**, we showed that T cells, in particular the number of $CD4^+$ tumor infiltrating lymphocytes (TILs), were a strong predictor for prognosis irrespective of molecular subtype. At present, the quantification of immune cells for prognostication is not feasible in a clinical setting. The emerging implementation of digital pathology may resolve this problem in the future.[44] Despite the rapid technology development, a more simplified scoring method based on the density and location of the $CD3^+$ or $CD8^+$ T cells was proposed in colorectal cancer.[45] Four T cell infiltration patterns were identified based on the number and distribution of immune cells in the epithelium and stroma. The inflamed, altered-excluded, altered-immunosuppressed, and deserted tumors had a different clinical outcome.[46] When we applied these patterns on early-stage VSCC, comparable clinical outcomes were observed for the two high T cell infiltrated patterns (inflamed and altered-excluded) and two low T cell infiltrated patterns (altered-immunosuppressed and deserted, **chapters 6 & 7**). Despite the simplicity of this scoring method, there are some constraints to use this method for VSCC samples. First, this two-tiered system ignores the complexity of the tumor microenvironment (TME) consisting of many cell types, chemokines, and cytokines that shape the immune contexture.[41] For instance, in vulvar high grade squamous cell lesions (vHSIL), a precursor lesion of HPVpos VSCC, $CD14^+$ inflammatory macrophages together with type 1 T helper cells orchestrate a pro-inflammatory TME which is associated with better response to therapeutic vaccination.[40] Secondly, the current T cell infiltration patterns only focus on $CD3^+$ or $CD8^+$ expressing cells, while our study indicates that differences in clinical outcome are mainly associated with $CD4^+$ T cells in VSCC (**chapter 6**). Thirdly, the T cell infiltration patterns focused on the number of

cells in the tumor core and invasive margin, while the role for stromal immune cells should not be neglected.[40, 47, 48] The use of the immune infiltration patterns are not standardized nor validated in another cohort yet, and should therefore be seen as the starting point of immunological characterization of VSCC.

For now, it seems attenable to first focus on the integration of conventional staging of VSCC with molecular subtypes rather than T cell infiltration patterns as this is more robust and closer to current clinical practice. There is a strong but not perfect overlap between the molecular subtypes and T cell infiltration patterns, because 80% of the HPVpos VSCC are highly infiltrated with T cells, followed by 60% of the HPVneg/p53wt VSCC and 40% of the HPVneg/p53mut VSCC (**chapter 6**). Based on these data, the prognosis of the early-stage HPVpos VSCC might be too optimistic in 20% of cases while the prognosis in 40% of the early-stage HPVneg/p53mut are too pessimistic. A more advanced and accurate tool for predicting prognosis could be a (modified) T cell-inflamed gene expression profile (GEP) score as shown in other cancer types (**chapter 7**).[49, 50]

In the light of clinical feasibility at this moment, adding the molecular subtype to the current FIGO staging system would refine prognosis as shown in oropharyngeal squamous cell carcinoma (OPSCC). This tumor type shows analogy with VSCC as it consists of two subtypes: one HPV-associated and one HPV-independent subtype. In addition, the local immune response determines clinical outcome, irrespective of molecular subtype and tumor stage.[51] Because of the convincing data that HPVpos OPSCC reflected a better prognosis than the HPV-independent counterpart, the American Joint Committee on Cancer (AJCC) decided to stage HPVpos OPSCC separate from its HPV-independent counterpart in 2017.[52] Such an innovative step should also be pursued in VSCC.

TOWARDS IMMUNOTHERAPY AS A NEW TAILORED APPROACH FOR VULVAR CANCER TREATMENT

Besides optimization of current diagnostics and clinical management of VSCC, new therapies should be developed which diminish surgical-related morbidity without compromising clinical outcome. VSCC tumors have high expression of programmed cell death protein ligand 1 (PD-L1)[53, 54,55] and 80% of the TILs express PD-1 (**chapter 6**), making a strong case for immunotherapy with anti-PD-1 antibodies. Indeed, this option of immunotherapy has been explored in VSCC. One case report described successful treatment with pembrolizumab in a patient with PD-L1 positive recurrent VSCC with unknown HPV status.[56] Two other reports described the treatment of a total of 23 VSCC patients with either nivolumab (CheckMate 358 trial, *n*=5) or pembrolizumab (KEYNOTE-028, *n*=18), both anti-PD1 therapeutics.[50, 57]

The KEYNOTE-028 trial included 18 VSCC patients, of which 8/18 patients showed high expression of PD-L1, 13/18 had a high T cell-inflamed GEP score, and 3/18 patients showed high mutational burden.[50] The T cell-inflamed GEP score is derived from an 18-gene signature that correlates with the responsiveness to anti-PD-1 therapy in other cancers.[50, 58] One out of 18 VSCC patients showed an objective partial response to pembrolizumab and six patients had progressive disease during treatment. The median progression-free survival was 3.1 months and overall survival 3.8 months for patients treated with pembrolizumab.[50] Unfortunately, the T cell-inflamed GEP score of each VSCC patient was not presented in respect to responsiveness to therapy. In our cohort, we applied the T cell-inflamed GEP score to 29 early-stage VSCC samples, and we found the highest score in inflamed tumors (**chapter 7**). These tumors are most likely to respond to checkpoint inhibition in colorectal cancer[46], and it would be of interest to confirm this in VSCC. The CheckMate 358 trial determined the efficacy of nivolumab monotherapy in recurrent or metastatic vaginal/vulvar carcinoma after all patients received radiotherapy and/or chemotherapy. Three of five treated patients had FIGO stage IVA or IVB stage of disease at baseline, and two of five were HPVpos VSCC. An objective response rate of 20% was described (one partial response, three stable disease, and one progressive disease), with five months duration of response.[57]

The low response rates in these clinical trials seem disappointing at first sight, but are in line with the overall reported effectiveness of anti-PD-1 monotherapy (10-35% response rate) compared to the use of anti-PD-1 as an adjuvant treatment.[59] This already indicates that additional mechanisms of peripheral immunosuppression may exist, for instance the presence of Tregs (**chapter 6**) and meyloid-derived suppressor cells (MDSC), and additional checkpoint inhibitors (**chapter 7**). Another potential explanation for the low responsiveness in these trials is the selection of the patients. Only patients with recurrent disease and/or high FIGO stage were included. Based on our data, the tumors of these patients are most likely HPVneg VSCC (**chapter 4**) of which 50% display low T cell infiltration. Moreover, the T cells in these tumors grossly lack the expression of PD-1 (**chapter 6**). The three clinical studies are exemplary for one of the major challenges in the field of immunotherapy; selection of patients that benefit from precision cancer immunotherapy that match the biology of the patient's tumor. Multiple variables that are important for responsiveness have been described such as pre-existing immunity.[46] Inflamed tumors have pre-existing immunity and would benefit from a priming therapy that counteracts tumor-induced T cell dysfunction.[60, 61] Whilst low T cell infiltrated tumors need a therapy that enhances the activation of a T cell response as well as their migration into tumors, in addition to the removal of co-inhibitory signals and/or invigoration of co-stimulatory signals.[60]

Actionable targets for immunotherapy in non-inflamed tumors

The cancer-immunity cycle (**chapter 1**) aids a systematical exploration of strategies that enhance immunity in low infiltrated tumors.[62] Here, the first three steps include the release

of tumor-specific antigens (TSA) and their presentation to T cells. One strategy to increase the number of TSA is low-dose radiation because it induces tumor cell apoptosis. The released TSAs will be engulfed and presented by APCs and prime and activate T cells, eliciting a more effective type 1 immune response.[63-65] This is also referred to as *in situ* vaccination. In a preclinical study, irradiation of tumor-associated draining lymph nodes in combination with anti-cytotoxic T lymphocyte associated protein 4 (anti-CTLA-4) in melanoma and non-small cell lung cancer (NSCLC) improved survival.[60, 66] Another generic *in situ* vaccination approach to turn low T cell infiltrated tumors into inflamed tumors are oncolytic viruses.[67] These viruses can selectively infect and lyse tumor cells which leads to release of TSAs and the induction of a tumor-reactive T cell response.[68] One oncolytic virus, named talimogene laherparepvec (T-VEC), has been FDA-approved for the treatment of melanoma with clinical success in early metastastic melanoma.[69] When used as a primer and combined with anti-CTLA-4 or anti-PD-L1 in melanoma it leads to better clinical outcome than anti-CTLA-4[70] or anti-PD-L1 as monotherapy.[71] T-VEC treatment increased the infiltration of mainly $CD8^+$ TILs and expression of PD-L1 and IFN-γ, suggesting that T-VEC is capable of turning cold tumors hot.[71] Alternatively, a topical creme with toll-like receptor (TLR) 7/8-agonists called imiquimod[72], led to the infiltration of both $CD4^+$ and $CD8^+$ T cells and tumor regression in breast cancer.[73] It remains to be determined what the functionality is of those TILs, because in vHSIL it has been shown that a desired pre-existing immune infiltrate comprising $CD4^+Tbet^+$ T cells and inflammatory myeloid cells is needed for clinical responsiveness to therapeutic vaccination but also to imiquimod treatment.[40]

Therapeutic vaccines can also be applied to enhance the differentiation from naïve T cells to tumor reactive T cells and to reactivate spontaneously induced tumor reactive T cells that may have become anergic or dormant[74], but this requires knowledge of the tumor antigens expressed in VSCC. For HPVpos VSCC it is obvious that the two HPV-encoded oncoproteins E6 and E7 can serve as tumor antigens recognized by immune cells (**chapter 8**). Therapeutic vaccination with HPV16 synthetic long peptide (HPV16-SLP) in immunosuppressed HPVpos OPSCC is successfully tested as an adjuvant to checkpoint inhibitor therapy.[75] In vHSIL, clinical responsiveness to HPV16-SLP vaccination was associated with a strong circulating type 1 T cell response to E6 and E7, and the presence of a pre-existing immune infiltrate comprising high numbers of $CD4^+Tbet^+$ T cells and $HLA\text{-}DR^+CD14^+$ inflammatory myeloid cells.[40] This makes HPV16-SLP vaccination less interesting for the low T cell infiltrated HPV16pos VSCC unless we are able to orchestrate an influx of those immune cells first. In HPVneg VSCC we are confronted with a low number of mutations and unknown TSAs.[76,36] Whole-exome and transcriptome sequencing are required to determine if and which (neo)antigens are present in HPVneg VSCC.[77] The isolation of tumor-reactive TILs[78] based on CD39 expression (**chapter 8**) may foster the identification of TSAs.[79] In advanced melanoma, personalized vaccine-based immunotherapy improved the immunogenicity prior to anti-PD-1 therapy.[80] The disadvantage of such a personalized therapy is the long bench-to-bedside time frame.

Therefore, a more general off-the-shelve available therapy would be desirable for HPVneg VSCC patients.

Activated tumor-reactive T cells need to migrate into the tumor in order to combat cancer cells. The trafficking can be blocked by immunosuppressive cells such as type 2 tumor-associated macrophages (TAMs), Tregs, and MDSCs. We observed a preferential expression of genes associated with the myeloid compartment in low T cell infiltrated VSCC. This suggests that local immune suppression may play a role and indicates that in-depth studies on the myeloid compartment are required. When performed in the same cohort as presented in **chapter 6**, more information will be gathered on the density and location of those cells and their prognostic impact and potential therapeutic consequences. For instance, the polarization of type 2 TAMs is inhibited by colony stimulating factor 1 receptor (CSF1R) blockers, and thereby inducing a $CD8^+$ T cell mediated anti-tumor response.[81] The same effect has been shown after depleting MDSCs with therapies such as low-dose chemotherapy and tyrosine kinase inhibitors. Also the recruitment of MDSCs to the tumors can be blocked by CCR-5 or CXCR-2 inhibitors.[82] Also myeloid checkpoint inhibitors such as LAIR1, LILRB1 and LILRB4 were upregulated in inflamed VSCC, which may open a new area of interest of the innate immune checkpoint inhibitors, and can be used as a primer or adjuvant to immunotherapy.

Actionable targets for immunotherapy in inflamed tumors

An alternative approach is the infusion of *ex-vivo* expanded tumor-reactive T cells, so called adoptive cell therapy (ACT), which has been very successful in melanoma and other tumors.[83, 84] ACT is a complicated procedure and the success is limited by the percentages of tumor-reactive T cells present in the infusion product.[85, 86] The data presented In **chapter 8** suggests that this can be improved by isolating the $CD3^+CD39^+$ TIL fraction and applied to the treatment of VSCC. It remains to be determined if we can also use these cells for the treatment of HPVneg VSCC as this will require a formal demonstration of their tumor-reactivity. The cytokines IL-2, IL-21, and IL-7 are described to improve $CD8^+$ T cell proliferation, differentiation, and activation.[87-89] Hence, these cytokines may be used to support the infused TILs. Furthermore, it is likely that co-treatment with checkpoint blockers is required in order for T cells to exert their anti-tumor function which are mainly upregulated in inflamed tumors (**chapter 7**).

Inflamed tumors contain high numbers of (activated) TILs[45, 46], which may be exhausted or dysfunctional due to the expression of co-inhibitory receptors (checkpoints) which suppress T cell functions.[90, 91] Some TILs may be tumor reactive and can be identified by their expression of CD39 (**chapter 8**) in HPVpos VSCC. In our study, checkpoint inhibitors such as PD-1 and LAG-3, CD39, CTLA-4, and TIM-3 were upregulated in inflamed VSCC (**chapter 7**). A synergistic effect is observed when different checkpoint molecules are blocked simultaneously.[92] Our study in **chapter 6** suggests that an improved influx and function of tumor-specific $CD4^+$

TILs, may be beneficial for clinical outcome in VSCC. The $CD4^+CD39^+$ TIL fraction of HPVpos VSCC mainly upregulated checkpoint molecule *PDCD1* (PD-1). The $CD8^+CD39^+$ TIL fraction upregulated *LAG3* (**chapter 8**). Therefore, a combination of anti-CD39, anti-PD-1 and anti-LAG-3 would be of high interest. A study on combination treatment with anti-PD-1 and anti-LAG-3 is underway in melanoma (NCT03743766). One can also envision that the combination of anti-PD-1 with anti-CTLA-4[93,94] or OX-40[94, 95] reinvigorates $CD4^+$ T cell reactivity and may be beneficial in VSCC treatment. In view of the above-mentioned collaboration between T cells and myeloid cells, it can be envisioned that blockade of myeloid cell associated checkpoints, such as LAIR and LILRB, found to be expressed in VSCC (**chapter 7**) may also improve clinical outcome.[96]

Some of the inflamed tumors represent the altered-excluded phenotype. These tumors are less likely to respond to immunotherapy as they express less checkpoint molecules (**chapter 7**), similar to what was seen in colorectal cancer.[46] It is hypothesized that particularly for altered-excluded tumors, T cells require stronger trafficking signals such as CXCL9, CXCL11, and CCL5.[97] Indeed, the expression of the genes for these molecules was lower in altered-excluded tumors when compared to inflamed tumors (**chapter 7**). In ovarian cancer it has been described that DNA methylation suppresses the expression of these genes, which may be reprogrammed by selective epigenetic modulators.[98]

FUTURE DIRECTIONS TOWARDS PROGNOSTIC REFINEMENT AND TAILORING CURRENT TREATMENT STRATEGIES

Future vulvar cancer care continues as a multidisciplinary process combining pre-operative clinical findings, imaging results, histopathological analysis, and consequently providing information to tailor (standard) treatment. Our future aims should be separated into prognostic refinement and identifying predictive markers for response to (standard) therapy. To bring order in the confusing mass of prognostic variables, we need to conduct a large (inter)national prospective study with standardized and detailed pathology instruction forms. Clinically feasible prognostic variables should be included together with p16-IHC and p53-IHC. We should strive to optimize, standardize, and validate a clinically feasible tool that represents the immune infiltrate of VSCC. This tool should be incorporated in a multivariate analysis of a large prospective trial. Consequently, based on the prognostically important variables, we should be able to categorize patients in low-, intermediate-, and high-risk groups for death or recurrent disease. The proposed prospective study would also aid the development of a nomogram, which is an easy graphical calculating tool that visualizes the weight of prognostic factors and computes an individual's probability on either survival or recurrence based on the combination of the inserted risk factors. It provides clear insight in the prognosis for both healthcare workers and patients[99] and encourages the active participation of patients in their treatment decisions.[100] In breast-[101], prostate-[102], pancreatic-[35], and oropharyngeal cancer (OPSCC)[34], nomograms are used to

make important decisions concerning (adjuvant) treatment. In OPSCC, the development and external validation of nomograms in a European Multicenter Study (OroGrams), led to the online publication of a nomogram where the overall survival and progression-free survival are calculated based on a few clinically available prognostic markers (www.orograms.org).[34, 103-105]

In order to predict the response to standard therapy, we need to report on clinicopathological variables in (pre)treatment biopsies. If several important clinicopathological variables can be assessed in biopsies, it should be envisioned that we can tailor treatment based on these variables. For instance, HPVpos VSCC (characterized by a 'block-type' p16-IHC) showed the best clinical outcome after standard therapy. These patients may benefit from minimal tissue removal, less stringent follow-up schedules, and a lower threshold to adjuvant radiotherapy as HPVpos VSCC are more radiosensitive than HPVneg VSCC.[106-108]

In the meanwhile, translational research should focus on a deeper understanding of molecular and immunological mechanisms underlying VSCC, as this will lead to new therapeutic options such as immunotherapy. Because we demonstrated that the intratumoral T cell infiltration has a prognostic role in early-invasive VSCC, it is time to determine the role of the TME in advanced stages of VSCC. When these results have been validated in a larger cohort of VSCC, we should develop clinically feasible tools that can be easily integrated in the field of immunopathology. An example of such a tool is the integration of the immunoscore with the conventional TNM-classification of colorectal cancers. The immunoscore is also used as a predictive tool for response to immunotherapy.[42, 44, 46, 109, 110] Alternatively, one may use the T cell-inflamed GEP score not only to determine the prognosis, but also the potential of immune checkpoint therapy for an individual patient.[50, 58, 111] The prognostic and predictive use of the T cell-inflamed GEP score should also be studied in a large cohort of VSCC, preferably in samples that derive from prospective studies where patients have been treated with checkpoint inhibitors. The key question is which immunomodulatory agents should be combined and are most promising. With our current knowledge, inflamed VSCC are the most eligible candidates to start with. These tumors show high upregulation of many checkpoint inhibitors, and should be treated with anti-CD39, anti-PD-1, -in combination with anti-LAG-3 or anti-CTLA-4 therapy. For HPVpos VSCC this may be combined with HPV16-specific therapeutic vaccination. Low T cell infiltrated HPVneg VSCC may benefit from priming therapy with for instance T-VEC, in combination with ACT using *ex-vivo* isolated and expanded $CD3^{+}CD39^{+}$ TILs.

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