Biomarkers and 3D models predicting response to immune checkpoint blockade in head and neck cancer (Review)

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Abstract. Immunotherapy has evolved into a powerful tool in the fight against a number of types of cancer, including head and neck squamous cell carcinomas (HNSCC). Although checkpoint inhibition (CPI) has definitely enriched the treatment options for advanced stage HNSCC during the past decade, the percentage of patients responding to treatment is widely varying between 14-32% in second-line setting in recurrent or metastatic HNSCC with a sporadic durability. Clinical response and, consecutively, treatment success remain unpredictable in most of the cases. One potential factor is the expression of target molecules of the tumor allowing cancer cells to acquire therapy resistance mechanisms. Accordingly, analyzing and modeling the complexity of the tumor microenvironment (TME) is key to i) stratify subgroups of patients most likely to respond to CPI and ii) to define new combinatorial treatment regimens. Particularly in a heterogeneous disease such as HNSCC, thoroughly studying the interactions and crosstalking between tumor and TME cells is one of the biggest challenges. Sophisticated 3D models are therefore urgently needed to be able to validate such basic science hypotheses and to test novel immuno-oncologic treatment regimens in consideration of the individual biology of each tumor. The present review will first summarize recent findings on immunotherapy, predictive biomarkers, the role of the TME and signaling cascades eliciting during CPI. Second, it will highlight the significance of current promising approaches to establish HNSCC 3D models for new immunotherapies. The results are encouraging and indicate that data obtained from patient-specific tumors in a dish might be finally translated into personalized immuno-oncology.

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1. Introduction

Patients with recurrent or metastatic (R/M) head and neck squamous cell carcinomas (HNSCC) have few treatment options with little or no permanent response to therapy. Novel treatment regimens are essential as conventional treatment pillars exert substantial toxicities and are associated with unfavorable outcomes. This limited response may be partly explained by distinct intratumoral and intertumoral heterogeneity of HNSCC represented by a complex mutational landscape. HNSCC carcinogenesis is propagated by frequent chromosomal instability and multiple genetic drivers undergoing evolution due to selective pressure during treatment (1). In a relevant amount of HNSCC cases, an inflammatory phenotype with tumor-infiltrating lymphocytes is apparent (2). Yet, often immunomodulatory molecules are expressed. For instance, a number of tumor entities, including HNSCC, express programmed cell death ligand (PD-L)1 and PD-L2, which interact with their programmed cell death-1 (PD-1) receptor to limit the function of activated T cells (3-5). At present there is growing knowledge of the molecular processes that induce the expression of PD-L1 and PD-L2 or modulate protein stability (6,7). However, the influence of established therapies such as radiotherapy (RT), chemotherapy (CT), combined radiochemotherapy (RCT) and the antibody against the epidermal growth factor receptor (EGFR) cetuximab on PD-L1 and PD-L2 expression is only insufficiently known.

That HNSCC frequently express immunomodulatory molecules and bear inflammatory phenotypes establish the potential effectiveness of immunomodulatory therapeutic agents (8). The development of so-called checkpoint inhibitors, which influence the specific T cell activity as part of the adaptive immune system, was a major breakthrough in immunotherapy. With the discovery of the first checkpoints cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and PD-1, a key regulatory principle of the immune system was revealed (9-11). PD-1 antibodies are the first immunotherapeutics that have been able to achieve a stable response and a reduced mortality rate in R/M HNSCC patients (12-15). The safety and clinical anti-tumor activity of pembrolizumab and nivolumab, two antibodies against PD-1, have already been confirmed in several clinical studies in patients with R/M HNSCC. The two antibodies have been approved by the FDA (Food and Drug Administration) since 2016 and have already established themselves as a new standard therapy option for patients with advanced HNSCC after failure of conventional approaches, especially after platinum-based CT. Despite these promising data, the number of patients responding to immune modulation is low. The overall response rate (ORR) in the CheckMate 141 study is only 13.3% (13) and 18% in the KEYNOTE-012 study (14). If a durable response is attained, which is rarely the case, this is mostly due to other types of anticancer therapies (16).

2. Biomarkers for immunotherapy in head and neck cancer

Emerging biomarkers for HNSCC in the age of immunotherapy. Biomarkers are useful tools and are meant to support stratification of patients when randomizing the cohort, assist monitoring under treatment and aid in predicting which subgroups of patients may derive the greatest benefit from specific treatments (17). The identification of these markers, whether they are molecular, histologic, radiologic, or physiologic, is challenging in a number of tumor types, including HNSCC. The ideal biomarker is required to encompass the presentation of molecular therapeutic targets considering the mutational spectrum but also the tumor microenvironment (TME) features affecting the clinical course and therapeutic sensitivity of the disease (18).

As only some patients respond to CPI, it is necessary to identify and select the subset that might benefit from CPI beforehand. For monitoring the response to standard oncological treatments of HNSCC, a variety of biomarkers has been described, demonstrating predictive value (17). For instance, Sailer *et al* (19) found that paired-like homeodomain transcription factor 2 (PITX2) methylation functions as an identifier for patients that potentially require additional measures such as intensified surveillance or adjuvant therapy. DNA repair protein expression including excision repair cross complementation group 1 (ERCC1) is also proposed as a predictive and prognostic marker with decreased overall survival (OS) in HNSCC (20) and in patients undergoing definitive platinum-based RCT for HNSCC, irrespective of human papillomavirus (HPV) status (21). Regarding EGFR blockade, phosphatase and hensin homolog (PTEN) loss exerts a negative prognostic impact in patients treated with cetuximab + CT, but not in the CT only group (22). Negative effects of CD44 and EGFR and positive effects of p16 on RT results have been published (23).

PD-1 and PD-L1 expression currently remain the most significant tissue biomarkers to predict success or failure of immuno-oncological approaches. Interferon (IFN)y induced upregulation of PD-1/PD-L1 suppresses the immune response by downregulating cytokine expression in the TME. PD-1/PD-L1 inhibitors induce tumor regression and this process is affected by TME-related parameters such as PD-L1 status and tumor-infiltrating lymphocytes (TILs) (24). Adaptive immune resistance is one mechanism by which tumors escape the immune system and is represented by the process of expression of PD-L1 in response to cytokines. IFN-y induces upregulation of PD-1/PD-L1, cytokines could be decreased and the immune response in the TME is consecutively impaired (25,26). Anti-PD-1/PD-L1 treatment causes tumor regression. Factors that are tumor cell intrinsic and related to the TME such as PD-L1 status and TILs are supposed to affect this process (27). Accordingly, PD-L1 expression and CD8+ T cell density are suggested as predictive biomarkers for anti-PD-1/PD-L1 efficacy There is an interrelation between docetaxel, platinum and fluorouracil (TPF) induction CT in advanced HNSCC and PD-L1 positivity on tumor-infiltrating immune cells as well as CD8+ lymphocytes density . These features suggest that combination strategies of concomitant cytotoxic therapies and anti-PD-1/PD-L1 therapies might be relevant for HNSCC (28).

Cytotoxic TILs, regulatory T cells (Tregs) and natural killer cells. HNSCC is a disease characterized by profound immunosuppression (16,29). Cytotoxic TILs are noted by their expression of CD8. By binding to major histocompatibility complex (MHC) class I molecules TILs are capable of directly attacking and destroying cancer cells (30). TILs and Tregs, that inhibit immune responses, act as antagonists. The prognostic value of CD8+ TILs, albeit still debated, is assessed in most trials as it is a more robust predictor than CD3 (31). According to a recent meta-analysis, high CD3+ TIL infiltration correlates with a favorable prognosis for both HPV-negative and HPV-positive head and neck tumors (31), which is in agreement with other types of cancer (32).

In esophageal and colorectal cancer, TIL and Treg cells are associated with a favorable outcome (33,34). Conversely, the expression of the forkhead box P3 (FoxP3) protein, the most specific Treg marker, in HNSCC tissue samples was significantly associated with inferior survival (35). An enhanced infiltration of Tregs in both intratumoral and stromal compartments is associated with improved clinical outcomes (36). By analyzing pre-therapeutic tissue samples of 280 patients with locally advanced HNSCC treated with RCT for expression of CD8+ cytotoxic T cells and FoxP3+ Tregs, Echarti *et al* (37) describe a classification into different subsets defined by intraepithelial and stromal cytotoxic T cells. They found opposing effects of Tregs between the groups. While in 'immune desert' and 'immune excluded' tumors high Tregs lead to worse survival, the OS was improved in 'inflamed' tumors with the same Treg constellation. This finding might in part explain why the prognostic significance of Tregs is inconsistent in earlier publications (37). Cho and Lim (38) refer to these discrepancies by differences in tumor type,

molecular features, distribution patterns of Tregs and markers of Treg cells. Recently it was shown that an increase in the levels of circulating Treg cells not only in tumor tissue but also in peripheral blood could serve as a prognostic factor of survival in patients with oral cancer. Notably, in this study, intratumoral-infiltrating Tregs had no prognostic significance, while circulating Treg cells in peripheral blood were associated with a favorable clinical outcome (38). When evaluating the impact of Treg cells on prognosis, HPV-association of the tumor should be considered as a relevant factor as the immunologic landscape of HPV-driven HNSCC is modified (1). Although PD-1 and PD-L1 expression is not altered by HPV status, HPV-infected tumors display higher expression of anti-CTLA-4 as well as Treg infiltration and Tregs/CD8 ratio, indicating that HPV positivity might enhance the sensitivity to CPI (1). In accordance, most studies report an increased density of TILs in HPV-driven HNSCC compared with HPV non-driven tumors. These observations imply a more effective anti-tumoral immune response and consecutively an improved clinical outcome for HPV-negative as well as HPV-positive patients with a more intense tumor infiltration of high CD8+ T cells This observation suggests a more effective anti-tumoral immune response and hence an improved clinical outcome for HPV-negative as well as HPV-positive patients with a more intense tumor infiltration of high CD8+ T cells (31). However, Cho and Lim (38) found the Treg cellular impact on clinical outcome and HPV status not to be interrelated. In contrast, Lukesova *et al* (39) state that oropharyngeal squamous cell carcinoma patients show improved survival when their tumors are HPV-associated and display elevated Treg levels in peripheral blood samples.

In addition to Tregs, natural killer (NK) cells serve an important role in the immune system. NK cells are characterized as CD56+ CD3- lymphocytes that are part of the innate immune system and the first line of defense against viruses, pathogens and cancer (40). Again, Lukesova *et al* (39) found a significant difference in the levels of NK cells between the groups of HPV-positive and HPV negative patients. Higher levels of NK cells are observed in HPV-positive patients with improved prognosis. According to Renoux *et al* (41) NK cells are stimulated to higher cytotoxicity and increased cytokine production by the binding of HPV-specific virus-like particles via the C16 receptor, which could mechanistically explain the finding from Lukesova *et al* (39). Furthermore, smoking as one of the main risk factors for HNSCC may also contribute to the sensitivity of the tumor towards PD-L1 agonists. Certain genetic smoking signatures of the tumor reflect smoking. In such tumors, immune infiltration is diminished, often combined with local immunosuppression and lower levels of cytotoxicity within the immune microenvironment. This is associated with an unfavorable prognosis (2,42,43). These subsets of patients may well respond to CPI. CPI could probably be combined with immune agonists targeting co-stimulatory molecules expressed on the surface of T cells, such as 4-1BB, OX40, CD40, GITR) thereby directly stimulating immune response (1,44,45). Suppressive effects on NK cells, CD8+ T cells and dendritic cells (DCs) are likely to be involved, but the precise mechanisms remain to be elucidated (46). In turn, a higher mutational load is associated with higher response rate towards anti-PD-1 therapy as shown in a study on lung cancer patients (47). Mandal *et al* speculate from their data on HNSCC that smoking signature-high HNSCC may benefit from immune modulators such as IL-2, toll-like receptor (TLR) and stimulator of interferon genes (STING) agonists. These compounds are already applied in other entities to enhance overall host immunity (1). De la Iglesia *et al* (48) analyzed a cohort of HNSCC for their expression of CD3, CD8, FOXP3, PD-1, PD-L1 and pan-cytokeratin by multiplex immunofluorescence. They report decreased numbers of cytotoxic T cells in tumors of current smokers and lower gene expression in the interferon cascades compared with former- and never-smokers. They conclude that the tumor immune microenvironment is actively modulated by smoking. This might be depicted by the presence of higher numbers of immune cells in certain areas of the tumor, such as the tumor margin (48). The data underscore the need to investigate novel agents that target modulators of Tregs [e.g., CTLA-4, Glucocorticoid-Induced TNFR-Related (GITR), inducible T cell co-stimulator (ICOS), IDO and vascular endothelial growth factor A (VEGF-A)] as well as NK cells (e.g., KIR, TIGIT and 4-1BB) in addition to anti-PD-1 compounds in the treatment of advanced HNSCC (1).

Tumor mutational burden and response to immunotherapy. Tumor mutational burden (TMB) has been investigated as a potential predictive biomarker to immune checkpoint blockade across 27 tumor types (49). TMB is defined as a median number of coding somatic mutations per DNA megabase (N mut/MB) (49). HNSCC is among the neoplasms with the highest TMB. However, why is TMB associated with the response to PD-1/PD-L1 inhibitors? In theory, an increased number of missense mutations is related to a higher number of tumor neo-antigens, which may induce a more substantial immune reaction and increase the response to CPI treatment. TMB, PD-L1 and T cell inflamed gene expression profile (GEP) are independently predictive of response to pembrolizumab in HNSCC patients, in general regardless of the HPV status. They were also correlated with progression-free survival (PFS). TMB, the combined positive score (CPS) and a T cell inflamed GEP were all associated with best ORs (50). By contrast, in a cohort of 126 HNSCC patients, responders to immunotherapy displayed significantly higher levels of TMB than did non-responders. Notably, virus-positive [HPV-positive)/Epstein-Barr virus (EBV)-positive] patients had a lower TMB ($P<0.01$) and improved OS ($P=0.02$) (51). High TMB is often used as a surrogate of immune response to tumors as it is associated with a larger number of tumor neo-antigens, due to a higher somatic mutation level. Those neo-antigens boost the development of the anti-tumor immune response by facilitating the immune recognition as foreign (52). Additionally, plasma-based TMB (bTMB) was evaluated as a prognosticator in the phase III EAGLE study for HNSCC. Patients with high bTMB levels had significantly improved OS and PFS after immunotherapy compared with patients who were administered platinum-based CT (53). In current smokers, a suppressive immune microenvironment is mirrored by a decreased numbers of cytotoxic T cells in the tumor and suppression of IFN response pathways. Notably, in this study there was no evidence for an association between smoking status and TMB and tumor clonality by the MATH

(mutant-allele tumor heterogeneity) score (48) which is not in line with former observations that carcinogens in tobacco smoke are expected to cause permanent DNA damage reflected in TMB (54).

The impact of the TME on response to immune CPI. A T cell inflamed TME persists in the major subset of patients with advanced solid tumor diseases. This phenomenon probably reflects an anti-tumor response resulting in a more favorable clinical outcome (55). Tumors with T cell infiltration into the TME are more likely to respond to immunotherapy including CPI. Hanna *et al* (51) investigated a cohort of 126 HNSCC patients treated with anti-PD-1/L1 therapy and found higher TMB and CD8+ T cell infiltrates to predict a potential benefit from anti-PD-1/L1 treatment significantly among virus-negative tumors. B cells and myeloid-derived suppressor cells are increased in PD-1 blockade responders (56) and the authors now hypothesize an expanded CD8+ effector memory T cell population among the responders. The inflammation-induced enzyme Indoleamine 2,3-dioxygenase (IDO) normally controls harmful inflammatory responses by propagating immunosuppression. IDO is increasingly expressed in tumor, stroma and immune cells and is hypothesized to contribute to cancer immune evasion (57). Accordingly, Jia *et al* reported that higher expression of IDO was associated with poorer OS in head and neck cancer patients (P=0.011) and classified it as a prognostic predictor in head and neck cancer (58). IDO activity has already been linked to resistance against PD-1 CPI in non-small cell lung cancer (NSCLC) (59). For HNSCC, Phase I/II clinical trials have been conducted and showed ORs (34-55%) and disease control rates (62-70%) for IDO1 inhibitor in combination with a PD-1 inhibitor, as recently summarized by Lin *et al* (60). Combining IDO inhibitors revealed similar safety profiles with the compound given as monotherapy. IDO gene expression is referred to as a predictive biomarker for response to PD-L1 therapy. Although there is a body of evidence for the application of IDO-based treatment, in all of the trials IDO inhibitors have been combined with anti-PD1/PD-L1 agents. Furthermore, the IDO immune-based regimen have not been compared with current standard of care (SOC) regimens for HNSCC (60).

In summary, it is necessary to unveil the molecular mechanisms causing a reduction of effector T cell infiltration in the TME and causing a decreased susceptibility towards CPI. Consequently, the development of novel compounds that permit to restore T cell infiltration will promote the efficacy of immunotherapy. The majority of HNSCC displays immune infiltration, which is an indicator of an ongoing natural immune response. Additionally it will be a major challenge to develop new therapeutic interventions that will amend the mode of action of immunotherapies towards enhanced efficacy in patients whose tumors bear the non-T cell-inflamed phenotype. In HNSCC, an inflamed cancer phenotype is very common, but even those tumors eventually manage to evade host immunity (61). Therefore Chen *et al* (62) propose to stratify HNSCC into the active and exhausted immune classes. The active immune class incorporates factors correlating with increased survival including HPV association, an abundance of TILs, increased cytolytic activity and pro-inflammatory M1 macrophage signature while the exhausted immune class is linked with enriched activated stroma, M2 macrophage signatures, activation of Wnt signaling and unfavorable outcome. Understanding the immune responses and their regulation by tumor-intrinsic and extrinsic factors in the TME is the prerequisite for optimizing the immunotherapeutic response. Patients assigned to the active immune class may benefit from CPI as a monotherapy while those with the exhausted immune class may benefit from combinations including TGF-β inhibitors or anti-CTLA-4 therapy, respectively, combined with anti-PD-1/PD-L1 agents (62).

The impact of signaling pathways on immune response in HNSCC. HNSCC harbor complex molecular pathology features and a distinct heterogeneity and vary between localization and etiology. HPV association in oropharyngeal HNSCC serves a major role in prognosis and treatment and has recently been classified as a separate disease entity by the 8th Ed UICC/AJCC TNM staging system (63). Signaling pathways are widely affected in HNSCC and are known to be involved in processes underlying immune evasion in HNSCC. Wondergem *et al* (64) explored three pathways: STAT3, PI3K/AKT/mTOR and Wnt, which are assumed to represent promising targets to possibly facilitate immunotherapy response. These pathways are of particular importance, because they are involved in cellular processes considered to account for primary or acquired resistance to immunotherapy. The authors hypothesize that immune responses will be augmented by pharmacological interference with the signaling components and their immune-modulatory features to enhance their sensitivity to CPI. It has been demonstrated that CD8+ T cell infiltration is pushed through inhibition of the PI3K/AKT/mTOR or Wnt pathways inducing immunologically 'hot' tumors. The aim is to stop the propagation of tumor cells and stimulate the immune response at the same time by blocking responsible signaling cascades. The emphasis should be on the comparison between HPV-driven and non-driven head and neck cancer patients as different pathways seem to be relevant for their respective immune response and response to drugs (64). Oncogenic pathways that are activated through gain-of-function alterations in oncogenes or loss-of-function alterations in tumor suppressor genes are known to influence the local anti-tumor immune response. Spranger and Gajewski (65) state that CPI is more likely to be effective if T cells infiltrate the TME. There are several pathways involved in the reduction of the effector T cell infiltration and new therapeutic strategies should aim on boosting the efficacy of immunotherapy by restoration of T cell infiltration by molecularly targeting these biochemical cascades. PI3K inhibitors, which have already been approved for cancers such as B cell lymphoma, leukemia and breast cancer, could help to overcome the dismal response rates to CPI in cancers. The idea is to boost a T cell-inflamed TME by inhibiting relevant pathways with specific compounds that should be administered in a combined regimen along with anti-PD-1 or anti-PD-L1 treatment to promote the response of cancer to these mAbs. Spranger and Gajewski (65) refer in their review to a synergism between a PI3Kβ isoform-preferential inhibitor and CPI in an *in vivo* model, pointing out that these inhibitors might be capable of boosting the susceptibility to immunotherapy. Peng *et al* (66) show that loss of PTEN in tumor cells of preclinical melanoma models lead to an inhibition

of T cell mediated tumor cell killing and diminishes T cell infiltration into the tumor. PTEN loss is known to be associated with a decrease of T cell infiltration and successful T cell expansion after tumor resection, concomitantly with lower susceptibility to PD-1 mAb and unfavorable clinical outcome. As a mechanism, immunosuppressive cytokine expression is stimulated by the loss of PTEN, with consecutively low levels of T cell infiltration in tumors and inhibition of autophagy by which T cell-mediated tumor cell killing is reduced. After a combined treatment with a selective PI3Kβ inhibitor and anti-PD-1/anti-CTLA-4 antibodies, respectively, the susceptibility to immunotherapy was enhanced *in vivo* (66). Using a triple negative breast cancer (TNBC) patient-derived xenograft (PDX) model, PI3K inhibition by BKM120 is followed by a more inflammatory tumor leukocyte infiltrate. Accordingly, the combined application of BKM20 and anti-PD-1 consistently inhibits the tumor growth compared with monotherapy. In conclusion, the susceptibility to CPI is boosted by PI3K pathway inhibition (67). This combination regimen might be a strategy for TNBC but also for other types of cancer with low response rates to immunotherapy including HNSCC. These studies warrant the need for new strategies to transform the TME of non-responsive patients into a milieu supporting T cell-based inflammation. However, one has to take into account that some of these pathways identified are critical for the maintenance of normal tissues. New compounds should therefore selectively target the relevant immune components while preserving globally relevant pathways (65).

The RAS/RAF/MEK/MAPK pathway. MAPK cascades activate a number of important cellular processes by inducing mediators leading to cell growth, proliferation, differentiation, migration, invasion and survival (68). The ability of immunocompetent MAPK mutations in HNSCC are proven to drive a CD8+ T cell-inflamed status in *in vivo* models (69). Patients with MAPK mutations consistent with CD8+ T cell-inflamed phenotypes were investigated. This subset of patients survived 3.3-4 times longer than wildtype patients under anti-PD-1/PD-L1 immunotherapies. The phenomenon was seen independently of the TMB. Notably, in this context pathway mutations were linked to remarkably long patient survival rates. The prognostic power was found to be independent of the HPV status. As a mechanism, phosphorylated human epidermal growth factor receptor 3 (p-ErbB3) regulation by MAPK pathway-mutants is featured. Low tumoral p-ErbB3 levels and elevated CD8+ T cell infiltrations are described as indicators of prolonged survival in HNSCC (70,71). The two events have now been shown to be governed by MAPK mutations and are likely to be independent of each other. These findings are likely to contribute to the ongoing search for predictive biomarkers in HNSCC. MAPK pathway mutations could help identifying HNSCC patients with CD8+ T cell-inflamed tumors that might benefit from immunotherapy (69).

Activation of the STING pathway. Another approach, which may offer new therapeutic opportunities, is the activation of the STING pathway through polymer-induced STING condensation. The cyclic guanosine monophosphate-adenosine monophosphate synthase-stimulator of interferon genes (cGAS-STING) is a major regulator of innate immune sensing of cancer, with potential to enhance tumor rejection through the induction of a pro-inflammatory response dominated by Type IIFNs. The first STING agonist is currently in phase I clinical development. Although in pre-clinical trials assessing a plethora of natural and synthetic cyclic dinucleotides and non-nucleotidyl STING agonists these compounds have been promising, clinical early phase studies on various tumor entities revealed only modest anti-cancer activity (72). A number of early phase trials are continuing. For R/M HNSCC, a phase II study is currently examining the combination of the STING pathway activator ADU-S100 plus pembrolizumab in patients with no prior systemic treatment. Endpoints include safety, preliminary anti-tumor activity, pharmacokinetics and immunomodulation. The results provided evidence for a robust toleration of ADU-S100 plus pembrolizumab (73). One limitation of these efforts is the inherent instability of dinucleotides. The administration of most of the STING agonists in ongoing clinical trials, namely directly intra-tumoral (i.t.) is not ideal, as their application is limited to a narrow spectrum of tumors. An promising exception is stable STING agonists as identified by Pan *et al* (74) and Chin *et al* (75) inducing the same 'closed' conformation as the natural STING ligand. The advantage of these small molecules over previously designed i.t. administered STING agonists is the possibility of an oral application. The agonists were shown to activate STING and diverse immune cell types thereby promoting antitumor immunity by activation of CD8+ T, natural killer and DCs and exhibiting anti-cancer activity. Notably, the compound SR-717 stimulates the expression of relevant target proteins including PD-L1 in a STING-dependent way (75). Efforts should be made in the progress towards the clinical practice of viable STING agonists as these compounds are hypothesized to be conveniently applied in a low-cost regimen.

Predictive tissue markers for PD-1/PD-L1 inhibitors. Unfortunately, it has not yet been possible to predict adequately the success of CPI for the individual patient. Clinical studies are searching for biomarkers that would enable such a prediction. In general, poor prognostic factors such as high tumor burden, rapidly progressive tumor growth and poor general condition and performance status seem to be rather unfavorable regarding the response to immunotherapy (76).

For individual indications, the expression rate of PD-L1 in tumor tissue has been proven to be a response marker to PD-1/PD-L1 therapy (i.e. PD-L1 as a predictive biomarker). In most cases, PD-L1 expression on tumor and/or immune cells is also associated with OS independently of treatment (i.e. PD-L1 as a prognostic biomarker) (76).

The KEYNOTE 040 study is an example for this assertion. Patients with R/M HNSCC after progression on previous platinum-based therapy were treated with either pembrolizumab or standard therapy of the investigator's choice (methotrexate, docetaxel, or cetuximab). Patients had a significantly improved survival in case their tumor tissue samples showed positivity for PD-L1. The positivity was defined by a TPS (tumor proportion score) of ≥50%, which means PD-L1 expression in tumor cells only. Those patients displayed both an improved response in the pembrolizumab treatment arm and poorer survival in the CT arm compared with patients with PD-L1 TPS <50% (77).

These differing responses in PD-L1 high and low-expressing tumors led to a restriction of approval for patients with PD-L1 high-expressing tumors. Szturz and Vermorken (5) give recommendations for the translation of the KEYNOTE-048 data in the clinical practice. They scrutinize the motivation to apply immunotherapy in tumors with lower PD-L1 expression as given cut-off values should not be regarded as a dogma and the results can be biased by various factors. They indicate that high CPS should not be considered equally with CPI response. Indeed several factors could influence the decision process in the R/M situation including biological age, tumor burden, or pace of disease (5). The KEYNOTE-048 study of pembrolizumab alone or with CT vs. cetuximab with CT in R/M HNSCC preceded the extended approval of pembrolizumab for first-line treatment. PD-L1 expression is most predictive when applying the CPS with a cutoff of ≥20 but already predictive in those with CPS ≥1 (78). Consequently, pembrolizumab is only approved for patients whose tumors show a ≥50% TPS for PD-L1 expression in the second-line treatment. Pembrolizumab as a monotherapy can be applied with a CPS ≥1 independently of a platinum-based CT as first-line option. In the CHECKMATE-141 study, nivolumab appears to be superior to SOC, regardless of tumor PD-L1 expression or p16 status (13). This is surprising as nivolumab is assumed to exhibit the same mode of action as pembrolizumab, but can be in part explained as follows: In principle, nivolumab and pembrolizumab are interchangeable (79). The incongruity in biomarker references (CPS, TPS, or none in CheckMate-141) is obviously driven by the composition of the respective trial (5,80). For nivolumab, it was observed that the compound is effective in both PD-L1-positive (PD-L1+) and PD-L1-negative (PD-L1-) patients (13). Similar to the conclusions made for pembrolizumab, these findings suggest that improved markers need to be identified to stratify patients for immunotherapy. The challenge in making the PD-L1 status a condition for CPI treatment is that PD-L1 status has been performed with different tests using different antibodies and different cutoffs for positivity. However these methods are not conclusive even if the same antibody is applied. It needs to be mentioned that in KEYNOTE 055, a phase II study of pembrolizumab as second-line treatment for R/M HNSCC, PD-L1 negative patients (besides those with positive PD-L1 status) also benefits from CPI at a statistically significant level although the response rate is higher in PD-L1+ patients. These data clearly indicate that stratification of patients for CPI is complex and challenging and should not be based on PD-L1 as the only determinant (81). Similar observations are reported from the phase III CHECKMATE 141 study investigating nivolumab treatment for R/M HNSCC. Patients with >1% TPS have a more favorable outcome in terms of improved PFS, while regarding OS, no significant difference is found between PD-L1+ and PD-L1-patients (13,82). Intratumoral heterogeneity, one of the landmark features in HNSCC, could at least in part explain these ambiguous clinical data on PD-L1 status as a predictor for immunotherapy. There are observations of Rasmussen *et al* (83) that could shed light on the response to CPI of PD-L1 negative tumors on the one hand and on treatment failure of CPI in PD-L1 expressing HNSCC on the other hand. They find PD-L1 varying positivity within the tumor, both with TPS and CPS, a finding that questions the applicability of the biomarker. The authors suggest using repeated biopsies or multiple tumor sampling to improve the predictive power of PD-L1 for response to CPI (80-83). It is comprehensible that the PD-L1 status alone should not determine patient exclusion from immunotherapy and alternative biomarkers need to be established in clinical routine. Despite these controversial results from clinical trials, PD-1 and PD-L1 expression still remains the most significant tissue biomarkers for response to immunotherapy. A total of 175 oral squamous cell carcinomas and 33 corresponding lymph node metastases were screened for expression levels of PD-L1 and PD-L2. Results were correlated with clinicopathological data and the study reveals worse OS in case of high expression levels of PD-L1 and a higher risk of developing neck node metastases, indicating that CPI may be appropriate even in early tumor stage to prevent further disease progression (84). Feng *et al* (85) perform a meta-analysis of the combination of PD-1/PD-L1 and CTLA-4 inhibitors in patients with malignant tumors to address the significance of PD-L1. Notably, they find higher PD-L1 expression to be associated with longer PFS. Tardy *et al* (86) present a case of an R/M HNSCC patient with negative PD-L1 or PD-L2 expression where a microsatellite instability (MSI)-high status is associated with durable complete response to anti-PD-L1 therapy. The continuing prospective trial PRECISION-01 (NCT03917537, www.clinicaltrials.gov) aims to eliminate ambiguities on response markers for CPI and to stratify a patient subgroup who could clearly benefit from nivolumab. The study defines mutational signatures by Whole-genome study analyses on archival tumor tissue samples from platinum-refractory patients who received at least four cycles of nivolumab.

In summary, the administration of CPI in the clinical routine is based on PD-L1 cutoffs corresponding with immunotherapy. However, a valid PD-L1 cutoff that is generally accepted has yet to be defined. Evrard *et al* (87) give an overview on clinical and preclinical studies about cutoffs for PD-L1 positivity in HNSCC. In most trials a 1% cutoff was applied as this value easily differentiates between a tumor with positive expression and one that does not express PD-L1 at all (14,81). In this regard, the importance of soluble immunological markers should be specifically considered.

Soluble immunological biomarkers in HNSCC. There are two forms of PD-L1. One is membrane-bound PD-L1 (mPD-L1) that is located mainly on the membrane of tumor cells, the other is soluble PD-L1 (sPD-L1) in the peripheral blood from cancer patients but also from chronically sick individuals (88,89). The soluble form is derived from the membrane-bound form after proteolytic cleavage (90). sPD-L1 has been suggested as a potential biomarker to predict response to immunotherapy in different types of cancer (91). High mPD-L1 expression is associated with worse survival in cancer patients (92,93). There is prognostic significance of circulating tumor cells (CTC) in head and neck cancer as well. A study by Strati *et al* (94) demonstrates that detection of CTC overexpressing PD-L1 may provide important prognostic information in HNSCC. In case of an overexpression of PD-L1 on CTC post treatment, patients had significantly shorter PFS and OS, an observation helping PD-L1 to emerge as an independent prognostic factor for PFS and OS. Patients whose CTC did not overexpress

PD-L1 had a more favorable prognosis in terms of complete response to immunotherapy (94). These data imply that biomarkers not only from tissue samples but also from liquid biopsies are clearly needed to improve customized treatment. Potential predictors for treatment response to immunotherapy are high blood TMB and low baselines levels of sPD-L1 in lung cancer, as recently reviewed (95). Patterns with high PD-1+/CD4+ T cell count are related to poor OS, while PD-1+/CD8+ T cells are associated with a favorable prognosis. CTC with PD-L1 expression are in most cases connected with a failed response to immunotherapy and consecutively with worse clinical outcome. Recently, the translational potential of plasma Semaphorin 4D (sSema4D) as an immune marker in plasma of HNSCC patients was assessed in matched blood and tumor tissue samples (n=104). The data suggest that HNSCC with elevated sSema4D could be a distinct phenotype. An association between sSema4D and the histological inflammatory subtype was assumed. Younis *et al* (96) hypothesize that changes in sSema4D can monitor the underlying dynamics of tumor and stromal inflammation in real time. Notably, the humanized anti-Sema4D antibody is currently assessed in advanced solid tumors (97,98).

Boschert *et al* (99) investigate whether serum levels of hepatocyte growth factor (HGF) and sPD-L1 can be potential markers of CPI therapy failure in HNSCC. Serum protein levels of 20 HNSCC patients before immunotherapy are correlated with clinical outcomes. They found the clinical data to be positively associated with both serum proteins (HGF and sPD-L1) in the sera of patients with HNSCC. In case of non-responsiveness to immunotherapy, the serum concentrations of sPD-L1 are significantly higher which indicate sPD-L1 functioning as a prognosticator for CPI treatment in HNSCC (99). It has been hypothesized that sPD-L1 in peripheral blood might be able to suppress T cell activity thereby impairing the antitumor immune response (100). Therefore, designing novel strategies to block circulating sPD-L1 before anti-PD-L1 antibody treatment should preserve the anti-PD-1-mediated immune response (101).

Altogether, these data indicate that improved tools are needed to predict patient outcome. The following sections will introduce and discuss different predictive systems for cancer therapy, especially in the light of immunooncology.

3. 3D models predicting response to immune checkpoint blockade in head and neck cancer

Predictive models for cancer therapy in different tumor types. In a review, Binnewies *et al* (102) speculate that it is necessary to focus on stratification schemes for patients not only based on their tumor type but also on their tumor immune microenvironment (TIME) type. Such schemes might enable to improve the response to CPI and also to identify new targets for therapeutic approaches by recording of new datasets (102). Malignant cells proliferate in the complex TME that is crucial for cancer progression. Multifaceted interactions between the tumor and the microenvironment promote tumor progression by cellular processes such as metastasis and resistance development eventually resulting in treatment failure. These features render the TME a key target in oncology. To understand these tumor/stroma processes, 3D models that closely mimic cell interactions are essential. Ideally, these platforms incorporate patient-derived cells from different compartments, i.e. tumor cells, immune cells, fibroblasts and endothelial cells and allow readouts over time. When developing 3D cancer platforms mimicking the immune system one needs to take different subclasses of immune environment into account that are known to influence tumor initiation and response to therapy. The tumor stroma is colonized with various immune cells. CD8+ T lymphocytes are capable of destroying cancer cells by detecting their surface antigens. After interactions between these T cells with tumor-associated macrophages (TAMs), cancer-associated fibroblasts (CAFs) and tumor cells, they become suppressed, exhausted and cannot reach the parenchyma of the tumor resulting in the proliferation of tumor cells (103). In general, the aim of multicellular 3D cancer models is to predict and guide immuno-oncologic treatment to improve sensitivity and identify new therapeutic targets. One can anticipate that 3D platforms might close the loop between 2D models that do not adequately represent tumor/stroma interactions and animal model studies whose results cannot be translated into the human situation one on one. Halfter *et al* (104) introduced an *in vitro* breast cancer spheroid model by which they intend to predict response to neoadjuvant therapy. 3D spheroids are generated from fresh breast cancer tissue and are exposed to equivalents of the therapeutic schemes the patients are about to receive. Cell survival is measured as a readout after treatment simulation. As the patients undergo surgery after neoadjuvant CT, the presence of the pathological complete response (pCR) is correlated with the experimental data. Below a certain threshold for cellular proliferation in the model, patients are likely to achieve pCR (65.6% of respective patients) with a high sensitivity and specificity. Increased cell survival in the model was associated with the extent of residual disease. The authors propose their model as a valuable predictor for post treatment pCR in breast cancer patients (104).

3D models for response to CPI. *In vitro* models cannot comprehensively represent the immense complexity of *in vitro* cancer growth and development. However, they might allow for testing novel treatment options in a controlled setting, therefore facilitating efforts in precision immuno-oncology. This is particularly the case for immunotherapeutic treatment in HNSCC as the response rate is limited (105). However, there is no established protocol for sensitivity testing of the individual patient's tumor before selection for CPI treatment so far. As the TME affects the immune system, challenges for 3D platforms are incorporation of TME features and representation of the dynamic response to CPI (Fig. 1).

Multicellular spheroids. In contrast to monolayer models, 3D culture systems where tumor and stromal cells can interact in a tissue-like architecture, have been created to assess sensitivity towards immunotherapies and to bridge the gap between 2D cell cultures and animal models. One obvious advantage is their increased stability and longer lifespan, By extended culturing periods, they are improved suited for the assessment of long-term effects, in particular of clonal outgrowth which is a major mechanism in resistance development and essential for the evaluation of therapeutic resistance (106). When it comes to

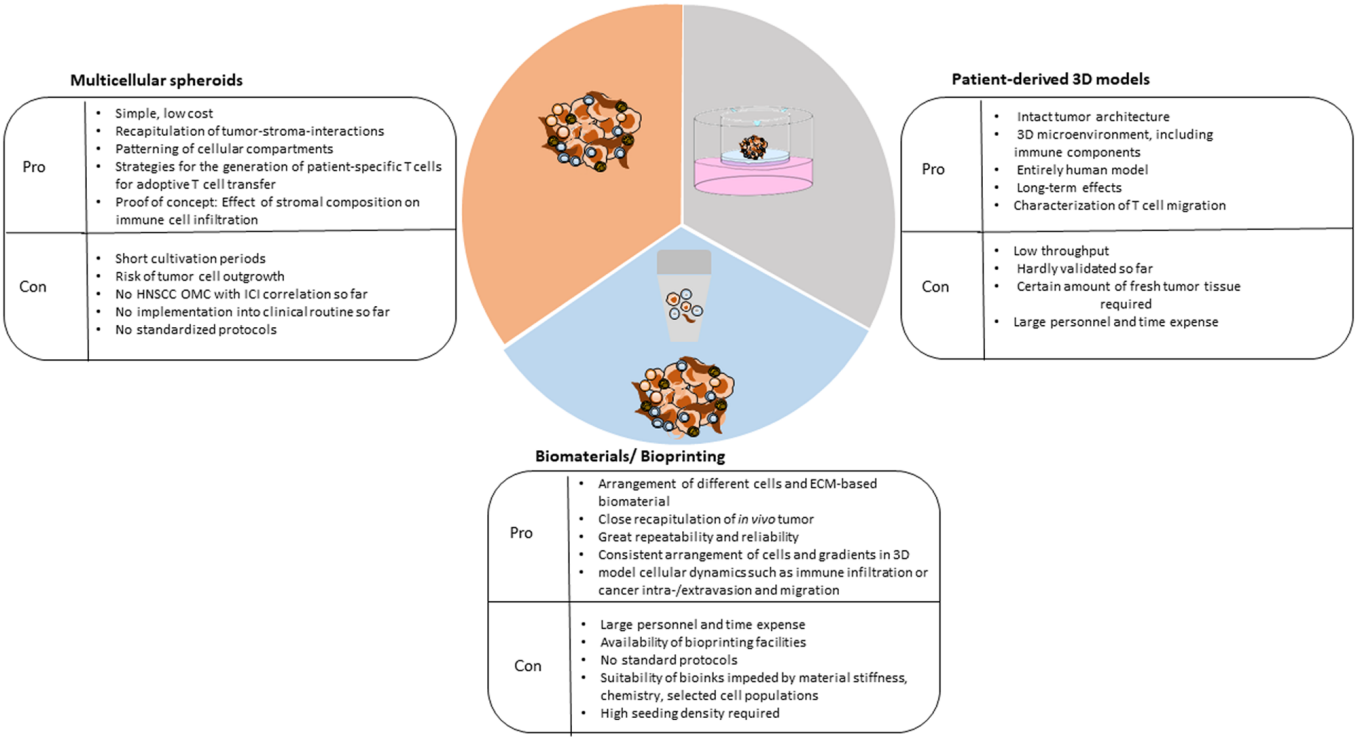

Figure 1. Benefits, limitations and drawbacks of current preclinical animal-free HNSCC models for immunotherapy. For each model, pros and cons are given. Parts of the figure were drawn by using pictures from Servier Medical Art (http://smart.servier.com/), licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

testing immunotherapy, it is of particular importance to choose the right model. For instance, Marrella *et al* (107) observed that immune-checkpoint molecules including B7-H3, PDL2 and PVR more closely resemble immunophenotypic variants of human tumors in 3D alginate-based hydrogel neuroblastoma cultures than under standard 2D culture conditions. Appleton *et al* (108) evaluate immune-related cell composition and PD-1/PD-L1 expression status in their 3D culture based on recomposing dissociated cells from vital ovarian cancer samples. They screen patient-specific alterations in the immune composition, activation, cytokine secretion and drug response with the spheroid models. Although the results are based on only two patients, they appear promising. The authors propose their 3D system as suitable to address basic biology questions and immune-oncological drug development (108).

One example for tissue derived tumor spheres, which are generated after enzymatic digestion of the original tumor tissue are colospheres from colorectal cancer tissue, established by Weiswald *et al* (109,110). Colospheres are implanted as patient-derived xenografts in mice, then extracted and taken in culture to build spheroids. To address the interaction between the TME and tumor immunologic processes, Koeck *et al* (4) propose a multicellular 3D co-culture system to address the effect between CAFs and TME-derived cytokines on the infiltration ability of CD3-CD8 cytotoxic T lymphocyte subpopulations. Cancer cell lines are co-cultivated with fibroblasts and peripheral blood mononuclear cells (PMBCs). Aggregation and chemokine/cytokine expression are assessed. Notably, in case of cancer cell monocultures, peripheral blood mononuclear cells (PBMCs) are able to invade the whole spheroid while in the presence of fibroblasts co-cultured with tumor cells, they locate at the margin. By contrast, infiltration is enhanced in activated CD69 and CD49d cells in the presence of fibroblasts suggesting that immune cell infiltration is affected by the composition of the tumor stroma. The incidence of fibroblasts seems to result in a shift from T lymphocyte infiltration to activated T lymphocytes (4). The immune compartment is been described in a pancreatic ductal adenocarcinoma culture slice model where CD8+ T cells (CD3+ CD8+), Tregs (CD3+, FoxP3+) and macrophages (CD68+, CD163+, HLA-DR+) are present throughout the culture period of six days (111). Herter *et al* (112) recognize that it is a challenge to mimic the complexity of the TME *in vitro*, particularly regarding tumor-host interactions. Therefore, they establish a 3D heterotypic spheroid model consisting of human colon adenocarcinoma cells, fibroblasts and immune cells that allow the assessment of cancer immunotherapy agents. So far, reports on the use of HNSCC organotypic multicellular spheroids to assess immunomodulatory aspects are lacking in the literature.

Patient-derived 3D models for immunotherapeutic response. As previously outlined, the major advances in the development of new drugs targeting cell mechanisms that control antitumor immunity have aroused an enormous interest in tumor immunobiology and immunotherapy. There are unprecedented numbers of preclinical and clinical studies. However, the percentage of responder patients remains very low (13,113) and due to this small percentage the majority of patients undergo ineffective therapies with avoidable side effects. 3D models to study tumor-immune interactions and responses to PD-1 blockade are clearly needed especially for

cancer entities with rather low response rates such as HNSCC. New approaches for CPI have been tested in patient-derived explant models for various cancer entities including pancreatic ductal carcinomas, endometrial cancer and prostate cancer (114-116). Recently a patient-derived *in vitro* model for colorectal cancer enabled induction and analysis of tumor-specific T cell response. Organoids and peripheral blood lymphocytes were co-cultured, the organoids embedded in Geltrex and the lymphocytes in suspension in the culture media. Tumor-reactive T cells expand after co-cultivation. These T cells kill the organoids derived from tumor tissue but not those from benign tissue. The authors aim at discovering personalized determinants of response to immunotherapy (117). The response of murine- and patient-derived organotypic tumor spheroids from various entities to immune checkpoint blockade was assessed using a 3D microfluidic device (118). Autologous lymphoid and myeloid cell populations are preserved in the presence of cancer spheroids. The authors modulate the response to PD-1 blockade by TBK1/IKKε inhibition, which is predictive for treatment response. Neal *et al* (119) demonstrate that the PD-1/PD-L1 axis is conserved in mouse and patient-derived organoids (PDOs) from NSCLC. These models mimic the immune compartment as CD3+CD8+ and CD3+CD4+T lymphocytes as well as B cells, natural killer cells and macrophages are visible. They use an air-liquid interface (ALI) method to propagate PDOs or mouse tumors in syngeneic immunocompetent hosts. The PDO model is generated preserving tumor epithelial cells with embedded endogenous immune cells including native TILs. The growth features of human PDOs correlate with the tumor grading and the condition of the biopsied tissue. The system allows modelling intratumoral aspects of CPI as anti-PD-1-dependent human TIL activation is assessed. TIL expansion and activation are evoked by PD-1 axis blockade within the TME in human and mouse PDOs. Correlation studies between patient and organoid treatment response are still pending (119). Augustine *et al* (120) describe the establishment of a 3D Matrigel system for the analysis of interactions between Treg lymphocytes and NK cells with breast cancer. They use luminal phenotype MCF-7 cells and basal phenotype MDA-MB-231 cells with regulatory T-lymphocytes and NK cells and found that breast cancer phenotype and immune stimulation affect the level of CCL4 secretion (120). For HNSCC some remarkable approaches have already been made. Majumder *et al* (121) propose a multi-compartment *ex vivo* platform maintaining the tumor architecture and heterogeneity as well as morphologic features and characteristics of signaling. The cultivation time of samples deriving from HNSCC and colorectal cancer is three days. During this period, anticancer drugs are applied to the co-culture of immune cells and autologous patient plasma and tumor matrix proteins. The utility of this system for addressing the biology of CPI treatments has yet to be studied (121). Al-Samadi *et al* (122) introduce a humanized *in vitro* microfluidic chip assay. The assay allows the testing of immunotherapeutic drugs against HNSCC patient samples. Freshly isolated cancer cells, patients' serum and immune cells are used on the chips. Immune cell migration towards cancer cells is assessed under the effect of a PD-L1 antibody and an IDO 1 pathway inhibitor. The IDO 1 inhibitor, but not the PD-L1 antibody, induces the migration of immune cells towards cancer cells. There is a patient-dependent efficacy of the PD-L1 antibody and the IDO 1 inhibitor. They conclude that the assay is helpful to predict the efficacy of immunotherapeutic drugs for individual patients (122). The variability of the drugs' efficacy apparently reflects the situation in the clinical practice. Aref *et al* (123) discuss the challenges in translating diagnostic assays such as their 3D microfluidic *ex vivo* culture of organotypic tumor spheroids to the clinic. Samples were taken from various solid tumors including HNSCC. They describe a screening tool for the response of patient tumors to immune checkpoint blockade therapy evaluating murine- and patient-derived organotypic tumor spheroids (MDOTS/PDOTS). By the use of this system, it is feasible to evaluate the requirement for 3D microfluidic culture in MDOTS and the sensitivity towards immune-checkpoint agents of PDOTS, Using RNA sequencing to extrapolate changes in the TME tumor-immune interactions were assessed. Although the results from this approach are promising, there are certain limitations. The current version of the spheroids, either murine or human, is only capable of evaluating tumor-immune interactions of immune cells that have already infiltrated the tumor. They cannot recapitulate T cell priming (which occurs primarily in lymph nodes) or recruitment of naïve immune cells to the TME. The usage of core needle biopsies and fine needle aspiration material instead of bigger samples such as wedge biopsies is also described as demanding (123). The authors of the present study and others (124-126) have demonstrated the feasibility to culture immune cells ideally in their original TME, which is the essential condition to study the individual tumor's susceptibility to immunotherapy. However, so far no data on the usability of head and neck cancer *ex vivo* models to assess sensitivity to immunotherapies in correlation to clinical response has been reported. Bougherara *et al* (127) use an *ex vivo* assay for lung and ovarian cancer to track resident immunostained CD8 T cells. T cell migration is influenced by the extracellular matrix and affecting the control of tumor growth. Such models will make quite a substantial contribution to the development of novel immunotherapeutics to boost T cell migration in cancers (Table I).

Applicability and limitations of animal models in general. Animal models are frequently employed (and also exploited) in preclinical examinations. At least historically, they serve an essential role in the exploration and characterization of disease pathogenesis and physiology for various diseases including cancer. They have also contributed to the identification of targets and the evaluation of new therapeutic agents.

However, there is a debate on the utility of animal models (128-131) which has triggered the search for alternative methods. It is widely accepted that animal models are limited in their ability to mimic the extremely complex process of human carcinogenesis, physiology and progression (132,133). Only little more than a third of highly published animal experimental data will take the decisive step into clinical trials later (134) and only 8% of pharmaceutical agents pass phase I trials with favorable results (132). This high rate of ineffective compounds should send a clear signal to establish more precise model systems for the prediction of therapeutic efficacy of new

Table I. Non-animal 3D models for immunotherapy.

Author, year	Tumor derivation	HNSCC	(Refs.)
Weiswald *et al*, 2009	Multicellular spheroids/organoids	No (colon)	(110)
Weiswald *et al*, 2013		No (colon	(110)
Halfter *et al*, 2015		No (breast)	(104)
Jiang *et al*, 2017		No (pancreas)	(111)
Herter *et al*, 2017		No (colon)	(112)
Koeck *et al*, 2017		No (lung)	(4)
Marrella *et al*, 2019		No (glioblastoma)	(107)
Appleton *et al*, 2021		No (ovarian)	(108)
Kross *et al*, 2007	Patient-derived explant models	Yes	(125)
Augustine *et al*, 2015		No (breast)	(120)
Majumder *et al*, 2015		Yes (and colorectal)	(121)
Bougherara *et al*, 2015		No (lung; ovarian)	(127)
Dijkstra *et al*, 2018		No (colorectal)	(117)
Jenkins *et al*, 2018		Various	(118)
Neal *et al*, 2018		No (lung)	(119)
Aref *et al*, 2018		Various	(123)
Al-Samadi *et al*, 2019		Yes	(122)
Klöss *et al*, 2015		Yes	(126)
Engelmann *et al*, 2020		Yes	(124)
Phuengkham *et al*, 2018	Biomaterials/scaffolds/ bioprinting models	No (breast)	(215)
Swaminathan and Clyne, 2020		No (breast)	(220)
Browning *et al*, 2020		No (skin)	(221)
Almela *et al*, 2018		Yes	(222)

HNSCC, Head and neck squamous cell carcinoma.

molecular entities (NMEs) before stepping into clinical trials. Despite this knowledge, the FDA and the European Medicines Agency EMA require that NMEs are tested on more than one animal species before being transferred into clinical trials.

As Alexander Pope exhorts, 'The proper study of mankind is man' (135); severe failures when translating animal experiments into clinical trials have been reported by various authors (136-138). These can primarily be explained by relevant species-specific discrepancies leading to misconception of animal-to-human predictability. Therefore, animal experiments in order to inform human health are not seen as ethically acceptable (139,140); 50% of all compounds fail in clinical trials due to unacceptable adverse events although those have been evaluated as safe in animal experiments (141) or are withdrawn from the market after passing clinical approval studies as happened in the case of Vioxx (Merck & Co., Inc.) (142). There are a number of cases of severe toxicities in humans that had not been predicted in animal models before. One of the best-known examples is Thalidomide where extensive animal experiments did not predict teratogenicity (141,143). Eventually only very few animal model data are transferable into early phase clinical studies and much more fail in human trials (114,144,145), a fact that increases the calls for human-relevant alternatives (146).

Currently, the advent of cancer immunotherapy particularly scrutinizes the validity of animal models by enclosing further intricacy. When it comes to modelling the immune system, despite some success, there are severe limitations of animal models. In order to minimize the subset of patients that are unlikely to respond to CPI, trustful immunocompetent tumor models that robustly recapitulate the contexture of immune cells within the TME are urgently required. In this regard, it is obvious that the traditional utilization of human tumor cells xenotransplanted into immunocompromised mice which is considered standard to evaluate pharmacology, efficacy and safety profiles of cytotoxic anticancer drugs (147) is not applicable in questions regarding immunotherapy. Here, only models with a functionally intact immune system can be employed. Nearly all animal models lack a functional TME, in particular the immune system environment, comparable to the human equivalent. The following aspects contribute to the defective representability of human immune responses in animal models. Mice are too young with a very active immune cell production to compare with the immune status of elderly patients receiving CPI and this limitation needs to be taken into account when analyzing the data (148). Another factor that is not generally considered is the influence of the sterile environment coming along with animal housing that retrains microbial exposure (146). There is evidence on an increasing role of the oral and intestinal microbiota discussed as a candidate marker of response to anti-PD-1/PD-L1 agents

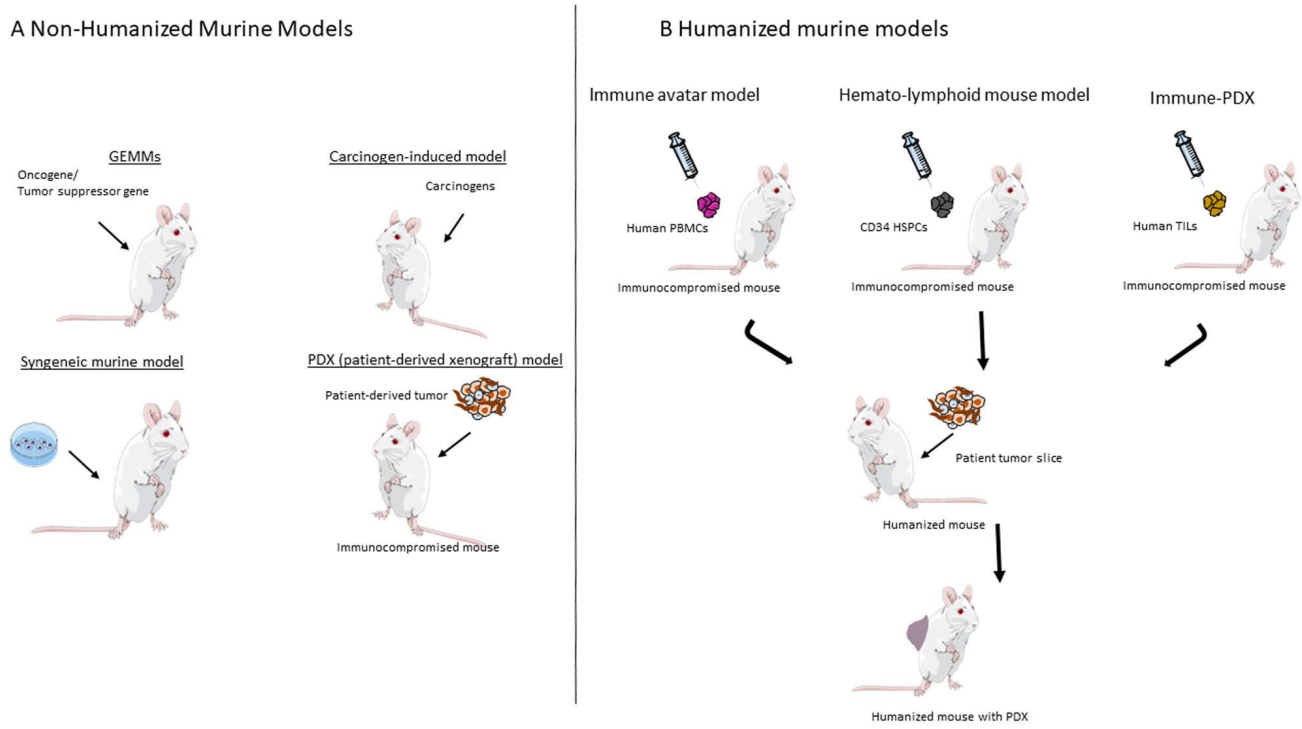

Figure 2. Murine models for immunotherapy. The principles of non-humanized (left panel) and humanized mouse models (right panel) are depicted. Parts of the figure were drawn by using pictures from Servier Medical Art (http://smart.servier.com/), licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/). GEMMs, *genetically engineered mouse models*; PBMCs, peripheral blood mononuclear cells; HSPCs, hematopoietic stem and progenitor cells PDX, patient-derived xenograft.

in HNSCC as this ecosystem correlates microenvironment and TME (149,150) and which entails deficiencies of immune functions and lymphoid tissue architecture (151).

In the emerging field of immunooncology, several preclinical murine cancer models have been described (Fig. 2) including syngeneic mouse tumor cell lines, autochthonous tumors that occur spontaneously in contrast to transplanted tumors, conventional xenograft models or immunologically humanized mice (148). PDX and cancer cell line-derived xenografts (CDX) are achieved by transplanting either cancer cell line or vital human tumor tissue into immunodeficient mice. CDX head and neck cancer models have been developed with different HNSCC cell lines (152-154). Brand *et al* (155) describe an HPV-associated CDX model by bilateral injection of HPV-positive HNSCC cell lines (UM-SCC47 (SCC47) and UPCI-SCC90 (SCC90) into athymic nude mice for evaluating anti-HER3 therapy. However, relevant drawbacks of this system are the lack of lymphocytes, the perpetuation of murine stroma and normal/reduced innate immunity (156). PDX and CDX have the potential to model individual patient tumors and different cancer subtypes and to study the efficiency of standard and novel agents for specific patient tumors preclinically. However, the engrafted tumors arise in immunocompromised animals, which hampers the information on the role of the immune system in carcinogenesis and response to treatment. In CDX, it is obvious that immune cells that infiltrate the grafted tumor are completely murine while the immune cell portion of the PDX from the first transplantation is lost in the course of passaging to other host mice.

Syngeneic mouse models. Syngeneic models consist of tumor cells from the same genetic background as the immune-competent recipient mouse-strain. The origin of transplanted cells, the TME and the host from the same strain is commonly indicated as an advantage. It is anticipated that mechanisms how cancer therapies perform could be evaluated in the presence of a functional immune system. However, cancer cells engrafted in syngeneic models show rapid growth, a kinetics that provides an inadequate time frame for assessing the susceptibility to immunotherapy (157). The established tumor constructs are barely heterogeneous, even though heterogeneity is one major characteristics in HNSCC that makes every tumor unique (158). On the contrary, in syngeneic mouse models tumor cells and the TME cells are abnormally homogeneous. This is due to the absence of progenitor cancer stem cells or due to the adaptation of tumor cells to their artificial environment after transplantation. Anti-PD-1/PD-L1 effects have been investigated in syngeneic mouse models. The combination of irradiation and immunotherapy is assessed by measuring tumor size, animal survival and animal body weight in a syngeneic murine melanoma model. It is observed that treatments with immunotherapy alone shows only modest effects while combination causes increased survival and decreased growth pace of the tumor and the model is considered as suitable for this question (159). Another report evaluated whether SGT-53, a tumor-targeting nanomedicine carrying the p53 gene, could enhance anti-PD-1 treatment in a syngeneic glioblastoma model (160). In this context, tumor cells interacting with a fully competent immune system are considered as the major benefit of syngeneic models. However, rapid tumor growth hampers the development of the chronic inflammatory environment, although this is a key feature of human cancers (161). Additionally, syngeneic tumors do not go

through the typical process of oncogenesis from initiation to a completely developed tumor (162). Syngeneic tumor models are also used to investigate the antitumor activity of other CPI, including anti-CTLA-4 (163). Yu *et al* (162) propose that four commonly used syngeneic models are an effective instrument as different tumor-immune landscapes enable to stratify into responders and non-responders to CPI. The features on which this segregation is thought to be based on depend on pre-existing immune infiltrates. However, injection of xenomaterial into mice will entail inflammation and thereby trigger the innate immune system. This previously activated immune reaction might inadequately mimic the TME (161). Finally, the mutational load in murine tumors is lower compared with human neoplasms. For instance, in mice the perturbation of only two signaling pathways involving p53 and RAF/MAPK seems to be sufficient for tumorigenic conversion of fibroblasts compared with five altered cascades in humans (164). In 1997, O'Malley *et al* (165) proposed an orthotopic immunocompetent murine model for head and neck cancer, where lung metastases were present but without the evidence of metastatic lymph nodes. This was explained by a potential spillage of transplanted tumor cells into the vasculature of the animals causing pulmonary lesions which did not undergo the normal process of metastases (166). Vahle *et al* (167) found neither distant nor lymph node metastases in their orthotopic murine model of HNSCC in fully immunocompetent mice although the tumors had a broad invasive front and were not encapsulated.

Genetically engineered mouse models (GEMMs). To optimize syngeneic models, GEMMs were evolved to create spontaneous tumors in a mouse model. GEMMs are supposed to depict the TME and the tumor configuration in a more sophisticated manner. Tumor development in these systems is due to the inclusion of certain molecular changes in the genome. In these models, mostly oncogenes are expressed and/or tumor suppressor genes are deleted by specific genetic engineering techniques (168). GEMMs provide the opportunity to introduce more than one transgene or *knock-in* gene for human checkpoints. The system is suggested to be suitable for examining the prospect of immunotherapy as fresh tumors develop against the backdrop of a functional immune system (168). GEMMS are used for evaluation of candidate drug targets, therapeutic effects and the identification of drug resistance in the presence of a competent immune system. Immunocompromised genetically-engineered models bear specific immune relevant characteristics as some models preserve NK cell response. In chemosensitivity assays, specific drugs for the human checkpoint molecule can be tested in the scenario of an intact immune system. Autoimmune effects observed in patients treated with anti-CTLA-4 agents can be modelled in CTLA-4 *knock-in* mice (169). Again, low mutational burden of the tumor model is an issue (170). After all, *knock-in* mice, GEMMs and syngeneic models share one severe disadvantage, as they are all based on a fully murine immune system, This might be in part overcome by the usability of the clustered regularly interspaced short palindromic repeats-CRISPR-associated 9 (CRISPR/Cas9) system (171,172). This technology enables the *knockout* of different genes in order to illustrate their contribution to cancer-related processes and response to therapeutics. Regarding immunotherapy, strategies have been developed where, through CRISPR/Cas9-based editing, PD-1 and CTLA-4 are removed in order to enhance the effect of T cell-based immunotherapy (173). The technology has already made the step into a phase I trial on metastatic non-small-cell lung cancer where CRISPR/Cas9-mediated PD-1 *knock-out* in T cells is currently under clinical evaluation (174).

Chemically-induced tumor models. Immunocompromised mice [athymic nude mouse (nu/nu)] and subsequently the severe combined immunodeficient (SCID) mice have provided the opportunity to screen anticancer drugs in human tumor xenografts in mice for decades (175). Due to the compromised immune function of the mice, these analyses have limited potential to assess tumor-immune interactions and investigations with checkpoint blockers. The usefulness of athymic mice in oncology research is due to their lacking mature T cells, which renders them unable to reject allogenic and xenogeneic engraftments (176). However, mice keep an innate immunity and immune functions apart from T cells are still existent.

In athymic mice and rats tumors tend to grow in a capsule without spreading to distant organs regardless of the manner they are implanted (orthotopically or heterotopically). It is noticeable that immune responses from innate components (DCs, neutrophils and monocytes/macrophages) are not only preserved but also enhanced compared with euthymic mice (177,178). This gives rise to the question of how this modified immune response is comparable with the one in euthymic humans when addressing immunotherapeutic questions. It is questionable whether these findings have relevance for testing immunotherapeutic approaches since the animals are immunocompromised. The complex dynamics of tumor-immune-surveillance and immune-mediated editing (179) is not adequately reflected (170). In parallel, attempts are being made to establish immunocompetent models, where DNA damage caused by chemicals is thought to convey heterogeneity of the constructs. Atypical precursor epithelial cells and chronic inflammation of the connective tissue, similar to the setting in HNSCC, are supposed to represent human tumor development. By use of the Epithelial Atypia Index Nauta *et al* (180) compare the successive stages of 4NQO-induced rat epithelial dysplasia with specimens of human oral epithelial dysplasia and find close histological similarity. One other example for chemically-induced head and neck tumor models is the hamster cheek pouch (181,182). Although the tissue in the human oral cavity histologically resembles the check pouch, the pouch lacks lymphatic drainage. Therefore, the model can only be utilized to a limited extent. The thin pouch skin is considered as specifically immune-privileged, with sparsely distributed lymphatics, preventing antigen escape and enabling engraftment and acquisition of blood supply for xenograft transplants (183). Variations by applying tumor cells in the tongue led to a broad histological inconsistency as reactive papilloma started to develop probably after causing an injury on the back of the tongue in order to enhance carcinogen exposure (184). When comparing the model with original oral epithelial lesions, certain similarities in the expression of cytokines/cytokine receptors and Cox2 and NF-κB activation were observed (185). However, these artificially developed

models do not show any metastases (186) and are usually less aggressive than human HNSCC. Eventually, this could be amended by a high increase in drug concentration in the drinking water of the animals and extension of treatment and observation times (187). However, there are sparse reports of metastatic spread in a chemically-induced mouse model to the best of the authors' knowledge. The model can be considered as a tool for evaluating cancer development in the early stages, however, various drawbacks such as additional unintended tumor development at other sites including paws due to, for example, grooming (156) restrict the applicability of chemically-induced tumor models. Another aspect that should never be ignored is the wellbeing of animals in chemically-induced models. Especially when establishing colon cancers, animals suffered from significant weight loss and diarrhea. Along with the extended observation periods such procedures are burdening for the animals and not in accordance with the concept of animal welfare (188).

Conclusions to draw from animal modelling. After all these attempts, it is still unclear whether animal models are a significant and reliable basis for therapies of individual patients. To date, comparisons of a sufficiently big cohort of PDX models with the clinical outcome of the respective patients are still lacking in HNSCC to the best of the authors' knowledge. The inability of defining one or more possible endpoints to determine drug efficacy in mice is considered as one major issue. Indeed, studies have shown that following alignment of all relevant factors, there is a high correlation between the responses of original tumor and avatar, as summarized by Durinikova *et al* (189). In case of HNSCC, the PDX model was used to represent genetic alterations and susceptibility to anticancer drugs such as cetuximab and the PI3K inhibitor PX-866, respectively, in a study from 2013 (190). However, PI3K inhibitors have not been approved for the treatment of HNSCC yet, although they are repeatedly tested in different clinical trial settings alone or in combination (NCT00897988; NCT04997902). Additionally, a PDX approach is not feasible in a number of patients as their tumors fail to engraft (191). Another issue is the long cultivation time as it takes up to 6 months for a PDX model to establish, which considerably impedes the synchronization of avatar and patient. HNSCC is a distinctly heterogeneous disease, a feature, which might be inadequately emulated in serial passaging if the heterogeneous pattern is not observed in the explanted and passaged tumor slices (192). High costs and high personnel expenditure also hinder the widespread use of PDX models (193). Moreover, the engraftment of HPV-driven HNSCC compared with HPV non-driven HNSCC appears to be complicated as reported in various studies. In particular, the establishment of HPV-induced HNSCC xenografts still encounters substantial technical challenges as summarized by Facompre *et al* (194). Altogether, the study of immunomodulatory effects of new anticancer drugs such as CPI in immunodeficient mice might have led to an inadequate prediction of clinical outcome in patients (132,170).

Generation of humanized mice for modeling immunotherapy. Humanized mouse models have recently been presented as an option for developing and testing immunotherapeutic regimens. Following whole body gamma irradiation, human PBMCs are engrafted into immune-deficient mice, followed by transplantation of human tumor tissue or cell lines. This so-called immune-avatar model is limited to a rather short lifespan, for instance compared with the implantation of hematopoietic stem cells (HSCs) and is reduced to 4-8 weeks to investigate immunotherapeutic effects (195). Furthermore, there is a constant risk of graft vs. host reactions in the presence of T cells (196). Mosier *et al* (195) described the so-called SCID-hu-peripheral blood leukocytes (PBL) model as a first attempt of a human-mouse model system in 1988. They set up a xenograft model where PBMC were i.v. injected into SCID mice in order to stably reconstitute a functional human immune system inside the mice. Immune responses were represented by spontaneous secretion of human immunoglobulin and through detection of human immune cells in the murine blood. As a side effect, mice rapidly developed EBV-positive B-cell neoplasms after engraftment with EBV-positive PBL. Eventually, the human immune cells were eliminated and supplanted by the murine innate immune system (195). The model was refined by various approaches, such as transplantation of combined human fetal thymus/liver and CD34+ cells (197) in order to represent the immune system at an advanced setting. Eventually, the high complexity of the models left them unsuitable for high-throughput assays. Hidalgo *et al* (198) suggested a so-called co-clinical trial concept where the avatar model is established from a patient enrolled in a clinical trial and treated synchronously to mimic drug sensitivity and clinical response. However, so far there are no reports about a successful synchronization. Remaining issues are on the one hand the time gap between engraftment of patient tumors in mice and the patient treatment schedule and lack of sufficient amounts of tumor tissue and low tumor take rates on the other hand. Additionally study designs and protocols need to be aligned and standardized (198).

Immune-PDX models. In the immune-PDX (iPDX) model severely immunodeficient mice are engrafted with patient-derived tumor slices after 'humanizing' the immune system of the mice. Alternatively, CD34+ human hematopoietic stem and progenitor cells (HSPCs) are used which can be obtained from different sources, including umbilical cord blood, bone marrow, fetal liver and peripheral blood, for the establishment of so-called human hemato-lymphoid mouse models (148,170,199). In an HNSCC engrafted model into HSC-NOG-hIL-6 Tg mice, human TAMs could be found that expressed CD163 as a marker of immunoregulatory myeloid cells and produced immunosuppressive molecules (200). iPDX models are thought to provide a platform to analyze tumor growth in SCID mice in a similar situation as they grow in patients. They allow the investigation of human immune responses by analyzing TILs, cytokines and antibodies (192). Prior to the first passaging, the human TME is still present so iPDX provides access to the analysis of tumor-stroma/immune interactions in the first engraftment. During consecutive passages, however, human stroma is replaced by murine stroma. In this model, human TILs are available in the TME and are suitable to be targeted with mAbs administered systemically to the mice (170). However, the xenografted tumor constructs take up to at least 1-2 months

Table II. Animal models for immunotherapy.

Author, year	Tumor derivation	Immunity	HNSCC	(Refs.)
Li *et al*, 2014	CDX	Immunocompromised	Yes	(152)
Bais *et al*, 2015			Yes	(154)
Brand *et al*, 2017			Yes	(155)
Brand *et al*, 2018			Yes	(153)
Garrido-Laguna *et al*, 2011	PDX		No (pancreatic)	(191)
Keysar *et al*, 2013			Yes	(190)
Facompre *et al*, 2017			Yes	(194)
O'Malley *et al*, 1997	Syngeneic	Immunocompetent	Yes	(165)
Vahle *et al*, 2012			Yes	(167)
Wang *et al*, 2019			Yes	(163)
Jiao *et al*, 2020			No (melanoma)	(159)
Kim *et al*, 2019			No (glioblastoma)	(160)
Eveson and MacDonald, 1981	Chemically-induced		Yes (lingual carcinoma production)	(186)
Matthews *et al*, 1986			Yes (oral)	(223)
Ghiabi *et al*, 1992			(Hamster cheek pouch)	(182)
Thomas *et al*, 1995			Yes (oral)	(224)
Nauta *et al*, 1995			Yes (oral)	(180)
Aromando *et al*, 2008			(Hamster cheek pouch)	(181)
Liu *et al*, 2012			Yes (oral)	(185)
Bürtin *et al*, 2020			No (colon)	(188)
Lute *et al*, 2005	GEMM		No (colon)	(169)
Ren *et al*, 2017			No (ALL, prostate)	(173)
Cyranoski, 2016			No (lung)	(174)
Mosier *et al*, 1988	Humanized immune avatar		Yes	(195)
Hidalgo *et al*, 2014			Yes	(198)
Matsumura *et al*, 2003	Humanized hemato-lymphoid		No (HSCs)	(199)
Hanazawa *et al*, 2018			Yes	(200)
Morton *et al*, 2016	Humanized immune PDX		Yes	(203)

HNSCC, Head and neck squamous cell carcinoma; CDX, cancer cell line-derived xenografts; PDX, patient-derived xenograft; GEMM, genetically engineered mouse models.

to start growing (201). Restrictions in patient tumor material is another issue as only very few animals can be transplanted per tumor sample. This is particularly true for small head and neck cancer specimens. These aspects limit the broader application of iPDX in the preclinical and clinical routine (170). Occasional approaches for HNSCC iPDX models have been proposed (202) where a correlation between immune therapy effects and HLA matching in preclinical models is described. It remains doubtful to what extent a reproduced human immune system in murine adequately resembles the human immune system. The novel technique of engineering so-called Xact mice has been proposed by Morton *et al* (203). Thereby HSPCs reconstitute the bone marrow from NSG mice that has been previously suppressed by irradiation. Patient tumor tissue is then engrafted into the mice and human HSPCs now form immune cells that grow into the xenograft and recreate the natural TME. Consecutively the expression patterns of epithelial, stromal and immunological genes in Xact mice tumors match the patient's tumor to a greater extent than tumors from non-humanized mice. This model is, however, limited by the fact that there are still murine stromal cells present and innate immune cells reside. Other issues are linked to the interaction of human with murine components, lack of HLA matching and heterotopic engraftment (156). Animal models discussed in the present review are presented in tabular form (Table II).

The chick chorioallantoic membrane (CAM) assay in the preclinical evaluation of immunotherapy. As an intermediate step between *in vitro* and *in vivo* platforms, the CAM assay has established itself as a fast, easy-to-use, cost and time efficient model. Indications are to study tumor-promoting processes, including angiogenesis, invasion, cancer progression and

metastasis, as well as for drug screening, therapy optimization and biomarker discovery. To a certain extent, CAM may serve as an alternative to *in vivo* PDX models. Therefore, CAM-PDX models have gained increased importance as a valuable platform in precision medicine (204). As an embryonic immune system is gradually developed, it could possibly provide a basis for addressing immune-oncological questions. Apart from that, due to the immunodeficiency of the chick embryo up to embryonation day 18, implantation of various cell types is feasible with only a minor risk of rejecting the engrafted material. Moreover, CAM models are in agreement with the 3R (replacement, reduction and refinement) principles, as the chicken embryo does not develop pain perception before the 17th day of incubation (205). As cytokines are secreted early, there is evidence that the model can be used for inflammation studies (206) and for immune-based questions as the chicken embryo manages to yield strong immune reactions (206,207). Notably, the CAM model is of special interest as there is a developmental dichotomy between cell-mediated (thymus-dependent) and humorally mediated (bursa-dependent) immune functions (208). So far the CAM assay has been used for investigation of perineural invasion in HNSCC (209). De Medeiros *et al* (210) demonstrate by the use of CAM assays that galanin secreted by HNSCC cells exhibits immune-suppressive and pro-tumoral effects. However, there are certain limitations of the CAM model. Advantages and disadvantages have been extensively summarized by two reviews (204,206). To sum up, a general overview of immune system development *in ovo* to gain closer insights into the suitability of the CAM assays as an interesting system to approach immune-based issues is still missing

High-throughput alternatives to animals to assess immune CPI. Against the background of questions concerning animal welfare or animal ethics, and in light of limitations and inaccuracies of murine models for immunotherapeutic options, there have been several new approaches published to comply with the 3R principle.

Combination of biomaterials as scaffolds to establish 3D tumor models and immunotherapy. As noted (150), various forms of biomaterials are already available serving as a scaffold for 3D cell culture to improve cellular functions (211-213). Recently there have been advances made in the delivery strategies of immunotherapies based on localized biomaterials, focusing on implantable and injectable biomaterial scaffolds. Injectable biomaterial scaffolds are transformable gel-like biomaterials. The tumor location or resection site can be injected to provoke systemic or local antitumor immune responses (214). Implantable biomaterials are based on the idea that the introduction of tumor antigens to DCs has shown efficiency in vaccine and immunotherapy. Accordingly, a 3D scaffold was developed by cross-linking collagen and hyaluronic acid to deliver gemcitabine and the immunostimulant poly(I:C). Following stimulation of Toll-like receptor 3 (TLR3) in DCs and macrophages, an intense immune response results (215). It appears essential to not only enhance immune activation mechanisms, as it has been in focus during the last decades, but also to explore processes that restore tumor-induced immune deficiency selectively in the TME (216). Sanmamed and Chen (216) point out that it may be reasonable to subdivide patients into certain subgroups according to their individual antitumor immune defect (lack of TILs; overreaction of TILs; dysfunction of TILs). Although this tumor immunity in the microenvironment (TIME) classification system is still at a preliminary stage, it might be a good alternative to using the same inefficient and costly treatment routine for all. Possibly 3D constructs based on biomaterials will help to understand specific deficiencies in tumor immunity to be able to develop strategies to 'normalize' them. Altogether, it is likely that combining biomaterials and immunotherapy will help overcome current deficiencies in patient stratification and application of this significant treatment pillar.

Bioprinting in immunotherapy. Bioprinting is emerging as a promising tool for creating 3D human cancer models that recapitulate critical features of the TME architecture in an advanced manner. It is a promising novel technique to more closely represent the TME, compared with current methods (217). 3D bioprinting enables the spatially defined placement of cells in a 3D microenvironment in order to create viable 3D constructs. Microfluidic platforms have already proven beneficial for representing spatial compartmentalization and migration of immune cells. Despite bioprinting becoming applied more and more, there are few approaches in oncology at present (218,219). As comprehensively summarized in a recent review, by using bioprinting protocols the TME could already be adequately and dynamically mimicked by microfluidic channels or by channels printed using sacrificial ink. Perfusing cells through channels allowed detecting migration properties or immune cells including PBMC, DCs, macrophages and cytotoxic CD8+ T cells, which have been shown to exert prognostic relevance in cancer (217). Drawbacks such as the secretion of matrix metalloproteinases causing degradation of the hydrogels limit the applicability of the techniques as certain features of immune cells, including long-term viability, is affected and highlights the need for a straight revision of current protocols. Swaminathan and Clyne (220) recently published a first description of bioprinting breast spheroids in their 3D architecture for co-culture with endothelial cells also in their 3D architecture. Despite limitations, such as migration tendency of cells out of the spheroids, the technique is anticipated as a powerful tool for precision medicine through testing of drug efficacy in a patient-specific model. Browning *et al* developed a 3D bioprinted skin model of cutaneous squamous cell carcinomas together with a microscopy assay allowing the testing of the efficacy and general toxicity of chemotherapeutics (221). Almela *et al* (222) established a 3D printed bone-mimicking scaffold, which was used to investigate the bone invasion of oral squamous cancer cells to develop a cancerous bone oral mucosal model. So far, a protocol to create a bioprinted head and neck tumor model has yet to be published. Unpublished data form our group already indicate a great potential of this technology to develop various naturalistic head and neck tumor models (Table I).

4. Conclusions

In the course of optimizing the response rates of HNSCC to immunotherapy reliable models that are predictive of clinical

efficacy remain few. At present, no description of a successful translation of drug sensitivity assays or predictive models into the clinical routine has been released for HNSCC. Certainly, none of the markers reviewed in the present review is adequate to sufficiently give overall predictive data on the response to CPI so far. It appears essential to establish predictive 3D tumor models to recapitulate the heterogeneity of clinical HNSCC and especially to assess the interactions between tumor and stroma and immune cells. In the context of optimizing immunotherapeutic response rates in HNSCC, human immune components need to be included to qualify the respective model to evaluate novel immunotherapeutic compounds as well as additional new drugs to be combined with CPI. Various approaches have already been developed and are discussed in the present review. So far, however, there are only anecdotal reports of accurate response prognostication. The aims of novel 3D models studying immunotherapeutic response are to unveil the underlying mechanisms of therapeutic resistance in order to develop strategies for circumvention. It has been hypothesized that combinatorial regimens of CPI and additional inhibitors might be advantageous. However, there are still no standardized tumor models available that allow the prediction of the efficacy of these combinations and the testing of the individual sensitivity of the tumor to be able to select from an abundance of therapeutic agents. Having identified a suitable therapeutic approach, the tumor should ideally be constantly monitored to discover the outgrowth of resistant clones as early as possible. The facilitation of a long overdue comparison between the clinical response of the original tumor and the model will pave the way for co-clinical trials. Unfortunately, short cultivation times of 3D HNSCC enabling the analysis of long-term effects limits their widespread use in precision medicine. Extension of the cultivation time of *ex vivo* cultures is therefore one of the most important directions. One major issue is the performance of clinical validation studies. The ambitious aim is to account for the guidance of patients' therapy using the response of *ex vivo* models in the frame of adaptive clinical trials or to conduct co-clinical trials that parallel ongoing human phase I/II clinical trials. Parallelization of pre-clinical and clinical trials will become of paramount importance in future decades. It is envisioned that eventually bioprinted or scaffold-based multicellular 3D cancer models could be applied 'from bench to bedside' to tumor and stroma material cells from patient tumor biopsies.

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Availability of data and materials

Data sharing is not applicable to this article, as no data sets were generated or analyzed during the current study.

Authors' contributions

All authors were involved in the conception of the study. AA, CS, JK and AL were involved in the literature search. AA, JK, CS and AL were involved in the writing and preparation of the original draft of the manuscript. All authors were involved in the writing and reviewing of the article. NR and KB supervised the study. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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