2nd International Symposium
on Tumor-Host Interaction in Head and Neck Cancer

in conjunction with the

3rd International Symposium on HPV Infection in Head and Neck Cancer

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**Invited lecture | Investigation of predictive biomarkers for response to PD1 checkpoint inhibitors**

A. Psyrri (Athens/GR)

**Background:**
Successful application of programmed death 1 (PD1) checkpoint inhibitors in the clinic may ultimately benefit from appropriate patient selection based upon predictive biomarkers. Molecular characterization of circulating tumor cells (CTC) is crucial for the investigation of molecular-targeted therapies while predictive biomarkers for response to PD1 checkpoint inhibitors are lacking. We sought to assess whether overexpression of PD-L1 in CTCs could be detected at baseline and at different timepoints during treatment in a prospective cohort of head and neck squamous cell carcinoma (HNSCC) patients and used to predict clinical outcome after treatment with curative intent.

**Patients and methods:**
We developed a highly sensitive, specific and robust RT-qPCR assay for PD-L1 mRNA expression in EpCAM(+) CTCs. In a prospective cohort of 113 locally advanced HNSCC patients treated with curative intent we evaluated PD-L1 expression in the EpCAM(+) CTC fraction at baseline, after 2 cycles of induction chemotherapy (week 6) and at the end of concurrent chemoradiotherapy (week 15).

**Results:**
PD-L1 overexpression was found in 24/94 (25.5%) patients at baseline, 8/34 (23.5%) after induction chemotherapy and 12/54 (22.2%) patients at the end of treatment. Patients with CTCs overexpressing PD-L1 at end of treatment had shorter progression-free survival (P = 0.001) and overall survival (P < 0.001). Multivariate analysis revealed that PD-L1 overexpression at end of treatment was independent prognostic factor for progression-free survival and overall survival. The absence of PD-L1 overexpression at the end of treatment was strongly associated with complete response with an odds ratio = 16.00 (95% CI = 2.76-92.72, P = 0.002).

**Conclusions:**
We demonstrate that detection of CTCs overexpressing PD-L1 is feasible and may provide important prognostic information in HNSCC. Our results suggest that adjuvant PD1 inhibitors deserve evaluation in HNSCC patients in whom PD-L1(+) CTCs are detected at the end of curative treatment.
2 Invited lecture | Neutrophils and MDSC mediate progression of head and neck cancer

S. Brandau (Essen/DE)
The NLRP3 inflammasome regulates neutrophil trafficking to HNSCC

L. Mittmann, J. Schaubaecher, K. Lauber, F. Krombach, B. Uhl, C. Reichel (Munich/DE)

There is emerging evidence that neutrophils substantially contribute to the pathogenesis of head and neck squamous cell carcinoma (HNSCC). The underlying mechanisms, however, remain poorly understood. Recently, the NLRP3 inflammasome has been identified to play a key role for the initiation of inflammatory processes. We therefore hypothesized that this intracellular protein complex is vital for neutrophil trafficking to HNSCC. Employing multi-channel in vivo microscopy, we found that neutrophil responses in squamous cell carcinoma (cell line SCC VII) implanted into the ear of C3H mice are almost completely abolished upon inhibition of NLRP3 inflammasome formation. Multi-channel flow cytometry analyses further revealed that neutrophils recruited by NLRP3 inflammasome activation (elicited by alum crystals) exhibit a pro-tumorigenic “N2” phenotype. Mechanistically, confocal laser scanning microscopy on tissue whole mounts demonstrated that formation of the NLRP3 inflammasome initiates the expression of ICAM-1/CD54 on the microvascular endothelium. Importantly, single cell multi-channel flow cytometry analyses indicated that NLRP3 stimulation does not directly activate neutrophils or endothelial cells, but potently induces synthesis of pro-inflammatory cytokines in macrophages that, in turn, activate endothelial cells. Thus, our experimental data suggest that the NLRP3 inflammasome critically shapes the inflammatory milieu in the tumor environment, thereby controlling neutrophil trafficking to HNSCC.

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4 Correlation of altered HLA class I component expression with immune cell infiltration in oral squamous cell carcinoma

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It has recently become evident that the progression of oral squamous cell carcinoma (OSCC) is associated with an evasion of tumor cells from the host immune surveillance. This finding is accompanied by a worse outcome of patients and might influence the efficacy of immunotherapies. Since little information exist about the molecular mechanisms leading to tumor immune evasion and its correlation with the immune cell contexture the expression of HLA class I antigens and components of the antigen processing machinery (APM) was analyzed in 4 OSCC cell lines and a large panel of human papilloma virus (HPV)-negative OSCC lesions and correlated to the immune cell infiltration and clinical parameters. Immunohistochemical analyses of 151 HPV-negative OSCC lesions revealed high cytoplasmic expression levels of (i) MHC class I components (β2-microglobulin, MHC class I heavy chain) as well as (ii) the chaperones calnexin and calreticulin, (iii) a low cytoplasmic expression of the peptide transporter associated with antigen processing (TAP)1 and (iv) a high nuclear expression of LMP2, which correlated with a poor overall survival (OS) of OSCC patients. Furthermore, a heterogeneous, but predominantly diminished expression of MHC class I APM components was found in the HNSCC cell lines analyzed, which was accompanied by a reduced HLA class I surface expression. With the exception of calnexin and calreticulin the impaired expression of the other APM component analyzed was reversed by treatment with IFN-γ, which resulted in an enhanced MHC class I surface expression. In order to determine whether the increased APM component expression was due to IFN-γ produced by CD8+ cytotoxic T lymphocytes (CTL) the APM expression levels of OSCC lesions were compared to the frequency and composition of immune cell infiltration. These data demonstrated differences between HLA class I and APM positive and negative tumors, related to the immune cell infiltration of the tumor microenvironment and OSCC patient’s prognosis. This knowledge might help to overcome immune escape and to improve the efficacy of immunotherapeutic strategies for HNSCC patients.
5 Immunological characteristics of circulating monocyte subsets in patients with squamous cell carcinoma of the head and neck

K. Sakakura, H. Takahashi, S.-I. Motegi, Y. Yokobori-Kuwabara, T. Oyama, K. Chikamatsu (Gunma/JP)

Introduction
Monocytes in circulation are classified into three subsets according to CD14 and CD16 expressions: classical, intermediate and non-classical monocytes. However, the characteristics of these subsets in cancer patients are still unclear.

Objectives
Here we aim to elucidate trends of the 3 subsets in patients with squamous cell carcinoma of the head and neck (SCCHN).

Materials and Methods
Peripheral bloods from SCCHN patients and age-matched normal donors (ND) were collected. Expressions of various HLA molecules, markers and proteins in each monocyte subset were tested by flow cytometry. Primary tumor samples were stained immunohistochemically with HLA-G MFG-E8 and CD34. Correlations to clinicopathological parameters and survivals were analyzed. Monocyte from ND was cocultured with SCCHN cell lines ex vivo for 3 days and tested by flow cytometry.

Results
Total 54 patients and 24 ND were enrolled to this study. In SCCHN patients, ratio of classical monocyte was increased, and mature intermediate and non-classical monocyte were decreased. HLA-G and PD-L1 expressions in intermediate monocyte from SCCHN patients were significantly higher than those from ND. The cases with fewer intermediate/nonclassical monocyte, lower expression of HLA-DR or CX3CR1 showed more frequent recurrence and death. Significant shorter disease free and overall survival were found in patients with lower CX3CR1, CD68 or CD163 (maturation markers) in intermediate/nonclassical monocytes. In ex vivo analyses, monocytes cultured with cancer cells also expressed lower HLAs and maturation markers.

Conclusion
Monocyte subsets in SCCHN patients were biased toward immature. Moreover, cases with monocyte in more immature feature were significantly associated with worse prognosis. Our study suggests that tendency of mature "patrolling" monocyte closely relates to immune surveillance and prognosis in patients with SCCHN.
6 Invited Lecture | HNC in the emerging era of immunotherapy

V. Grünwald (Hanover/DE)

Single agent immune checkpoint inhibition has become a clinical reality in the treatment algorithm of relapsed/metastatic (RM) squamous cell head and neck carcinomas (SCCHN). Based on its favorable risk profile and the survival benefit has led to an early adoption of this novel type in RM-SCCHN after platinum-failure. The novel mechanism of action and the perspective to derive long-term clinical benefit or cure drives the clinical development. Currently, only a limited number of patients derive benefit from single agent checkpoint inhibition and current clinical research focuses on the development of combinational strategies, which may boost anti-tumor activity. Given the tremendous costs associated with such approaches it becomes evident that therapy has to be tailored according to the individual disease of a patient, implying a proper patient selection. Hence, predictive biomarkers and rational combinations are key areas of interest and drive its current clinical development.
7 Invited Lecture | Enlisting myeloid effectors cells in the fight against cancer

M. van Egmond (Amsterdam/NL)
Toll-like receptor agonists improve cetuximab-mediated immunotherapy of head and neck cancer

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C. R. Leemans, M. van Egmond, R. H. Brakenhoff (Amsterdam/NL)

**Background**
Efficacy of immunotherapies are hampered in many cancer patients by a key barrier, the immune suppressive tumor micro-environment, that lowers induction of adequate anti-tumor responses via patients’ own immune cells. This may underlie the low success rate of immunotherapy with cetuximab in patients with HNSCC. This therapeutic antibody can directly block epidermal growth factor induced tumor outgrowth. However, antibodies can also bind Fcg-receptors (FcgRs) on immune cells and as such immune cells bound to tumor cells become activated and execute their tumoricidal functions. However, the immune suppressive tumor micro-environment lowers these anti-tumor immune responses. Thus, immune stimulatory agents that overcome this immune suppression would augment efficacy of immunotherapies.

Therefore, our aim is to investigate whether agonists for the inflammatory Toll-like receptors (TLRs) combined with cetuximab treatment overrules the immune suppressive environment and improves tumor elimination.

**Methods**
We performed immune cell mediated cytotoxicity assays with HNSCC tumor cell lines with or without TLR agonists and analysed tumor elimination and changes in cytokine and chemokine profiles. Also, efficacy of cetuximab with and without the addition of TLR agonists was tested in tumor bearing mice.

**Results**
Cetuximab in combination with TLR agonists improves tumoricidal functions of immune effector cells. This improved capacity of immune cells to eliminate tumor cells is linked with induction of a pro-inflammatory cytokine and chemokine profiles. Importantly, tumor-bearing mice treated with cetuximab and TLR agonists displayed most regression in tumor outgrowth and improved overall survival.

**Conclusion**
Based on our results we anticipate that combining tumor targeting antibody-based immunotherapy with TLR agonists represents a novel treatment strategy that may improve efficacy of current cetuximab treatment of patients with HNSCC.
9 Tumor-derived exosomes do not disturb a novel EBV peptide-based immunotherapy for nasopharyngeal carcinoma

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Background
Epstein-Barr virus (EBV) is constantly present in nasopharyngeal carcinoma (NPC). Despite the abundant presence of immunosuppressive NPC exosomes, we propose a peptide immunotherapy able to induce a CD4+ Th1-response specific of EBV antigens.

Methods
Human EBV-specific CD4+ T c.l. were generated from autologous CD4+ T lymphocytes. The function of both cells was evaluated with NPC cell- and patient-derived exosomes. Immunotherapy was assessed in humanized NPC xenotransplanted SCID mice. NPC Patients’ PBMC were challenged with EBV peptides.

Results
EBV peptides stimulation induced PBMC proliferation and IFNγ secretion after pre-incubation with NPC cells. EBV-specific CD4+ T c.l. induced lysis of NPC c.l., even in the presence of autologous exosomes. EBV peptides restrained in vivo tumor growth. All NPC patients’ PBMC recognized the EBV peptides, eliciting a strong Th1 response.

Conclusion
EBV peptides could be used as tumor vaccine for immunotherapy of nasopharyngeal carcinoma, regardless the immunosuppressive properties of NPC exosomes.
Aberrant promoter methylation of the PD-1/PD-L1/PD-L2 immune checkpoint axis in head and neck squamous cell carcinomas

A. Franzen, F. Bootz, D. Dietrich (Bonn/DE)

Immune checkpoint inhibitors targeting the programmed cell death 1 (PD-1) receptor and its ligands PD-L1 and PD-L2 are currently of great clinical interest. This study aimed to investigate the association of PD-1, PD-L1 and PD-L2 methylation with mRNA expression, protein expression and human papilloma virus (HPV)-status in head and neck squamous cell carcinomas (HNSCCs).

PD-1/ PD-L1 and PD-L2 promoter methylation and its mRNA expression were analyzed using Infinium HumanMethylation450 BeadChip and RNA-Seq (both Illumina, Inc.) technologies in a representative HNSCC patient cohort (n = 528) enrolled by The Cancer Genome Atlas (TCGA) Research Network. Furthermore, a validation cohort consisting of 168 HNSCC patients treated at the University Hospital Bonn was analyzed regarding PD-1/PD-L1 and PD-L2 promoter methylation. PD-L1 protein expression in the validation cohort was quantified via immunohistochemistry (PD-L1 antibody clone 22C3, Dako/ Agilent Technologies, Inc.).

PD-1 hypermethylation was associated with a shorter overall survival after surgical resection in both the TCGA (HR = 2.24 [95%CI: 1.08-4.64], p = 0.029) and the validation cohort (HR = 1.54 [95%CI: 1.08-2.21], p = 0.017). DNA methylation of PD-L1 and PD-L2 correlated inversely with mRNA expression (PD-L1: Spearman’s ρ = -0.444, p ≤ 0.002; PD-L2: ρ = -0.15 p ≤ 0.014). Methylation of PD-1, PD-L1 as well as PD-L2 were further significantly associated with HPV-status in the TCGA cohort. In the validation cohort, PD-L1 protein expression was associated with PD-L1 hypomethylation (p = 0.012).

PD-1 methylation as a prognostic biomarker might aid the identification of HNSCC patients potentially benefitting from a radical or alternative treatment, particularly in the context of immunotherapies targeting PD-1/PD-L1. Additional studies are warranted to test PD-1, PD-L1 and PD-L2 methylation as predictive biomarkers for response to immunotherapies that target the PD-1/PD-L1/PD-L2 immune checkpoint axis.
11 Invited Lecture | Molecular Biology of HPV-induced HNC – recent findings and implications

G. Wichmann (Leipzig/DE)

The infection of epithelial cells lining the upper aerodigestive tract and in particular epithelial cells in the crypts of the palatine and lingual tonsils with oncogenic subtypes of the human papillomavirus (HPV) can essentially contribute to neoplastic transformation and development of head and neck squamous cell carcinoma (HNSCC). The deviating molecular biology and the lower mutational load of this distinct HNSCC entity together with differences regarding immunologic features are linked to a superior outcome in the majority of HPV-related HNSCC. However, HPV-related HNSCC are characterized by lower genetic instability and fewer mutations together with different DNA methylation and gene expression patterns are associated not only with the replication of the virus but also activation of certain signaling pathways and the suppression of cell cycle regulators hence leading to increased proliferation. The presentation besides detection of HPV and its surrogate p16INK4A focusses on newly emerging biomarkers for HPV-driven HNSCC including diagnostic, prognostic and predictive biomarkers derived from the tumor itself and in body fluids.
12 Invited Lecture | HPV carcinogenesis in the upper aerodigestive tract

P. Snijders (Amsterdam/NL)
13 Upregulation of Aldo-Keto-Reductase 1C1 and 1C3 is associated with poor prognosis in OPSCC independent of HPV status


Introduction
Different studies have shown that HPV16-positive OPSCC can be subdivided based on integration status (integrated, episomal and mixed forms). Previously, we showed that integration did neither affect the levels of viral genes, nor those of virally disrupted human genes (Olthof et al., PlosOne 2014).

Objectives. To perform a genome-wide screen to identify human genes which expression is influenced by viral integration and to determine their clinical relevance.

Materials and Methods
Total RNA was collected from 33 fresh-frozen HPV-16 positive OPSCC samples (9 integrated, 4 mixed, 20 episomal), and analyzed by mRNA expression profiling using Agilent Whole Human Genome 4644K Microarrays. Non-hierarchical clustering and pathway analysis was carried out to identify genes of interest. Aldo-keto-reductases 1C1 and 1C3 (AKR1C1, AKR1C3) expression was confirmed by RT-qPCR and Immunohistochemistry. Additionally, 141 OPSCC, including 50 HPV-positive cases, were used to validate gene expression by immunohistochemistry. Results were correlated with clinical and histopathological data.

Results
Non-hierarchical clustering resulted in two main groups of mRNA expression patterns corresponding to OPSCC with either exclusively integrated or episomal viral DNA. Several deregulated cellular pathways were identified, in which AKR1C1 and AKR1C3 were predominantly involved. Upregulation of gene expression was observed in OPSCC with exclusively integrated viral DNA. Survival analysis of 141 additionally immunostained OPSCC showed unfavorable survival rates for those patients with tumors exhibiting upregulation of AKR1C1 or AKR1C3 (both p < 0.0001) both in HPV-positive (p ≤ 0.001) and –negative (p ≤ 0.017) tumors.

Conclusion
Our findings show that OPSCC with integrated HPV16 show upregulation of AKR1C1 and AKR1C3 expression, which very strongly correlates with worse survival rates. Moreover, also in HPV-negative tumors, upregulation of these proteins correlates with poor outcome. This is in agreement with data from other tumors, making these genes promising candidates as indicators of prognosis. In addition, the availability of inhibitors of these gene products may be utilized for drug treatment.
Mass spectrometric comparison of HPV+ and HPV- HNSCC tumors and cell lines

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M. Wurlitzer, A. Münscher (Hamburg/DE)

Experimental setup
To identify protein expression differences between HPV+ and HPV- HNSCC in an open, unbiased approach we conducted a mass spectrometric comparison of a panel of 10 HPV+ and 10 HPV- OPSCC with similar characteristics as well as a comparison of 8 HPV+ and 8 HPV- HNSCC cell lines. In the case of tumor tissues formalin-fixed paraffin-embedded samples were cut in 6µm slices followed by macrodissection of tumor areas and deparaffinization and trypsination prior to mass spectrometric analysis.

Results
We identified a total of 2051 proteins from tumor tissues and a total of 3262 proteins from HNSCC cell lines. As expected, the HPV surrogate marker p16 was identified to be expressed solely (tumors) or at a far higher level (cell lines) in HPV+ samples. A random forest analysis identified 24 proteins to be differentially expressed in tumor tissues with half of these proteins belonging to 3 functional groups: 1) Cytoskeletal regulators, 2) Replicative helicases and 3) Proteins of the nuclear envelope and lamina. Furthermore, a component of various chromatin editing complexes and a proposed radiosensitivity factor was expressed at a higher level in HPV+ tumors. Of note, only 2 of the 24 differentially expressed proteins were expressed at a higher level in HPV- tumors, among them a proposed stem cell factor with negative prognostic value in HNSCC. Preliminary analyses of the cell line comparison confirm some of the results obtained with tumor samples but differed in others (e.g. no difference in replicative helicases).

Conclusions
We identified differences between HPV+ and HPV- HNSCC, some of which will be further investigated for their mechanistic role and translational relevance as prognostic/predictive biomarkers, using in vitro studies and TMA analyses. The feasibility of cell culture models and the questions why and in which aspects they do or do not reflect tumor behavior and characteristics will also be further investigated.
**Session 4 – Host-directed mechanisms of therapy**

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**15  Synchronous, bilateral tonsillar carcinomas – patient characteristics and human papillomavirus genotypes**

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  C. von Buchwald (Copenhagen/DK)

**Introduction**

The incidence of oropharyngeal squamous cell carcinoma (OPSCC) is increasing, but data on the incidence of synchronous, bilateral tonsillar squamous cell carcinomas (BiTSCCs) is sparse. In this study, we report the incidence and tumour characteristics of BiTSCCs in a population-based, consecutive cohort of OPSCCs.

**Methods**

We identified all patients diagnosed with tonsillar squamous cell carcinoma (TSCC) in eastern Denmark during a 15-year period to detect the incidence of synchronous BiTSCCs. The tumours were assessed for p16\(^{INK4a}\) expression, the presence of HPV DNA and HPV genotypes. Furthermore, we systematically reviewed the literature examining BiTSCCs.

**Results**

Of the total of 1119 TSCCs diagnosed in eastern Denmark from 2000 to 2014, we identified 12 BiTSCCs, nine of which initially presented as a cancer of unknown primary (CUP) in the neck. Nine cases were bilaterally HPV16 positive (HPV16+), while two cases were HPV16+ in one tonsil and respectively, HPV33 and HPV35 positive in the contralateral tonsil. One case was bilaterally HPV-negative. We also identified an increase in the incidence of BiTSCCs after 2012 when histological examination of the entire tonsil tissue became routine, suggesting that BiTSCCs might be underdiagnosed. In the literature, we identified 15 studies from six countries, encompassing 25 cases in total.

**Conclusions**

BiTSCCs were primarily HPV16+ and were most often diagnosed as part of the diagnostic work-up for CUP. We found an incidence of 9% BiTSCCs in patients with TSCC after 2012 and we therefore recommend focusing on putative BiTSCC with total embedding and histological examination of tonsils harvested by bilateral tonsillectomies.
16 Invited lecture | TGF-beta at the intersection of immunotherapy and radiotherapy in HNSCC

M. H. Barcellos-Hoff (San Francisco, CA/US)

Introduction
The substantially better prognosis of human papilloma virus (HPV) positive head and neck squamous cell carcinoma (HNSCC) in response to treatment suggests that HPV creates an intrinsic molecular vulnerability that is exploited by standard of care therapy. Transforming growth factor beta (TGFβ) is an extracellular cytokine that regulates aspects of proliferation, phenotype and differentiation in all cells, and plays an underappreciated role in regulation of the DNA damage response (1). As a consequence, TGFβ inhibition synergizes with radiation treatment to improve tumor control in preclinical models of breast, brain and lung cancer (2-4).

Objectives
Here we determined that HPV positive cancers are unresponsive to TGFβ and that loss of TGFβ signaling compromises DNA damage recognition and homologous recombination repair. HPV negative HNSCC cells in which TGFβ signaling is compromised pharmaceutically replicate this defect, which increases sensitivity to radiation, cis-platin, and PARP inhibition.

Conclusion
These studies identify the mechanism by which impaired TGFβ signaling in HPV-positive head and neck cancer alter default settings of DNA damage repair pathways, resulting in increased sensitivity to radiation, cis-platinum and PARP inhibitors. Thus, HPV positive head and neck cancer is an 'experiment of nature' that supports the potential for significant benefit of pharmaceutical inhibition of TGFβ during radiotherapy, but also points to under explored aspects of HPV-initiated cancer.

References
17  Long-term inhibition of EGFR in OSCC cells leads to a partial EMT/stem cell phenotype

A. Berndt (Jena/DE)

Introduction
Oral squamous cell carcinoma (OSCC) is the most frequent head and neck cancer with still poor prognosis and limited therapeutic options. Although OSCC shows an increased EGFR expression, EGFR inhibitor therapies were only partially successful. Cellular processes like epithelial mesenchymal transition (EMT) and human cancer stem cells (HCS) progression may promote resistance development. Until now, the mechanisms behind are not fully understood.

Objectives
Therefore, a Gefitinib resistant cell line was established and characterized as a model of EGFR inhibitor induced phenotype changes in OSCC.

Methods
The cell line UPCI-SCC-026 was used. To induce treatment derived phenotype changes, the cell line was cultivated under Gefitinib supplementation (5μM) for more than 12 months (SCC026Gef). To detect differences in Gefitinib sensitivity, phenotype and behavior, cell invasion / migration assays, xCELLigence® RTCA proliferation assay and flow cytometry were performed. Protein and RNA expression of EMT and HCS markers were detected by immunofluorescence and RT² Profiler PCR Arrays.

Results
In comparison to controls, SCC026Gef cells showed a degreased sensitivity to Gefitinib and developed a spindle shaped and scattered growth pattern, increased migration and invasion, and reduced proliferation. Immunofluorescence and mRNA expression analyses revealed an EGFR upregulation, only minimal down-regulation of E-Cadherin, increased expression of collagen 3α1, fibronectin, and cytokeratin 14. Additionally, several HCS markers were up-regulated: AXL, DLL1, FLOT2, KIT, KIT ligand, NOS2.

Conclusions
We provide an in vitro model of therapy induced selection of resistant cell clones in OSCC. The long-term EGFR inhibitor treatment of OSCC cells leads to the development of drug resistance associated with the development of a partial EMT / HCS phenotype. The tumor biological significance of this phenotype is the subject of further studies.
AKT and ERK1/2 inhibition can reduce cancer stem-like cells and epithelial-mesenchymal transition induced by multiple radiation in HNSCC

M. Buchberger, D. Schüttler, G. Piontek, A. Pickhard (Munich/DE)

Introduction
Irradiation is one of the standard therapies in HNSCC and has been linked to an enhanced tumor migration. We previously reported that this increased tumor migration upon irradiation is dependent on GSK3β inactivation and can be reversed by inhibition of either AKT, ERK1/2 or p38 MAPK signaling. Most experimental studies investigate effects induced by single dose irradiation.

Method and Material
In this study, we tried to discover the effects promoted by a multiple radiation dose of 2 Gy on 5 consecutive days regarding cell proliferation, migration, invasion and cell signaling. This irradiation regime we used is clinically more realistic and the purpose was to find possible links to therapy problems like local and distant recurrences which occur around 50 % following radiotherapy.

Results
We detected that multiple irradiated cells in HNSCC express features which are linked to epithelial-mesenchymal-transition (EMT) in Western Blot analysis and in spheroids. Furthermore, they show a significantly increased behavior of invasion and express markers which are linked to cancer stem-like cells. Inhibition of AKT or ERK1/2 signaling reverses EMT and cancer stem like markers under multiple irradiation in HNSCC.

Discussion
These findings could be of use in the development of new treatments to prevent local or distant recurrences in the future.
Dual inhibition of PARP1 and the intra-S/G2 cell cycle checkpoints as a novel strategy for highly effective radiosensitization of HPV+ HNSCC

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Background
Enhanced radiation sensitivity of HPV+ HNSCC is also observed on cellular level when comparing HPV+ and HPV-HNSCC cell lines. We could show that the underlying mechanism is a defect in DNA double-strand break repair associated with a profound and sustained G2-arrest. This defect can be exploited by molecular targeting approaches additionally compromising the DNA damage response of these cells to further enhance their radiation sensitivity. We now tested a novel approach of combined targeting of PARP1 and intra-S/G2 cell cycle checkpoints to achieve highly efficient radiosensitization.

Methods
Mechanistic proof of efficacy of the inhibitors and functional analyses were performed using Western blot, immunofluorescence microscopy, colony formation assay, assessment of cell cycle distribution and flow cytometric assessment of γH2AX. PARP1 was inhibited using olaparib; intra-S/G2 checkpoint inhibition was performed using Wee1-inhibitor AZD1775 or a combination of AZD1775 and Chk1-Inhibitor prexasertib.

Results
Enhancing CDK1/2 activity through AZD1775 resulted in reduced proliferation rates and severe replication stress in HPV+ HNSCC cells. The latter was apparent from an accumulation of cells in the S-phase as well as a strong increase of γH2AX marker especially in S-phase cells. When combined with radiation, both olaparib as well as AZD1775 induced radiosensitization as observed previously. Combined inhibition resulted in a markedly enhanced radiosensitization as compared to single inhibitor usage. An alternative checkpoint inhibition approach using dual Wee1/Chk1-inhibition with severely reduced concentrations to limit toxicity yielded similar results.

Conclusion
Combined inhibition of PARP1 and intra-S/G1 checkpoint is a highly effective approach for radiosensitization of HPV+ HNSCC cells. It may therefore represent a viable alternative for the current standard of concomitant cisplatin-based chemotherapy and may even allow a reduction in radiation dose.
Introduction
Plasma-derived exosomes are emerging as promising non-invasive correlates of cancer progression. In patients with solid tumors or hematological malignancies, plasma exosomes carry a cargo enriched in immunosuppressive proteins. As immune suppression is one of the hallmarks of cancer progression, circulating exosomes rich in inhibitory molecules such as PD-L1 are implicated in mediating systemic immune suppression.

Objectives
The objectives were to determine whether total plasma exosomes in HNSCC patients are PD-L1+ and to correlate PD-L1 exosome levels with clinical endpoints. Also to separate CD3+ from CD3(-) T-cell derived exosomes and correlate their cargo to disease activity.

Materials and Methods
We isolated exosomes from plasma of HNECC patients by size exclusion chromatography. We identified PD-L1high and PD-L1low exosomes by on-bead flow cytometry and co-incubated them with PD-1+ effector T cells. Also by immunocapture, we isolated CD3+ and CD3(-) exosomes from plasma and studied their cargo and immune functions.

Results
PD-L1+ exosomes from plasma of HNSCC patients mediated checkpoint inhibition. The PD-L1 levels on plasma-derived exosomes and suppressive effects of these exosomes on immune cell subsets correlated with the disease activity and tumor stage. CD3+ exosomes were also enriched in immunoinhibitory ligands, and this cargo was associated with the presence of advanced disease, high tumor stage and nodal involvement. CD3(-) exosomes were enriched in the tumor-derived cargo and were also immunoinhibitory.

Conclusions
Our data suggest that plasma-derived exosomes fractionated into tumor-derived and T-cell derived vesicles could be useful in the future as non-invasive biomarkers of disease progression and of immune dysfunction in HNSCC. Upon future validation, plasma-derived exosomes have a potential to cancer serve as non-invasive biomarkers of disease progression and outcome.
Introduction: Cancer-associated fibroblasts (CAF) are a poorly defined cell population that most commonly have a contractile, myofibroblastic phenotype, expressing alpha smooth muscle actin (αSMA) and secreting extracellular matrix. CAF promote many of the ‘hallmarks of malignancy’, which has led to their emergence as potential therapeutic targets.

Objectives: To characterise CAF molecular and functional heterogeneity; to identify the mechanisms regulating CAF differentiation; to develop therapeutic strategies based on CAF targeting.

Materials and Methods: RNA sequencing was used to investigate gene transcription during myofibroblast differentiation and examine heterogeneity in CAF isolated ex vivo. Mechanisms regulating fibroblast-to-myofibroblast transdifferentiation were investigated in vitro using RNA interference/pharmacological inhibitors followed by PCR, Western blotting, immunofluorescence and functional assays. RNA-Sequencing/bioinformatics and immunohistochemistry was used to analyse CAF differentiation and prognostic effects in human tumours. CAF function and targeting strategies were assessed in vivo —using xenograft and isograft tumour HNSCC models.

Results: Myofibroblastic CAF have heterogenous transcriptional profiles, particularly in expression of collagen-related genes; this can be modelled in vitro using different differentiation stimuli. In vivo, CAF promote invasion and metastasis, are associated with ‘immune cold’ tumours and are highly prognostic in human HNSCC. CAF differentiation is regulated by a reactive oxygen species-generating enzyme, NOX4, which strongly correlates with myofibroblastic-CAFs in multiple human cancers. Genetic/pharmacological inhibition of NOX4 reverts the myofibroblastic-CAF phenotype ex-vivo, and prevents myofibroblastic-CAF accumulation in vivo, slowing tumour growth, suppressing metastasis and promoting an intratumoral immune response.

Conclusions: Myofibroblastic CAF are transcriptionally heterogenous, but NOX4 is a common mechanism regulating differentiation. The tumour-promoting properties of CAF can be suppressed by NOX4 inhibition, which may have broad applicability for stromal targeting across cancer types.

References
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22 Microenvironment of head and neck squamous cell cancer as important factor influencing the tumor biology

K. Smetana (Prague/CZ)

Incidence of malignant tumors is increasing worldwide that seems to be associated with ageing of population (Anticancer Res 36: 5009-5018, 2016). This non favorable trend represents the great challenge for future. One of new research visions can be study of tumor microenvironment. Malignant tumors are consisted not only from cancer cells but they also contain many stromal elements (fibroblasts, inflammatory cells…) and their products that influence biological properties of tumor (Int J Mol Sci 16: 24094-24110, 2015). Study of tumor microenvironment needs combination of many methodological approaches such as classical histology, immunocytochemistry, in vitro modeling as well as genomics and proteomics. In this lecture we will summarize our data in comparison with results of other authors concerning the squamous cell cancer of the head and neck. The results demonstrate a remarkable similarity between cancer microenvironment and granulation tissue of the wounds. Molecules such as endogenous lectin-galectin-1, chemokines (IL-8, CXCL-1) and cytokines (IL-6) can be a potential therapeutic targets in future (Biol Cell 104: 738-751, 2012; Int J Cancer 131: 2499-2508, 2012; Anticancer Res. 37: 2275-2288, 2017).
**Introduction**

Prognosis of HNSCC remains poor despite advances in therapeutic modalities. HPV+ HNSCC exhibit a better prognosis than HPV− cancers, which may be due to differences in their aetiology or microenvironment. Leukocytes, such as macrophages and neutrophils, are recruited to HNSCC and their increased numbers are associated with poor prognosis. Mechanisms of how these cells are recruited to HNSCC and if there are differences between HPV+ and HPV− cancers is not known. This *in vitro* study aimed to examine differences in the chemoattractant capacity of HPV+ and HPV− HNSCC.

**Methods**

Levels of gene and protein expression of chemoattractants were measured in HPV+/− HNSCC cell lines and also in tonsillar fibroblasts (TF) stimulated with HNSCC HPV+/− conditioned medium. Gene and protein expression of IL-1R on TF was determined by qPCR and confocal microscopy, respectively. TF were incubated with the specific IL-1R antagonist, anakinra, and then cultured with HNSCC HPV+/− conditioned medium and chemoattractant production measured by ELISA. Levels of leukocyte recruitment to HNSCC HPV+/− conditioned medium were measured in a 3D model.

**Results**

HPV+, HPV− cell lines and TF alone expressed low levels of the chemoattractants analysed. TF cultured with conditioned medium from HPV− cell lines expressed significantly (*p*<0.05) higher levels of leukocyte chemokines (CXCL8, CXCL1, MCP-1, CCL5) and caused more leukocyte recruitment than from HPV+ cell lines. Chemokine secretion was significantly (*p*<0.05) reduced when TF were pre-treated with the IL-1R antagonist anakinra in a dose-dependent manner in both 2D and 3D in vitro models.

**Conclusion**

HPV− HNSCC cells stimulate TF to secrete higher levels of leukocyte chemoattractants and recruit more leukocytes than HPV+ cells, and this process is mediated by activation of IL-1R expressed by TF. This mechanism may explain differences in the amount and types of leukocytes in HPV− compared to HPV+ HNSCC.
Invited Lecture | HPV in non-oropharyngeal head and neck cancer

L. Alemany (Barcelona/ES)
The 8th edition of TNM classification does not correctly predict survival in a German cohort of 415 patients with locally advanced oropharynx cancer

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G. Wichmann (Leipzig/DE)

Background
Optimal prognostic accuracy is mandatory to define the best treatment. The 8th ed. of AJCC/UICC TNM staging (TNM 2017) modifies staging of p16+ oropharyngeal squamous cell carcinomas (OSCC) and neck nodes with extracapsular extension (ECE).

Methods
Prognostic accuracy of TNM 2017 was retrospectively assessed in 415 OSCC patients of stage III-IVB (according to TNM 2010) treated 2007-2016 in our hospital; p16 was evaluated by IHC in 189 (45.5%) OSCC. Tumor-specific (TSS) and overall survival (OS) were analyzed.

Results
According to TNM 2010, 58 (14.0%) OSCC were stage III, 308 (74.2%) IVA, and 49 (11.8%) IVB; 84 (20.2%) were p16+ and 105 (25.3%) p16-. In 383 N+ patients 136 (35.5%) had ECE. TNM 2017 reclassified 30 (7.2%) OSCC as stage I and 26 (6.3%) as II; numbers in III increased to 74 (17.8%), in IVB to 123 (29.6%), whereas 162 (39.0%) remained IVA. Kaplan-Meier curves revealed TNM 2010 as clearly discriminating UICC III, IVA and IVB with 70.7, 68.4 and 21.2 months median OS (p=0.00002); 75% TSS was 48.6, 28.2 and 10.6 months (p=0.0002); median TSS in IVB was 35.2 months. TNM 2017 fails discriminating UICC III, IVA and IVB (68.3, 53.9 and 41.9 months median OS). The 5-year TSS of all was above 50%; their crossing curves showed 75% TSS of 28.2, 17.3 and 29.0 months. Worsened discrimination of survival of III, IVA and IVB is linked to downstaged 23 pN2a-pN3 p16+ OSCC in III and upstaged 84 IVA OSCC in IVB due to ECE (now pN3b). Downstaging of p16+ OSCC to I or II exactly reflects improved survival. Within 5 years no UICC I and only 1 of II died cancer-related, whereas downstaged 28 p16+ OSCC (5 T4b and 23 IVA including 18 T4a) in III had significantly too low median TSS and OS of 36.1 months.

Conclusion
Whereas correctly predicting improved survival of patients now staged in UICC I or II, the new staging of p16+ OSCC is suboptimal for survival estimation in T4. Upstaging of patients with ECE to IVB failed to correctly reflect their survival.
Introduction
Oropharynx cancer (OCP) has increased over the past 20 years in spite of declining tobacco use. High risk HPV (hrHPV) and p16 positive tumors account for 70-80% OPCs at our centers. HIV-infected persons are at higher risk for development of virally induced cancers, including cancer of the head and neck. HRHPV types are the cause of nearly all cervical cancers. Early detection of hrHPV and treatment has reduced the rate cervical cancers such that there are now more incident OPCs than cervix cancers in the US.

Objectives
Persistent genital high risk HPV (hrHPV)is associated with elevated risk of cervix cancer. We sought to examine oral HPV infection, persistence and clearance in HIV+/- subjects. We postulated that HIV infection may predispose a person to acquire and experience persistence of oral hrHPV infections.

Materials and Methods
HIV infected and HIV-negative subjects recruited from 5 academic centers provided serial saliva samples and social/sexual behavior questionnaires and were compensated each time. Saliva was collected in RE-100 kits (OraGene) for DNA isolation. Fifteen hrHPV types, 2 low risk HPV types (HPV6,11) and one potentially hrHPV type (HPV90) were assessed by HPV-PCR MassArray assays. Base line and follow-up samples and secure online Qualtrics questionnaires were obtained at 3-6 month intervals.

Results
Data from 345 subjects was available as of August 2017. Subjects in the 45 to 65-year age range predominated matching the population at greatest risk of OPC. Oral HPV infection prevalence was 20.1% in the consortium study compared with 9.1% in a concurrent study of healthy volunteers. HPV prevalence by site varied from 30.6% to 13.0% depending on the population studied at each site. HPV prevalence and persistence were much greater in the HIV+ that HIV- participants.

Conclusion
Oral hrHPV infection is more common and more persistent in HIV-infected subjects consistent with increased risk of OPC.
29 Extracellular vesicle cargo is related to HPV status in oropharyngeal carcinoma

B. Peacock, D. W. Lambert, K. Hunter, S. Hunt (Sheffield/GB)

Introduction
Viruses are capable of manipulating host endosomal-exosomal pathways which can aid in tumourigenesis. Human papilloma virus (HPV) encoded proteins can alter the production and cargo of extracellular vesicles (EVs) secreted by cervical cancer cells. However, the extent of HPV’s oncogenic properties relating to EV release in oropharyngeal carcinoma (OPC) is not well understood. Here, we aimed to evaluate differences in size, quantity, and molecular contents of EVs released by HPV positive (HPV+) and HPV negative (HPV-) OPC cell lines.

Methods
EVs were purified from the conditioned medium of OPC cell lines by size exclusion chromatography (SEC). EV size and concentration was measured by Tuneable Resistive Pulse Sensing (TRPS). Transmission electron microscopy was used to validate size measurements made by TRPS. Vesicular protein and RNA were extracted for subsequent mass spectrometry and small RNA sequencing, respectively.

Results
There was no significant difference in the modal diameter of vesicles released by HPV+ compared to HPV- cell lines (n=9). However, HPV- cells produced significantly more EVs (up to 2-fold) than HPV+ cells (n=9, P value <0.05). 90 proteins were identified that showed a significantly different abundance based on HPV status (P value <0.05). EGFR was only detected in EVs from HPV- cells. Bioinformatics analysis of miRNA abundance data revealed that samples clustered based on the HPV status of the producing cell.

Conclusion
The current study highlights that the molecular EV cargo (protein and miRNA) is correlated with the HPV status of the cell of origin, suggesting a differing role in the tumour microenvironment and their potential use as a source of circulating biomarkers in OPC.
Introduction

Human Papilloma Virus (HPV) infection is a prognostic factor in oropharyngeal squamous cell carcinoma (OPSCC). We aimed to develop an updated prognostic model for OPSCC, including HPV status, based on a large consecutive series of patients diagnosed and treated in three international multi-institutional cohorts.

Methods

A total of 1339 patients were curatively treated for OPSCC at 5 different medical centers. Three centers were located in the Netherlands and provided information for 2 cohorts of patients. One consisted of n=311 patients diagnosed between 1984 and 2011, the other of n=723 patients diagnosed between 2000 and 2006. The other two centers were located in the USA and provided information for the last cohort of n=305 patients, diagnosed between 1996 and 2009. The presence of HPV was determined by p16 INK4A immunostaining, followed by a high-risk HPV DNA PCR. Prognostic factors included gender, age, cTNM classification (both 7th and 8th), comorbidity and HPV status. Model performance was measured by Harrell’s Concordance index in an internal – external cross validation procedure. Decision curve analysis was performed to evaluate the reliability of decisions based on predictions derived from the prognostic model.

Results

The 5-year overall survival (OS) estimates were 70.7% in the HPV+ group and 38.7% in the HPV- group. The four different prognostic models (7th versus 8th TNM classification, without HPV and with HPV, either measured by high-risk HPV DNA PCR or p16 INK4A) were cross-validated over the three cohorts, leading to a total of 12 performance assessments. The Harrell’s Concordance Indices of these models differed between 0.64 and 0.74. Models containing 8th TNM and a separate variable for HPV as a prognostic factor performed better than models without HPV. Performance of the final model, incorporating 8th TNM classification and HPV measured by p16 INK4A, revealed a C-index of 0.70 after internal validation using bootstrapping.

Conclusion

HPV is an important marker in OPSCC and needs to be considered in counselling patients about their individual prognosis.
Invited Lecture | Impact of HPV status in head and neck cancer staging

S. Huang (Toronto/CA)

Introduction
In 2007, the WHO acknowledged the etiologic role of high-risk HPV in head and neck cancer (HNC). An HPV+ specific staging system has been introduced in the 8th edition TNM classification.

Objectives
To outline the relevance of tumour HPV status in head and neck cancer staging.

Materials and Methods
Literature search identified publications on HPV+ HNC staging. HPV+ HNC disease sites and potential prognostic value of HPV status were described.

Results
Although high-risk HPV was detected in all mucosal sites, the incidence was low (<5%) and prognostication remained unconfirmed. In contrast, the prognostication of HPV was unambiguous in oropharynx. Due to early nodal involvement, some HPV+ OPC present as CUP. Thus, HPV status is applicable for staging OPC and CUP in the 8th edition TNM. The T-category definitions remain the same as HPV-negative OPC excepting elimination of subdivision of T4a and T4b. Different criteria exist for the clinical and pathological N-classification. Clinical N-classification for HPV+ OPC and CUP is similar to nasopharyngeal cancer (NPC) but excluding lower neck nodes. Pathologic stage classification should provide guidance in addressing the need for adjuvant radiation or chemoradiation and the pathological N-classification is based on lymph node number (<5 or ≥ 5 lymph nodes) without considering involved nodal side (ipsilateral vs bilateral) or size (<6 vs ≥6 cm). Whether these should be considered in the future is unresolved since they were under-represented in the surgical series used to derive the classification. Finally, for the first time in HNC staging non-metastatic (M0) T4 or N3 disease are not considered stage IV disease.

Conclusion
With the differential prognostication of tumour HPV status, 8th edition TNM has introduced HPV+ specific stage classification for OPC and CUP. It is anticipated that the new staging will significantly impact the management of these diseases.
A small fraction of oral premalignant lesions is etiologically driven by human papillomaviruses

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Background
Oncogenic human papillomaviruses (HPV) cause up to 70% of oropharyngeal and 5-10% of oral cavity cancers. However, no causally HPV-driven premalignant lesions could be detected in the head and neck region to date. Diffuse overexpression of the cellular protein p16\textsuperscript{INK4a}, a sensitive and specific marker of transforming HPV infections in the anogenital tract and invasive head and neck cancers, occurs in a proportion of oral premalignant lesions (OPL). However, it is presently unclear whether those p16\textsuperscript{INK4a}-expressing lesions are truly induced by HPV.

Methods
A cohort of mild, moderate and severe OPL from British Columbia, Canada, was subjected to HPV DNA detection by PCR and genotyping, HPV DNA in-situ hybridization and p16\textsuperscript{INK4a}/Ki-67 immunohistochemistry. Oncogene E6*I mRNA detection, the gold standard to diagnose a transforming HPV infection, was performed on HPV DNA-positive samples. Comprehensive longitudinal clinical data were correlated to the molecular data.

Results
Data from the interim analysis revealed HPV DNA from oncogenic types (16, 18 and 52) in 15/236 OPL. HPV DNA-positive OPL more frequently showed diffuse p16\textsuperscript{INK4a} overexpression compared to HPV DNA-negative OPL (33% vs. 8%; p=0.004). Of note, patients with HPV DNA-positive OPL were more likely to be current smokers (73% vs. 27%; p=0.001). First results from the E6*I transcript analyses indicated that a small proportion (2/236) of the OPL samples were indeed etiologically driven by oncogenic HPV. These lesions also demonstrated diffuse p16\textsuperscript{INK4a} overexpression.

Conclusions
We demonstrate for the first time that a small fraction of premalignant lesions in the oral cavity is causally driven by oncogenic HPV. While these lesions showed diffuse p16\textsuperscript{INK4a} expression, it has to be considered that this expression pattern was also found in a proportion of HPV-unrelated OPL. The biological and clinical characteristics of the HPV-driven OPL will be analyzed in more detail within this study.
Detection of serological HPV16 Status in the course of therapy in patients with oropharyngeal squamous cell carcinoma


Introduction
Advanced HPV-associated oropharyngeal carcinoma is related to a better prognosis and a lower recurrence rate compared to equivalent HPV-negative tumors. This awareness led to studies focusing on eventual de-escalation of therapy to possibly raise the quality of life of those patients.
The incidence of HPV-related oropharyngeal carcinoma ascends provably in industrial countries. An early diagnosis crucially effects prognosis. Beside classical risk factors such as smoking and alcohol, which can be easily ascertained, the detection of a tumor-relevant HPV infection proves to be difficult.
A competitive ligand assay for the detection of HPV16 L1 antibodies may provide new perspectives in risk stratification and early diagnosis of HPV-related oropharyngeal carcinoma.

Material and Methods
We analyzed 30 patient serums with histologically proven oropharyngeal SCC at the time of diagnosis and during treatment by using a competitive ligand assay (Abviris) to detect HPV16 L1 antibodies. Furthermore we quantified antibodies by using photometry. Additionally PCR and hybridization were performed to detect HPV-DNA including genotyping of 32 HPV-subtypes.

Results
The majority of HPV16-DNA-positive patients showed “classic” trends of declining antibody titers during treatment. Occasional patients showed interesting trends with decreasing titers initially and an increase during follow-up lacking of clinical evidence of recurrence so far.

Conclusion
The detection of HPV16 L1-antibodies using a competitive ligand assay (Abviris) during treatment shows potential for monitoring therapy success in patients with HPV-associated oropharyngeal carcinoma. A longer follow-up period is necessary to see whether a sudden increase of antibody titers is of any prognostic significance.
Antibodies against HPV as diagnostic and prognostic biomarker for patients with neck squamous cell carcinoma from unknown primary tumor


Question
Patients with neck squamous cell carcinoma from unknown primary tumor (NSCCUP) present with lymph node metastasis without evidence for a primary tumor. Treatment of NSCCUP patients is challenging due to the risk of missing occult tumors or inducing toxicity to unaffected sites. Human papillomavirus (HPV) is a promising biomarker given its causal link to oropharyngeal SCC and superior survival of patients with HPV-driven oropharyngeal SCC and NSCCUP. Antibodies against HPV are strongly associated with oropharyngeal SCC, but have not yet been studied in NSCCUP patients. For the first time, we assessed HPV antibody patterns in NSCCUP patients, as well as their diagnostic and prognostic value to identify HPV-driven NSCCUP.

Methods
Antibodies against HPV E6 and E7 (HPV16/18/31/33/35), E1 and E2 (HPV16/18) were assessed in sera collected at time of diagnosis from 46 NSCCUP patients. For comparison, HPV tumor status was determined in 28 patients using molecular markers (HPV DNA, mRNA and cellular p16INK4a).

Results
Thirteen (28%) NSCCUP patients were HPV-seropositive for HPV16, 18, 31, or 33. Of eleven patients with HPV-driven NSCCUP, ten were HPV-seropositive, while all 17 patients with non-HPV-driven NSCCUP were HPV-seronegative, resulting in 91% sensitivity (95%CI: 59-100%) and 100% specificity (95%CI: 80-100%). HPV-seropositive patients had a better overall and progression-free survival with hazard ratios of 0.09 (95%CI: 0.01-0.42) and 0.03 (95%CI: 0.002-0.18), respectively.

Conclusion
Antibodies against HPV are present in patients with HPV-driven NSCCUP and high sensitivity and specificity for HPV-driven NSCCUP are demonstrated. Moreover, HPV seropositivity predicts better overall and progression-free survival and thus appears to be a reliable diagnostic and prognostic biomarker for patients with HPV-driven NSCCUP.
HPV associated immune escape in head and neck cancer

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Introduction/Background
Human papillomavirus (HPV)-driven cancer differs from HPV-negative cancer regarding clinical and biological aspects. This relies on drivers of carcinogenesis, which is activity of viral oncoproteins or genetic and epigenetic alterations. The balance between immune response and evasion contributes to regression or progression of cancers. Tumor infiltrating lymphocytes and immune factors of the tumor microenvironment are critically related to cancer patient's prognosis. We investigated immune cell infiltrations and expression of cellular markers associated with squamous cell carcinoma of the oropharynx (OPSCC).

Material and Methods
OPSCC FFPE samples were analyzed by immunohistochemistry (IHC) for the presence and activity of immune cells and expression of cellular markers. HPV-status was determined by bead-based hybridization following PCR (Luminex technology) and by p16INK4a IHC.

Results
We found loss of cell surface beta-2 microglobulin (β2M) expression and significantly higher numbers of CD56+ immune cells in HPV-associated compared to HPV-negative OPSCC. Detection of Granzyme B (GZMB)-positive immune cells correlated with positive HPV-status and improved survival. Immunofluorescence localization of granular GZMB within CD56+ cells and coexpression of CD16 and CD56 suggests that the detected CD56+ cells represent cytotoxic NK cells.

Conclusion
Our results point towards HPV-related immune escape mechanisms triggered by reduced functional cell surface HLA I expression. Conventional treatment of patients with OPSCC appears to shift the balance between immune response and evasion in HPV-associated OPSCC, which involves cytotoxic NK-cells and GZMB induced activation of apoptosis in cancer cells. This might contribute to the improved prognosis of patients with HPV related OPSCC.
Reduced MHC class II expression on dendritic cells from human papillomavirus E7 transgenic mice

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Cervical cancer caused by human papillomavirus (HPV) is one of the leading causes of morbidity and mortality among women worldwide. The virus infects basal keratinocyte and persistent high-risk HPV infection along with an elevated level of E6 and E7 oncoproteins associated with malignancy. As a model of HPV-mediated precancers, we have utilized K14E7 transgenic mice which express HPV16 E7 oncoprotein in keratinocytes and as a result of that display epithelial hyperplasia and suppressed local immune responses. To assess the effect of HPV16 E7 expressing skin on DC activation and function, we characterized DC surface molecule expression and MHC-II-restricted antigen presentation. As measured by flow cytometry, DCs from the epidermis and skin-draining lymph nodes of K14E7 mice have significantly increased levels of CD80 and CD86 expression but reduced MHC-II expression relative to C57BL/6 mice. In contrast, skin DCs from K14E7 mice deficient in lymphocytes (Rag1-/- x K14E7) contain high-levels of costimulatory and MHC-II molecules. After intradermal OVA immunization of K14E7 mice, CD4 T cell priming is impaired compared to non-transgenic mice. Therefore, the data suggest that lymphocytes within K14E7 mice impair the expression of MHC-II on dendritic cells with an associated reduction in CD4 T cell function. This may have implications for the generation of CD4 T cell help in HPV-mediated cancers.
HPV-associated head and neck cancer is characterized by distinct profiles of CD8 T cells and myeloid-derived suppressor cells

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S. Brandau (Essen/DE)

Question
Patients with HPV-positive localized HNSCC show favourable outcomes after surgery and radiochemotherapy compared to non-virally associated cancers. The underlying mechanisms remain elusive, but more active immunity is implicated.

Methods
In this study, we analysed immune profiles of CD8 T cells and myeloid-derived suppressor cells (MDSC) in HPV-positive versus HPV-negative disease.

Results
The overall frequency of CD8 T cells was reduced in HNSCC versus healthy donors but substantially increased after curative therapy (surgery and/or radiochemotherapy). In HPV16-positive patients this increase was associated with a marked induction of peripheral blood CD8+/CD45RA-/CD62L- effector memory cells. The frequency of HPV-antigen-specific CD8 cells was low even in patients with virally associated tumors, and dropped to background levels after curative therapy. Expression of PD-1 and TIM-3 was below 20% in most patients and not affected by therapy or HPV status. HPV-positive tumors showed enhanced infiltrates of CD8+ and CD45RO+ immune cells over HPV-negative tumors. Importantly, pre-therapy counts of monocytic MDSC, but not PMN-MDSC, were increased in patients with HPV-negative disease. This increase was accompanied by reduced numbers of terminally differentiated CD8+ effector cells.

Conclusions
Distinct baseline immune profiles and therapy-associated immune activation may contribute to the improved clinical outcome of patients with HPV-positive HNSCC. This study provides a basis for the future development of adjuvant T-cell-directed immunotherapies in this tumor entity.
Interference between mutational load, HPV status, immune signatures and outcome in patients with head and neck cancer treated with definitive chemoradiotherapy: A multicentre study of the German Cancer Consortium Radiation Oncology Group (DKTK-ROG)

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Background
Recently, we have shown that the extent of CD8+ T cell infiltration and the expression of PD-1/PD-L1 in squamous cell carcinoma of the head and neck were correlated with human papilloma virus 16 (HPV16) status and clinical outcome after concurrent chemoradiation (cCRTX). The influence of mutational burden and/or spectrum on the composition and expression profiles of tumor immune infiltrates as well as their role in the efficacy of cCRTX remain unknown.

Methods
Archival FFPE tumor samples from 118 patients with locally advanced squamous cell carcinoma of the oropharynx, hypopharynx and oral cavity collected within the framework of a retrospective multicenter biomarker trial of the DKTK Radiation Oncology Group (ROG) were available for molecular profiling. All patients had been treated with definitive cCRTX according to standard protocols. Mutational profiling was performed using an in-house gene panel targeting 327 genes with the Haloplex amplicon-based enrichment system. The genes have been selected based on the results from publicly accessible SCCNH whole exome sequencing (WES) datasets and the COSMIC database. The complete coding sequence of all exons in the 327 genes was covered, resulting in a target region of 1.45 megabase pairs in total. Only somatic mutant variants at an allele frequency of ≥0.05 and annotated in the COSMIC database were considered for further analysis. Expression levels of 730 immune-related genes were measured by the Nanostring PanCancer Immune panel on the Nanostring nCounter platform. The mutational burden was correlated with HPV status, TP53 genotype, immune signatures and outcome.

Results
Overall, 1283 single nucleotide variations (SNVs) affecting 234 (72%) of the 327 genes were detected. The median number of mutations per individual patient was 10, ranging from 0-42. The median number of somatic mutations per Mbases was 6 (range 0-29) which is well in line with the previously reported mutational burden in SCCHN established by TCGA / ICGC WES studies (Alexandrov et al, Nature 2013). We observed a trend for higher number of mutations in HPV-p16- compared to HPV+p16+ as well as TP53 mutated compared to TP53 wildtype cases. More striking however was the correlation of the mutational load with distinct immune signatures, previously established to be correlated with outcome after cisplatin-based cCRTX (Hess AK et al, ms in preparation). While we did not observe any influence of the mutational load on overall survival or locoregional control, the immune signature was significantly associated with both outcome parameters.

Conclusion
tNGS using our 327-gene panel was established as useful tool for estimation of overall mutation burden in SCCHN. The observed interference between mutational load and immune signature deserves further studies.
Intravital spatiotemporal analysis of the dynamics of tumor associated neutrophils reveals differential migratory patterns in early versus advanced tumor lesions

S. Sody, A. Klingberg, M. Gunzer, S. Brandau (Duisburg-Essen/DE)

Tumor-associated neutrophils (TAN) are an important functional element of the tumor microenvironment and of prognostic relevance in patients. A high frequency of TAN in tumor lesions is associated with adverse prognosis in the majority of tumor entities, including head and neck cancer (HNC). Nevertheless, for some tumors and in the context of therapeutic interventions anti-tumoral functions of TAN are also described. To shed light onto the dichotomous biology of TAN we established an intravital multiphoton imaging model for murine transplantable HNC. This system allows us to study neutrophil dynamics in an untouched, longitudinal manner in the living animal. Our results describe the activity of TAN during very early tumor formation in different tumor compartments, which is not achievable with classical histology. Furthermore, we monitored the effect of chemokine receptor blockade on tumor formation and TAN dynamics. Our results show a clear reduction of TAN mobility during tumor progression and differential TAN migratory patterns in peritumoral versus intratumoral regions.

Acknowledgement
This work is supported by Else Kröner-Fresenius-Stiftung and AstraZeneca
**Session 9 – Biomarkers and novel technologies**

**Chair:** S. Brandau (Essen/DE)

**40 Invited lecture | Identification of prognostic biomarkers by integrative multiscale omics analysis**

*J. Hess (Heidelberg/DE)*

**Introduction**

Genomic and epigenetic alterations are driving forces in the multistep process of head and neck squamous cell carcinoma (HNSCC) development and result from complex interactions of environmental exposures and endogenous cellular processes. The mutational landscape of HNSCC has been unraveled and provides a comprehensive view on underlying oncogenic principles. In addition, HNSCC subgroups with distinct clinical features have been identified based on characteristic patterns in their global transcriptome or epigenome. However, the identification of reliable prognostic biomarkers has been hampered by a high level of tumor heterogeneity in combination with a certain degree of cellular plasticity despite common mutational patterns.

**Objectives**

Major objectives of our research are the identification of key nodes in gene regulatory and signaling networks as reliable prognostic biomarkers and promising new drug targets for more effective and less toxic treatment.

**Materials and Methods**

Integrative multi-scale analysis was performed on global omics data, which were available from a patient cohort with advanced HNSCC and pre-clinical tumor models. New findings were confirmed with data from the TCGA-HNC cohort.

**Results**

Computational analysis identified common mutational signatures with distinct relationships to most prominent etiological risk factors. PCA analysis based on mutational signatures revealed four patient subgroups with statistically significant differences in clinical and pathological features as well as survival. Moreover, integrative omics analysis unraveled not only new putative tumor suppressor genes but also highlighted DNA-methylation as an important regulator of tumor cell plasticity by fine tuning the expression of genes with recurrent copy number gain.

**Conclusion**

Advances in omics technologies and new computational tools enable to jointly profile multiple molecular layers and to decipher reliable prognostic biomarkers as well as new promising drug targets.
Invited lecture | The emerging role of the microbiome in head and neck cancer

D. Thurnher (Graz/AT)
Unravelling cetuximab resistance in patient derived xenograft models of head and neck cancer

K. Klinghammer, J. D. Raguse, R. Otto, A. Albers, T. Eder, J. Hoffmann, K. Jöhrens, I. Tinhofer
U. Keilholz (Berlin/DE)

Background
Cetuximab is a monoclonal antibody approved for the treatment of head and neck cancer (SCCHN) and predictive biomarkers have not been established.

Methods
Cetuximab response was assessed in 47 patient derived xenografts (PDX) of SCCHN. All 47 models were assessed for mutational profile by employing Illumina cancer panel sequencing or a 327 gene panel designed for head and neck cancer by Tinhofer et al. 28 models were subjected to Affymetrix gene expression studies on HG U133+ 2.0. Based on the expression of 821 genes, the subtype of each of the 28 models was determined by integrating gene expression profiles. Receptor tyrosine kinase activation profiling was evaluated for 39 models by employing phospho-tyrosine array.

Results
Treatment results were heterogeneous with 29 tumors responsive to cetuximab treatment. 18/47 models were considered refractory. The 28 models evaluated for gene expression signature distributed over the 3 signature-defined subtypes: 5 mesenchymal/inflamed phenotype (MS), 15 basal type (BA), 8 classical type (CL). Cluster analysis revealed a strong correlation between response to cetuximab and the basal subtype and non response in the mesenchymal subtype. Relative Tumor Volume at the end of treatment MS 3.32 vs BA 0.78 (MS vs BA, unpaired t test p<0.0002). Cetuximab responder were distributed as following: 1/5 in MS, 5/8 in CL and 13/15 in the BA group. Mutational profile did not reveal a clear association of resistance in regard to any, but especially PI3K and TP53 mutation. Phospho array revealed a high number of ERBB2 (11/39) phosphorylation next to ERBB1 (35/39). We observed a significant association between primary resistance to cetuximab and missing phosphorylation for ERBB1/ERBB2 (p 0.04).

Conclusion
A single predictive marker for cetuximab resistance or response is unlikely to be established in heterogeneous tumors such as SCCHN. Best prediction was possible through definition of molecular subtypes.
Disruption of PUMA-associated apoptosis in mucosal melanoma

A. Knopf, H. Bier, K. Fritsche (Munich/DE)

Background
Mucosal melanomas (MuM) represent a rare variant of common melanoma (MM) with predilection in the sinonasal system. Limitation in extensive local surgery due to complex midfacial anatomy and failure of apoptosis inducing chemotherapeutic agents contribute to a highly aggressive phenotype. Molecular mechanisms underlying the chemo-resistance remain unclear.

Material and Methods
p53 up-/downstream was analyzed in melanocytes, 4 MuM, and 5 MM cell lines via Western Blot experiments (WBE, with/without cisplatin-pre-incubation). Differential analysis of PUMA function was done by WBE and sequencing as well as after autophagy-inhibition by siRNA-ATG5-knockdown. Post-transcriptional/translational modification of the PUMA protein was analyzed by PUMA mRNA- and protein-stability tests. Protein stabilization was further analyzed by miRNA knockdown constructs.

Results
After cisplatin pre-incubation WBE experiments demonstrated regular p53 upstream and p21 stabilization in both, MuM and MM. In contrast to MM, PUMA protein expression was absent in control and pre-incubated MuM. Sequence analysis excluded PUMA mutation. However, ATG5 knockdown by siRNA failed to stabilize PUMA protein. PUMA RNA stability test demonstrated a rapid RNA-degradation mediated by high miR-221 and miR-222 expression level in MuM. miR-221 and miR-222 knockdown resulted in PUMA protein stabilization after cisplatin-treatment.

Conclusion
PUMA RNA degradation is mediated by miR-221 and miR-222 with derogation of the corresponding protein. miRNA knockdown constructs resulted in a PUMA protein stabilization suggesting therapeutic implications in agents inducing p53-dependent apoptosis.
Detection of treatment induced phenotype changes in OSCC cells by MALDI imaging


Introduction
The survival rate of oral cancer remains poor with limited therapeutic options. Early detection of transdifferentiated tumor cells may contribute to more individualized treatment protocols and fast treatment changes. Matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) enables a label-free detection of hundreds of molecules within cancer tissue. Since the spatial distribution remains preserved it allows the characterization of tumor cells in their environment.

Objectives
The aim was to establish a protocol to analyze cultured cells by MALDI-MSI and to determine, if treatment relevant phenotype changes in OSCC could be detected by MALDI-MSI on cellular level.

Methods
The OSCC cell lines UPCI-SCC-026, UPCI-SCC-029A and the fibrosarcoma cell line HT1080 were used. Phenotypes were defined by immunofluorescence technique. UPCI-SCC-026 (SCC026) cells were incubated with 5µM Gefitinib for more than 12 months to induce treatment derived phenotype changes (SCC026Gef). The cells were cultured on Indium-Tin-Oxide slides and subjected to MALDI-MSI. For tryptic protein digestion (100ng/µl) and application of matrix, 2,5-Dihydroxybenzoic acid, the SunCollect and the SunDigest (SunChrom) were used. MALDI-MSI measurements were performed with UltrafleXtreme MALDI-TOF mass spectrometer (Bruker) (spatial resolution: 50µm, mass range: 400-7000Da, data analysis: SCiLS software).

Results
A protocol for processing cultured cells for MALDI-MSI analysis was developed. Discriminating imaging patterns could be revealed. Based on pre-dominant peptide peaks, the different cells could be grouped to epithelial or mesenchymal phenotype. In contrast to control cells (untreated or DMSO treated SCC026), SCC026Gef could be classified as mesenchymal phenotype.

Conclusions
We could show that MALDI-MSI is an appropriate tool to investigate treatment-induced phenotype changes. In further studies the data will be validated by using tumor tissue from patients.
Rapid nodal staging of head and neck cancer surgical specimens with flow cytometric analysis


Purpose
Metastatic spread to cervical lymph nodes is characteristic for head and neck cancer and significantly affects prognosis. It is also essential for oncological therapy. Neck dissection is used to evacuate lymph nodes at risk and for staging purposes. All removed lymph nodes are investigated by specialised pathologists. Evaluation of multiple lymph node specimens is both time-consuming and expensive. Our goal is to develop a reliable, inexpensive and fast method to detect lymph node metastases in head and neck cancer.

Experimental Design
Sixteen patients, diagnosed with oral squamous cell carcinoma and treated by surgery, including resection of the primary tumour and neck dissection, were enrolled in the study. Fresh perioperative lymph node biopsies from several anatomical regions of the neck were collected. Lymph node tissue was dissociated, stained for the carcinoma cell markers cytokeratin 5/8 (CK5/8), epithelial cell adhesion molecule (EpCAM) and epithelial mucin (MUC-1), and analysed with flow cytometry for the presence of cancer cells. Flow cytometry data was compared with the clinical pathology diagnosis, and enhanced histopathology, including serial sectioning and immunohistochemistry, was also performed to confirm the nodal staging results. Six normal cervical lymph nodes from cancer-free patients were used as reference.

Results
In total, 36 lymph nodes from cancer patients were analysed. Flow cytometry analysis detected 6 metastases and could also identify non-metastatic lymph nodes. Clinical histopathology diagnoses corresponded precisely with flow cytometry results. Results from flow cytometry analysis could be obtained within 6 hours of the time of biopsy.

Conclusions
We show that flow cytometric analysis of gross nodal tissue is reliable in detecting metastases. Flow cytometry is also inexpensive and fast, providing a possibility of perioperative diagnostics and rapid treatment planning.
**Invited lecture | The role of early antigen HPV serology in head and neck cancer**

*T. Waterboer, M. Pawliita (Heidelberg/DE)*

**Introduction**
Both the proportion of oropharyngeal cancers caused by HPV16 infection (HPV16-OPC) as well as the incidence rate of these tumors are rising, resulting in an increased need for early biomarkers. Thus, we have developed high-throughput multiplexed assays to quantify antibodies to HPV16 early antigens.

**Objectives**
The presentation will summarize published and ongoing prospective cohort studies, and discuss clinical implications of the findings.

**Materials and Methods**
Antibodies to HPV16 L1, E1, E2, E4, E6, and E7 proteins in serum or plasma samples were analyzed by multiplex serology. Tumor HPV status was determined by HPV in-situ hybridization, HPV DNA detection, HPV RNA patterns, and/or p16 immunohistochemistry.

**Results**
We have shown that antibodies to HPV16 E6 are a prospective serological biomarker for the development of HPV16-OPC [1] that can be stably and strongly detected more than 10 years prior to diagnosis [2]. Also, HPV16 E6 antibodies are almost exclusively present in cases with molecularly defined HPV16-OPC (both sensitivity and specificity >95%) [3]. Antibodies to other HPV16 proteins, especially E1, E2 and E7, were also associated with OPC, albeit less strongly, based on higher prevalence among controls and/or lower prevalence in OPC cases. The prevalence of HPV16 E6 antibodies in healthy controls has been repeatedly shown to be <1%, i.e. the disease specificity of this biomarker exceeds 99% [4].

**Conclusion**
HPV16 serological markers, especially antibodies to E6, are a highly sensitive and specific biomarker for detection and prediction of HPV16-OPC. To date, the trigger for seroconversion (e.g., yet to be described premalignant OPC lesions) is not understood, and the clinical implications of early HPV16-OPC detection are under debate, given the lack of treatment options. Based on the rarity of the disease, serology-based HPV16-OPC screening requires careful considerations.

**References**
[3] Holzinger et al., Int J Cancer 2017
[4] Lang Kuhs et al., Cancer Epidemiol Biomarkers Prev 2015
47  Invited lecture | TORS as treatment strategy for HPV-positive tumors

C. Simon (Lausanne/CH)
Efficacy of the antiviral agent Cidofovir to inhibit the growth of HPV-positive and -negative head and neck squamous cell carcinomas *in vitro* and the role of DNA-damage

*F. Verhees, D. Legemaate, R. Jacobs, M. Rousch, B. Kremer, E.-J. M. Speel (Maastricht/NL)*

**Introduction**
There is an urgent need to improve outcome and quality of life after treatment of head and neck squamous cell carcinoma (HNSCC) patients. Cidofovir (CDV) has been reported to have anti-viral as well as anti-proliferative properties.

**Objectives**
To explore the effect of CDV on the growth of human papilloma virus (HPV)-positive and –negative HNSCC cell lines and the effect of CDV treatment on expression of proteins involved in the DNA-damage pathway.

**Material and Methods**
Three HPV-positive HNSCC (UD-SCC-2, 93-VU-147T, UM-SCC-47), two HPV-negative HNSCC (UM-SCC-72, UPCI-SCC-003), two HPV-positive uterine cervical carcinoma (CasKi, SiHa) and one immortalized normal oral keratinocyte (NOK) cell line were treated with different concentrations of CDV for 3, 6 and 9 days. MTT assays were performed to assess the drug dose causing 50% growth inhibition. IC50 values were calculated with GraphPadPrism. The effect of CDV on expression of proteins involved in the DNA-damage pathway was further studied in 93-VU-147T, UM-SCC-47, UM-SCC-72 and the NOK cell line. The presence of DNA double strand breaks (DSBs) and activation of the DNA-damage pathway were analyzed using immunofluorescence (γ-H2AX) and western blotting (γ-H2AX, p-Chk1, p-Chk2, p53, p21).

**Results**
CDV inhibited cell growth in all cell lines and was most effective between 6 and 9 days. The IC50 values ranged from 1.6-168.3 µM. DNA DSBs accumulated upon CDV treatment as shown by γ-H2AX immunofluorescence. The proteins involved in the DNA-damage pathway became activated in most of the cell lines.

**Conclusion**
Treatment with CDV was most effective between 6 and 9 days. The anti-proliferative properties of CDV may be attributed to the accumulation of DSBs upon treatment. Although the DNA-damage pathway appears to be activated upon the accumulation of DNA-damage, further studies are needed to elucidate which mechanisms are responsible for the cell growth inhibition by CDV in HNSCC cell lines.
Influence of HPV status and hypoxia on HER receptor expression in HNSCC – an in vitro study on intrinsic cetuximab resistance and the potential of afatinib

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A. Wouters (Wilrijk/BE)

The epidermal growth factor receptor (EGFR) is an important therapeutic target in head and neck squamous cell carcinoma (HNSCC). Oxygen deficiency is a common characteristic of HNSCC tumours and these hypoxic tumour regions often contain cells that are more resistant to treatment. It has already been established that human papillomavirus (HPV) has a prognostic role in HNSCC, while its predictive significance concerning the efficacy of EGFR inhibition is still unclear. The objective of this study was to investigate whether HPV status and hypoxia influence the expression of HER receptors and the efficacy of afatinib, a second-generation irreversible EGFR-tyrosine kinase inhibitor, in intrinsically cetuximab resistant HNSCC cell lines.

We included three HPV-negative and two HPV-positive intrinsically cetuximab resistant HNSCC cell lines. The basal expression level of EGFR, HER2, HER3 and HER4 under normoxia and hypoxia (1% O2) was assessed using flow cytometry. Cytotoxicity of afatinib (72h, 0–5 μM) was investigated with the colorimetric SRB assay. Cell cycle distribution and induction of apoptotic cell death were determined using the Vindelov method and Annexin V-FITC/PI assay, respectively.

In general, we observed high expression of EGFR, HER2 and HER3 and rather low expression of HER4. Receptor expression was not significantly influenced by HPV status (p>0.114). In contrast, hypoxia resulted in a significant increase of EGFR and HER2 expression (p<0.005). Afatinib showed a clear concentration-dependent cytotoxic effect in both HPV positive and negative cell lines. Treatment with afatinib induced a G0/G1 phase cell cycle arrest and an increase in the percentage of AnnV+/PI- and AnnV+/PI+ cells.

In conclusion, our results suggest that HPV status does not influence the expression of HER receptors. Moreover, our data support the hypothesis that afatinib might be a promising novel therapeutic strategy to treat HNSCC patients experiencing intrinsic cetuximab resistance.
Evidence-based (level I evidence) treatment approaches for patients with squamous cell carcinoma of the head and neck (SCCHN) include single modality treatment for early (stage I-II) disease and combined modality therapy (CMT) for locoregionally advanced disease stages, i.e. surgery followed postoperatively by radiation (RT) or concurrent chemoradiation (CCRT), definitive CCRT, hypoxic modification (only in Denmark), while both altered fractionated RT (AFRT) and bioradiation (BRT) with cetuximab are superior over conventionally fractionated R (CFRT), and BRT also over AFRT (only in subanalyses). Apart from an accepted role in larynx preservation protocols (followed by RT) for advanced hypopharynx and larynx cancer, the exact role of induction chemotherapy (ICT) has still not been defined. A new role to select those patients (with HPV-associated tumors) in whom locoregional therapy could be de-intensified is on the horizon, as well as a possible role in patients with oligo-metastatic disease. Immunotherapy has no established place in the primary disease setting (although intensely studied), and has gained momentum in recurrent/metastatic (R/M) SCCHN, in particular after survival benefit has been observed in the second-line R/M disease setting in patients failing platinum-based chemotherapy or who fail early (within 6 months) after platinum-based CCRT. Long-term survival data are, however, lacking.

Optimal locoregional disease control (LRC) with the least side effects/morbidity should be our main focus. Both newer RT techniques/optimal dosing and targeting, optimal dosing of traditional RT enhancing agents (platinum, 5-FU, gemcitabine, taxanes, cetuximab) as well as the use of novel radiosensitizing agents should get attention, with emphasis on those with highest translational promise. Tumor hypoxia leads to radiation resistance by reversing radiation-induced DNA-damage. Nimorazole is the most tested hypoxic cell hypoxic cell radiosensitizer. Previous studies in Denmark have shown that nimorazole improves outcome of radiation, whether given by CFRT or AFRT. The positive effect on LRC and survival was particularly evident in the more strongly hypoxic tumors. HPV/p16 positive tumors do not seem to benefit from hypoxic modification. The ongoing EORTC 1219 ROG-HNCG/DAHANA trial is trying to confirm the benefit of adding nimorazole to platinum-based CCRT. In addition, the study will test whether the benefit is restricted to patients with a hypoxic gene profile.

Precision medicine is coming up as a new form of treatment of SCCHN through comprehensive analysis of patient's individual variations in genes, environment and lifestyle. Advanced understanding of the molecular mechanism of cancer has heightened targeted therapies as more promising than the traditional agents. Five major methods of precision medicine in SCCHN patients include 1) inhibiting targeting cell surface signaling receptors, 2) inhibitors targeting cellular signaling pathways, 3) precision genetic manipulation therapies, 4) precision medicine combining with surgery and 5) precision targeted radionuclide therapy. A pilot study of personalized biomarker-based treatment strategy or immunotherapy in patients with recurrent/metastatic squamous cell carcinoma of the head and neck (UPSTREAM)/EORTC-1559-HNCG. Such projects might lead to development of therapies with significantly enhanced efficacy.
Postersession 1 – Tumor immune system interplay & Immunotherapy
Chair: B. Seliger (Halle, Saale/DE)

P 1  
A high density of CD45-positive lymphocytes in resection margins of primary OSCC predicts local recurrence

B. Kremer, E.-J. M. Speel (Maastricht/NL)

Question
The local recurrence rate of oral SCCs (OSCCs) hardly decreases. This is partly due to the presence of (pre)malignant cells in the remaining tissue after resection, which may lead to the development of a new tumor in time. Because histopathologic detection of (pre)malignant cells in tumor resection margins is unreliable to recognize patients at risk for recurrence, we determined if CD45, CD8 and/or PD-L1 expression in OSCC and/or the adjacent resection margins may be used for this purpose.

Methods
FFPE tissue sections of 41 primary OSCC, the histopathologically confirmed tumor-free resection margins and 11 recurrences were analyzed. Inclusion criteria comprised a minimal 5y follow-up period and surgery without adjuvant radio- and/or chemotherapy. Immunohistochemistry was performed using CD45-, CD8- and PD-L1-specific (clone 22C3) antibodies on a Dako Autostainer Link 48.

Results
Immunostaining revealed that 1) a low density of CD45-positive lymphocytes (<3.54%/0.977mm2) in OSCCs is associated with recurrence (p=0.03), 2) a high density of both CD45- and CD8-positive lymphocytes (≥7.72% and ≥4.77%, respectively) just below the squamous epithelium in tumor resection margins correlated with recurrence (p=0.001 and p=0.003, respectively), 3) CD45 combined with chromosomal instability (Pierssens et al., Oral Oncology 2017) in tumor resection margins is the most optimal predictor for recurrence (p<0.001), 4) neither tumor PD-L1 expression nor histopathologic classification correlates with recurrence. Both a high density of CD45- and CD8-positive lymphocytes alone, or (CD45) combined with chromosomal instability in tumor resection margins correlated with unfavorable recurrence-free survival (p≤0.002).

Conclusions
A low density of CD45-positive lymphocytes in OSCC or a high density in tumor resection margins, either alone or in combination with chromosomal instability, can reliably identify patients at risk for developing a local recurrence.
Induction of tolerogenic dendritic cells by nasopharyngeal carcinoma-derived exosomes

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O. Moralès, N. Delhem (Lille/FR)

Background
A characteristic of the nasopharyngeal carcinoma (NPC) micro-environment is the presence of immunosuppressive exosomes released by tumor cells. Our team has recently shown that NPC-derived exosomes, which carry Galectine-9, favor the recruitment and suppressive activity of human regulatory T cells (Treg), thus contributing to NPC immune escape (Mrizak et al, JNCI, 2015).

Question
In this study, our objective is now to evaluate whether these NPC-derived exosomes could promote the emergence of tolerogenic semimature dendritic cells (tolDC) able to induce regulatory T cells from naive CD4+ T cells ultimately contributing to the tolerance of tumor cells.

Methods
We performed a complete phenotypical and functional study comparing the effect of NPC and healthy donor-derived exosomes on DC maturation. This study includes (i) cell morphological analysis by photonic microscopy, (ii) transcriptomic study by RTqPCR, (iii) flow cytometric analysis of the expression of specific makers (phenotypic DC and Treg markers), (iv) a preliminary DC functional study by western blotting (IDO) and HPLC dosage of tryptophan metabolites, (v) a secretome analysis by ELISA (IL-10; TGF-β, TNF-α, IL-6 and IL-12) (vi) and finally a functional assay where the CNP exosome-exposed tolDCs are co-cultivated with naive T cells in order to determine the type of T cells generated.

Results
Taken together our results strongly suggest that the presence of NPC-derived exosomes favors the emergence of semi-mature tolerogenic DCs.

Conclusions
Despite the importance of immature DCs as mediators of cancer immune escape, no other studies have shown the impact of tumor exosomes on the maturation of human DCs. Thus, these promising results should open new prospects for antitumor immunotherapies based on the inhibition of factors involved in the emergence and activation of Tregs.
P 3  **Association of PD-L1 expression in peripheral blood and tumor specimens of oral cancer patients with histomorphologic parameters**


**Question**
Inhibitory immune checkpoints like the PD/PD-L1 pathway (PD: programmed cell death, PD-L1: PD ligand 1) are involved in immune escape oral squamous cell carcinomas (OSCC). PD/PD-L1 inhibitors are approved and successfully used for treating advanced OSCC. However, the physiological relevance of PD/PD-L1 signaling in OSCC is still insufficiently understood. As PD-L1 negative tumors respond to PD/PD-L1 blocking, it is unclear if the tumor based of the immune cell based expression of PD-L1 is of higher biological relevance. The aim of the study was to analyze if PD-L1 expression in tumor tissue and peripheral blood samples of OSCC patients is associated with histomorphological parameters (T-, N-, L-, Pn-status, grading).

**Methods**
PD-L1 mRNA expression was analyzed in tumor specimens and in peripheral blood samples of 45 OSCC patients using RT-qPCR analysis. Testing for statistical significance was performed using the Mann-Whitney U-test. An association between positive test results and histomorphological parameters was calculated using the Chi-square test ($\chi^2$ test).

**Results**
OSCC cases with high grading and cases with lymph node metastases (N+) had a significantly ($p<0.05$) increased PD-L1 expression in peripheral blood samples. Upregulation of PD-L1 mRNA in blood samples of N+ cases was more than 2-fold. The $\chi^2$ test revealed that an increased expression of PD-L1 mRNA in peripheral blood of OSCC patients was significantly ($p<0.05$) associated with the presence of lymph node metastases (N+).

**Conclusions**
Increased malignancy of the tumor might be associated with a PD-L1 mediated state of systemic immune tolerance. Thus, PD-L1 expression in peripheral blood might be a predictive marker for metastatic disease (N+) in OSCC. These results underline the potential relevance of immune cell based PD-L1 expression.
P 4  Altered PD-L1 expression in tumor tissue and peripheral blood of OSCC patients compared to healthy controls


Background
Immune checkpoints like the PD/PD-L1 pathway (PD: programmed cell death, PD-L1: PD ligand 1) are involved in immune escape of solid malignancies like oral squamous cell carcinomas (OSCC). Hence inhibitors of the pathway are promising candidates for treating especially advanced OSCC. However, the physiological relevance of PD/PD-L1 signaling in OSCC is insufficiently understood. The aim of the study was to analyze if PD-L1 expression in tumor tissue and peripheral blood samples of OSCC patients is different compared to healthy controls.

Material and methods
PD-L1 mRNA expression was analyzed in tumor specimens respectively healthy oral mucosa and in peripheral blood samples of 45 OSCC patients and 36 healthy control persons using RT-qPCR analysis. Groups were tested for statistical significance using the Mann-Whitney U-test. A cut-off point (COP) for the discrimination between positive and negative cases was determined. An association between positive test results and malignancy was calculated using the Chi-square test ($\chi^2$ test).

Results
OSCC tumor specimens showed a significantly higher PD-L1 expression than oral mucosa of healthy controls ($p<0.05$). Upregulation of PD-L1 mRNA in tumor specimens was more than 3-fold. The $\chi^2$ test revealed that an increased expression of PD-L1 mRNA in tissue specimens was significantly ($p<0.05$) associated with malignancy. There was no significant association between PD-L1 expression in peripheral blood and the diagnosis of OSCC.

Conclusion
OSCC tumor tissue shows and increased PD-L1 expression compared to healthy oral mucosa. This might be an expression of a PD-L1 mediated local immune tolerance in OSCC.
**Introduction**

PD-L1/L2 expressed by antigen-presenting cells binds to the PD-receptor on immune cells and reduce their activity under physiological conditions. PD-L1/L2 are upregulated on cell surface by several cancers to escape immune response, preventing the tumor cells from being destroyed by the immune system. Upcoming immune checkpoint inhibitor therapy has revealed promising results in several malignancies. Still the clinical relevance of PD-Ligand expression remains unclear and reliable biomarkers are lacking. The objective of the study was to evaluate the prognostic impact of PD-L1/L2 expression in OPSCC.

**Methods**

FFPE tissue samples from $n=207$ OPSCC were used to perform Tissue Micro Arrays. Slices of this array were immunohistochemically stained and evaluated for PD-L1/L2 expression. PD-L1/L2 expression was correlated according to HPV-status and clinical data of patients.

**Results**

In HPV-related OPSCC, we found significantly higher rates of PD-L1 and PD-L2 ($p<0.001; p=0.034$) expressing tumors. PD-L1 expression was associated with improved overall survival (OS) independent of HPV-status ($p<0.001$). In contrast, expression of PD-L2 was associated with an unfavorable outcome in the absence of smoking and alcohol.

**Conclusion**

Conventional therapy seems to interfere with checkpoint activation in HPV-related and HPV-negative OPSCC, which is reflected by the independent prognostic value in our retrospective cohort. PD-L1 expression presents as the most validated biomarker in our cohort of OPSCC according to prediction of survival. Nevertheless, investigation of further biomarkers and HPV-stratified study designs are advisable to optimize prediction of therapy response in checkpoint inhibitor therapy.
P 6 Complement key proteins in head and neck squamous cell carcinoma

U. Werner, R. Pries, B. Wollenberg (Lübeck/DE)

Question
Head and neck squamous cell carcinoma (HNSCC) is one of the most common cancers worldwide. The complement system is supposed to play an important part in the maintenance and progression of cancer cells, but the distinct role for HNSCC remains completely unclear. This study aimed to describe the expression patterns of important complement key components in HNSCC.

Methods
Four different human HNSCC cell lines (UT-SCC-16A/16B/60A/60B) and primary cancer tissue samples were examined using molecular biological and biochemical methods whereas healthy mucosa was used as internal control.

Results
Varying expression patterns for C3 and C5 were determined for the examined cell lines and a higher amount of these factors was detected in healthy tissue compared to cancerous samples. Also anaphylatoxin receptors are heterologous distributed for the cell lines.

Conclusions
Understanding the role of the complement cascade in the processes of maintenance and immune modulation developed by HNSCC may head to promising and innovative therapy approaches.
HLA traits are risk factors for development of head and neck squamous cell carcinoma differentially affecting the progression-free survival of patients


Background
Personalized medicine and treatment stratification of patients with head and neck squamous cell carcinoma (HNSCC) mostly ignore genetic heterogeneity in HNSCC but also the patient’s genetic background. We hypothesized that HLA traits influence development of HNSCC and could be prognostic factors for relapse after curative treatment and may introduce uncertainty regarding progression-free survival (PFS) into clinical trials.

Patients and methods
90 HNSCC patients treated between 08/2010 and 05/2011 at the University Leipzig underwent low resolution typing of HLA-A, B, Cw, DR, and DQ. The cohort included 21 study patients of DeLOS-II (NCT00508664) and TISOC-1 (NCT01108042). Antigen and haplotype frequencies were compared to those in German blood donors. Effects on PFS were analyzed using Kaplan-Meier curves and Cox proportional hazard models.

Results
HNSCC patients had overall altered HLA-B frequencies (\( P<0.05 \)); frequencies of B*44 were lower, those of B*13, B*52, and B*57 increased (\( P<0.05 \)). Almost all other antigen frequencies showed no deviation. Homozygosity in HLA-Cw and DRB4 were frequent and associated with reduced PFS (\( P<0.05 \)). Altered haplotype frequencies were common and particular haplotypes accompanied by differing PFS. B*13/Cw*06 carriers had the poorest outcome (\( P=0.011 \)). Multivariate Cox analysis revealed significant different PFS associated with 3 clinical covariates (localization oropharynx, loco-regional metastasis, and T4 category), HPV16-DNA positivity, and 10 HLA traits as independent predictors for PFS. Building a HLA score from 10 traits, HNSCC patients with high and low risk regarding relapse are distinguishable. HLA scores \( \geq 0 \) and \( <0 \) of 12 DeLOS-II patients (all HPV16 negative) exactly predicted 7/7 vs. 0/5 relapses within 3 years of follow up.

Conclusions
HLA traits constitute critical prognostic factors. Their use as stratification factors may improve comparability in outcome assessments e.g. within clinical trials.
HLA-G polymorphism mediates HNSCC risk in Indian population

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Human leukocyte antigen-G (HLA-G) is a non-classical MHC class I, an immuno-modulatory molecule that exhibits important tolerogenic functions by inhibiting different immune-competent cells and modulating immune system. HLA-G gene has been identified as a disease susceptibility locus in cancer genome-wide association studies. Though, the DNA sequence variation that lies under the association of HLA-G with tumorigenesis has not been defined in Head and neck squamous cell carcinoma (HNSCC). Although, all DNA variations occurs on different sites, may possibly influence biological functions of HLA-G, those present at the 3’ untranslated regions (UTR) have been particularly studied in pathological conditions.

We conducted a genetic association study in North-Indian patients with HNSCC (n=383) and similar-aged controls (n=383) between two polymorphisms - Del/Ins (rs371194629) and +3142G/C (rs1063320). The genotyping study was documented using DNA-PAGE and RFLP-PCR method. Furthermore, the combined study of both sites was also seen.

Logistic regression analysis indicated that the heterozygote and homozygote 14-bp ins/ins genotype confer a lower risk of HNSCC (aOR = 1.71, 95% CI: 1.14–2.58; OR 1.81, 95% CI: 1.12–2.97, respectively). For G/C SNP, C/C genotype (aOR=1.93, 95% CI: 1.22–3.03) and C allele were more pronounced in HNSCC patients in comparison to controls. Likewise, HLA-G gene carrying both variants (Del/Ins-Ins/Ins & G/C-C/C) were shown to have a significant risk (OR = 2.78) of having HNSCC disease compared to gene carrying one variant (Del/Del-G/C or Del/Del-C/C or Ins/Ins-G/G). On comparing with different clinical parameters, allele C was found to be associated with tumor stage IV. Besides, both polymorphisms showed positive correlation with tobacco consumers (OR = 1.91, 95% CI: 1.18–3.07) on comparing with non-tobacco consumers.

Our study suggests that polymorphism Del/Ins and +3142 G/C of HLA-G gene may be essential for carcinogenesis of HNSCC. However, HLA-G polymorphisms have not been investigated in the context of HNSCC till date. Further investigation for its underlying role in this disease is warranted.

Keywords: HNSCC, HLA-G polymorphism, North-Indian Population.
P 9 Suppressive function of CD4+CD25hiFOXP3+ regulatory T cells is mediated by Tim-3 expression in HNSCC patients

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Background
Checkpoint receptor blockade has shown efficacy against different cancer entities including Head and Neck Squamous Cell Carcinoma (HNSCC) with durable response, however, only being achieved in a fraction of patients (15-20%). Persistent immune escape is achieved via other co-inhibitory or co-stimulatory receptors, one of them being Tim-3. Previously, we could show, that PD-1 resistance in HNSSC TIL is mediated by Tim-3 upregulation, which can in turn be overcome by Tim-3 blockade. Regulatory T cells (Treg) play an important role in modifying the tumor microenvironment and express Tim-3, the functional relevance of this being unclear.

Methods
To investigate the role of Tim-3 in Treg TIL of HNSCC patients, phenotypic and functional differences between Tim-3+ and Tim-3- Treg were compared using flow cytometry. Also, gene expression profiling and NanoString were used to evaluate the relevance of Tim-3 expression on a gene level. Finally, the efficacy of IFN-γ as a way to overcome Treg suppression was assessed.

Results
The gene expression profile and NanoString of Tim-3+ Treg show a more effector-like phenotype. In the phenotypic analysis Tim-3+ Treg express higher levels of CD39, CTLA-4, PD-1, Granzyme B, IFN-γ receptor and IFN-γ than Tim-3+ Treg. Also Tim-3+ Treg exhibit a higher suppressive ability for inhibiting naïve CD8+ T cell proliferation, which can be partially reversed when pre-treated with IFN-γ.

Conclusions
Tim-3+CD4+CD25hiFOXP3+ cells comprise a population of inhibitory T cells that suppress effective tumor defense in the tumor microenvironment. This effect can be overcome by IFN-γ treatment. Tim-3 is thus an intriguing target for cancer immunotherapy aiming to overcome persistent immune escape, especially with the advent of combinational immunotherapy.
P 9a  Methodological aspects of mass spectrometry-based exosomal studies on head and neck cancer cells

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Exosomes are small extracellular vesicles with phospholipid bilayer membrane and diameter around 100 nm. They are formed by inward budding of late endosomes and secreted after their fusion with plasma membrane. Besides nucleic acids, exosomal cargo includes also proteins which makes these nanovesicles an appreciated object of interest for proteomics. Exosomes can be isolated from different body fluids like blood (plasma/serum) or urine as well as from in vitro cell culture media. Therefore, standardization of sample preparation is necessary. For proteomic analysis, a particularly important aspect is significant reduction of contaminants like protein components of intercellular compartments or high abundant proteins, for example from cell culture media supplements, like albumin delivered with FBS. Since all prevalent methods of exosome isolation are based rather on exosome enrichment than on highly selective separation, it is necessary to minimalize the influence of background molecules on mass spectrometry measurement.

In our studies, we identified several issues potentially influencing results of mass spectrometry analysis of exosomal proteins. We show that cell culture media-derived protein contaminants significantly reduce the number of identifications of exosomal-specific proteins and we suggest size exclusion chromatography as an effective tool for exosomal sample purification. Moreover, we indicate that apart from the properties of the sample itself and the selected method of vesicle isolation/purification, also the method of exosomal proteins extraction and further sample processing can determine the final list of identified proteins. Therefore, we compared four different methods of exosomal sample preparation for mass spectrometry analysis, especially focusing on the range of obtained identifications.

These studies allow us to present an alternative method of effective exosomal protein extraction based on an organic solvent, fully compatible with mass spectrometry requirements.

This work was supported by the Polish National Science Centre Grants 2013/11/B/NZ7/01512 and 2016/22/M/NZ5/00667.
Postersession 2 – Molecular biology of HPV-associated disease & host-directed mechanisms of therapy  
Chair: R. H. Brakenhoff (Amsterdam/NL)

P 10 The VX2 carcinoma of the rabbit as a suitable animal model for papilloma virus associated head and neck squamous cell carcinomas

M. Bette (Marburg/DE)

Background
For more than 20 years, the rabbit VX2 carcinoma serves as an animal model for HNSCC. VX2 tumor development is associated with the cottontail rabbit papilloma virus (CRPV). VX2 cells therefore can be considered as an equivalent to HPV positive HNSCC cells. Here we evaluate how the VX2 animal model can be deployed in investigations to better understand the biology of HPV positive HNSCCs.

Methods
Solid VX2 tumor tissues were generated by s.c. inoculation of a VX2 cell suspension into the rabbit ear followed by extraction and validation of whole cellular RNA, which was used for RNASeq as well as for qPCR with CRPV gene specific primers. Immunocytochemistry of cultured VX2 derived cells was performed under laboratory standard conditions.

Results
Transcriptome analysis of VX2 tumor tissues demonstrated around 0.3-0.5% of all reads to match the CRPV genome. Mapping of the reads revealed a low representation of viral genes coding for E1, E5, L1 and L2, whereas a high covering rate was observed for the E7 gene. A major coverage was also observed for regions representing the long (LE6) and short (SE6) versions of E6 as well as E8 genes. The overlapping E2 and E4 genes exhibited very high coverage rates but only in the distal half of their genes pointing to a major disruption in the function of E2/E4. Furthermore, VX2 cells were cultured and relative viral gene expression levels were monitored. Viral transcripts could also be detected in the peripheral blood of VX2 bearing animals. Immunocytochemistry was deployed to help in the characterization of cultured VX2 cells.

Conclusions
The VX2 tumor represents a phenotypically stable papillomavirus associated animal tumor, allowing for the reproducible monitoring and quantification of viral transcripts. Next to tumor immune response studies one could also envisage studies testing the stage of tumor disease as well as the efficacy of antitumor therapies by screening for peripheral tumor markers using liquid biopsies.
P 11 HPV16 oncogene expression leads to an increase in the number of migratory cancer stem cells

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Question
HPV16 is a major risk factor for development of oropharyngeal squamous cell carcinoma (OPSCC). Although HPV+ OPSCC have a better prognosis, they metastasize faster than HPV-cancers which indicates an essential role of HPV in carcinogenic processes, eventually driven by an increase in the number of cancer-stem-cells (CSC). With the help of the epithelial CSC markers CD44 and EpCAM we examined whether expression of HPV16 E6 and E7 oncoproteins target the number of migratory and stationary CSC and whether aberrantly expressed miRNAs may serve as biomarkers for invasive tumours.

Methods
For FACS analysis and cell sorting, HPV16-E6E7 positive primary keratinocytes, H357 cells and OPSCC samples were stained against CD44 and EpCAM. RNA from sorted H357 cells was analyzed in a comparative miRNA microarray and quantified by real-time PCR. In situ hybridisation of HPV16+ and HPV- OPSCC samples was performed with LNA detection probes. Inhibition of specific miRNAs was achieved through transfection of miRCURY LNA inhibitor.

Results
Expression of HPV16-E6E7 led to an increase in the number of stationary stem cells (CD44high/EpCAMhigh) in primary keratinocytes, but to an increase in the number of migratory CSC (CD44high/EpCAMlow) in the cell line H357. This increase in migratory CSC pool could also be confirmed in HPV16+ primary OPSCCs. Differentially expressed miRNA panel from HPV16-E6E7 positive migratory CSCs was validated by qRT-PCR and in situ hybridization on HPV- and HPV16+ OPSCCs and led to the identification of upregulated miR-1281 and miR-3194-5p in HPV+ cancers. A reduction of migratory CSC could be achieved through inhibition of miR-1281 in cell culture.

Conclusion
Our findings highlight that HPV16 can change the phenotype of stem cells in oropharyngeal tissue. Strategies targeting miR-1281 may represent options to increase treatment success and patient survival.
P 12  Upregulation of pAKT(Ser473) expression is involved in progression of HPV-positive oropharyngeal squamous cell carcinoma

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Background
PIK3CA alterations have been shown to be a frequent event in oropharyngeal squamous cell cancer (SCC), especially in human papillomavirus (HPV)-related tumors.

Methods
Tissue microarrays (TMAs) were used to evaluate pAKT(Ser473)/(Thr308), total protein kinase B (AKT)(pan) and phosphatase and tensin homolog (PTEN) expression in primary tumors and corresponding nodal disease in oropharyngeal SCC. The HPV status was determined in regard of HPV16 DNA and RNA. Survival analysis was performed by using Kaplan-Meier curves, log-rank testing, and multivariate Cox regression analysis.

Results
HPV16 is a prognostic predictive marker for advanced oropharyngeal SCC. pAKT(Ser473) and PTEN are highly expressed in HPV-related oropharyngeal SCCs in contrast to pAKT(Thr308). The pAKT(Ser473) expression increased from primary tumors to progressive nodal disease (21.1%; P < .011).

Conclusion
Activation of phosphoinositide 3-kinase (PI3K)/pAKT(Ser473) frequently occurs in advanced HPV-positive oropharyngeal SCC and elevated pAKT(Ser473) levels represent a feature during progression of oropharyngeal SCC, indicating a critical role of the mammalian target of rapamycin (mTOR) complex. Further studies are required to evaluate specific drugs targeting PI3K/AKT/mTOR in consideration of PIK3CA alterations.
P 13 Characterization of HPV integration sites in oropharyngeal squamous cell carcinomas

L. Pinatti, H. Walline, T. Carey (Ann Arbor, MI/US)

Introduction
Most HPV-positive oropharyngeal squamous cell carcinoma (OPSCC) patients respond to intensive therapy, but treatment is often associated with high morbidity. To improve patient care and survival, our goal is to develop molecular biomarkers that distinguish patients who will respond to current or reduced therapies from those who will progress or recur, and will require additional or alternate therapies. Recent studies have shown that HPV integration into the human genome can cause alteration of cellular genes through several mechanisms including tumor suppressor loss of function, enhanced oncogene expression, and gene rearrangement. The exact mechanisms and consequences of integration on cellular gene expression remain unknown.

Objectives
Our hypothesis is that responsive tumors are driven primarily by HPV oncoprotein expression, but recurrent tumors harbor additional carcinogenic drivers as a result of HPV integration into the host genome. Therefore, our objective is to characterize the integration events of OPSCCs to determine whether integration status or site contributes to response to therapy.

Materials & Methods
My research is focused on the characterization of HPV integration sites in OPSCC tumors at the University of Michigan by Detection of Integrated Papillomavirus Sequences (DIPS-PCR), and assessment of subsequent cellular gene alterations using PCR, RTR-PCR, and sequencing methods.

Results
My results show integration events throughout the host genome in the majority of OPSCC tumors examined. Equal rates of intergenic and genic integration were seen, some of which involved cancer-related genes.

Conclusion
DIPS-PCR is an effective tool to characterize viral integration events in OPSCC and can be used to identify potential alternate drivers of carcinogenesis. Future work will include correlative studies on patient outcome and survival with HPV integration status and site to test viral integration as a clinically relevant biomarker.
P 14  Tumor extracellular vesicles include distinct oncogenic factors in HPV-positive and –negative head and neck squamous cell carcinoma

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Introduction
Despite advances in treatment for head and neck squamous cell carcinomas (HNSCC), no significant improvement has been seen in the 5-year survival rate over the last 40 years. Factors that contribute to poor survival in HNSCC include late stage diagnosis, lack of reliable markers for early stage detection, high level of biologic heterogeneity, and local recurrence/distant metastases after treatment. While some elements involved in progression and response of HNSCC are known, the underlying mechanisms that contribute to tumor behavior remain uncertain. Extracellular vesicles (EV) secreted from tumor cells are able to transmit signals and transfer cellular contents distantly to recipient cells. EV cargo represents the composition of the originating tumor cell, and also non-randomly includes materials directed for horizontal transfer to recipient cells. Nucleic acids and proteins within EVs are protected from degradation in biological fluids; this not only allows for successful delivery of the EV cargo to recipient cells, but also enables isolation and evaluation of EVs from patient liquid biopsies.

Objective
Identify oncogenic biomarkers contained within HNSCC EVs that may be developed for diagnostic and prognostic use in patient liquid biopsies.

Methods
Using PCR, sequencing, RT-PCR, and tandem mass spectrometry, we evaluated genomic DNA, RNA transcripts, and proteins isolated from HNSCC and normal cell line exosomes to identify oncogenic factors contained in EV cargo.

Results
HPV-positive and –negative HNSCC EVs contain mutant genomic DNA and oncogene transcripts that recapitulate the oncogenic profile of the originating cells. EV proteomic signatures differ in HPV-positive HNSCC cells compared to normal cells, and based on HPV subtype.

Conclusion
Tumor EVs contain biomarkers that may be developed for translational utility in HNSCC using liquid biopsy testing for early diagnosis, monitoring of tumor progression, and response to therapy.
P 15  *In-vitro* effect of cytostatics in combination with radiation on the proliferation and migration behavior of HNSCCs as a function of HPV status

**V. Guarda, M. Buchberger, G. Piontek, A. Pickhard (Munich /DE)**

**Introduction**
Although survival in locally advanced HNSCCs is improved by radiochemotherapy, locoregional recurrences are still a critical problem. We investigated the proliferation and migration behavior of newer and standard cytostatics in combination with irradiation, depending on HPV status, in vitro.

**Material and Methods**
The proliferation behavior of two HPV-negative and one HPV-positive HNSCC cell lines was tested by treatment with the cytostatics 5FdU, Ecyd and cisplatin + 5FU in combination with irradiation (2Gy; 5Gy) by means of the crystal violet proliferation assay. The migration tendency was investigated using a wound healing assay and biochemical effects by Western blot analyzes. In addition, inhibition experiments (AKT inhibitor MK2206 and MEK1 / 2 inhibitor U0126) were performed for the more accurate evaluation of activated signaling pathways.

**Results**
All cell lines showed a good anti-proliferative response to cytostatics and an additive irradiation effect. In addition, migration induced by chemotherapeutic agents could be identified in addition to the known radiation-induced migration, especially in the HPV-positive cell line, which was effectively inhibited by the inhibitors. Western blot analysis revealed a cytostatic-induced phosphorylation of Akt, Erk1 / 2 and p38 with subsequent activation of GSK3β, which could explain the increased migration.

**Discussion**
The increased migration behavior after cytostatic administration could be a reason for the development of local recurrence so that the observed inhibition of this effect by Akt and MEK1 / 2 inhibitors is a possible therapeutic approach which should be further investigated.
P 16 Tumor-stroma interaction in porcine matrix-based tissue-engineered advanced human ex vivo oral carcinoma equivalents

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Solid tumors represent complex formations able to instruct the surrounding tissue to promote their own progression. They are dependent on signals from the adjacent environment, which decisively influences their biology. That is why in vitro or ex vivo test systems mimicking solid tumors must combine both, tumor and non-malignant areas in order to avoid translational failure of therapeutical experiments. In this study, we introduce an advanced human ex vivo equivalent for oral cavity squamous cell carcinoma (OCSCC) based on a biological matrix.

We used tissue biopsies from tumor formations and autologous healthy oral mucosa of OCSCC patients. From these specimens, primary oral fibroblasts (FB), healthy oral keratinocytes (KC) and tumor cells (TC) were isolated. Segments of the so-called BioVaSc® (biological vascularized scaffold) which were generated from de-cellularized porcine jejunum were subsequently seeded with these cells. After initial submerged cultivation, the models were transferred into air-liquid conditions for 14 d. Each series was evaluated by immunohistochemistry. FB migrated into the scaffold and KC developed a characteristically differentiated multilayer epithelium including a basal membrane. Tissue models that contained TC developed various tumor nodules including typical cellular alterations. Nodules invaded the intact epithelium and all of them developed horny bulks. A particularly high in vivo-in vitro correlation was achieved. The equivalents could be cultured over several weeks.

The study reports on the successful establishment of a highly advanced OCSCC equivalent representing major characteristics found in vivo. Since primary mucosa and tumor tissue from the same donor and from the same anatomical site is cultured together, personalized long-term ex vivo test systems are generated reflecting the heterogeneity of OCSCC. Furthermore, the model merges human tumor-stroma communication to assess therapies addressing tumor-host interactions.
P 17  Myofibroblasts induce multiple functions and potentiate several signaling pathways in tumor cells of head and neck squamous cell carcinoma

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Question
Myofibroblasts are detected by alpha smooth muscle actin (SMA), which is also the best marker for carcinoma-associated fibroblasts (CAFs) in head and neck squamous cell carcinoma (HNSCC). Secretome of SMA+ cells was hypothesized to significantly influence cell growth, survival, migration and differentiation of HNSCC tumor cells.

Methods
Commercial human gingiva fibroblasts were cultured under different pH conditions (7.4 and 7.7), as well as were mixed 1 : 4 with SCC-25 tumor cells. Conditioned media of fibroblasts and mixed culture was used to treat SCC-25 HNSCC cells. Cell growth and survival were assessed by cell counting, migration by scratch assay, differentiation by mRNA gene expression analysis, flow cytometry and western blot. Secreted factors were investigated by ELISA and a cytokine array, and induced signal pathways by a tyrosine kinase array.

Results
At pH 7.7 the fibroblast culture contained 84.26 ± 2.36 % SMA+ cells while at pH 7.4 it was only 16.31 ± 0.4 %. Conditioned medium (CM) of fibroblast cultures and SCC-25 tumor cells / fibroblasts mixed culture induced cell proliferation, cell migration and epithelial – to mesenchymal transition (EMT) in SCC-25 tumor cells, where the highest effect was found with CM derived from fibroblast culture containing the most SMA+ cells. TGF-β1 was involved in tumor cell dissemination and EMT, while interleukin – 6 in cell proliferation. Signal transduction array analysis revealed that CM did not induce any specific new targetable signaling pathway, just increased the activity of several basically active signal pathways.

Conclusions
SMA+ myofibroblasts seem to contribute to the mortality of HNSCC, not by switching on a novel well targetable signaling pathway in tumor cells, but by potentiating an assortment of several functions at the same time.
**P 18** Expression profiling and immunohistochemical analysis of squamous cell carcinoma of head and neck (tumor, transition zone, normal) by whole genome scale sequencing

*V. Zivicova (Prague/CZ)*

The possibility to determine genome-wide expression profiles of cells and tissues opens a new level of analysis in the quest to define dysregulation in malignancy and thus identify new tumor markers. Toward this long-term aim, we here address two issues on this level for head and neck cancer specimen: i) defining profiles in different regions, i.e. the tumor, the transition zone and normal control and ii) comparing complete data sets for seven individual patients. Special focus in the flanking immunohistochemical part is given to adhesion/growth-regulatory galectins that upregulate chemo- and cytokine expression in an NF-κB-dependent manner, to these regulators and to markers of differentiation, i.e. keratins. The detailed listing of up- and downregulations, also available in printed form (1), not only served to unveil new candidates for testing as marker but also let the impact of the tumor in the transition zone become apparent. The extent of interindividual variation raises a strong cautionary note on assuming uniformity of regulatory events, to be noted when considering therapeutic implications. Thus, a combination of test targets (and a network analysis for galectins and their downstream effectors) is (are) advised prior to reaching conclusions on further perspectives.

**Keywords:** galectins, genome scale sequencing, squamous cell carcinoma, transition zone
P 19 Prognostic role of tumor associated macrophages and regulatory T-cells in EBV positive and EBV negative nasopharyngeal carcinoma

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Aims
Tumor-associated macrophages (TAM) and regulatory T-cells (Tregs) form a special niche supporting tumor progression, and both correlate with worse survival in head and neck cancers. However, the prognostic role of TAM and Tregs in nasopharyngeal carcinoma (NPC) is still unknown. Therefore, we determined differences in TAM and Tregs in different NPC subtypes, and their prognostic significance.

Methods
Tissue of 91 NPCs was assessed for TAM and Tregs by determination of CD68, CD163, CD206 and FOXP3 expression in the tumor’s micro-environment. Clinicopathological correlations were assessed using Pearson X2 test, Fisher’s exact test, ANOVA and Mann-Whitney U test. Survival was analyzed using Kaplan-Meier curves and Cox regression.

Results
CD68 and FOXP3 counts were higher in EBV positive NPC, while CD68-/FOXP3-, CD163+/FOXP3- and CD206+/FOXP3- infiltrates were more common in EBV negative NPC. In the whole NPC group CD68-/FOXP3- correlated with worse OS, and after multivariate analysis high FOXP3 count showed better OS (HR 0.352, 95%CI 0.128-0.968). No difference in M2 counts existed between EBV positive and negative NPC.

Conclusions
FOXP3, a Treg marker, seems to be an independent prognostic factor for better OS in the whole NPC group. Therefore, immune-based therapies targeting Tregs should be carefully evaluated. M2 spectrum macrophages are probably more prominent in EBV negative NPC with also functional differences compared to EBV positive NPC.
P 20  Soft tissue metastasis or regional neck metastasis? An undefined pathological entity

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Background
Head and neck cancer patients with advanced disease, sometimes show contiguously located soft tissue metastasis (cSTM) in the postoperative neck dissection specimens. These specimens contain histopathologically metastatic tissue without evidence of primary lymph node tissue. Currently, for these cases there is no distinct staging according to the UICC/AJCC TNM system.

Methods
A total of 610 consecutive patients with head and neck cancer operated between 01/14-12/16 were analyzed retrospectively. 17 (2,7%) showed cSTM. Histopathological slides were assessed twice by achieved by independent examiners, to differentiate cSTM and metastatic lymph nodes of the neck.

Results
The average follow-up of patients with cSTM was 33,2±9,3 months after diagnosis. Mean age at diagnosis was 65,6±11,7 years (f n=2; 11,8%). 3 patients (17,6% showed a distant metastasis at time of primary diagnosis; Primary tumor subsites were: oral cavity (n=1), oropharynx (n=12), Hypopharynx (n=2), Nasopharynx (n=2), Gl. Parotidea (n=1)). In Patients with oropharyngeal squamous cell carcinoma N=6 (50%) were p16 – positive. Three (17,6%) patients showed a recurrence of disease. Squamous cell carcinoma was detected in 15 (88,2%) patients, further adenocarcinoma (n=1) und undifferentiated carcinoma (n=1)was found.

Discussion
Currently there is no clear guideline whether cSTM should be staged as regional or distant metastasis, a difference in diagnosis which changes the management of the patient completely. This study exclusively reports on a cohort with STM contiguously located to the primary tumor and results will be compared to current literature.
P 21 Microbiota and TLR signaling in HNSCC

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Introduction
Toll like receptors (TLR) play an important role in the progression and maintenance of HNSCC. TLRs are pattern-recognition receptors which have been identified on various cells of the immune system as well as in HNSCC. They recognize various endogenous ligands from dead or damaged cells as well as bacterial components. It is known that each bacterial niche in the human body harbours very specific compositions of characteristic microbes and that these closely interact with the surrounding cellular environment and the immune system, whereas the influence of the microbiome on the tumor environment is poorly understood.

Objectives
We comprehensively analyzed the microbiome profiles of various solid HNSCC from different localisations. Subsequently, different characteristic identified bacterial species were analyzed concerning their potential to trigger TLR signalling cascades in HNSCC.

Materials and methods
The Microbiome from solid HNSCC samples was analyzed by 16S sequencing. The HEK-Blue reporter system was used to analyze the activation of NF-κB, based on an inducible SEAP (secreted embryonic alkaline phosphatase) reporter gene.

Results
Microbiome analysis revealed an enormous variety of different bacterial families in solid HNSCC. Each individual tumor manifests an individual microbiome signature, whereas consistent characteristics could be found throughout the analyzed probes. Our data indicate that the different microbial species have strongly deviating effects on TLR signalling, NFκB activation and target gene expression.

Conclusion
Different microbiota have different effects on the protein expression profiles in HNSCC and thus different impact on tumor progression and therapy response. These findings strongly suggest microbiome profiling as an innovative and promising module towards an individualized therapy of patients with HNSCC.
P 22  Mitochondrial reprogramming and co-evolution in oral squamous cell carcinoma and stromal cells

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Background
Emerging evidence indicates that cancer is a metabolic disease involving disturbances in the energy metabolism of both transformed cells and the normal cells from the adjacent microenvironment. The aim of this study was to investigate the metabolic alterations of cancer associated fibroblasts (CAF) in oral squamous cell carcinoma (OSCC).

Methods
Normal oral fibroblasts (NOF) and keratinocytes (NOK), as well as CAF and OSCC cells were isolated from five patients after informed consent. Mitochondrial morphology and volume, and mitochondrial membrane potential (MMP) were analyzed by confocal microscopy of living cells and by flow cytometry after TMRE and MTG staining in a co-culture system when NOF/CAF were grown in transwells, at distance from NOK/OSCC cells. Energy production (ATP), ROS and L-lactate production were also determined. Expression of caveolin 1 (Cav1) and monocarboxylate transporter 4 (MCT4) were assessed by western blotting.

Results
After co-culture with OSCC, but not NOK, both NOF and CAF showed decreased MMP and expression of cav1 with increased expression of MCT4. In addition, NOF showed increased production of ATP, ROS and L-lactate. Confocal microscopy showed mitochondrial transfer from both NOF and CAF to OSCC, but more often in NOF-OSCC co-cultures.

Conclusions
OSCC were able to induce the "reverse Warburg effect" in both NOF and CAF, but more potently in NOFs, by promoting mitochondrial dysfunction and oxidative.


P 23 Stromal versus tumoral inflammation differentially contribute to metastasis and poor survival in laryngeal squamous cell carcinoma

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Introduction
In solid tumors the biology and clinical course are strongly influenced by the interaction of tumor cells and tumor infiltrating stromal host cells. The aim of this study was to assess the relative importance of stromal vs. tumoral inflammation for metastasis and survival in patients with laryngeal squamous cell carcinoma.

Materials and methods
110 patients with histologically proven laryngeal squamous cell carcinoma were included. Immunohistochemical staining of CD45, CD11b, CD3, MMP-9 and COX-2 was performed and expression was semiquantitatively analyzed in stromal regions and tumor nests.

Results
CD45, CD11b, CD3 and MMP-9 positive cells were more abundant in stroma (CD45: p < 0.001, CD11b: p = 0.001, CD3: p < 0.001, MMP-9: p = 0.036), whereas COX-2 was predominantly expressed in epithelial tumor nests (p = 0.021). High expression of stromal CD45 was positively correlated with high stromal CD11b (p = 0.001), MMP-9 (p=0.024) and tumoral COX-2 (p = 0.025) expression. High levels of CD45 in stroma as well as CD11b and COX-2 in tumor nests were associated with increased metastasis compared to a low CD45 (p = 0.001), CD11b (p = 0.033) or COX-2 (p = 0.001) expression. In contrast, high tumoral CD3 expression was associated with lower rates of metastasis (p = 0.039). Survival analysis indicated significantly reduced survival of patients with high stromal CD45 (p = 0.047), high tumoral CD11b (p = 0.043) and high tumoral COX-2 (p = 0.009) expression.

Conclusion
This is the first study which separately analyzes peritumoral stroma and tumor core area in laryngeal squamous cell carcinoma in terms of CD45, CD11b, CD3, MMP-9 and COX-2 expression. Our results indicate that stroma and tumor islands need to be considered as two separate compartments in the inflammatory tumor microenvironment. Inflammatory leukocytic stromal and high expression of CD11b and COX-2 in tumor nests are linked to metastatic disease and poor overall survival.
Spatial profiling of neutrophils and T cells in the human head and neck cancer microenvironment

Y. Si, A. Squire, S. Lang, S. Brandau (Essen/DE)

The tumor microenvironment is a spatially organized landscape with immune cells located both in the tumor and stroma regions. To improve current tumor immunotherapies, it is essential to understand the spatial patterns and interactions of immune cells in those tumor regions. We used multi-color immunohistochemistry together with digital image analysis to obtain quantitative information on tumor-infiltrating immune cells with spatial resolution.

In this study, the tumor tissue of patients with stage I–IV head and neck cancer, who were scheduled for surgical resection, and consented to tissue collection of a portion of their tumor, was analyzed. Immunofluorescence was used to explore the specific phenotype and spatial distribution of tumor infiltrating neutrophils (TAN) and T cells (TIL). To this end the neutrophil marker CD66b and T cell marker CD3 were combined with markers indicative of cellular differentiation and activation states. The whole slide was scanned with Apotome, and subjected to digital image analysis using the Definiens platform, tissue studio ® and R analysis. We established multi-color protocols and analysis tools to obtain a high resolution picture on the functional interaction of neutrophils and T cells in head and neck cancer. Initial results revealed hot spots of functional interaction with relevance for overall survival and patient outcome.
Objective
Laryngeal cancer is the most common type of cancer in the head and neck. Human papilloma virus (HPV) represents a group of more than 150 related viruses. Infection with certain types of HPV can also cause some types of cancer. This study aimed to evaluate the socio-demographic and histopathological characters of squamous cell carcinoma of the larynx and its relationship to Human papilloma virus subtype-16 (HPV-16).

Study Design
Cross – Sectional

Setting
Tertiary university hospitals at five Districts in Egypt

Patients and Method
It is a cross – sectional study that was conducted on 50 adult patients with laryngeal cancer who were admitted at 5 different tertiary care hospitals in Egypt from January 2014 through December 2014. All patients were subjected to a comprehensive preoperative assessment, histopathological assessments of tumor biopsies and immunohistochemical staining for HPV-16.

Results
HPV-16 immunostaining was positive in 9 patients (18%). A significant correlation between HPV-16 immunoreactivity and tumor grade (P<0.001) was detected with no significant correlation between HPV-16 immunoreactivity and other clinical and pathological variables. (age, gender, smoking, alcohol consumption, tumor site, tumor stage)

Conclusion
The frequency of HPV-16 in laryngeal carcinoma is 18%, and there is significant correlation between HPV-16 and Tumor grade
P 26  Assessment of HPV DNA detection and typing between tissue biopsies and formalin fixed paraffin embeded (FFPE) of head and neck cancer

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Introduction
As incidence of human papillomavirus (HPV) – associated oral cancers is increasing, dental hospitals pose an unique advantage in implementation of programs targeted to early detection and control of oral malignancies.

Objectives
Present investigation was aimed to assess utility of Formalin Fixed Paraffin Embedded (FFPE) tissues and to evaluate HPV infection in such settings.

Material and Methods
We examined 20 prospectively-collected FFPE tissues from histopathologically-confirmed oral cancers reported in a regional dental hospital for evaluation of different DNA isolation protocols using xylene-based and xylene-free methods and subsequent detection of intra-lesional HPV infection. Following performance evaluation of these DNA in PCR-based amplification of internal controls, different HPV primer-based PCRs were performed. DNA-isolated from freshly-collected, paired biopsies used as reference.

Results
FFPE DNA showed concordant results with reference protocol for internal control or HPV detection only when short fragment length-PCRs were performed. Inclusion of FFPE samples from another 30 retrospective oral cancer cases from dental hospital for validation revealed a comparable HPV positivity (23%) which was similar to the HPV prevalence in concurrent prospectively-collected oral cancers (n=96) reported at tertiary cancer care hospitals (TCCH). However, these cases differed particularly with respect to the representation of the high risk HPV types. Cases reported in dental hospitals showed exclusive presence of HPV18 whereas HPV16 was detected in OSCC cases reported in TCCH which could be overrepresentation of selective oral sub-sites.

Conclusion
Overall, DNA isolated from FFPE using xylene-free protocol could be a promising strategy for PCR-based HPV detection at dental hospital settings for OSCC early detection and management.
P 27 Neuroendocrine carcinoma of the head and neck: HPV-status and co-expression of immunomodulating receptors


Introduction and Question
Lately, HPV-association of head and neck tumors has frequently been described in the literature, occurring not only in squamous cell carcinomas, but also in the less common neuroendocrine carcinomas (NEC) of the head and neck region. The PD-1/PD-L1 checkpoint was found to be associated with a better outcome in patients with HPV-positive squamous cell carcinoma. It is source to antibody-therapies, such as Pembrolizumab.

Methods
The aim of this study was to determine the HPV-status of NEC of the head and neck region that were collected at our department during surgery in the course of the last 11 years. Additionally, the samples were analyzed in terms of their expression of PD-1, PD-L1 and PD-L2.

Results
Eight different NEC tumor samples of the head and neck region were analyzed, three of them (37.5%) showed HPV-association. All three HPV-positive samples showed HPV type 18 on molecularpathological analysis. Expression of PD-1 and PD-L1 differed widely between the samples and showed no correlation to the HPV-status. PD-L2 showed a stronger expression in HPV-positive samples.

Conclusions
HPV type 18 appears to be frequently associated with NEC. PD-L1 und PD-L2 expression varies widely in NEC. Its role in NEC to date remains unclear. Multicentric studies are required to further analyze their impact on this rare tumor entity.

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Question
Wether 8th edition of the AJCC/UICC TNM staging system (UICC) precisely differentiates between stages and reflects disease outcome in human papilloma virus (HPV)-associated oropharyngeal squamous cell carcinoma (OPSCC).

Methods
OPSCC patients that were diagnosed between 2000 and 2016 were included and HPV status was determined by combined DNA and p16 testing. Stratification was done according 7th and 8th UICC staging rules. Incidence trends of HPV-associated tumorigenesis, 5-year overall survival (OS) according tumor stages and the influence of therapy and prognostic factors towards the outcome were calculated.

Results
A significant increase [2000-2010 (21%); 2011-2015 (53%); \(p = 0.002\)] in HPV-associated OPSCC was seen in the observation period. 150/599 (25.0%) patients had HPV-driven OPSCC and 64.7% of curative treatments in all OPSCC patients included upfront surgery of the primary and the neck. 7th edition staging rules led to no discrimination in all UICC stages in HPV OPSCC underlining the need for new staging rules. Only discrimination between stages I vs. II and III vs. IV was significant in our patients with HPV-OPSCC (94.4 vs. 77.5%; \(p = 0.031\) and 63.9 vs. 25.0%; \(p = 0.013\)), and stages II vs. III did not differ in OS rates (\(p = 0.257\)), when applying new staging rules. For HPV-negative OPSCC, significant outcome differences were only seen between UICC stages III vs. IV (57.6 vs. 35.2%; \(p = 0.012\)).

Conclusions
While the 7th edition of UICC shows invalid discrimination between stages, the 8th edition is more suitable for HPV-associated carcinoma. Due to lack of differentiation between stages II and III further adaption is needed.
P 29 The relationship between tumor and nodal stage of laryngeal carcinoma infected by HPV-16

M. Gomaa (Minia/EG)

Objective
Laryngeal cancer is the most common cancer in the head and neck. Human papilloma virus is a group of over 150 related viruses. Infection with certain types of HPV can also cause some forms of cancer.

The aim of the study to evaluate the tumor and nodal stage of Squamous cell carcinoma of the larynx and Human papilloma virus subtype-16.

Patients and Method
The study was conducted on 47 patients who were admitted at the otolaryngology-head and neck surgery department in Minia University Hospital, and other four tertiary University Hospitals during the period from January 2015 till December 2015 All patients were subjected to a preoperative assessment protocol that include history taking, general examination, otolaryngological examination, laryngeal imaging, laryngeal biopsy and biopsy assessment to identify the tumor stage and Immunohistochemical staining for HPV-16.

Results and Conclusion
HPV-16 immunostaining was positive in 19% (9/47) cases studied. There was no statistically significant difference between the HPV-16 immunoreactivity and tumor size, tumor stage nor lymph node metastasis.
P 30  **HPV-E6/7 oncogene-expressing circulating tumor cells in oropharyngeal squamous cell cancers**


**Introduction and Objectives**
Human papillomavirus-related oropharyngeal carcinoma (HPV-OSCC) is increasing in incidence in the United States and in Europe. Although HPV-OSCC has favorable prognosis, 10% to 25% of HPV-OSCCs recur. Circulating tumor cells (CTCs) are considered indicators of residual disease and thus are associated with an increased risk of metastasis. HPV- E6/E7 oncogene expressing CTCs might be a useful biomarker for tumor surveillance. We sought to assess whether CTCs expressing HPV E6/E7 oncogenes could be detected at baseline and at the end of treatment in a cohort of patients (pts) with HPV-OSCC.

**Methods**
16 patients with locally advanced (n = 14) or recurrent/metastatic (n = 2) OSCC were included in this analysis. Pre-therapy tumor biopsies (FFPE tissue) were assessed for high risk (HR) HPV infection by p16 immunohistochemistry, PCR (GP+ and MY systems) and quantitative PCR (qPCR) for HPV 16, 18 and 31. HPV DNA+ tumors were subjected to HPV E6/E7 oncogene expression analysis by quantitative reverse transcriptase. HPV E6/E7 oncogene expression analysis was performed in immunomagnetically positive selected CTCs from 14 of these pts, isolated before and after treatment.

**Results**
HPV infection was detected in 14 of 16 OPC (87.5%) by p16 and PCR. 10 of 14 patients (71.4%) were HPV16+, while HPV16 E6/E7 expression was detected in 8 patients (80%). HPV16 E6/E7+ CTCs were detected in 7 patients (50%), all of which were p16+/HPV DNA+, but only 3 of them (42.9%) were HPV E6/E7 mRNA+.

**Conclusions**
Detection of HPV E6/E7+ CTCs might be a useful noninvasive test for determination of a clinically relevant HPV infection in HPV-OSCC; its potential utility as a surveillance biomarker deserves evaluation in clinical trials.
P 31  FAM107A gene is silenced in laryngeal tumors by combined DNA methylation and deletion

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Laryngeal squamous cell carcinomas (LSCC) are characterized by complex genotypes, with numerous abnormalities found in various genes. Application of high throughput technologies has largely extended the knowledge of altered genomic regions and their molecular background, however despite this progress 5-year survival rates in LSCC remain unsatisfactory. Therefore, extended studies are still conducted, with the aim to find genes, potentially implicated in this cancer.

In our study, we focus on yet poorly analyzed FAM107A (3p14.3) gene, since we found its significantly reduced expression in LSCC by microarray profiling. This finding encouraged us to identify the mechanisms involved in transcriptional silencing of the gene.

With the use of RT-PCR we have confirmed complete FAM107A downregulation in laryngeal cancer cell lines (15/15) and primary tumors (21/21) in line with microarray results. This finding was further supported by immunohistochemistry, where FAM107A downregulation was found in all analyzed cases (15/15). Using available array CGH profiles we have found recurrent deletions of the short arm of chromosome 3, resulting in loss of one copy of the FAM107A gene. Next, we sequenced the coding regions of the gene, with the aim to find the second-hit mutation but no clear novel inactivating mutations were detected. Therefore, we have analyzed the DNA methylation level of the gene’s promoter region by bisulfite pyrosequencing, finding hypermethylation of FAM107A in 9/15 cell lines and in 15/21 primary tumors in contrast to 0/8 non-tumor controls. As a proof of principle, we show that induced hypomethylation using Decitabine restores FAM107A expression in a studied cell line.

Taken together, we have demonstrated that a combined and recurrent two hit mechanism including loss of 3p and hypermethylation of FAM107A promoter region results in the gene transcriptional loss. Therefore, FAM107A may be a promising tumor suppressor candidate involved in LSCC development.
P 32  EGFR detection in serum and saliva as a diagnostic and prognostic tool in oral cancer

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Question
Epidermal growth factor receptor (EGFR) is a type I transmembrane glycoprotein that is overexpressed in a wide variety of malignancies, including oral squamous cell carcinoma (OSCC). Our objective was to assess the EGFR diagnostic and prognostic value in OSCC by investigating its expression in serum and saliva of patients in comparison with healthy subjects in a prospective case-control study.

Methods
Serum and saliva samples were collected from a cohort of 63 treatment-naïve OSCC patients before surgery and a matched group of 60 healthy subjects. EGFR concentrations in serum and saliva were quantified by an enzyme-linked immunosorbent assay.

Results
OSCC patients showed lower values of serum EGFR compared with controls (p=0.0002). Conversely, saliva EGFR concentrations were higher in OSCC patients than in controls (p=0.0014). Saliva EGFR levels were also increased in patients with higher T category (pT4 vs. pT<4, median 6.0 vs. 3.8 ng/ml; p=0.02). Considering 9.2 ng/ml (fourth quartile) as the cut-off value, patients with higher levels of saliva EGFR had worse prognosis in terms of overall survival (p=0.04). Conversely, no association was found between serum EGFR and clinical outcomes in OSCC patients.

Conclusion
Saliva EGFR can be considered as a potential tumor marker for OSCC with both diagnostic and prognostic values. Serum EGFR levels, on the other hand, were lower in OSCC patients, but did not show any prognostic impact. Saliva EGFR levels are worthy of further investigation as a potential diagnostic and prognostic biomarker for OSCC.
P 33 Epigenetic biomarker for improved detection of head and neck cancer by quantitative real time PCR


Introduction
Head and neck cancer (HNC) constitutes the sixth most frequent cancer world-wide with an overall 5-year survival rate of less than 50%. The tumour entity comprises a heterogeneous group of malignancies and mostly arises in the oral cavity, pharynx and larynx. Further, HPV-positive patients show a higher overall survival rate. Aberrant DNA hypermethylation is a common event during carcinogenesis and the detection of a cancer specific methylation signature could complement the current state of diagnostic for HNC.

Methods
We determined the performance of three methylation markers Z6, Z7 and Z8 with bisulfite-converted DNA from a cohort of 63 HNCs and 56 normal tissues via methylation specific real-time PCR. Z7 and Z8 predominantly detected HPV16-positive HNC and here mainly tonsillar carcinomas, whereas Z6 was a marker detecting all types of HNC. Furthermore, a subcohort was tested utilizing five methylation markers in order to increase marker performance by combination and to detect cancer independent from HPV status.

Results
Z6 proved detection of HNCs with sensitivity of 0.71 and specificity of 0.96, whereas Z7 and Z8 were identified as putative detectors of HPV-positive tonsil cancer (sensitivity 0.88, specificity 0.94). But single markers were unlikely to function alone for distinction between normal tissue and HNCs, even due to the heterogeneous nature of HNC. Therefore the combination of Z6, Z8 with Z1 improved sensitivity to 0.79 with specificity of 0.96.

Conclusion
A DNA methylation marker set of three shows a high potential for overall detection of HNC. Further investigation with a larger cohort is necessary to validate the biomarkers. For the intended non-invasive sample collection we currently compare results from tissue with matching swabs from the oral cavity. These specific DNA-methylation markers represent the basis for further development of marker tests and are an important tool for improving HNC diagnostics in the future.
P 34 Circulating cell-free DNA methylation of SHOX2 and SEPT9 is a versatile biomarker in head and neck squamous cell carcinoma patients

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Circulating cell-free DNA methylation testing in blood has recently received regulatory approval for screening of colorectal cancer. Its application in other clinical settings, including staging, prognosis, prediction, and recurrence monitoring is highly promising, and of particular interest in head and neck squamous cell carcinomas (HNSCC) that represent a heterogeneous group of cancers with unsatisfactory treatment guidelines.

SHOX2 and SEPT9 DNA methylation in plasma from 649 prospectively enrolled patients (training study: 284 HNSCC / 122 control patients; testing study: 141 HNSCC / 102 control patients) was quantified prior to treatment and longitudinally during surveillance.

In the training study, 59% of HNSCC patients were methylation-positive at 96% specificity. Methylation levels correlated with tumor and nodal category (p<0.001). Initially increased methylation levels were associated with a higher risk of death (SEPT9: HR=5.27, p=0.001, SHOX2: HR=2.32, p=0.024). Disease recurrence/metastases were detected in 47% of patients up to 377 days earlier compared to current clinical practice. The onset of second cancers was detected up to 343 days earlier. In the testing study, sensitivity (52%), specificity (95%), prediction of overall survival (SEPT9: HR=2.78, p=0.022, SHOX2: HR=2.50, p=0.026), and correlation with tumor and nodal category (p<0.001) were successfully validated.

Methylation testing in plasma is a powerful diagnostic tool for molecular disease staging, risk stratification, and disease monitoring. Patients with initially high biomarker levels might benefit from intensified treatment and post-therapeutic surveillance. The early detection of a recurrent/metastatic disease or a second malignancy could lead to an earlier consecutive treatment, thereby improving patients’ outcomes.
P 35  hTERT promoter methylation status as a molecular marker of cancer progression in head and neck cancer patients

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Introduction
The head and neck squamous cell carcinoma (HNSCC) is the sixth leading cause of cancer worldwide, representing over half a million incidents every year. Cancer cells, including HNSCC, are characterized by an increased telomerase activity. This enzymatic complex is active in approximately 80-90% of all malignancies, and is regulated by many factors, i.e. methylation status of hTERT gene promoter. hTERT gene is also surmised to be differentially methylated in cancer patients than in controls.

Objectives
The aim of this study was to analyze the hTERT gene promoter methylation status in blood leukocytes of HNSCC patients.

Materials and Methods
DNA was extracted from PBMC (Peripheral Blood Mononuclear Cells) of 92 patients with histologically diagnosed HNSCC and 53 healthy volunteers. Methylation status of 19 CpG islands was estimated using bisulfide conversion technique followed by sequencing of PCR products.

Results
Close to the significant \( p=0.0532 \) differences in the general frequency of hTERT CpG sites methylation was detected between patients and healthy controls. However, it was discovered that some of analyzed positions (CpG islands: 1 \( p=0.0235 \), 5 \( p=0.0462 \), 8 \( p=0.0343 \)) are significantly more often methylated in HNSCC patients than in controls. The opposite finding was observed in case of CpG position 2 \( p=0.0210 \). Furthermore, closer analysis of single CpG positions revealed differences in methylation status dependent on anatomical site and TNM classification.

Conclusion
Analysis of hTERT promoter methylation status (all or single CpG positions) may be used as a molecular markers of HNSCC progression.
P 36 hTERT C250T promoter mutation and telomere length as molecular markers of cancer progression in patients with head and neck cancer

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Background
The head and neck squamous cell carcinoma (HNSCC) is the sixth leading cause of cancer worldwide. Cancer cells are characterized by an increased telomerase activity. This enzymatic complex, active in 80-90% of all cancers, is responsible for lengthening of telomeres. Recently, highly recurrent point mutation in hTERT promoter have been reported in multiple human malignancies.

Objectives
The aim of the study was to analyze the frequency of the hTERT promoter C250T mutation and the telomere length in blood leukocytes of 61 HNSCC patients.

Methods
DNA was extracted from PBMC (Peripheral Blood Mononuclear Cells) of 61 patients with histologically diagnosed HNSCC and 49 healthy volunteers. Telomere length was assessed using quantitative PCR-based technique. To identify C250T hTERT promoter mutation, the HRM analysis was performed. Statistical analysis of the results was performed using the Student’s, ANOVA, Chi-square, and Fisher’s exact tests.

Results
No significant difference was observed in the relative telomere length between the studied and control groups. Telomeres in PMBC from individuals with T2 HNSCC cancer were significantly shorter compared to telomere length in leukocytes of healthy individuals (P=0.0001). There was also significant difference in telomere length between T2 and T3 (P=0.0063), and T2 and T4 (P=0.0028). hTERT promoter mutation was identified in 36% of HNSCC patients and in 27% of healthy individuals. There was significant correlation between frequency of mutation and grade of tumor (P≤0.0001) in contrast to the wild allele.

Conclusions
C250T hTERT promoter mutation is a common event during cancerogenesis in HNSCC patients and together with telomere length may be one of the molecular markers of HNSCC progression. The finding of long or short telomeres in PBMC of HNSCC patient does not necessarily indicate the presence or absence of hTERT promoter mutation, and both parameters should be considered to characterize patients status.
P 37 Initiation of an international multicenter study on SNSCC and a possible influence of viral oncogenesis

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Sinonasal squamous-cell carcinomas (SNSCC) are relatively rare. Thus, data regarding the rate of lymph node metastases are inconsistent in contrast to well-known high metastasis rates in squamous-cell carcinomas of the head and neck (HNSCC) (oral cavity, pharynx and larynx). Hence, the indication for elective neck dissection is difficult in SNSCC. The aim of this multicenter study is to assess common genetic alterations as well as the EBV and HPV status as a function of metastasis in SNSCC.

So far, we have acquired 33 European centers to join this study. The ethical application had already been approved for 10 centers so that the material could be further processed. As in the pre-study of a domestic intergroup, a relatively high rate of induction with EBV and HPV was shown, correlating with the likelihood/rate of metastasis.

We now postulate that viral oncogenesis plays an important role in the biology of SNSCC.
P 38  Detection of tumor-specific mutations and viral DNA in liquid biopsies of patients with human papillomavirus (HPV)-associated oropharyngeal squamous cell carcinoma (OPSCC)

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Introduction
Head and neck cancer is the sixth most common cancer worldwide and shows an increasing incidence. Infection with human papillomavirus (HPV+) has been identified as an additional risk factor, especially for oropharyngeal squamous cell carcinoma (OPSCC) and composes an independent tumor entity compared to HPV-unrelated (HPV-) OPSCC. Even though HPV+ OPSCC patients often present with more and larger metastases than HPV- OPSCC, they have a significantly better outcome.
We aim to identify genetic alterations responsible for unfavorable outcome in a subgroup of patients with HPV+ OPSCC as well as patient-specific biomarkers to monitor treatment failure and to detect disease recurrence.

Materials and Methods
Patients with HPV+ OPSCC and poor outcome (n=13) and best matching patients with a favorable outcome (n=13) were included. Blood was drawn at sequential time points before and after treatment during follow-up. DNA was extracted from FFPE tumor tissue, lymphocytes (control) and plasma (liquid biopsy). HPV genes E6 and E7 were quantified by qPCR. A panel for targeted next generation sequencing (tNGS) with mutations frequently observed in HPV+OPSCC was designed. Libraries for the tNGS panel and the fragment analyses were generated and sequenced with the Ion PGM System.

Results
HPV genes E6 and E7 were detected by qPCR in cell-free DNA (cfDNA) from plasma and could be correlated with course of disease and treatment. Tumor mutations detected with the tNGS panel were abundant in pre-treatment cfDNA. The relative amount of HPV genes and the tumor mutations decreased after treatment.

Conclusion
HPV-specific genes can be detected in cfDNA from plasma and can be correlated with treatment strategies at sequential time points. Therefore, follow-up plasma from patients will be analyzed in an upcoming study. Accordingly, tumor-specific mutations from various patients will be investigated.
CD31 and VEGF are prognostic biomarkers in early-stage, but not in late-stage, laryngeal squamous cell carcinoma

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Objectives
Patients suffering from squamous cell carcinoma of the larynx (LSCC) with lymphatic metastasis have a relatively poor prognosis and often require radical therapeutic management. The mechanisms which drive metastasis to the lymph nodes are largely unknown but may be promoted by a pro-angiogenic tumor microenvironment. In this study, we examined whether the number of microvessels and the expression level of vascular endothelial growth factor (VEGF) in the primary tumor are correlated with the degree of lymph node metastasis (N-stage), tumor staging (T) and survival time in LSCC patients.

Material and Methods
Tissue-Microarrays of 97 LSCC patients were analyzed using immunohistochemistry. The expression of VEGF was scored as intensity of staining (low vs high) and the number of CD31-positive vessels (median ≤7 vessels per visual field) was counted manually. Scores were correlated with N-stage, T-stage and 5-year overall survival rate.

Results
A high expression of angiogenic biomarkers was not associated with poor overall survival in the overall cohort of patients. Instead, high CD31 count was associated with early stage cancer (p=0.004) and in this subgroup high VEGF expression correlated with poor survival (p=0.032) while a high vessel count was associated with an increased recurrence rate (p=0.004).

Conclusion
Only in the early stage subgroup a high expression of angiogenic biomarkers was associated with reduced survival and an increased rate of recurrence. Thus, biomarkers of angiogenesis may be useful to identify high risk patients specifically in early stage LSCC.
P 40  Role of narrow band imaging in detection of head and neck unknown primary squamous cell carcinoma

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Question
There is no general consensus on what kind of examination is mandatory to include in an optimal detection strategy for head and neck squamous cell carcinoma of unknown primary (SCCUP). This study investigates the diagnostic accuracy of Narrow Band Imaging (NBI) in SCCUP not detectable by standard white light (WL) endoscopy and state of the art imaging.

Methods
Between 2007 and 2016, 29 untreated patients affected by SCCUP were referred at two academic institutions. Selection criteria were: lymph node fine-needle-aspiration citology positive for SCC; no evidence of any primary tumor at WL endoscopy; CT or MR, and PET non-diagnostic for any primary; no contraindication to general anesthesia. Each patient underwent office-based NBI evaluation. If a suspicious area was identified, we performed a guided-biopsy for histological confirmation. By contrast, if no suspicious area was detected, patients underwent WL and NBI panendoscopy under general anesthesia, with bilateral tonsillectomy and base of the tongue (BOT) mucosectomy. We defined as pT0 patients in whom primary tumor remained undetectable after all these diagnostic steps.

Results
Office-based NBI endoscopy identified 10 SCCs (34.4%): 1 in the nasopharynx, 3 in the palatine tonsil, 4 in the BOT, and 2 in the supraglottis. In only one (3.5%) patient we found the primary in the BOT even if the NBI panendoscopy under general anesthesia was negative. In one (3.5%) patient we found an NBI suspicious area during panendoscopy under general anesthesia, but the histological examination did not confirm such finding. Seventeen (58.6%) patients remained true pTx. Sensitivity, specificity, positive, and negative predictive values of NBI were 91%, 95%, 91%, and 95%, respectively. Diagnostic accuracy was 90%.

Conclusions: Office-based NBI increases the detection rate of head and neck SCCUP and should be considered as an adjunctive tool in the diagnostic work-up of these patients.
P 41 Establishment of a 3D-organotypic culture model of head and neck mucosal melanoma

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Question
Although new therapeutic options are constantly improving the overall survival in cutaneous melanoma, its evil twin, the mucosal melanoma remains a rare tumor disease with poor clinical outcome. Current efforts to investigate the biological and genomic characteristics of these tumors have been constrained by their low incidence and the fact, that there is no commercially available cell line and preclinical mouse model for experimental analysis. In this study, we addressed the need for preclinical mucosal melanoma models that can be used to better characterize this aggressive disease as well as to identify therapeutic targets and empower testing of candidate pharmacologic drugs.

Methods
We established a 3D-organotypic co-culture model of head and neck mucosal melanoma (HNMM). Overall, 14 HNMM samples from two different patients were grown on dermal equivalents (DEs) reinforced by a scaffold of modified viscose fibers (non-woven Bemcot-M3) and colonized with skin fibroblasts, producing genuine dermis-type matrix.

Results
These 3D-HNMM-OTC-models could be successfully cultivated up to 40 days. The HNMM cells showed migrating and proliferative activity which commenced a few days after tissue specimens were placed on top of the DEs. Outgrowing cells harbored mucosal melanoma markers such as HMB-45, Melan-A and S100 equivalent to the primary tumor at day 0 and to mucosal melanoma in vivo.

Conclusions
To the best of our knowledge, we are the first group to present a preclinical model for long-term cultivation of head and neck mucosal melanoma. We could demonstrate that this model has the potential to study the biology of this rare yet aggressive tumor entity. Besides, it might, in the future, combine the advantages of rapid evaluation of therapies as well as long-term observation of cultured tumors, ultimately aiming to integrate patient-specific preclinical models into clinical application and to increase our knowledge on this deadly tumor entity.
Feasibility and outcome after proton beam therapy for head and neck tumors at the West German Proton Therapy Center Essen (WPE) – early results of the prospective registries ProReg and KiProReg

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Question
Proton therapy (PT) is used to protect normal tissue in the nasopharynx, nasal cavity and paranasal sinuses. Since 2013, PT is offered to patients with head and neck tumors at WPE. We report early data on feasibility and outcome after PT.

Methods
Between October 2013 and August 2017, 69 patients (63 adults, 6 children; 49 m, 20 f), median age 58.2 years (13.0-82.5) with tumors of the nasopharynx, nasal cavity and paranasal sinuses were included in the register study. Histologies were squamous cell carcinoma (ca) (n=16), adenoid cystic ca (n=15), undifferentiated ca (EBV+ n=16), adeno ca (n=6), esthesioneuroblastoma (n=6), mucous membrane melanoma (n=3) and miscellaneous ca (n=7).
PT was administered with either pencil beam (58%) or uniform scanning (37.7%) or both (4.3%). 17.7% received photons and proton boost. Patients were predominantly treated definitive (66.7%) and in 47.8% with concomitant chemotherapy. The median total dose was 70 Gy (50.0-72.0) applied in a median of 35 fractions (10-40) at a single dose of median 2.0 Gy (1.65-2.2), 5x/week. Side effects were documented according to CTCAE V4.0.

Results
Median follow-up (FU) time after initial diagnosis was 14.5 months (0.3-131.8). Adverse reactions of grades 1/2 during PT were predominantly erythema (n=53) and fatigue (n=52). Grade 3/4 toxicities during PT were mainly dermatitis (n=4), anorexia (n=3), dysphagia (n=3) and oral mucositis (n=11). Toxicity data after 3 and 12 months are available for 42 and 19 patients, respectively. No new onset of grade 3/4 toxicities occurred. Tumor control was achieved in 82.6%. Local recurrence or progression occurred in 8.8%, dissemination in 10.5%. 10.1% died so far, the majority due to the tumor. 1 patient had a second malignancy (out of field).

Conclusions
Early results suggest that PT was effective and feasible when irradiating head and neck tumors. Data on toxicity are promising. However, longer FU is needed to evaluate long-term toxicities and final results.
P 43 Evaluation criteria for chromosome instability detection by FISH to predict malignant progression in premalignant glottic laryngeal lesions

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Question
Chromosome instability (CI) detected by Fluorescence in Situ Hybridization (FISH) for chromosome 1 and 7 centromeres predicts malignant progression in premalignant head and neck lesions in which routine histopathological evaluation does not provide sufficient information. The aim of this study was to determine objective, clinically applicable evaluation criteria for CI detection.

Methods
We performed dual-target FISH for chromosome 1 and 7 centromeres on 4 µm formalin-fixed, paraffin embedded tissue sections of 87 laryngeal premalignancies (severe dysplasia and carcinoma in situ (CIS) excluded) to detect copy number variations (CNVs). Thirty-five randomly selected, normal head and neck squamous cell samples were used as a control. The chromosome 7:1 ratio (CR) and percentage of aberrant nuclei (PAN) were established in each lesion. The normal range of CRs (≥ 0.84 ≤ 1.16) was based on the mean CR +/- 3 x SD found in the normal population, the cut-off value for PAN was set at ≥10%.

Results
PAN showed a stronger correlation with malignant progression than CR (resp. OR 5.6, P= 0.001 and OR 3.8, P= 0.009). PAN combined with dichotomized histopathological diagnosis (HP) resulted in a prognostic model with an area under the ROC curve (AUC) of 0.75 (s.e. 0.061, sensitivity 71%, specificity 70%).

Conclusions
Evaluation criteria for FISH 1c/7c based on PAN ≥10% provide the best prognostic information in the risk-management of premalignant laryngeal lesions.