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ABSTRACT

The adhesion molecule NECTIN1 is a novel suppressor of metastasis in melanoma

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Metastasis is the leading cause of death in melanoma. Better understanding its molecular basis is critical to develop new therapies. Here, we found that the third most significant deletion in melanoma (chr11q23, frequency: 53%, q = 2.7E-21) only contained the gene *NECTIN1*, encoding an adhesion molecule required for the establishment of adherens junctions. Staining of human tissue microarrays showed that NECTIN1 levels were lower in melanoma metastases than in primary tumors (average score: 4.8 vs. 6.0, p = 0.01). We recently developed a method to functionally test any genetic alterations found in human melanoma in adult zebrafish (Ablain et al, Science 2018). Here we used it to generate *nectin1*-knockout tumors *in vivo*. In a zebrafish subcutaneous transplantation assay that visualizes cancer cells as they disseminate, *nectin1* inactivation increased cell spreading 6 fold (p < 0.001). Surprisingly, *nectin1*-knockout cell lines established from primary fish melanomas appeared exquisitely sensitive to serum stress and failed to propagate outside of rich media. In human cell lines, *NECTIN1* inactivation by shRNA or CRISPR similarly reduced cell proliferation by 20% (p < 0.001) and impaired spheroid formation in low-adhesion conditions, but increased cell migration 4 fold in the absence of serum in a transwell assay (p < 0.001). Mechanistically, serum starvation triggered the formation of adherens junctions between wild-type, but not *NECTIN1*-knockout cells, as evidenced by cadherin/catenin staining. Phospho-kinase analyses and cell surface proteomics revealed that NECTIN1-deficient cells instead activate a distinct adhesion program that includes an integrin/FAK/src axis. Combining human cancer genomics, functional testing in zebrafish and cell line studies, we thus uncovered a role for NECTIN1 in controlling a new link between serum sensing and cell-cell contact that balances growth and migration in melanoma.

Results from a checkpoint inhibition-naive cohort of patients in a phase 1b study of PV-10 and anti-PD-1 in advanced melanoma

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PV-10 (rose bengal disodium) is a small molecule oncolytic immunotherapy in development for solid tumors; intralesional injection can yield immunogenic cell death and induce tumor-specific reactivity in circulating T cells.

PV-10-MM-1201 (NCT02557321) is a phase 1b/2 study of PV-10 in combination with anti-PD-1 (pembrolizumab, "pembro") for pts with advanced cutaneous melanoma. Eligibility for Phase 1b required pts to have at least 1 injectable lesion, be naïve to checkpoint inhibition (CI), and be candidates for pembro. The combination was administered q3w for 5 cycles followed by pembro alone for up to 24 months; the primary endpoint is safety and tolerability with objective response rate (ORR) and progression free survival (PFS) key secondary endpoints (assessed by RECIST 1.1 after 5 cycles then q12w).

Full accrual of the main cohort was reached in April 2018, with 21 CI-naïve pts (2 IIIC/IIID, 8 M1a, 7 M1b, 4 M1c; median age 69 years, range 28–82) receiving at least 1 dose of PV-10 and pembro (2 pts with prior CI treatment enrolled are not included here). Treatment-Emergent Adverse Events were consistent with established patterns for each drug, principally Grade 1–2 injection site reactions attributed to PV-10 and Grade 1–3 immune-mediated reactions attributed to pembro, with no significant overlap or unexpected toxicities. Among the mostly Stage IV population a best overall response of CR was achieved by 10% of pts (1 each M1a and M1b), 57% of pts achieved PR (including all M1c pts), with PFS estimated at 51 weeks. Response assessment is ongoing and updated data will be presented.

The primary endpoint for phase 1b was met, with acceptable safety and tolerability and no unexpected safety issues identified. Two phase 1b expansion cohorts (24 pts each) are enrolling pts refractory to prior CI and pts with in-transit or satellite disease.

The aged tumor microenvironment promotes melanoma metabolic plasticity and therapy resistance

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"Aged" melanoma patients (>55 years old) have poorer prognosis and reduced response rates to targeted therapy relative to "young" patients (<40 years old). Here, we report an altered metabolic landscape in the aged tumor microenvironment (TME) as critical for melanoma aggressiveness. Aged fibroblasts uptake high levels of glucose compared to their young counterparts, which fuels lipid production. Melanoma cells cultured in an aged TME *in vitro* or *in vivo* display elevated intracellular lipid levels and increased metastatic potential relative to melanoma cells in a young TME. Further, lipidomics reveal an elevated lipid profile (i.e., triglycerides, ceramides, and cholesterol) preferentially secreted by aged fibroblasts relative to young. In turn, we show that melanoma cells adaptively increase extracellular fatty acid scavenging via the lipid transporter FATP2 in the lipid rich aged TME. Melanoma cells cultured in the aged TME also display an increased oxygen consumption rate (OCR) relative to those grown in young TME, which could be ablated when fatty acid transport into the mitochondria is blocked with etomixir. Notably, melanoma cells in the aged TME treated with BRAFi/MEKi in combination with a FATP2 inhibitor display decreased OCR relative to BRAFi/MEKi inhibitor alone, which paralleled the increased overall survival of aged mice treated with BRAFi/MEKi/FATP2i relative to either agent alone. To rule out off-target effects of the FATP2 inhibitor, we used a doxycycline inducible system for FATP2 knockdown in melanoma cells and reproduced the abrogation of tumor growth and extension of survival in aged immune-competent mice when combined with BRAFi/MEK therapy. Additionally, elevated FATP2 levels correlate with worse response in human patients. We hypothesize the aged TME triggers adaptive metabolic plasticity of melanoma cells critical for therapy escape.

Age-related changes in the extracellular matrix regulate melanoma resistance to targeted therapy

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Older patients with advanced cutaneous melanoma have a poorer prognosis than patients under the age of 55, and respond poorly to targeted therapy. While this may be due to several external cues, the aging tumor microenvironment may play a key role. We have extensively shown that dermal fibroblasts regulate melanoma cell invasion and metastasis, as well as the response to therapy. Using a combination of artificial 3D skin reconstructs, cell-derived matrices (CDM), and by analyzing mouse dermis, we show that the architecture of the extracellular matrix (ECM) produced by aged fibroblasts (~45–65 years old) is very different from the ECM produced by young fibroblasts (~18–35 years old): it is more anisotropic, with fibers that are thicker and less cross-linked. By using atomic force microscopy (AFM), we performed an elasticity analysis of aged CDMs and found that aged CDMs are stiffer than young CDMs. How these changes impact resistance to MAPK inhibition is yet unknown. Therefore, we seeded WM983B melanoma cells onto aged CDMs and analyzed the expression of resistance markers by western blot. When in contact with aged CDMs, WM983B express higher levels of AXL, EGFR and NGFR in comparison with young CDMs, all of which are established resistance markers. To analyze cell survival under BRAFi, live-cell imaging was performed on fluorescent labelled melanoma cells (nuclear histone marker H2B-GFP). On the aged CDMs, there was a higher number of cells in comparison with the young CDMs, which suggests that in the aged CDM there was less cell death. Our data demonstrates that the biomechanics of the ECM, which changes with age, regulates resistance to BRAFi in melanoma cells.

Real world outcomes of ipilimumab (I) and nivolumab (N) in patients with metastatic melanoma

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Background: The combination of I+N is approved for the treatment patients with advanced melanoma. Data collected in the clinical practice can deepen the understanding of these drugs in the real-life setting.

Objectives: To assess real-world outcomes (ORR, survival) and toxicity profile of I+N in patients with advanced melanoma.

Methods: We retrospectively collected data on patients treated at our institute from January 2014 through June 2018. Factors associated with response and survival were evaluated using chi square and Cox PH regression.

Results: 99 patients were treated with I+N, of whom 67% in first line. Mean age was 57.2 (+/-12.9y). At treatment initiation 21% had CNS involvement, 51.5% had normal LDH and 88% had ECOG PS 0–1.

After a median follow up of 12.2 m, 22% had to stop treatments due to toxicity, and 49.5% due to disease progression; 23.2% were still undergoing therapy.ORR and CR rates were 44% and 23.2% for

the whole cohort and 57% and 33.3% in the first-line population, respectively. mPFS was 6.8 m (95%CI 0.7–12.9 m) and mOS was17.7 m (95%CI 7.8–27.5 m).

Frequent (≥5%) immune related adverse events were hepatitis (16.5%), rash (14%), thyroid disorders (13%), colitis (10%), pneumonitis (8.5%) and arthritis (5%). Grade 3–4 toxicity rate was 33% and two patients died due to toxicity (pneumonitis and hepatitis).

Factors associated with tumor response were cutaneous melanoma (p = 0.01), ECOG PS 0–1 (p = 0.03) and first line (p = 0.01). Factors associated with OS were LDH level (p = 0.05) and first line (p = 0.02). mOS for M1c with and without liver involvement were 7.8 m and 23 m.

Conclusions: The results of this study reflect the efficacy and toxicity, as observed in the real-life setting. Severe toxicity was less frequent than previously reported, and CR rate was higher than predicted. Low disease burden is a confirmed predictive factor, where liver disease represents poorer prognosis.

Phase II single-arm multi-center study of adjuvant ipilimumab in combination with nivolumab in subjects with high-risk ocular melanoma

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Background: Treatment of primary ocular melanoma is often very effective, with local recurrence rates of < 5%. However, distant recurrence is as high as 50% depending on features of the primary tumor. These data emphasize the need for effective adjuvant therapy for patients with locally treated ocular melanoma. Several adjuvant treatments have been developed for patients with high-risk cutaneous melanoma, including ipilimumab and nivolumab monotherapies and an ongoing trial is exploring the nivolumab/ipilimumab combination (CA209-915), but patients with high-risk ocular melanomas have been excluded from these trials. As yet there is no approved adjuvant treatment for high-risk ocular melanoma patients.

Methods: We are conducting a Phase II single-arm multi-center study of adjuvant ipilimumab in combination with nivolumab in subjects with high-risk ocular melanoma. The primary endpoint is 3-year relapse-free survival rate. Secondary endpoints are median relapse-free survival, median overall survival, 3-year overall survival rate and safety. All patients will receive nivolumab 240 mg IV every 2 weeks plus ipilimumab 1 mg/kg IV every 6 weeks. Subjects may receive up to 25 doses of nivolumab and 8 doses of ipilimumab. The accrual goal is 50 patients across all participating institutions. Subjects treated in this study will be matched with controls selected from a contemporaneously collected OM registry, "contemporaneous control" in order to better assess efficacy. Control subjects will be from institutions not participating in this trial, will otherwise meet the trial eligibility criteria and will be further matched with trial participants for various demographic and risk factors to the extent possible. The study is enrolling in 6 comprehensive cancer centers in the US. NCT03528408.

Five-year survival outcomes in patients (pts) with *BRAF* wild-type advanced melanoma who received nivolumab (NIVO) monotherapy in the phase 3 CheckMate 066 study

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We previously reported a 3-year overall survival (OS) rate of 51% for pts who received NIVO monotherapy in the CheckMate 066 study (NCT01721772). Here, we report the 5-year survival outcomes from this study. In this multicenter, double-blind, phase 3 trial, pts with previously untreated, unresectable stage III/IV melanoma without a tumor *BRAF* mutation were randomized 1:1 to NIVO 3 mg/kg every 2 weeks (n = 210) or dacarbazine 1000 mg/m^2 every 3 weeks (n = 208). The primary endpoint was OS. At a minimum follow-up of 60 months for all pts, the 5-year OS rate was 39% in the NIVO group

and 17% in the dacarbazine group, with a median OS of 37.3 months (95% confidence interval [CI], 25.4–51.6) and 11.2 months (95% CI, 9.6–13.0), respectively (hazard ratio [HR], 0.50; $p < 0.0001$). Progression-free survival (PFS) rates at 5 years were 28% in the NIVO group and 3% in the dacarbazine group, with a median PFS of 5.1 months (95% CI, 3.5–12.2) and 2.2 months (95% CI, 2.1–2.5), respectively. The objective response rate was 42% (89/210 pts) in the NIVO group and 14% (30/208 pts) in the dacarbazine group; the complete response rate was 20% (42/210 pts) in the NIVO group. Median duration of response (DOR) has not been reached in the NIVO group, and 30% (27/89 pts) of responders had a DOR of at least 5 years. Median DOR was 6.0 months in the dacarbazine group, with no responder having a DOR of 5 years or more. Treatment-related grade 3/4 adverse events were reported in 16% of pts in the NIVO group and 18% of pts in the dacarbazine group. This follow-up analysis from CheckMate 066 shows that first-line treatment with NIVO monotherapy results in durable responses and long-term survival benefit in pts with *BRAF* wild-type advanced melanoma without any new safety signals.

Interim Results of Keynote 695/OMS-I103, a Phase II prospective multicenter clinical trial of intratumoral IL-12 combined with pembrolizumab for patients with unresectable stage III or stage IV melanoma with definitive progression on single agent or combination nivolumab or pembrolizumab

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Background: Intratumoral plasmid IL-12 (tavokinogene telseplasmid; tavo) with electroporation (IT-tavo-EP) is a plasmid DNA approach that forces localized expression of the proinflammatory cytokine IL-12, capable of converting poorly immunogenic/low T cell infiltrated tumors into highly inflamed immunologically active lesions. The primary objectives of this study is to evaluate the efficacy of pembrolizumab plus IT-tavo-EP in unresectable Stage III/IV melanoma patients who have definitively progressed on approved checkpoint inhibitors, and secondary objectives are to assess the immunomodulatory effects and the safety profile of therapy.

Methods: KEYNOTE 695 OMS-I103 (PISCES) is a phase 2, multicenter, open-label trial of IT-tavo-EP 0.5 mg/mL administered Day 1, 5 and 8 of each 6 week cycle and Pembrolizumab 200 mg IV every 3 weeks with pembrolizumab in patients with unresectable stage III/IV melanoma. Patients were required to have progressed on either pembrolizumab, nivolumab or ipilimumab/nivolumab and to have at least 12 weeks of therapy with either PD-1 antibody. The primary objective is objective response by RECIST v1.1 as determined by independent central review.

Results: Up to 100 patients are expected to be enrolled. TAs of July, 2019, approximately 70 out 100 patients have been consented and enrollment is ongoing. We will present the results of investigator response assessment as of September 2019. Safety data including irAE and AE of special interest is being collected and will be presented as well. Correlative analysis includes FACS and gene expression on pretreatment samples and post treatment samples.

$CD103^+CD39^+PD\text{-}1^+$ $CD8^+$ T cells are associated with a reduced risk of recurrence in adjuvant PD-1 treated-stage III melanoma patients

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Adjuvant anti-PD-1 therapy (adj-PD1) significantly prolongs recurrence-free survival in high-risk resected melanoma. However, patients (pts) can recur following therapy. This study investigated clinical and immunological factors associated with recurrence in consecutive pts receiving adj-PD1, with median follow up of 13 months (range 5–37 mo). Pt demographics and disease characteristics prior to adj-PD1 in pts who recurred (recurrence) ($n = 31$) and those who did not recur (control) ($n = 72$) were compared. Intratumoral $CD8^+$ T cells were quantified in pre-treatment tumour tissue in a subset of patients ($n = 25$ recurrence vs 15 control) using multiplex fluorescent immunohistochemistry. CD8 + T cell density was significantly higher in control pts (mean = 244.33 vs 796 cells/mm^2, $p = 0.0006$). We identified 8 phenotypically distinct $CD8^+$ T-cell populations based on CD103, PD-1 & CD39 expression.

While all populations were significantly higher in control pts, a $CD103^+CD39^+PD\text{-}1^+$ $CD8^+$ T cell population was most significantly higher in controls (mean = 24.92 vs 160.4 cells/mm^2, $p = 0.0003$). Examining the composition of the $CD8^+$ T cell compartment found two significant populations. $CD103^+CD39^+PD\text{-}1^+$ cells comprised a higher proportion of $CD8^+$ T cells in controls (6.2% vs 16.4% of total $CD8^+$ T cells, $p = 0.0042$), and $CD103^-CD39^-PD\text{-}1^-$ cells were higher in recurrence (26.7% vs 16.9% of total $CD8^+$ T cells, $p = 0.0464$). These data suggest that the presence of baseline $CD103^+CD39^+PD\text{-}1^+$ $CD8^+$ T cells may be associated with a lower risk of recurrence in melanoma pts treated with adj-PD1, and warrant further study as a potential biomarker determining response to anti-PD-1 therapy.

Metformin use is associated with decreased recurrence in diabetic patients with early stage melanoma

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Several retrospective studies involving lung and colorectal cancers have demonstrated improved clinical outcomes in pts taking metformin (Met), but the impact of this drug in diabetic melanoma patients has not yet been described. The ability of Met to inhibit complex I and disrupt tumor oxidative phosphorylation is an important requirement for tumorigenesis inhibition, and a recent pre-clinical study showed that Met reduces intratumoral hypoxia and results in improved T cell-function when combined with anti-PD1 therapy. Using an IRB approved protocol, we reviewed records from 2,798 melanoma patients (pts) treated at our institution between 1997–2018; 293 pts were diabetic. Pts were grouped by stage (AJCC 7): 72 (I), 92 (II), 85 (III), and 44 (IV). Notably, the incidence of recurrence for stage I/II pts taking Met (adjusted for age, sex) was significantly lower than pts not taking this drug (47.3% vs 71.8%, $p = 0.003$). This difference was also seen in stage III pts treated with immunotherapy +/- Met (69% vs 81.8%), but the results did not reach statistical significance. OS was significantly improved in stage III pts taking Met (132.8 vs 46.1 months at 50% survival probability, $p = 0.017$), but this was not significant after age adjustment. Stage IV pts treated with Met had increased OS as well (90.5 vs 49.8 months at 50% survival probability), but the difference was not statistically significant. Overall, this study shows that pts in earlier stages of disease appear to benefit from decreased recurrence when treated with Met. Differences in outcome with later disease stage may be more apparent with a larger cohort. Prospective studies of anti-PD1 + /- Met with rigorous translational endpoints are ongoing.

Reversing metabolic insufficiency in the tumor microenvironment: A trial of anti-PD1 plus metformin in advanced melanoma

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T cells have high metabolic requirements to carry out effector functions, a crucial variable when augmenting the immune response to cancer. T cells activated in the tumor microenvironment (TME) develop a phenotype of metabolic insufficiency; increased tumor oxidative (ox) metabolism in particular is associated with impaired T cell function. We have recently shown that increased ox metabolism in patients (pts) with advanced melanoma (mel) is associated with decreased response rates, progression-free survival (PFS), and overall survival (OS) on anti-PD1 immunotherapy (IMT). The type II diabetes drug metformin (Met) remodels the TME by inhibiting tumor cell ox metabolism, and preclinical models show robust synergy between anti-PD1 and Met. This is a single-site, investigator-initiated, randomized phase IB translational trial of pembrolizumab (P) vs. P + Met for non-diabetic pts with unresectable stage III/IV mel. Pts are randomized 1:1 to P vs. P + Met ($n = 15$ each arm). Met is 500 mg daily for 9 weeks (wks), with dose based on pre-clinical data. P is given per standard FDA approved regimen. Pts may receive P for up to 2 years; they are eligible if anti-PD1 naïve or if already on anti-PD1 with confirmed partial response or stable disease for 12 wks at randomization. The primary objective is to determine the change in cell cycle status (Ki-67) of tumor infiltrating CD8 + lymphocytes (TIL) in each cohort. Secondary objectives include measurement of hypoxia in the tumor (CA-IX, HIF-1a by IHC) and mitochondrial functional restoration of TIL (mitoFM). Punch/surgical biopsies are obtained at baseline and wk 9. Blood for research studies is drawn at baseline and wks 6, 9 and at disease progression. Restaging scans are obtained at baseline, wk 9, then q12 wks. Strategies to metabolically remodel the TME may have far reaching implications for pts receiving IMT.

Phase 1 trial of MEK1 inhibitor E6201 Plus Dabrafenib in patients (pts) with *BRAF V600*-mutated metastatic melanoma (MM) with central nervous system (CNS) metastases (mets)

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E6201 is a MEK1 inhibitor with activity against *BRAF V600E+* MM cell lines, including MAPK pathway resistance (*MEK1-C121S*). Brain distribution is unaffected by P-gp and BCRP efflux transport, in contrast to other MEK and BRAF inhibitors. A Ph 1 trial in 55 pts with advanced solid tumors confirmed an MTD of 320 mg/m^2 intravenously (IV) 1x/wk x 3 wks q 28 days (d) and activity in MM pts, including 2 *BRAF V600E+* pts with CNS mets and 1 pt with an ongoing near CR 9 + years.

This Phase 1 trial in *BRAF*-mutated MM with CNS mets (NCT03332589) includes a total of N = 28–34 subjects. The study is evaluating 320 mg/m^2 IV over 2 h 2x/wk x 3 weeks q 28d and dabrafenib 150 mg twice daily PO in monotherapy and combination Safety Run-in Phases followed by an Expansion Phase. Evaluations include cranial response rates (RR) by RANO-BM and RECIST 1.1, non-cranial systemic RR, cranial duration of response, progression-free survival and overall survival. Eligibility includes age ≥ 18 years; 1 prior immunotherapy and no prior BRAF/MEK for systemic disease allowed; prior SRS and surgical excision allowed.

Four pts (2M, 2F), *BRAF V600E+* (2), *V600K+* (1) and *K601E+* (1), have been treated in the Monotherapy Safety Run-In Phase. The median (range) age is 48.5 years (36–71), number of cycles received 1.4 (0.75–4), prior regimens 3.5 (3–7), CNS target lesions 2.5 (1–5) and systemic lesions 1 (0–5). One pt who received prior RT, pembrolizumab (pembro) and nivolumab (nivo)/ipilimumab (ipi) achieved CNS SD and a PR in lung mets after 2 cycles. A second pt who received prior RT x 2, dabrafenib/trametinib, pembro, nivo/ipi achieved CNS SD and a CR in a leg melanotic lesion for 4 cycles. E6201 has been well tolerated; 1 drug-related AE, thrombocytopenia (Gr 2) was observed, reversible. The Combination Safety Run-in Phase is open.

Correlation between system-specific irAEs, associated glucocorticoid exposure, and anti-PD-1 monotherapy efficacy in advanced melanoma

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Background: Anti-PD-1 antibody is front line therapy in advanced melanoma. Severe immune-related adverse effects (irAEs) require immunosuppressive treatment with glucocorticoids (GCCs). Data suggests that irAEs may correlate with better efficacy, but the predictive value of different system-specific irAEs and the relationship of GCC exposure to survival remains elusive.

Patients & Methods: Multicenter retrospective cohort study from Massachusetts General Hospital (MGH), Vanderbilt University Medical Center (VUMC), and Peking University Cancer Hospital (PUCH). Clinical notes and trial data were reviewed for irAE and associated GCC exposure, their correlation with survival was analyzed.

Results: Of the entire cohort of 647 patients, 344 (53.2%) developed irAE(s). Endocrine, musculoskeletal, and skin irAEs were correlated with improved PFS (HR = 0.41, 0.41, 0.41; 95% CI 0.22–0.77, 0.23–0.76, 0.23–0.76; p = 0.005, 0.004, 0.004; respectively); musculoskeletal irAEs also with longer OS (HR = 0.29, 95% CI 0.12–0.67; p = 0.004) in the MGH exploratory cohort (n = 169). High GCC treatment was associated with poorer post-irAE PFS (HR 2.19, 95% CI 1.15–4.19, p = 0.018) compared with irAE without high GCC exposure in the MGH cohort. These findings were validated in the VUMC cohort (n = 246). Data from PUCH (n = 232) suggested that high GCC exposure itself, rather than discontinuation of anti-PD-1 monotherapy, may be the main contributing factor for differences in post-irAE PFS between patients with different GCC exposure. No significant association with survival was found for gastrointestinal, pulmonary, and hepatic irAEs.

Conclusion: Endocrine, skin, and musculoskeletal irAEs were correlated with better survival. High GCC exposure mitigated the survival in irAE(+) advanced melanoma patients. Judicious use of GCC during anti-PD-1 monotherapy should be considered.

Should warfarin be the preferred anticoagulant for melanoma patients? The association between warfarin & survival in melanoma

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Introduction: Warfarin has been shown to reduce cancer risk, partly due to the anticancer effects of AXL tyrosine kinase inhibition by warfarin. AXL is implicated in disease progression & therapy resistance in preclinical melanoma models; however, there are no clinical human models evaluating the impact of warfarin on melanoma prognosis. We sought to evaluate the relationship between warfarin and survival in melanoma.

Methods: We performed a retrospective analysis of 10,611 melanoma patients ≥ 65 years of age diagnosed between 2009–2013 from the Surveillance, Epidemiology, & End Results-Medicare database. Patients were grouped according to warfarin therapy 6 months prior to/after diagnosis. Multivariable Cox proportional hazards & Fine & Gray competing risk models were used to compare overall (OS) & disease-specific survival (DSS) between groups.

Results: Only 13.4% of patients were prescribed warfarin with atrial fibrillation as the most common indication (74.1%). There were significant demographic & clinicopathologic differences between warfarin treatment groups ($p < 0.05$). Warfarin was associated with older age, greater comorbidities, & male gender ($p < 0.001$). There were no differences in surgical therapy, chemotherapy or radiotherapy associated with warfarin ($p > 0.05$). Warfarin was associated with greater OS in the multivariable model (aHR 0.89, 95%CI 0.79–0.99, $p = 0.04$), although not associated with improved DSS ($p > 0.05$). Subgroup analysis of patients with atrial fibrillation & by stage showed similar results.

Conclusion: Although warfarin was associated with improved OS among melanoma patients, the role of AXL inhibition remains ill-defined in melanoma as this study suggests that competing comorbidity risks may obscure melanoma-specific survival benefits. Future analyses will evaluate the impact of warfarin on melanoma incidence & recurrence.

Epidermal melanocyte subpopulations are defined early in human skin development

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Epidermal melanocytes are often considered a homogenous group of cells. However, human skin is phenotypically diverse with distinct morphological and functional characteristics based on anatomic location. For example, fibroblasts have topographical dependent gene expression patterns that are defined early in development and retained in adulthood. Epidermal melanocytes also exist across all anatomical locations yet baseline pigmentation differs from location to location. We hypothesize that, like fibroblasts, distinct subtypes of human epidermal melanocytes occupy distinct anatomical locations. In this study, we assessed the transcriptional diversity of freshly isolated human fetal, neonatal, and adult melanocytes from multiple anatomical locations using single cell RNAseq. Analysis of 6760 epidermal melanocytes from 22 patients identified distinct transcriptional programs that change with development and age. Within age groups, we have identified distinct subtypes of human melanocytes from lineages that diverge by day 67 of human development. At the single cell level, each subtype is enriched, but not exclusive to, either hair-bearing, glabrous or foreskin. Our study is the first to profile human epidermal melanocyte development and diversity at the single cell level and our discoveries suggest that major subtypes of melanoma - including acral and cutaneous - derive from distinct cells of origin.

Endocrinopathies in immunotherapy: a retrospective review to evaluate immunotherapy associated hypoglycemia

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Immunotherapy with checkpoint inhibitors in particular, has changed the way advanced Melanoma is treated. This evolution in treatment is leading to advanced knowledge on how to manage autoimmune side effects of immunotherapy. Endocrinopathies remain a potentially permanent complication of immunotherapy with hypothyroidism, hyperthyroidism, and hypophysitis being the most common. Post marketing research has demonstrated the additional risk for Type I Diabetes Mellitus often presenting in diabetic ketoacidosis. However, to my knowledge, the incidence of hypoglycemia has not been documented.

I performed a retrospective chart review of melanoma patients receiving checkpoint inhibitors between 1/1/2008 and 9/17/2018 with a random serum glucose or point of care glucose less than 70. Patients on medication for diabetes mellitus were excluded. I identified 47 patients who met this criteria. Glucose values ranged between 34–69. Median time to documented hypoglycemia is 114.31 days with 95% confidence interval (88.87, 233.06) days. Of the patients included in this analysis, 31.9% had a history of hypothyroidism, 27.7% thyroiditis and 19.1% adrenal insufficiency. The most common workup included thyroid function tests and adrenal axis testing. Future areas of research could seek to identify the incidence of true hypoglycemia as defined by Whipple's triad.

Safety and efficacy of immune checkpoint inhibitors (ICI) in patients with autoimmune conditions

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Autoimmune conditions (AIC) are common in patients (pts) with melanoma. Previous studies indicate that patients treated with ipilimumab (ipi) or PD-1 inhibitors are at high risk for exacerbation of the AIC, but clinical activity is preserved. Little is known about long-term outcomes or about the safety and efficacy of ipi+nivolumab (nivo) in these pts. We conducted a retrospective study of pts with unresectable stage III/IV melanoma and a history of pre-existing AIC (e.g. rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, psoriasis) treated at Memorial Sloan Kettering Cancer Center who received ≥ 1 dose of anti-CTLA-4 and/or anti-PD-1 therapy. Any systemic immunosuppression was recorded. Complete response (CR) was defined radiographically or pathologically by negative biopsy. Partial response (PR), stable disease (SD), and progressive disease (PD) were determined by the treating clinician. Disease control rate (DCR) was defined as CR + PR + SD.

We identified 52 pts with a median follow up of 20 months. 15, 28, and 9 pts got ipi, PD-1, and ipi+nivo respectively. Overall DCR was 71%. CR rate was 40% (21 pts). 23 pts (44%) discontinued ICI due to immune-related adverse events (irAEs), but only 5 (10%) were due to flare of the AIC. There were no deaths from irAEs. 9 pts received ipi+nivo; all had disease control (5 CR, 3 PR, 1 SD). 8 (89%) discontinued due to toxicity. 6 pts had a flare of the underlying AIC, but only 1 discontinued ICI due to the AIC.

In our cohort, only a small percentage (5%) of patients required discontinuation of ICI due to flare of AIC. In this small series with long follow up, CR, DCR, and toxicity are higher than that reported for pts without AIC, particularly those treated with ipi+nivo. Larger series are needed, but the results justify further study on pt outcomes as well as mechanisms of efficacy and toxicity in pts with AIC.

Repurposing chemotherapy to alter methylation, DNA repair and immune pathways to prime treatment-resistant melanoma for immunotherapy

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Melanoma is difficult to treat once resistance to immune checkpoint inhibitors occurs. Overcoming resistance may be achieved by repurposing chemotherapies, such as decitabine and carboplatin. We previously reported that methylation may cause of the lack of DNA repair in melanoma resulting in resistance to DNA-damaging therapies such as carboplatin. When used at low-dose, decitiabine results in global loss of methylation and re-expression of genes. We hypothesised that decitabine could be used to overcome resistance to carboplatin; increase expression of tumour neoantigens and immune-related pathways resulting in 'priming' for increased sensitivity to immune checkpoint inhibitors.

16 melanoma cell lines were treated with decitabine or carboplatin alone, or in sequential combination. Genome wide methylation changes were identified using MethylationEPIC arrays and global transcript expression levels identified using RNA-seq. Preliminary analysis of differentially methylated regions in responder cell lines identified enrichment at the HLA-locus and in cancer-related pathways including melanogenesis, endocytosis, and WNT signalling. RNA-seq confirmed down-regulation of cell cycle, telomere and chromosome maintenance and DNA replication. Most interestingly, upregulated pathways included immune system, class A/1 (Rhodopsin-like receptors) cytokine and chemokine signalling.

These results confirm that demethylation and DNA damage using decitabine and carboplatin elicits an apoptotic response and increased immunogenicity in melanoma cells. A Phase 2 clinical trial to test this combination with the addition of immunotherapy (PRIME002) has commenced. This will allow further analysis to determine if this combination can be used for treatment resistant advanced melanoma.

A scalable genomics-based approach to functional characterization of melanoma GWAS loci identifies the HIV-1 resistance gene *MX2* as a susceptibility gene

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Genome-wide association studies (GWAS) of cutaneous melanoma (CM) have identified 54 significant loci marked by 68 independent SNPs, however, the causal variants and genes underlying the majority of these loci have not been established. In order to identify susceptibility genes and functional variants underlying risk, we performed an

integrative analysis of cell-type specific quantitative trait loci (QTLs) and high-throughput functional assays. We performed multiple QTL analyses from a set of 106 primary melanocyte cultures based on SNP array and RNAseq (eQTL and spliceQTL), micro-RNAseq (miQTL), and DNA methylation (meQTL) data. Combining these with data from a new melanoma GWAS meta-analysis of 36,760 cases and 375,188 controls, we demonstrate that CM heritability is significantly enriched in genes specifically expressed in melanocytes, that imputation of gene expression onto GWAS (TWAS) identifies multiple additional loci, and that a majority CM GWAS loci (56%) colocalize with melanocyte eQTL or meQTLs, nominating candidate risk genes. We integrated these QTL data with those from a high-throughput sequencing-based reporter assay (MPRA), identifying functional risk variants for multiple loci. Among these, we show that a locus encompassing the HIV-1 restriction gene, *MX2*, is explained by a *cis*-regulatory variant (rs398206) binding YY1 in an allele-specific manner, where the rs398206-A risk allele correlates with higher melanocyte *MX2* levels. Melanocyte-specific transgenic expression of human *MX2* in a zebrafish model demonstrated accelerated melanoma formation in a $BRAF^{V600E}$ background. Thus, using an efficient scalable approach to streamline GWAS functional studies, we identified multiple candidate melanoma susceptibility genes and variants, and uncovered a pleiotropic function of *MX2* in melanoma susceptibility.

UV light degrades the dermis and inhibits primary melanoma invasion

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Melanoma incidence and mortality primarily affect the elderly population. Previous studies have identified the aged cutaneous microenvironment, specifically; aged dermal fibroblasts can contribute to melanoma metastasis. However, human skin ageing varies depending on anatomic site, and areas that accumulate UV damage present profound morphological changes in the dermis and prominent degradation of the extracellular matrix (ECM). In this study we investigated the effect of UV ageing on dermal fibroblasts and the effects of a photo-aged dermis on primary melanoma progression.

We examined differentially expressed pathways between donor-matched photo-damaged and photo-protected dermal fibroblasts from healthy individuals. The most up-regulated pathway in dermal fibroblasts from UV damaged sites was the collagen catabolism pathway, and the expressed levels of the main dermal collagenase MMP-1 significantly correlated with DNA damage in aged fibroblasts. We developed isogenic human dermal fibroblast cell lines with and without UV-driven DNA damage and confirmed that chronic UVB radiation leads to MMP-1 expression and secretion. In the absence of acute UV stimulation, UV-damaged fibroblasts degraded more collagen than UV-naïve fibroblasts. We confirmed this finding in dermal fibroblasts from healthy aged skin of elderly melanoma patients. We used spheroid models of melanoma to investigate if collagen density and degradation affect melanoma behaviour, and show low collagen density and degraded collagen both decrease melanoma invasion. Finally, we scored ECM degradation in 500 primary aged melanoma samples and confirmed that UV-induced dermal degradation is an independent prognostic factor in old patient survival, and we establish a significant inverse correlation between the number of melanoma cells singly invading in the dermis, detaching from the vertical growth component, and the amount of ECM degradation.

Clinical outcomes of melanomas associated with Germline mutations in cancer predisposition genes

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Background: Several pathologic germline mutations are known to increase risk of developing melanoma, including *BAP1*, *BRCA2*, *CDK4*, *CDKN2A*, *MITF*, *POT1* and *TERT*. Differences in clinical outcomes and characteristics between heritable and sporadic melanomas are not clearly defined. We aim to identify differences between familial and sporadic melanomas.

Methods: Eligible participants (n = 129) from a familial melanoma registry underwent pan-cancer gene panel testing of 81 genes to identify pathogenic germline variants. Mutation negative was defined as having a variant of uncertain significance or no known pathogenic germline variant. Mutation positive and mutation negative groups were compared against various clinical and demographic factors with χ^2 or *t*-test, depending on data type. Kaplan-Meier curves were generated for melanoma-specific survival and recurrence-free survival.

Results: Between mutation positive and mutation negative groups, no significant differences were identified for family history of cancer (p = 0.52), family history of melanoma (p = 0.44), histology (p = 0.28), ulceration status (p = 0.39), stage at diagnosis (p = 0.31), development of additional melanomas (p = 1), or location of primary tumor (p = 0.64). A significant increase was identified in mutation positive patients for development of additional cancers (p = 0.005). No significant difference was identified in recurrence-free survival (p = 0.5) or melanoma-specific survival (p = 0.19).

Conclusions: Melanomas associated with germline mutations have similar clinical characteristics and outcomes in comparison to

sporadic melanomas that do not harbor germline mutations. Patients carrying germline mutations are at higher risk of developing additional cancers, but not specifically additional melanomas. There may be a trend towards longer melanoma-specific survival among those who are mutation positive.

Coxsackievirus A21 (CVA21) and PD-1 blockade promote durable tumor regression in melanoma and responses are enhanced by prior host exposure to CVA21

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Oncolytic viruses are promising candidates to enhance the effect of immune checkpoint blockade inhibitors that have recently seen success in clinical trials and have gained FDA approval for treatment of advanced melanoma. These checkpoint inhibitors take advantage of intrinsic host immune surveillance by blocking negative interactions between tumor cells and T cells that would otherwise prevent an immune response to abnormal tumor tissue. Although this strategy has proven to be effective, a subset of patients do not respond and/or develop resistance to these immunotherapies, necessitating alternative interventions to improve patient responses. It is hypothesized that viral infection and oncolysis, coupled with immune checkpoint blockade, will enhance recognition and elimination of tumors via direct destruction of tumor tissue and by promoting tumor-specific adaptive immunity. Coxsackievirus A21 (CVA21/CAVATAK) is a wild-type virus which has a natural tropism for melanoma cells that express its known receptor, ICAM-1, on the cell surface. Cells expressing ICAM-1 can be efficiently infected and lysed by the virus, resulting in tissue destruction and the release of cytokines and tumor antigens that are important for activation of innate and adaptive immune responses. We are developing mouse models to test the efficacy of CVA21 infection in combination with αPD-1 blockade in melanoma. We also aim to better understand the underlying mechanisms whereby viral infection can be leveraged into anti-tumor immune responses. As tumor relapse is expected in a subset of mice, we further look to leverage these models to understand mechanisms of resistance to these therapies in order to develop improved treatment strategies.

Cooperation in heterogenous cell clusters accelerates melanoma metastasis

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Metastasis is the primary cause of cancer death, yet we know little about what determines its timing and aggressiveness. In melanoma, multiple studies have documented the coexistence of MITF+/proliferative (PRO) and AXL+/invasive (INV) cell states, but how these two states interact to promote metastasis is poorly understood. We utilized a zebrafish model of melanoma to longitudinally study these populations *in vivo* at single-cell resolution. From a $BRAF^{V600E}$ zebrafish melanoma we isolated two melanoma cell populations enriched for either proliferative (ZMEL1-PRO) or invasive (ZMEL1-INV) phenotypes. Intravital imaging revealed that the INV population formed cell clusters, extravasated collectively and metastasized more aggressively. Unexpectedly, we found that the INV and PRO cells self-aggregated into heterotypic clusters, with a central core of INV cells surrounded by PRO cells on the periphery. These heterotypic clusters co-extravasated and PRO and INV cells frequently co-metastasized. Strikingly, these two populations cooperated, with the less metastatic PRO cells metastasizing earlier when co-transplanted with INV. Mechanistically, RNA-seq and CRISPR-Cas9 validation established the TFAP2 transcription factors as master regulators of the PRO vs. INV state, with loss of TFAP2 both increasing cluster formation and driving metastasis. To find clinically actionable modulators of clusters, we performed a high throughput chemical screen and identified multiple FDA approved compounds that increase cluster formation, suggesting these drugs might increase the risk of metastasis in patients. Our data suggests a coherent framework for the co-existence of these two divergent cell populations in melanoma as they collectively cooperate to promote metastasis. Future work aims to understand the role of interrupting cooperation in clusters as a novel approach to prevent metastatic progression.

Loss of SOX10 induces a transition to an invasive/dormant-like phenotype in Melanoma.

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Melanoma heterogeneity and plasticity allow tumors to alter the invasive/proliferative balance, adapt to foreign microenvironments and resist targeted inhibitors. The mechanisms that underlie plasticity of melanoma cells remain poorly understood. We show that the neural crest transcription factor, SOX10, is heterogeneously expressed in melanoma. Using *in vivo*and 3D *in vitro*models, we demonstrate that SOX10 knockout/depletion induces a switch from a proliferative state to an invasive/dormant like state, associated with expression of an epithelial-to-mesenchymal transition (EMT) gene set including fibronectin and genes associated with cell cycle arrest. Consistently, TCGA data set analysis confirmed a reduction in SOX10 expression in regional lymph node and in cutaneous and sub-cutaneous regional metastasis. SOX10 loss of expression was detected in models of acquired resistance to targeted inhibitors, however its role has not been fully characterized. Herein we show that BRAF melanoma cells resistant to BRAF and MEK inhibitors (BRAFi/MEKi) loose SOX10 expression and show a similar phenotype to SOX10 knockout cells. Results were further confirmed in patient samples resistant to BRAFi and BRAFi/MEKi. Together, these data suggest that the level of SOX10 is associated with different cell states and thus, SOX10 plasticity may allow melanomas to adapt to altered microenvironments and drug treatments.

A phase 1/2 study of IDE196 in patients with metastatic uveal melanoma or solid tumors harboring *GNAQ/11* mutations or *PRKC* fusions

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Activating mutations in *GNAQ* & *GNA11* (*GNAQ/11*) Gα subunits of G protein coupled receptors have been seen in > 90% of patients with uveal melanoma (UM) and are considered genetic disease drivers. *GNAQ/11* mutations in other solid tumors, including cutaneous and mucosal melanoma, have also been seen in The Cancer Genome Atlas (TCGA) and FoundationOne databases. IDE196 (previously known as LXS196) is a selective protein kinase C inhibitor which has demonstrated preliminary anti-tumor activity in patients (pts) with metastatic UM (MUM). Pts with other solid tumors harboring *GNAQ/11* activating mutations may also benefit from IDE196.

In a phase 1 study (NCT02601378) in which 30 MUM pts were treated with IDE196 at doses of 200, 300 or 400 mg BID, 4 partial responses (PR) and 2 unconfirmed PRs were reported, for an overall response rate (ORR) of 13% (95% CI, 4%–31%), and a disease control rate of 73%. The most frequent DLT was transient hypotension, which was manageable. As of May 2019, 5 of 30 pts on BID dosing remain on IDE196 > 18 months, and 3 have been on > 2 years.

Study IDE196-001 (NCT03947385), a phase 1/2, multicenter, open-label study, will evaluate the safety & efficacy of IDE196 in pts with MUM and other solid tumors harboring *GNAQ/11* mutations or *PRKC* fusions. Approximately 40 MUM & non-MUM pts will be enrolled in dose escalation starting at 300 mg BID on a 28-day cycle. For phase 2 there will be a dedicated cohort for MUM and exploratory expansion cohorts for non-MUM pts with *GNAQ/11* mutations or *PRKC* fusions. The 1° endpoints are safety, pharmacokinetics, and investigator-assessed ORR by RECIST v1.1; 2° endpoints are centrally reviewed ORR, PFS, duration of response, pharmacodynamic biomarkers, and central confirmation of genetic alterations. This will be a global study.

Phase 3 LEAP-003: first-line (1L) Pembrolizumab (pembro) + Lenvatinib (len) in patients (pts) with advanced melanoma (MEL)

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Pembro (PD-1 inhibitor) confers robust and durable antitumor activity and survival benefit in pts with advanced MEL. Antitumor effect of anti–PD-1 + len (inhibitor of VEGFRs 1–3, FGFRs 1–4, PDGFR α, RET, and KIT) was superior to either agent alone in a preclinical study. Pembro+len showed antitumor activity and was well tolerated in pts with advanced MEL (phase

1b/2 KEYNOTE-146). LEAP-003 compares the efficacy and safety of pembro+len vs pembro alone in pts with advanced MEL (NCT03820986). Key eligibility: age ≥ 18 y; histologically/cytologically confirmed unresectable, untreated stage III-IV MEL; documented *BRAF*^{V600} status; ECOG PS 0/1; ≥1 measurable lesion per RECIST v1.1; provision of baseline tumor sample. Prior 1L standard-of-care targeted therapy only allowed for *BRAF*^{V600}-mutant disease. Prior neo-/adjuvant targeted therapy or immunotherapy was allowed if no relapse occurred during treatment (tx) or ≤ 6 mo after discontinuation. Pts (N~660) will be randomized 1:1 to pembro (200 mg Q3W IV) + len (20 mg QD PO; arm A) or pembro+placebo (QD PO; arm B), stratified by *BRAF* status (mutant/WT), prior adjuvant PD-1 inhibitor (yes/no), geographic region (China, yes/no). Pembro tx will continue for ≤ 2 y. If clinically beneficial, len or placebo may continue beyond 2 y until PD or unacceptable toxicity. Response (per RECIST v1.1) will be assessed Q9W to wk 54, Q12W to wk 102, Q24W thereafter; pts will be followed up for survival status Q12W. Pembro may be discontinued in pts with CR after ≥ 24 wk of pembro and ≥ 2 pembro doses after initial CR. Tx beyond initial RECIST-defined PD is allowed in eligible pts. AEs (graded per NCI CTCAE v4.0) will be assessed for ≤ 90 d. Dual primary end points: PFS (by BICR per modified RECIST v1.1) (max target lesions: 10; 5 per organ) and OS. Key secondary end points: ORR, DOR (both by BICR per modified RECIST v1.1), and safety.

Tumor-secreted MIDKINE drives immune checkpoint blockade resistance and defines poor patient prognosis

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Cutaneous melanoma is the most lethal form of skin cancer, characterized by high metastatic potential and a remarkable ability to evade immune surveillance. Therapies aimed at the deactivation of immune checkpoints have improved clinical responses. However, about 40–50% of patients still succumb to this disease. Resistance to immunotherapy is often observed in tumors that do not recruit cytotoxic T cells and/or that contain a high fraction of immunosuppressive cells, such as macrophages or T regulatory cells. Yet, mechanisms that define tumor immunogenicity and biomarkers of response, are still unmet needs in the field. We have previously identified a melanoma-secreted protein, called MIDKINE (MDK), with critical roles in lymphangiogenesis and metastasis. Here we will present the mechanistic characterization of novel immunomodulatory functions of MDK and their impact on patient prognosis and immunotherapy response. Loss- and gain-of-function studies of MDK were combined with computational studies in clinically-annotated melanoma datasets. Transcriptome and proteome analysis of melanoma cells were complemented with in vivo characterization of immune profiles analyzed in the context of vaccination and anti-PD1/PDL1 treatments. This approach uncovered an MDK-associated gene expression profile that is predictive of survival in melanoma and other tumor types. Importantly, we found MDK to rewire the transcriptome of melanoma cells ultimately favoring an immune-suppressive secretome that in turn, resulted in pro-tumoral macrophages and dysfunctional T cells. These autocrine and paracrine effects of MDK favored an enhanced resistance to vaccination and to anti-PD1/PDL1 treatments. These results provide insight on long-pursued mechanisms of tumor-immune evasion in melanoma and support MDK as a possible target for therapeutic intervention in immunotherapy-resistant patients.

Novel Epigenetic Regulators in Melanocyte Malignant Transformation and disease progression

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More than 80% percent of melanomas harbor mutations in either NRAS or BRAF. Our goal was to identify druggable targets that are key in the process of melanocyte tumorigenesis and molecules that can be used to prevent this event. No small molecule treatment for NRAS-mutant melanoma currently exist.To identify targets specific for the melanomagenesis driven by NRAS mutation, we integrated RNASeq data from primary human melanocytes (PHM) with and without an NRAS^{Q61} mutation and NRAS melanoma cell lines.To further select druggable and clinically relevant targets among potential targets identified by RNASeq data analysis, we used TCGA (The Cancer Genome Atlas) patient data to filter and found lncRNAs is up-regulated in > 90% of NRAS-mutant melanomas. Our analysis led to finding of a list of 28 unique lncRNAs differentially expressed in all comparisons between melanoma with and without NRAS mutation. To test the functional impact of the identified transcripts, we designed endoribonuclease prepared siRNA (esiRNA) libraries. After analyzing *in vitro* and *in vivo* data, we selected lncRNA BX470102.3 and AC004540.4 out of our eight top therapeutic targets for further functional and mechanistic studies. Their inhibition using siRNA or antisense oligonucleotide (ASO) specifically targeting these two lncRNA *in vitro* and *in vivo* was shown to stop progression to malignancy through impairing invasion, proliferation and colony

formation of NRAS-mutant cells, and ultimately inhibiting tumor growth and tumor progression of all tested NRAS-mutant melanomas. Moreover, inhibition of these two lncRNAs had a synergistic effect with MEK inhibitors. We believe these two lncRNA transcripts represent key components involved in progression of PHM^{Q61} cells from pre-malignant state to melanoma and new potential therapeutic targets in blocking NRAS mutant melanoma initiation, progression and metastasis.

Talimogene laherparepvec (T-VEC) in combination (combo) with ipilimumab (ipi) versus ipi alone for advanced melanoma (MEL): 3-year landmark analysis of a randomized phase 2 trial

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This is the 1st and only randomized trial testing the addition of an oncolytic virus to an immune checkpoint inhibitor in advanced MEL. The study met its primary endpoint: the combo of T-VEC plus ipi resulted in a significantly higher objective response rate (ORR) vs. ipi alone (39% vs. 18%; OR, 2.9; 95% CI, 1.5–5.5; $p = 0.002$) (Chesney et al. *J Clin Oncol*. 2017). Here, we present 3-yr analysis.

Pts with unresectable, stages IIIB to IV MEL were randomized 1:1 to combo or ipi alone. T-VEC was injected intratumorally on d 1 of wk 1 at 10^6 plaque-forming units (PFU)/mL followed by subsequent doses at 10^8 PFU/mL on d 1 of wk 4, and every 2 wks thereafter. IV ipi (3 mg/kg) was given every 3 wks starting on d 1 of wk 6 for up to 4 doses. Response was assessed by investigators per irRC until PD. The primary endpoint was ORR; key secondary endpoints were PFS, OS, and safety. This analysis occurred 36 months (mos) after the last pt was randomized.

198 pts were randomized (98 combo; 100 ipi). As of 25 Feb 2019, the median follow-up was 40.0 mos in combo arm and 34.3 mos in the ipi arm. ORR was 36.7% with combo and 16.0% with ipi (OR, 3.0; 95% CI, 1.6 to 6.0; $p = 0.002$). Median PFS was 13.5 mos with combo and 4.5 mos with ipi (HR, 0.78; 95% CI, 0.55–1.11; $p = 0.159$). Median OS was not reached in either arm (HR, 0.85; 95% CI, 0.55–1.32; $p = 0.480$). 45 pts (45.9%) in combo arm and 64 (64%) in ipi arm received subsequent anticancer therapy, with median time from randomization to the 1st subsequent therapy being 27.7 mos and 8.3 mos, respectively. No new safety signals were detected.

At 3-yr follow-up, T-VEC plus ipi combo continued to provide durable and statistically superior ORR vs. ipi alone. PFS was numerically longer with the combo than ipi. Survival is likely confounded by subsequent therapies.

A novel mechanism elevates Growth Hormone Receptor expression in melanoma and promotes migration and therapeutic resistance

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Melanoma is the most aggressive form of skin cancer and remains one of the most therapy-resistant forms of cancer despite recent success with targeted therapies. In this regard, adaptive upregulation of receptor tyrosine kinases (RTKs) are well documented, while the role of cytokine receptors in melanoma biology have been overlooked. Numerous clinical studies have reported a distinct association of human GH (hGH) with melanocytic nevi and melanoma incidences with significantly elevated hGH receptor (GHR) levels observed across different stages of melanoma tumours, yet the underlying mechanism prompting this increase is unknown. Here, we show for the first time that Ser/Thr kinases GSK3 and ERK1/2, both of which are phosphorylated in melanocytic nevi and melanoma tumours due to the driver mutations in RAF and RAS, regulate GHR on two different sites in the intracellular domain, at the post-translational level. Elevated ERK1/2 activation and GSK3 inhibition (via AKT activation) independently resulted in increased GHR availability on cell surface consequently amplifying signalling from autocrine GH. Melanoma cells subjected to acute GH treatment regulated key intracellular signalling pathways JAK/STAT, Src/ERK, and AKT, while sustained GH signalling resulted in a dose-dependent reduction in MITF levels. This concomitantly increased metastatic and de-differentiation markers (AXL, BRN2) resulting in phenotype switching. GH-GHR signalling in melanoma cells also provided a survival advantage to melanoma cells against nutrient deprivation cues, conventional therapy (BRAFi, MEK/ERKi) as well as UVR treatment. Thus, deciphering GHR-mediated melanoma progression is critical for developing new therapeutic strategies and understand the basis for melanoma susceptibility during pregnancy and in acromegaly, both of which have high levels of circulating GH.

Targeting AMPK/ACC in BAP1-deficient Uveal melanoma

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BAP1 is a major tumor suppressor gene on chromosome 3. It encodes a de-ubiquitinating (DUB) protein reported to regulate cellular processes in cancer including cell division, chromatin remodeling, DNA damage repair and stem cell pluripotency. Monosomy 3 is a marker of poor prognosis in uveal melanoma (UM) and loss of BAP1 expression or unmasking of BAP1 inactivating mutations are associated with elevated risks of metastasis of UM. Metastatic UM is aggressive and currently, there is a lack of effective treatment options. We explored the 80 UM sample dataset in the Cancer Genome Atlas (TCGA) for BAP1-specific dependencies and found that phosphorylation of AMP-dependent kinase (AMPK), an ATP:ADP sensor, and its downstream target, acetyl-CoA carboxylase (ACC), are elevated in BAP1-deficient samples. pAMPK and pACC levels were also higher in BAP1-deficient/mutant ($BAP1^{MT}$) UM cell lines than in BAP1-expressing/wild-type ($BAP1^{WT}$) lines. In two $BAP1^{MT}$ cell lines, MP46 and MP65, we found that AMPK is activated primarily by the calcium/calmodulin dependent protein kinase kinase 2 (CAMKK2), suggesting the role of Ca^{2+} signaling. However, the CAMKK2 inhibitor, STO-609, which decreases pAMPK levels, did not alter MP46 and MP65 2D growth. Activation of AMPK could be regulating tumor cell motility/invasion or promoting autophagy and resistance to targeted therapies. ACC, which regulates fatty acid metabolism, is shown to be phosphorylated in both an AMPK-dependent and -independent manner. We are testing ACC inhibitor effects. We also identified in the TCGA dataset that genes/proteins involved in fatty acid transport such as FATP2 and FATP6 were upregulated in BAP1-deficient samples and may be targetable by Lipofermata, an inhibitor of fatty acid transport. Overall, our studies aim to understand the role of AMPK and ACC activation and potentially uncover novel targets that may be therapeutically targetable in $BAP1^{MT}$ UM.

High intracellular lipid droplet concentration is associated with melanoma cell aggressiveness and phenotype switching

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Although treatment of metastatic melanoma has substantially prolonged overall survival, therapeutic resistance is inevitable. Thus, interventions to prevent therapeutic resistance or progression of melanoma to metastatic disease are needed. Using stimulated Raman scattering (SRS) and transient absorption (TA) imaging, increased amounts of lipid droplets and reduced pigmentation were detected in metastatic melanoma tissues compared to primary melanoma or normal adjacent tissues. This correlation was validated in eight genetically defined human melanoma cells, where lipid droplet-rich/pigment-poor melanoma cells displayed increased migratory capacity compared to lipid droplet-poor/pigment-rich melanoma cells. Lipid droplet-rich melanoma cells possessed the aggressive $MITF^{low}$/AXL^{high} phenotype whereas the lipid droplet-poor cells were $MITF^{high}$/AXL^{low}, suggesting that lipid content is involved in melanoma phenotype switching. Phenotype switching is characterized by the ability of melanoma cells to change from differentiated, non-aggressive cells to undifferentiated, invasive cells through altered molecular features. Incubation of 1205Lu $MITF^{low}$/AXL^{high} lipid-droplet rich cells in delipidized media decreased cell migration and altered cell morphology to resemble that of differentiated melanocytes compared to control cells. Further, AXL expression and protein were reduced. These results suggest that limiting lipid uptake through dietary or pharmacologic interventions could be useful to prevent melanoma phenotypic switching and avert metastatic progression.

Delayed immune-related toxicity after anti-PD-1 based therapy in melanoma patients

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Immune checkpoint inhibitors (ICI) have significantly improved the survival of patients with metastatic melanoma. However, treatment with ICIs is associated with immune-related adverse events (irAEs). Many irAEs occur early during treatment, but comprehensive data regarding delayed toxicities is sparse.

We retrospectively analysed all patients who received anti-PD-1-based therapy for advanced melanoma between 2013 and 2017 at our institution, defining delayed toxicities as irAEs that developed 1 year or more after the first dose of ICI.

Of 168 patients that were still alive at 1 year after commencing ICI, 59 were still on treatment. 20/59 patients (34%) developed irAEs of any grade, 6/59 (10.2%) grade 3 and 2/59 (3.4%) grade 4. 5 patients experienced > 1 irAE.

Of the 27 documented toxicities, 59% (16/27) were grade 1–2, 26% (7/27) grade 3 and 15% (4/27) grade 4. Amongst grade 3–4 irAEs, patients experienced colitis (n = 4), dermatitis (n = 3), hepatitis (n = 1), diabetes (n = 1) arthritis (n = 1) and haematological toxicity (n = 1). 1 patient experienced 3 distinct grade 4 toxicities, and required a subtotal colectomy for grade 4 colitis. Median time of onset of delayed toxicity was 22 months. 18 patients (90%) were still on treatment when experiencing delayed irAEs, while 2 had already completed ICI treatment.

Currently, ICIs are licensed for use for as long as there is clinical benefit, or until treatment is not tolerated. Most responses occur in the first year and can continue even after treatment is stopped for toxicity or patient choice. Our study suggests that 10.2% and 3.4% of patients who continue ICI beyond 1 year experience grade 3 and 4 toxicities respectively and this should be considered in decision-making regarding duration of therapy.

Impact of liver metastasis on outcomes in patients with stage IVc metastatic melanoma treated with immunotherapy

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Exploratory analyses have suggested that the presence of liver metastasis in patients with metastatic melanoma conveys a poorer response to treatment with immunotherapy.[1] Patients with stage IV M1c metastatic melanoma includes all patients with visceral metastases outside of the lung but excluding cerebral metastases. We performed a retrospective audit of patients with stage IV M1c metastatic melanoma treated with first line immunotherapy at a single institution in Australia between September 2015 and September 2018 and compared the outcomes in those with liver metastases versus those without liver metastases. 72 patients were included. 43 patients (60%) had liver metastasis and 29 patients (40%) did not have liver metastasis. Of the patients who did not have liver metastasis, the other sites of metastases included peritoneal, small bowel, adrenal, spleen and thyroid. We assessed response to first line immunotherapy. The overall response rate (ORR), including complete response (CR) and partial response (PR) in the liver metastasis cohort was 37% (CR in 10 patients and PR in 6 patients) compared with an ORR of 66% (CR in 13 patients and PR in 6 patients) in the cohort without liver metastasis. Stable disease was seen in a further 9% in the liver metastasis cohort as compared with 14% in the cohort without liver metastasis. These results are in keeping with previous reports that the presence of liver metastasis conveys a poorer response to immunotherapy. Further prospective research is required to determine if liver metastases are an independent predictive factor for immunotherapy outcomes within the stage IV M1c patient population.

[1] Tumeh P, Duad A et al. Liver metastasis and treatment outcome with anti-PD-1 monoclonal antibody in patients with melanoma and NSCLC, Cancer Immunol Res. 2017 May; 5(5): 417–424.

Harnessing *Sleeping Beauty* transposon mutagenesis to model tumor heterogeneity in melanoma preclinical models

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Many clinical challenges for treatment of patients with advanced cutaneous melanoma —incomplete drug repertoire, variable patient response rates, and inevitable drug resistance— are caused by hallmark levels of inter-/intra-tumor heterogeneity (ITH) which limits durable positive survival outcomes. Preclinical models are needed to design and test new treatment strategies that may advances precision melanoma treatment. Clinical challenges remain due to a lack of accurate preclinical systems that model the complexity and ITH of melanoma. We have created enhanced melanoma genetically engineered mouse models (GEMMs) that include the *Sleeping Beauty* transposon mutagenesis system (SB-GEMMs). In these mice, SB is used to both induce driver mutations and genetically labels cells to study clonal dynamics of ITH during melanoma progression. Using SBCapSeq, a genome-wide quantitative capture-hybridization enrichment sequencing method with single base pair resolution, we characterized ITH from bulk tumor and single melanoma cells from SB-GEMM mice. Genome-wide SB transposon mutagenesis, within melanocytes/melanoblasts *in vivo*, contain surprising and previously unknown levels of ITH that change based on fitness adaptation to defined selection pressures (e.g. limiting nutrients or drug treatments that alter cellular fitness. This feature allowed derivation of a SB|BrafV600E mutant immortalized melanoma cell line (SB-H80.1) from bulk tumor cells of an SB-induced cutaneous melanoma. Continuous SB mobilization within SB-H80.1 cells resembles the dynamics of population heterogeneity of human melanomas, and permits lineage tracing and population sampling by quantitative sequencing. SB-GEMMs are novel tools for discovering the heterogeneous genetic events that drive melanoma evolution and test effective preclinical treatment strategies to advance cutaneous melanoma research.

Identification of melanoma patients with low risk of sentinel lymph node positivity and favorable prognosis using a 31-gene expression profile test

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The 31-gene expression profile (31-GEP) test stratifies cutaneous melanoma (CM) patients based on 5-year metastatic risk as low (Class 1A), intermediate (Class 1B/2A) or high (Class 2B) and is an

independent prognostic factor. A Class 1A result was previously shown to identify patients with T1-T2 CM who have a low rate of sentinel lymph node (SLN) positivity, and low risk for metastasis and death in archival studies. This study describes SLN positivity rates in an expanded cohort and 3-year outcomes in a subset of this population. Clinicopathologic features and SLN status of 2152 CM patients tested with 31-GEP were collected prospectively using institutional review board (IRB)-approved protocols from 3 multicenter cohorts (731 previously unreported). CM patients prospectively enrolled in the EXPAND (NCT02355587) and INTEGRATE (NCT02355574) registries (n = 342) were evaluated for 3-year recurrence-free survival (RFS), distant metastasis-free survival (DMFS), and overall survival (OS). Seventy-one percent of T1-T2 patients ≥ 65 years were assessed by SLN biopsy, 63% of whom had a Class 1A result. Of those assessed by SLN biopsy (n = 497), 2.6% (95%CI 1.1–5.0) of Class 1A patients had a positive SLN versus 10.8% (95%CI 6.7–16.1) of Class 1B-2B patients (p = 0.0002). When considering all T1-T2 patients ≥ 65 years in the intention-to-treat population, 1.7% (95%CI 0.7–3.3) of Class 1A patients had a positive SLN. In the registries, Class 1A patients ≥ 65 years (n = 67) had 3-year OS, DMFS, and RFS of 98.1% (95%CI 94.6–100), 100%, and 98.4% (95%CI 95.3–100). The 31-GEP test can identify Class 1A T1-T2 CM patients ≥ 65 years at low risk for SLN positivity with favorable outcomes. Use of the test to guide SLNB decisions could improve health outcomes by identifying patients ≥ 65 years unlikely to benefit from additional surgical procedures.

Collaborative Ocular Oncology Group #2: Prognostic factors of uveal melanomas

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Only 5% of all melanomas are primarily uveal with distinct metastatic behavior compared to cutaneous melanoma. We prospectively compare the gene expression profile (GEP) and PRAME status of small and large uveal melanomas (UM), and analyze the clinical features of small tumors by GEP class.

The Collaborative Ocular Oncology Group Study 2 (COOG2) is a 22 center, prospective observational study of UM patients undergoing prognostic testing using the CLIA-certified DecisionDx-UM test (Castle Biosciences, Inc) between July 2017 and April 2019. De-identified data entered into a REDCap online database provided GEP and PRAME results for a small tumor cohort (STC), defined as tumor thickness£3 mm, and a large tumor cohort (LTC), tumor thickness > 3 mm. Statistical significance of comparisons between groups was calculated by MedCalc software (v19).

We enrolled 921 patients with UM, 272 STC patients (30%) and 649 LTC patients (70%). GEP was class 1A in 55%, class 1B in 28%, and class 2 in 17% in the STC, versus class 1A in 38%, class 1B in 22%, and class 2 in 40% of the LTC (p < 0.0001). PRAME was positive in 21% of STC tumors versus 37% of LTC tumors (p < 0.0001). Among the STC, PRAME was positive in 17% of class 1A, 21% of class 1B, and 40% of class 2 tumors (p = 0.004). Among the LTC, PRAME was positive in 27% of class 1A, 31% of class 1B, and 50% of class 2 tumors (p < 0.0001). Overall, STC was associated with class 1, spindle cytology, closer proximity to the optic disc, and PRAME negativity; LTC was associated with ciliary body involvement, epithelioid cytology, and PRAME positivity (p < 0.001). Clinical risk factors were not associated with any particular GEP class in STC.

Our study shows that most UM ≤ 3 mm thick are class 1 and PRAME negative, and thus have low metastatic potential. Traditional clinical nevus risk factors do not improve the accuracy of identifying small UM with high metastatic risk.

Prognostic biomarker AMBLor in surveillance strategies for AJCC Stage 1 Melanoma: a cost-effectiveness analysis

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The role of prognostic biomarkers tests in the surveillance of stage 1 melanoma is contentious. The argument for providing less-intense surveillance strategies is that they may be more acceptable to patients, clinicians and decision-makers if biomarkers such as AMBLor can differentiate accurately between high and low risk patients for recurrence that goes beyond AJCC staging as either 1a or 1b.

We developed a life-time microsimulation cost-effectiveness model that uses clinical, epidemiological, cost and quality of life data to assess what is the most cost-effective strategy [assessed in terms of the incremental cost per quality adjusted life year (QALY)] to follow up people treated for either stage 1a and 1b melanoma in the United Kingdom.

At low values for society's willingness-to pay (WTP) for health (<£5,000 per QALY gained), the model identified low-resource intensive surveillance by dermatologists (i.e. one visit at 12-months) as being the best value strategy for both 1a and 1b patients. As WTP thresholds increased, more resource-intensive strategies become more cost-effective. F™or 1b patients, the current recommended surveillance strategy for the UK (i.e. scheduled follow-up every 3 months for 3 years and then every 6 months up to 5 years by dermatologists) would have an incremental cost per QALY below

£20,000, the threshold value often adopted by the NHS in the UK. However, our model also predicts that the AMBLor test that stratifies patients into high and low surveillance regimens would be even more cost-effective and affordable compared to current surveillance recommendation.

The strategy of using the AMBLor prognostic biomarker test that can identify people as high and low risk of recurrence could allow people to be triaged into higher and lower intensity surveillance regimens, safety, effectively and efficiently.

Prognostic risk models following AJCC stage 1 Melanoma: a systematic review

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A risk prediction model is a statistical tool that uses multiple predictors to estimate the probability that an outcome will occur in an individual. We assessed the performance of published models to predict recurrence, new primary tumours and metastases of AJCC Stage 1 melanoma. In a systematic review we searched 10 bibliographic databases, grey literature and guidelines from 2000 to May 2018.

Eleven different risk prediction models were identified. The number of predictors per model ranged from 3 to 11, the most common were age, tumour site, tumour thickness, sex/gender and ulceration. Discrimination (ability to differentiate between high and low risk) was reported in 6 studies and the area under the operating curve [where 0.5 is fail and 1 is perfect ranged from 0.59 to 0.88. Calibration (agreement between observed and predicted risk) measures was reported in 3 studies. One study reported a calibration slope of 0.88 (p = 0.5), another reported concordance correlation coefficients of 0.9 and 0.93 for 5 and 10 year survival rates, both demonstrating high accuracy of the models. One study measured the overall performance of the model by assessing the Brier score (statistical measure of the accuracy of the measure; a higher score means higher inaccuracy). The proposed model showed a slightly better Brier score than the AJCC scheme. Eight studies conducted internal validation using data from their development set, so were at high risk of bias. Quality of all studies was low.

Existing prediction models have the potential to predict recurrence, new primary tumours and metastases of AJCC Stage 1 melanoma. However, it is unclear if most of the models would produce the same results in other populations, as none of these tools have undergone rigorous validation. To ensure clinical relevance risk prediction models need to adhere to standard methods for model development and validation.

Surveillance strategies for AJCC Stage 1 Melanoma: The health technology assessment (HTA) approach

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The majority (72%) of melanoma cases diagnosed in the UK are AJCC stage 1. However, risk of recurrence, metastasis and second primaries is heterogeneous within this group. The optimal intensity of surveillance regimens of these patients' remains an open question; one that a Health Technology Assessment (HTA) process can help answer.

A HTA combines clinical, epidemiological and economic data using rigorous, reproducible evidence synthesis methodology, which include systematic reviews, economic evaluation and potentially a value of Information analyses. We undertook systematic reviews to inform the development of an economic evaluation microsimulation model. Such a model estimates cost and health outcomes based on individual event histories associated with key components of a disease process.

Following on from our systematic reviews, we liaised with local clinical experts to establish plausible surveillance strategies. These strategies varied by which speciality was responsible for follow-up care [i.e. Dermatologist, Surgeons and Clinical Nurse Specialists], frequency of scheduled appointment (Every 3, 4, 6 or 12 months) and follow-up duration (1–20 years). We incorporated epidemiological and clinical data such as recurrence and mortality rates, speciality and tests accuracy, utility values, opportunistic self-examination diagnosis, false alarms and systematic therapy costs associated with advanced stage diagnosis.

Our analysis showed that as society's willingness-to-pay (WTP) for more health increased, more intensive strategies for longer follow-up duration with dermatologists would be worthwhile for both 1A and 1B patients.

Decreased antiviral type I IFN signaling in mucosal melanoma is associated with resistance to anti-PD-1 immunotherapy

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Cutaneous melanoma (CM) is the most common type of melanoma and occurs on UV-exposed skin. However, rare subtypes of

melanoma, such as acral (AM) and mucosal (MM), arise from melanocytes in non-sun exposed sites. Despite strong genomic similarity between AM and MM, including low tumor mutational burden (TMB), AM and MM respond differently to immune checkpoint blockade (ICB). AM responds to anti-PD-1 treatment similarly to CM (50% vs. 52%, $p > 0.88$) despite its extremely low TMB, whereas MM has a low response rate (9%, $p < 0.01$). Since genomic features do not correlate with ICB response, we analyzed transcriptomic profiles across melanoma subtypes by performing RNA sequencing on 45 melanoma patient-derived xenograft (PDX) models. These models included 14 AM and MM, and 20 ICB- treated melanomas. Surprisingly, CM and AM were similar transcriptionally, and MM differed from CM and AM, corresponding to trends in ICB response. Half of all genes differentially expressed in MM were also dysregulated in ICB non-responders. Gene ontology analysis identified downregulation of the antiviral type I IFN pathway across the majority of MM, and in CM ICB non-responders. The type I IFN pathway has many critical functions in innate and adaptive immunity. Frequently, type I IFN gene promoters are hypermethylated in cancer. Treatment of MM and ICB NR cell lines with hypomethylating agents (5'-Azacitidine or Decitabine) induced expression of type I IFN response genes and reduced cell viability. In summary, ICB response is associated with transcriptomic, not genomic, features across melanomas. Decreased antiviral type I IFN signaling likely contributes to ICB resistance in MM and a subset of CM. Therefore, pharmacologic re-activation the type I IFN pathway, alone or in combination with anti-PD-1, represents a new treatment strategy for MM or CM with primary ICB resistance.

A tag SNP in PDGFRA is associated with reduced PDGFRα expression and better survival in Chinese melanoma

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Purpose: Polymorphisms of PDGF signaling pathway can predict cutaneous melanoma survival in the United States population. The aim of the study is to characterize the functional effect of tag SNP rs2228230 and assess its association with the clinical outcomes of Chinese melanoma.

Methods: The PDGFRA mRNAs and PDGFRα expression in FFPE samples were measured using RNAscope and IHC. The association of rs2228230 genotype with survival was analyzed in a high-dose interferon a-2b (HDI), and a chemotherapy cohort. The functional effects of rs2228230:C>T were analyzed by *in silico* and *in vivo* analyses.

Results: The exspression level of PDGFRA was significantly reduced in melanoma tissues with rs2228230:T genotype. In the chemotherapy cohort, univariate and multivariate Cox regression analyses revealed that the PFS and OS of melanoma patients with rs2228230:T allele were significantly longer than patients with CC genotype. In the HDI cohort, the PFS and OS of patients with rs2228230:T allele also tend to be longer than patients with CC genotype. The mRNA folding analysis showed that the Minimum Free Energy of PDGFRA with rs2228230:T allele was higher than rs2228230:C allele, and the relative synonymous codon usage value of GTT (T allele) is lower than GTC (C allele) in both genome and PDGFRA gene level. The *in vitro* results demonstrated that the genotype of rs2228230 affected the expression of PDGFRA in both mRNA and protein level. Rs2228230:T allele reduced the mRNA and protein stability of PDGFRA compared to rs2228230:C allele. Furthermore, the signaling activity of MAPK and PI3K/AKT pathway was decreased in cells with rs2228230:T allele than cells with rs2228230:C allele.

Conclusion: The rs2228230:T is associated with better outcome in Chinese melanoma patients through reducing the expression of PDGFRA and decrease the activity of downstream signaling pathway.

Elucidating the molecular mechanisms regulating delayed-early genes in BRAF-mutant melanoma

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BRAF is the most common mutated proto-oncogene in cutaneous melanoma. Mutationally active BRAF signals through MEK->ERK MAP Kinase pathway to regulate key cancer cell hallmarks including entry into the cell division cell cycle, reduced program cell death, and enhanced cell motility. Amongst the genes regulated are those encoding integrins, heterodimeric transmembrane proteins that regulate cell adhesion to the extracellular matrix. Altered integrin expression has been linked to the acquisition of metastatic behavior by melanoma cells leading to diminished patient survival. We have previously documented the ability of RAF-activated MAPK pathway to induce the expression of *ITGB3* encoding integrin β3 in several different cell types. RAF-mediated induction of *ITGB3* mRNA requires sustained, high-level activation of RAF→MEK→ERK signaling mediated by oncogene activation and is classified as "delayed-early", in that it is sensitive to the protein synthesis inhibitor cycloheximide. However, to date, the regulatory mechanisms that allow for induced *ITGB3* downstream of sustained, high-level activation of MAPK signaling remain obscure. We have identified over 300 delayed-early genes (DEG), including those expressing additional cell surface proteins, that display similar regulatory characteristics as *ITGB3*. We use integrin β3 as a model to test our hypothesis that there is a different mechanism of regulation for DEG compared to the canonical regulation of Immediate-Early genes. We are relating the chromatin changes

seen during RAF activation to active enhancer histone marks. To elucidate the essential genes of this regulation process, we are employing the use of a genome-wide CRISPR knockout screen. The work presented from this abstract will help elucidate the regulatory properties of oncogenic progression in BRAF mutated melanoma that could lead to the identification of biomarkers.

Oncogenic gene fusions in malignant melanomas

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Oncogenic gene fusions have been identified in several hematological malignancies and solid tumors, including melanomas. Gene fusions occur when two independent genes form a hybrid gene through genomic rearrangements, which often leads to abnormal expression and function of the resulting proteins. Gene fusions may be targeted by small molecule inhibitors and thus may be clinically relevant genetic alterations. Approximately 34% of melanoma tumors are *"pan-negative"* for known recurrent driver mutations in five genes: *BRAF; NRAS; KIT; GNAQ*; and *GNA11*. Herein we report the observed gene fusions from screened melanoma samples.

Data was accessed from a retrospective database of 2552 melanoma samples profiled from October 2015 to October 2018 as part of routine clinical testing from FFPE. A subset of samples were screened for fusions using an RNA-based anchored multiplex PCR targeted enrichment panel for 52 genes with the ability to detect fusions and 3 variant transcripts. Additional biomarkers were evaluated using a DNA-based next generation sequencing panel of 592 genes, including TMB and MSI. In addition, PD-L1 protein expression was evaluated by IHC. Eight in-frame oncogenic gene fusions were identified from *n* = 314 tumors (2.5%) involving *BRAF* (*n* = 4), *RAF1*, *MET*, *ALK*, and *MAML2* genes. Three fusions have not been previously reported: *VIM-BRAF*; *KHDRBS3-BRAF*; and *PRKD3-ALK*. Except for one sample with a co-occurring activating mutation in *NRAS*, no fusion positive patient harbored known driver mutations. Among these eight cases, PD-L1 was expressed in three cases and average TMB was 15.5 mutation per megabase.

These data suggest that comprehensive molecular tumor profiling of melanomas may reveal rare gene-fusions, potentially targetable with existing drugs. Efficacy of such targeting is unknown. However, knowledge of these gene fusions could lead to biomarker-driven clinical trials in the future.

Activation/Exhaustion Profiling of CD8$^+$ cells shows distinctive patterns correlating with response in melanoma.

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Background: T cell exhaustion markers have been used prognostically for immunotherapy. We explored activation markers such as CTLA-4 in CD8 + cells to develop activation/exhaustion profiles for CD8 + cells and correlate these to response.

Methods: We identified 148 patients with stage III unresectable or stage IV melanoma that were treatment naïve and subsequently got PD-1 therapy following biopsy and 61 patients progressing on PD-1 prior to biopsy. Approx 2 × 10^6 T cells/patient were stained with anti-hCD3, anti- hCD8, anti-hCD45, anti-CD4, anti-Foxp3, anti–hCTLA-4 (14D3), anti–PD-1, anti–HLA-DR, anti–PD-L1, and LIVE/DEAD Fix- able Aqua Dead Cell Stain.Objective Responses were evaluated by RECIST 1.1, CR/PR were classified as "responders" and SD/PD as "non-responders".

Results: The percentage of CD8 + cells with dual expression of PD-1/CTLA-4 (cpCTL) as a % of all CD8 + cells correlated with response. Mean cpTIL was 27.1% for treatment naïve responders(R), 16.52% for treatment naïve non-responders (NR) and 8.59% for PD-1 resistant (post) patients (ANOVA p = 0.0003 for R/NR, <0.0001 for R/post). No differences were seen with Age, Gender, Stage or LDH. No differences were seen in Treg frequency by response or demographic variables. CTLA-4 expression (CTLA-4 MFI) showed a trimodal distribution and was correlated with response (ANOVA p = 0.0002). The ORR was 34.3% for CTLA-4 MFI < 250, 70.1% for CTLA-4 MFI 251–800 and 30.76% for CTLA-4 MFI > 801 (ANOVA p = 0.0002). For PD-1 MFI, < 400, the ORR was 30% vs 63% for PD-1 MFI > 400. Based on these 2 variables, we have made a 6 zone model.

Conclusions: CD8 + activation (CTLA4 MFI) can be separated from dysfunction (PD-1 MFI) and optimal zones exist for both as markers for PD-1 response. We define a 6 activation/exhaustion zones in tumor CD8 + cells and show that optimal response correlates with these zones.

A Phase 2, open-label, randomized, multicenter trial of Encorafenib + Binimetinib evaluating a standard-dose and a high-dose regimen in patients with *BRAF*V600-mutant melanoma brain metastasis (MBM) (POLARIS)

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Background: Melanoma that metastasizes to the brain has a poor prognosis, and accounts for up to 54% of melanoma deaths. Clinical data show that treatment with *BRAF*-targeted agents induces responses in *BRAF*V600-mutant MBM. The *BRAF*/MEK-targeted combination encorafenib + binimetinib demonstrated favorable efficacy and safety for patients with *BRAF*V600-mutant melanoma in the COLUMBUS study, but excluded patients with active MBM. The aim of this study is to evaluate encorafenib + binimetinib in patients with *BRAF*V600-mutated active MBM. A higher dose of combination therapy will be studied versus a standard dose to evaluate whether greater efficacy may be achieved with acceptable safety for patients with *BRAF*V600 MBM.

Methods: Eligible patients in this multicenter, randomized, open-label phase 2 study adults with *BRAF*V600-mutant MBM will have at least 1 measurable MBM, no prior local MBM therapy, no corticosteroids for MBM, and no prior *BRAF* or MEK inhibitors in the metastatic setting. One prior line of checkpoint inhibitor or adjuvant *BRAF* or MEK inhibitors is permitted. Patients will be randomized (1:1) to either the standard dose (450 mg orally QD and binimetinib 45 mg orally BID) or high-dose (encorafenib 300 mg BID and binimetinib 45 mg BID) stratified by baseline tumor burden (1–2 vs. ≥3 brain lesions at baseline) and prior checkpoint inhibitor (yes vs. no). The first 9 evaluable patients in the high-dose arm will constitute the safety lead-in cohort. If the high-dose is not tolerated, subsequent patients will receive standard-dose therapy. Assessments include intracranial response (per modified RECIST), extracranial response, global response rate, DCR, DOR, PFS, OS, PK, and safety. The study will enroll approximately 100 patients. (NCT03911869)

Preliminary efficacy and safety of first-line atezolizumab monotherapy in patients with BRAF^{V600} wild-type, locally advanced or metastatic melanoma

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The anti–PD ligand-1 (PD-L1) antibody atezolizumab is approved for the treatment of a variety of solid tumors. The objectives of this cohort (Cohort C) of a multisite, multicohort phase Ib study were to evaluate the efficacy and safety of 1200 mg atezolizumab every 3 weeks in adult patients (pts) with BRAF^{V600} wild-type, histologically confirmed, advanced or metastatic melanoma who had not received prior systemic therapy for advanced disease. The co-primary endpoints were confirmed objective response rate (ORR) per RECIST v1.1 and disease control rate (DCR; complete response, partial response [PR], or stable disease [SD] at 16 weeks). Safety was a secondary endpoint (CTCAE v4.0). The majority of the 52 pts enrolled had lactate dehydrogenase levels less than upper limit of normal (77%) and had PD-L1–positive tumors (55%). At data cutoff (14 March 2019), median follow-up was 6.3 months and 27 pts (52%) were continuing treatment. Investigator-assessed confirmed ORR was 35% (95% CI 22–49%) and included 3 CR (6%) and 15 PR (29%). The DCR was 46% with 16 of the 18 pts (89%) having ongoing response at data cutoff. Tumor response by independent review was similar with an ORR of 31% and DCR of 43%. Median PFS as assessed by the investigator was 3.7 months (95% CI 2.1–7.3). All pts experienced at least 1 adverse event (AE) and 21 pts (40%) had a grade 3/4 AE. Only 1 pt discontinued treatment because of an AE. The most common any grade AEs were anemia (27%), headache (19%) hypertension (19%), constipation (17%), diarrhea (17%), hypothyroidism (17%), asthenia (15%), and pain in extremity (15%). Together these data show that first-line atezolizumab monotherapy is safe and tolerable and has antitumor activity in pts with BRAF^{V600} wild-type, advanced or metastatic melanoma.

Whole genome landscape of conjunctival melanoma

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Melanomas are broadly classed as cutaneous (common, acral), mucosal and uveal. Common cutaneous melanoma is generally associated with exposure to ultraviolet radiation (UVR), whereas the other subtypes are rarely driven by this environmental insult. Conjunctival melanoma is a rare mucosal melanoma that arises from the conjunctiva and ocular external mucosa, but whose genomic landscape has not yet been fully explored.

We performed whole genome sequencing on 10 primary conjunctival melanomas and matched blood samples. This revealed a broad range of total mutations (8025 to 227551) and a UVR signature in 9 of 10 tumours, with the non-UVR signature tumour coming from a sun-protected location in the eye. Six of the 10 tumours carried *BRAF* mutations (3 V600E, 2 S467L, one G466E) and distinct from cutaneous melanoma, *NRAS* Q61 mutations were absent. Intriguingly, the common uveal melanoma mutations (*GNAQ*, *GNA11*, *BAP1*, *EIF1AX*, *SF3BP1*) were also absent. In 5 of the 10 samples, there were significant copy number alterations and structural variants. Curiously, we did not observe missense/nonsense mutations, indels, or large copy number alterations in known oncogenes in the non-UVR signature melanoma, suggesting a similar aetiology to the so-called triple-wild-type subtype of common cutaneous melanoma.

Taken together, although conjunctival melanoma is a mucosal melanoma, our results reveal a genomic landscape more similar to that of common cutaneous melanoma and distinct from other mucosal melanomas, or indeed other rare melanoma subtypes. Rather, conjunctival melanoma is characterised by particularly high numbers of total mutations, with UVR imprinted mutational signatures in 9 of our samples. Thus, although it is a mucosal melanoma, our data suggest that conjunctival melanoma should be treated in a similar fashion to common cutaneous melanoma.

Nephrotoxicity related to anti-PD1 treatment: histopathologic features driving the optimal clinical management

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Treatment with immuno-checkpoint(s) inhibitors (ICI) has enabled to identify novel immune-mediated (im) side effects. Among these, the im-nephrotoxicity(im-N) accounts for less than 1% but it can cause significant morbidity, sometimes mortality. Treatment guidelines are not always univocal, clinical features or laboratory parameters may not be sufficient to drive optimal therapeutic management.

We report the clinical management of patients (pts) treated with ICI, who developed an im-N. All pts received a renal biopsy, a pathological review was performed. Im-N was graded according to CTCAE v4.0, Banff working schema 2013 and to KDIGO (Kidney Disease Improving Global Outcomes) Guideline for Acute Kidney Injury (AKI) Three male pts with metastatic melanoma, treated with anti-PD-1 monotherapy or combination with IDO or BRAF/MEK inhibitors at the Center for Immuno-Oncology of Siena, showed a biopsy-proven AKI graded according to Banff score (1b in 2 pts; borderline in 1 patient). The median time to onset and resolution of AKI was 39 and 11 weeks, respectively. Histopathologic (hp) features showed signs of tubule-interstitial inflammatory cell infiltration, a scenario similar to acute T-cell-mediated rejection in transplanted kidneys. In light of this hp characterization, the therapeutic management of allograft rejection was activated by administering methylprednisolone at 250–500 mg i.v. for 6 days with slow tapering, if Banff score ≥ 1a (2/3 pts). This treatment led to a fast improvement of renal function and 2/3 pts were able to resume treatment.

Im-N can induce AKI due to acute interstitial nephritis. Renal biopsy should be recommended and hp features, according to Banff score, utilized to drive a more effective clinical management. A tight collaboration between oncologist, pathologist and nephrologist seems to be advisable for the optimal management of im-N.

Ambra1 impacts on melanoma development and metastatic potential.

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Despite the successful application of immune and targeted therapies, the treatment of advanced melanoma as well as the molecular mechanisms underlying its development and progression are still in need of further investigation. Indeed, drug resistance invariably develops and melanoma still remains the deadliest form of skin cancer. Recently, we have found a promising candidate for melanoma biology in the protein Ambra1. Ambra1 is a multifunctional scaffold protein regulating several oncogenic processes (*e.g.* autophagy, cell proliferation and cell death). Moreover, Ambra1 plays a critical role in the development of the central nervous system, with its functional deficiency in mouse embryos leading to neuroepithelial hyperplasia associated with autophagy impairment. By applying the genetically engineered mouse model of melanoma BrafV600E/Pten-depleted – a highly predictive preclinical model resembling the human disease – and a panel of human melanoma cell lines, we discovered that Ambra1 deficiency impacts on melanocytic nevi formation and promotes both melanoma development and metastatic progression. In line with these findings, expression of Ambra1, which was frequently found low in human melanoma cells, inversely correlates with expression of EMT-promoting factors as well as an aggressive phenotype. Altogether, our preliminary data reveal a potential role of Ambra1 in melanoma ontogenesis and progression. Further studies will help evaluate the responsiveness of Ambra1-deficient melanomas to current therapies and assess the prognostic relevance of AMBRA1 in melanoma patients.

Copy number alterations (CNAs) in primary melanoma and survival

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Our previous work reported a large-scale copy number (CN) study of primary melanoma [1]. Next generation sequencing (NGS) data from 303 formalin fixed paraffin embedded (FFPE) samples from the Leeds Melanoma Cohort (LMC) were generated. Libraries were generated by random shearing and then sequenced (1.7x coverage). In this study, problematic regions and common germline variations in the genome were identified and excluded accounting to approximately 13% of the autosomal genome. CN was generated by read count accumulated into 10k bp windows, adjusted jointly for sequence mappability and GC-content and comparison with Caucasian genomes (n = 312) from the 10k Genome Project [5, 6]. Minor difference in terms of frequency which may be due to the type of samples processed (frozen vs FFPE), platform used (NGS vs SNPS Array), or disease stage (primary vs metastatic) offer opportunity for discovery of novel CNA in melanoma. The Fraction of Genome Altered (FGA) was calculated as proportion of the genome length with aberration (deletion or amplification at a cut-off of absolute value of 0.10) in relation to the whole somatic genome, and was tested for association with survival using Cox proportional hazards model. FGA significantly predicted poorer melanoma specific survival after adjusting for age, sex and stage (HR = 1.5 for high FGA vs low FGA based on a dichotomy, Log rank $p = 3 \times 10^{-8}$). We found internal deletion of *CCSER1*, located at chromosome 4q22.1, to be associated with poor survival (HR = 2.5, $p = 3 \times 10^{-10}$), and significantly discriminated sample groups in terms of genomic instability. Whole genome correlation of 10K window CN with the transcriptome using Spearman correlation identified *PPP6C* gene expression at Chromosome 9q33.3 as the most associated with copy number (Rho = 0.60, $p = 3 \times 10^{-27}$).

Defining the differential packaging of microRNAs into extracellular vesicles after targeted therapy.

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Cell-to-cell communication between cancer cells and the surrounding/distant microenvironments is critical for survival, angiogenesis, and metastasis of tumorigenic cells. Extracellular vesicles (EVs), play an important role as cell-cell mediators when released into the extracellular space. EVs can carry proteins, metabolites, mRNA and small non-coding RNA molecules. Among these, microRNAs have great potential, as they have been shown to be dysregulated in cancer, involved in cancer progression, and in resistance to targeted therapy. Due to the stability of microRNAs inside EVs in body fluids, their potential as biomarkers of progression and response to therapy could be exploited. Previous experiments have shown an increase in several microRNAs detected in the EVs of melanoma patients at the time of therapy resistance, specifically miR-4454, miR-548o-5p and miR-4663.

Using multiple human melanoma cell lines (both sensitive and resistant to targeted therapy), we show different expression patterns

of these microRNAs in the EVs compared to their parental cells depending on their sensitivity or resistance to targeted therapy. We use the BRAF inhibitor, PLX4720, to evaluate cellular and EV microRNAs in human melanoma cell lines. Our results show an increase of miR-4454, miR-548o-5p and miR-4663 in the resistant cell line compared to the sensitive one, but a decrease, or even absence of miR-4663, in the packaging of these microRNAs in the EVs. Furthermore, treatment of a sensitive cell line with PLX4720 decreases the packaging of both miR-4454 and miR-548o-5p in the EVs.

These results suggest a highly regulatory and complex mechanism of expression of microRNAs after treatment with targeted therapy, and a subsequent selection of these molecules to be secreted into the extracellular space, suggesting a non random mechanism of microRNAs packaging in exosomes.

Myeloid-Wnt5a influences melanoma phenotype and tumor immunogenicity

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Despite breakthroughs in targeted therapies and immunotherapies, over 75% of patients either do not respond to therapy, or relapse. This may be due to changes in their tumor microenvironment (TME). Wnt5A has been shown to influence melanoma phenotype, promoting a slow-cycling, but more invasive cancer. Wnt5A was thought to be produced from tumor cells and fibroblasts in the TME. However, our data now suggest that unique pathologically induced myeloid derived suppressor cells (MDSCs) produce a major proportion of Wnt5A in the TME which is taken up by melanoma cells to increase invasiveness. This finding highlights the diverse roles that MDSCs play in melanoma, going beyond immunosuppression. Using novel transgenic animals with MDSC-specific knockdown of Wnt5A we demonstrate a clear decrease in the Wnt5A expression within the TME. Wnt5A-deficient MDSCs caused a decrease in tumoral MDSCs and Tregs. Tumor infiltrating lymphocytes had a significantly decreased PD-1 expression, suggesting a less exhausted phenotype, and increased ratios of effector T-cells to immunosuppressive cells suggesting in a more responsive immunogenic TME. Wnt5A promotes a switch from proliferative to an invasive melanoma cell state. Myeloid-specific Wnt5A knockdown caused decreased lung metastasis and significantly increased tumor growth *in vivo*, with the lack of MDSC infiltration negatively correlating to tumor volume. Tumor-infiltrating MDSCs from control animals had a strong positive correlation with Tregs, whereas, this was completely ablated in animals with Wnt5A-negative MDSCs. Overall, our data suggests that while MDSCs contribute to an immunosuppressive and less immunogenic environment, they may have an additional function as the major source of Wnt5A in the tumor microenvironment. These two functions likely synergize to drive a highly metastatic and therapy-resistant phenotype altering immunogenicity.

Spartalizumab (S) + dabrafenib (D) + trametinib (T) in first-line treatment (tx) of *BRAF* V600–mutant melanoma: clinical outcomes and biomarker analyses from COMBI-i parts 1 and 2

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The impact of BRAF/MEK inhibition on the tumor microenvironment (TME) suggests that combined targeted therapy and immunotherapy may improve outcomes. We present updated efficacy, safety, and biomarker data from the safety run-in and biomarker cohorts, pooled due to similar eligibility criteria and tx regimen, of the phase 3 COMBI-i trial (NCT02967692) of first-line S + D + T in patients (pts) with unresectable or metastatic *BRAF* V600–mutant melanoma. At data cutoff (8 April 2019; median follow-up, 19.9 mo), tx was ongoing in 13 of 36 pts (36%). Objective responses were confirmed in 28 of 36 pts (78%), including 15 (42%) complete responses (CRs). CR was associated with low baseline (BL) levels of circulating tumor DNA and immunosuppressive (IS) TME gene signatures. Median response duration and progression-free survival (PFS) were 20.7 and 23.7 mo, respectively. In pts with stage IV M1c disease (20/36 [56%]) or high BL LDH (15/36 [42%]), respective median PFS was 12.9 and 10.7 mo. Early PFS events (before 12 mo) occurred in 5 of 22 pts with paired DNA-seq and RNA-seq data available and were associated at BL with low tumor mutational burden and T-cell inflamed gene expression profiles (TI-GEPs) or high levels of IS TME signatures. TI-GEPs increased and MAPK pathway activation decreased early on tx in all pts regardless of

subsequent progression. Grade ≥ 3 tx-related AEs occurred in 72% of pts; 47% discontinued any study drug. AEs were consistent with those of each individual drug; pyrexia (89%) was the most common. S + D + T yielded a high and durable response rate, including in pts with poor prognostic features, and manageable toxicity. Predictive biomarkers of CR or progression were identified but need further validation. The randomized, placebo-controlled part 3 of COMBI-i is ongoing.

CD74 regulated inflammatory pattern on evaluating the risk of CNS metastasis and survival in Stage IV melanoma

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Innate inflammatory features in melanoma tumors at all stages have identified specific inflammatory proteins expressed by tumors cells in patients with the worse prognosis. We have previously observed impaired outcomes in patients with constitutive expression of inducible nitric oxide synthase (iNOS), Macrophage Migration Inhibitory Factor (MIF) and improved outcomes with CD74 expression in Stage III melanoma. In this study, we tested our hypothesis on CD74-regulated inflammatory markers' expression in stage IV melanoma tumors are associated with survival outcome and risk of developing CNS metastasis.

We retrospectively identified 315 patients with stage IV melanoma and used IHC analysis to measure the expression of cells with CD74, MIF, iNOS, Nitrotyrosine (NT), cyclooxygenase (COX)-2 and microsomal PGE synthase-1 (mPGES1). We analyzed the association of those inflammatory markers with overall survival (OS) and time to first CNS metastasis.

Tissue sections from patients with distant metastatic melanoma were included in the TMA of which 169 (54%) did not have CNS metastasis at the time of the last follow-up. The combination of tumor expression of CD74 and the lack of MIF expression showed the advantage of OS in stage IV melanoma patients (p = 0.0264). The expression of CD74 tend to be predictive of time to CNS metastasis. However, the expression of NT significantly affected time to CNS metastasis (p = 0.0008). We further investigated the survival associations in the melanoma Stage IV TCGA dataset for our markers of interest and showed only high CD74 expression predicted better prognosis in stage IV melanoma patients (p = 0.0265).

Our data validates CD74 as a useful prognostic tumor cell protein marker associated with favorable OS in stage IV melanoma. The tumor NT expression strongly predicts an increased risk of developing CNS metastasis in those patients.

Plasma proteomics in patients with metatatic melanoma treated with immune checkpoint inhibitors

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Immune checkpoint inhibitors (ICIs) have significantly improved the outcome in metastatic cutaneous melanoma (CM). Therapy response is limited to a subgroup of patients and clinically useful treatment predictive biomarkers are lacking. Serial plasma samples from 24 patients with metastatic CM patients receiving ICIs were analyzed with a mass-spectrometry proteomics method (HiRIEF LC-MS/MS) and proximity extension assays (PEA), and compared to a control group with metastatic CM patients undergoing targeted therapy. The aim was to investigate systemic biological processes and circulating tumor-derived proteins associated with treatment outcome. Unbiased MS proteome analysis indicated plasma levels alterations related to treatment in 80 out of 1,160 quantified proteins in patients with ICIs. PD-1 had the highest increase in plasma levels in the ICI cohort (log2-FC = 2.03, p = 0.0008) and iCI responders (log2-FC = 2.09, p = 0.005), but not in controls. Targeted, antibody-based proteome analyses by PEA confirmed the observations. Furthermore, we discovered new associations between plasma proteins and progression free survival during ICIs treatment. With a total of 1,911 proteins identified and 1,237 proteins analyzed, this is the first and most comprehensive study of the plasma proteome for CM patients receiving ICIs usingboth global and targeted proteomics.. For the first time, an increase in circulating PD-1 in response anti PD-1 is reported.

Circulating tumor (ct)DNA dynamics as a predictor of outcome in metastatic melanoma(mMEL)

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Plasma ctDNA has shown promise in monitoring response to cancer treatment (tx). Serial ctDNA samples were collected from 30 pts with BRAF-mutant mMEL treated with BRAF+MEK-inhibitors. Digital droplet PCR was used to quantify BRAF ctDNA levels at baseline and with samples obtained on-tx (median 4 time points per pt). The 1st ctDNA drop ratio was calculated as the 2nd ctDNA value (collected median of 4 weeks on-tx), divided by baseline ctDNA. 8 pts had a ctDNA below level of detection at baseline.

In 22 pts with elevated baseline ctDNA, median follow-up was 10 months and 14 pts eventually progressed (PD). Pts with a lower 1st ctDNA drop ratio (more steep drop) had a 78% less chance of PD vs pts with higher drop ratio (cut point 0.03). Median PFS for pts with low vs high ctDNA drop ratio was > 560 days (95% CI:371,NA) vs 111 days (95% CI:95,NA; $p = 0.0165$). Pts with ctDNA clearance had median PFS of 371 days vs 49 days ($p = 0.03$). The serial ctDNA values and CT tumor measurements were combined to calibrate the parameters of a mathematical model that simulates personalized intermittent (adaptive) BRAF+MEKi dosing to explore how it might prolong PFS in comparison to continuous tx. The aim is to prolong PFS by maintaining tumor sizes with timed drug breaks to allow drug sensitive cells to compete with resistant cells. This differential equation based model has 3 cell compartments: drug sensitive, drug resistant, and drug resistance not accounted for by BRAF ctDNA. The model may guide tx decisions at time of each ctDNA collection by simulating tx responses on/off drugs based on expected ctDNA levels on/off tx. By generating a virtual cohort to predict tumor responses in these pts, personalized intermittent therapy significantly delayed PFS vs continuous BRAF+MEKi. Plasma ctDNA dynamics may predict efficacy of BRAF+MEKi, and may inform personalized tx regimens to improve clinical outcomes.

Stromal and immune changes in the aged lung microenvironment create a permissive niche for the metastatic outgrowth of melanoma

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Our previous data has indicated that while tumors grow more slowly in aged mouse skin, they form more lung metastases. We queried whether the lung microenvironment undergoes age-related changes that provide a permissive niche for metastatic outgrowth. Our previous data has implicated dermal fibroblasts as the drivers of age-related changes in the skin, and so we analyzed young vs aged healthy human lung fibroblasts. The secreted changes in aged lung fibroblasts suggest that the lung microenvironment evolves during aging to promote proliferation of melanoma cells. Specifically, these changes involve the secretion of factors that suppress Wnt5A and AXL signaling, which typically drives a metastatic but slow-growing phenotype. Interestingly, this switch promotes activation of the MER tyrosine kinase pathway, increasing proliferation. Consistent with this, our in vivo studies indicate that tumor cells disseminate to the lungs at the same rate in young and aged mice. However, they persist as single cell populations in the young mouse lungs, whereas they grow out rapidly in the aged mouse lung. We next queried whether the immune microenvironment also played a role in the differential outgrowth of lung metastases in young and aged mice. We find that aged lung fibroblasts secrete higher levels of immunosuppressive factors ARG-1 and CXCL1, which are both necessary for efficient metastatic outgrowth in vivo. Analysis of the aged lung microenvironment reveals that immunosuppressive MDSCs and Treg populations are significantly increased compared with the young lung, while effector CD4 and CD8 T cells are decreased. Importantly, depletion of MDSCs in the aged lung significantly decreases metastasis. Overall, we find that aged-induced changes in fibroblast secretory phenotypes and immune subpopulations promote efficient metastatic progression in the lung.

Preclinical modeling of leptomeningeal disease (LMD) from melanoma to facilitate therapeutic development

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Leptomeningeal disease (LMD) is a devastating complication of metastatic melanoma that is associated with limited treatment options and dismal prognosis. A critical current limitation to the development of more effective treatments for LMD is a lack of preclinical models. Thus, we have developed an immunocompetent murine model of melanoma LMD, and have initiated studies to evaluate the safety and efficacy of systemic and intrathecal (IT) anti-PD1 for it. We accessed the leptomeninges by IT injection into the cisterna magna. Using this route, we showed that IT administration of anti-PD1 in non-tumor bearing mice was safe by observation of animal survival and by histological assessment of brain tissue for neurotoxicity, including IHC for CD3, CD8, CD163, CD56, CD15, PD1, and PD-L1, which identified no significant changes versus control IgG. IT administration of B16 cells consistently resulted in the establishment of LMD, which was evaluated by *in vivo* bioluminescent imaging, pathological analysis (gross and histological) of brain and spine specimens, and survival curves. Pilot studies showed that combined IT and systemic administration of anti-PD1 significantly improved survival versus sham treatment in mice with LMD (HR = 0.269, $p = 0.023$). Immunohistochemical analysis of harvested brain and spinal tissue revealed that IT anti-PD1 resulted in increased CD8 + T-cell infiltrate relative to the isotype antibody controls. Together the findings establish a new model to facilitate the testing and optimization of new immunotherapy strategies for melanoma patients with LMD.

MacroH2A as a novel chromatin regulator of the melanoma microenvironment

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Deregulation of epigenetic states has emerged as a critical driver of aberrant transcriptional programs promoting melanoma development and therapeutic resistance. Our laboratory showed that macroH2A, a histone variant associated with transcriptional repression, is downregulated in melanoma vs. benign nevi and suppresses melanoma cell line proliferation and metastatic potential. However, its role as a barrier to tumorigenesis has not been investigated *in vivo*. We crossed macroH2A1/2 constitutive double knockout (dKO) mice with a melanoma model driven by BRAF mutation and PTEN deletion. Whereas dKO primary tumors initiated similarly, their subsequent growth was significantly enhanced compared to WT. Bulk RNA-seq identified upregulation of genes associated with transformation and the myeloid lineage and downregulation of genes associated with skin differentiation and cytotoxic lymphocytes. Strikingly, dKO tumors were differentially infiltrated by immune cells, with increased monocytes and decreased CD8 + T cells. Intratumoral CD8 + T cells were less prone to activation *in vitro* and had a dysfunctional transcriptional program. Altogether, our data suggests loss of macroH2A induces immune suppression, which could stem either from melanoma cells eliciting tolerance, or from functional deficiencies intrinsic to immune cells. Regarding the latter, we observed hyperactivation of tumor-naïve CD8 + T cells by *in vitro* stimulation or *in vivo* chronic antigen exposure, which could contribute to faster exhaustion and loss of antitumor activity in the context of melanoma. To address how macroH2A-deficient melanoma cells shape their microenvironment, we generated macroH2A1 KO B16 cell lines to monitor tumor graft infiltration by WT immune cells. Our ongoing efforts could highlight macroH2A as a marker of response to checkpoint blockade and/or as an epigenetic regulator of tractable molecular targets for therapy.

Stable isotope metabolomics identifies autocrine feedback loop in melanoma

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Metabolomics in combination with stable isotope tracing provides a mechanistic understanding on the source of metabolite production and signaling in oncogenesis, allowing to effectively target mitogenically-activated tumors. Elevated tumor levels of the amino acid glutamate have been reported not only in central nervous system tumors, where glutamate is abundant, but also in melanoma and other solid tumors. In the context of metabolomics, the neuroektodermal origin of melanocytes, pigment producing cells that migrated and differentiated from the neural crest into the skin, might be of particular relevance in malignant melanoma. Melanoma is the third most common source of brain metastases following lung and breast cancer and more than 60% of patients with metastatic melanoma either present with or develop brain metastases during the course of their disease.
The glutamate metabotropic receptor 1 (GRM1) drives oncogenesis when aberrantly activated in melanoma and several other cancers. Glutamate, the natural ligand of GRM1, is one of the most abundant amino acids in the human body and the predominant excitatory neurotransmitter in the vertebrate central nervous system. Stimulation of GRM1 by glutamate leads to activation of mitogenic signaling pathways, which in turn increases the production of glutamate, fueling autocrine feedback.
Metabolomics reveals that patient-derived xenografts with GRM1-positive melanoma cells exhibit elevated plasma glutamate levels associated with spontaneous metastatic melanoma *in vivo*. Using a rational drug-targeting strategy, we critically evaluate metabolic bottlenecks with the goal to cut off tumor glutamate bioavailability. Using stable isotope tracing and GCMS analysis, we determine the flux contribution into glutamate. First in patient trials indicate that elevated circulating glutamate levels were significantly reduced by limiting glutamate bioavailability.

Reprogrammed tumor microenvironment by intratumoral LCMV vector therapy promotes T cell-dependent melanoma eradication

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Tumor cells grow in a complex and heterogeneous tumor microenvironment (TME) that consists of diverse cellular components including immune cells, stromal fibroblasts, and endothelial cells. The TME can influence antitumor immune responses and tumor outcome. Therefore, targeting the TME has become one of the main goals in anti-cancer therapy. Here, we demonstrate that a single intravenous or intratumoral administration of a recombinant replication –attenuated LCMV vector expressing the melanoma-associated antigen TRP2 (TheraT(LCMV)-Trp2) confer T-cell dependent control of melanomas, whereas only intratumoral but not an intravenous injection of the vector leads to T cell-dependent eradication of melanoma. Despite the high number

of tumor-infiltrating T cells in intravenously treated mice, this did not correlate with tumor control. However, we found preservation of T cell functionality in mice treated intratumorally with TheraT(LCMV) vectors. This suggests that intratumoral injection of TheraT(LCMV)-Trp2 vectors reprogram the TME to sustain T cell fitness. Using single-cell transcriptomics, we are currently setting out to dissect the changes in the TME after intratumoral TheraT(LCMV) treatment. Thereby, we are going to analyze cell populations in the hematopoietic as well as the non-hematopoietic compartment. Collectively, our data show that intratumoral injection of TheraT(LCMV) vectors is an efficient way of reprograming the TME landscape to enhance antitumor T cell responses resulting in tumor eradication.

Co-inhibition of autophagy and oncogenic signaling in NRAS-driven melanoma

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Melanoma is the deadliest form of all skin cancers and the 6th most common cancer diagnosed in the U.S.Unlike BRAF-mutant melanoma, NRAS-driven melanoma patients have no signaling pathway-targeted therapy options available, and immunotherapy is only efficacious in a subset of these patients, severely limiting their treatment options. Oncogenic NRAS results in the downstream activation of the MAPK®MEK®ERK and PI3K®AKT signaling pathways.Our lab has recently shown that autophagy,a conserved metabolic process of self-digestion that recycles intracellular components, is increased in NRAS-driven melanoma upon MEK1/2 inhibition. We further showed that co-inhibition of autophagy and MEK1/2 leads to tumor regression of human NRAS-driven melanoma PDXs in mice. Our proposed combination therapy is currently being translated into a phase I/II clinical trial for unresectable NRAS-driven melanoma patients with advanced disease. Moreover, our data indicatethat single inhibition of PI3K®AKT signaling downstream of oncogenic NRAS results in induction of autophagy, to an extent that is greater than autophagy induction upon MEK1/2 inhibition. Combined pharmacological blockade of autophagy and PI3K or AKT resulted in synergistic anti-proliferative effects in human NRAS-driven melanoma cell lines. Altogether, these data suggest that co-inhibition of autophagy and oncogenic signaling may represent potential new treatment strategies for NRAS-driven melanoma patients. Future experiments aim for a better mechanistic understanding of the combination treatment using*in vitro* and *in vivo* tools, with the objective to propose novel therapeutic strategies for NRAS-driven melanoma patientsin the clinical setting.

Blockade of bioenergetic function of skin mitochondria (MC) by chronic neurogenic pain

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Skin cells and subcellular structures – mitochondria – are involved in melanoma pathogenesis. The malignant process is often accompanied by pain. Our purpose was to study the effect of chronic neurogenic pain (CNP), B16/F10 melanoma and their combination on the MC antioxidant system in female mice.

The study included female C57BL/6 mice (n = 28): intact (n = 7), controls (C), CNP (n = 7), B16/F10 melanoma (n = 7) and CNP+B16/F10 (n = 7). Levels of reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione peroxidase-1 (GPO-1), glutathione peroxidase-4 (GPO-4), glutathione reductase (GR), glutathione-S-transferase (GT) and superoxide dismutase-2 (SOD-2) were determined by ELISA in skin MC.

Levels of components of the antioxidant system in skin MC of female mice with CNP were significantly elevated: GSH by 1.3 times, GPO-1 by 2.9 times, GPO-4 by 1.9 times, GR by 2.8 times and SOD-2 by 2.4 times. B16/F10 melanoma was characterized by the opposite changes: decreased levels of GPO-1 by 1.9 times, GPO-4 by 3.7 times, GR by 3.9 times, SOD-2 by 3.8 times and increased levels of GSSG by 1.36 times. CNP+B16/F10 resulted in an increase in GSSG by 1.52 times, GSH by 1.5 times, GPO-1 by 3.6 times, GT by 1.28 times, GPO-4 by 1.6 times and SOD-2 by 1.8 times.

CNP causes the blockade of the bioenergetic function in skin MC by reprogramming redox mechanisms and thereby dramatically changing the pathogenesis of melanoma.

Dysfunction of mitochondrial antioxidant systems of the heart in response to chronic neurogenic pain and melanoma

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Cancer is often associated with pain syndrome and cardiovascular disorders. Reactive free radicals damage both cardiomyocyte membranes and mitochondria. Our purpose was to study the antioxidant system of cardiac mitochondria in female mice with B16/F10 melanoma (M) and chronic neurogenic pain (CNP), alone and in combination.

The study included C57BL/6 female mice (n = 28): intact animals (n = 7), controls with a 3-week model of CNP (n = 7), comparison

group with a 3-week growth of transplantable B16/F10 melanoma (n = 7) and main group with CNP+M, where M was transplanted 3 weeks after CNP creation (n = 7). Animals were observed for up to 3 weeks. Levels of reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione peroxidase-1 (GPx1), glutathione peroxidase-4 (GPx4), glutathione reductase (GR), glutathione-S-transferase (GT) and superoxide dismutase-2 (SOD2) were determined in cardiac mitochondria (MC) by ELISA.

CNP alone was characterized by low levels of GPx1 – by 1.4 times, SOD2 – 1.6 times and high levels of GSSG – by 1.6 times, GR – by 1.36 times. M was characterized by low MC levels of GPx1, GR and SOD – by 1.6, 2.8 and 2 times respectively, while GSSG was increased by 1.58 times. CNP+M decreased GSH by 1.5 times and increased GSSG by 1.3 times. Enzymatic component of the glutathione system in cardiac MC with combined CNP+M was characterized by an accumulative potential: GPx1 increased by 2.1 times, GR by 5.8 times, GT by 1.2 times.

CNP and malignant process have systemic effect on the animal body, but affect different components of the regulation in the system of lipid peroxidation-antioxidant protection. Detected changes in the energy system of heart MC can be considered as stress ones.

Effect of chronic pain syndrome on receptor status of skin and tumor in female mice with growing B16/F10 melanoma

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Modulating effect of chronic pain syndrome on the receptor status in melanoma growth is poorly studied. The purpose of the study was to determine the effect of chronic pain syndrome on levels of sex hormone receptors in the skin and tumors in the dynamics of B16/F10 melanoma growth in female mice.

The study included female C57BL/6 mice divided into an intact group (n = 7), controls (n = 7) with chronic neurogenic pain (CNP), comparison group with standard transplantation of B16/F10 melanoma (n = 22) and main group (n = 28) with B16/F10 melanoma+CNP. After 1, 2 and 3 weeks of the experiment, animals were decapitated. Levels of receptors of estrogens (ERα, ERβ), progesterone (RP4) and androgens (RA) were determined in tumor and skin by standard ELISA test systems.

Mice of the main group showed an early tumor onset, active metastasis and poor survival, compared to the comparison group. Chronic pain affected the receptor status of the skin, upregulating estrogen receptors and downregulating androgen receptors. The tumor onset in animals of both groups caused similar changes in the skin levels of estrogen receptors and opposite changes in androgen and progesterone receptors. Melanoma samples were characterized by elevated levels of all the studied steroid receptors, similar to the perifocal tissues in animals of the main group.

Hormonal imbalance caused by neurogenic pain and changes in the content of steroid hormone receptors in the skin contributes to an earlier onset of transplantable melanoma and enhances its biological aggressiveness shown through active metastasis.

Gender characteristics of stimulating effect of chronic neurogenic pain on malignant process

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An experiment provides answers to many questions, including the effect of chronic neurogenic pain (CNP) on malignant growth. The vast majority of preclinical studies of pain involve male rodents only, and this poses a serious problem for the translational significance of preclinical studies. Our aim was to reveal the effect of CNP on the course of malignant B16/F10 melanoma in male and female mice.

The study included male and female C57BL/6 mice (n = 64). The CNP model was created by the sciatic nerve ligation on both sides. Main groups (males, females) received subcutaneous injections of B16/F10 melanoma cells below the right shoulder blade 2 weeks after the surgery. Controls (males, females) received subcutaneous transplantation of B16/F10 melanoma without CNP modeling.

All animals with CNP, regardless their gender, shared some common characteristics of B16/F10 melanoma course: early-onset tumors, poorer survival, melanoma ulceration and metastases at a certain stage. Gender differences in males were: 10 times higher rates of tumor growth – by 22.3 times in males compared to 2.3 times in females; single focus tumors, compared to common bifocal tumors in females; development of amelanotic melanomas since the first, or at least the second week of malignant process, unlike black tumors in females; later (from week 2) development of metastases, while in females metastases were registered since the first week of carcinogenesis; typical metastatic sites (lungs, spleen), unlike both typical (lungs, spleen, liver) and atypical ones (heart, uterus) in females.

The effect of CNP on the malignant growth of B16/F10 melanoma, together with common characteristics, has its gender differences, which allows us to insist on the feasibility of research aimed at studying mechanisms of the CNB effect on carcinogenesis in animals of both genders in parallel.

Influence of chronic neurogenic pain on the dynamics of endothelin-1 and components of the NO-system during B16/F10 melanoma growth

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Chronic neurogenic pain (CNP) is a pathogenic factor that triggers the mechanisms of homeostasis disorders, affects biological characteristics of B16/F10 melanoma. Aim: to determine levels of the vasoactive endothelin-1 and components of the NO-system in mice during the growth of B16/F10 melanoma combined with CNP.

64 female mice used in the study. B16/F10 melanoma was transplanted to animals of the main group 2 weeks after creating CNP model. Levels of endothelin-1, NOS-2, NOS-3, L-arginine, citrulline, total nitrite, nitrotyrosine, and asymmetric dimethylarginine (ADMA) were determined by ELISA in the intact skin and in tumor tissue 1, 2, and 3 weeks melanoma growth.

In animals with CNP and tumor growth level of endothelin-1 increased in the skin by up to 2.5 times and in tumor by up to 3.3 times. Level of NOS-3 in the skin decreased by up to 3.5 times by week 3, but ADMA increased on average by 53%. Stable elevated levels of NOS-3 by up to 3.8 times, NOS-2 by up to 78% were detected in tumor, ADMA increased on average by 43% at weeks 1–2 and its decrease by 33% by week 3.

The dynamics of the studied parameters differed in tumors with and without CNP. The growth of melanoma with CNP during 3 weeks was characterized by a steady increase in the level of endothelin-1 in the skin, while the NO-system activity decreased from the 2nd week. Tumor tissues showed increasing concentration of endothelin-1 and activity of NO-system by the 3rd week of the melanoma growth together with CNP. CNP contributes to the activation of the endothelin-1 and the NO-system in the tumor. The forming conditions favor the further development of melanoma. Changes in ADMA levels during the tumor growth with CNP may indicate a more subtle control of the NO level supporting the increased invasiveness of melanoma.

Neurogenic chronic pain increases malignancy of melanoma and contributes to early metastasis through accumulation of growth factors in perifocal tissues of the tumor

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The purpose of the study was to analyze levels of some growth factors in tumor, perifocal and intact tissues of female mice with chronic neurogenic pain (CNP) during the development of experimental B16/F10 melanoma.

The study included 64 female C57BL/6 mice. Animals were divided into an intact group (n = 7), controls (n = 7) with CNP, comparison group with standard transplantation of B16/F10 melanoma (n = 22) and main group (n = 28) with B16/F10 melanoma+CNP. After 1, 2 and 3 weeks of the experiment, animals were decapitated. The skin and tumor were isolated on ice, and 10% homogenates were prepared. Levels of EGF, EGFR, IGF1, IGF2, FGF21 and TGF-β1 were measured using standard ELISA test systems. Statistical analysis of the results was performed using the Statistica 6.0 program (Stat-Soft, 2001).

Chronic pain increased skin levels of IGF1 (by 24 times), IGF2 and EGF (by 3 times). 1 week after melanoma transplantation, levels of all growth factors in perifocal tissues of mice with CNP increased: IGF1- by 10 times, IGF2 - by 8 times, FGF21– y 4 times, TGF-β1 and EGFR - by 2 times, EGF – by 1.4 times. At the same time, tumor tissues showed increasing levels of EGF and EGFR – on the average by 1.8 times, and IGF1– y 1.4 times. Changes were maintained until the end of the study, to the death of mice after 3 weeks. The influence of chronic pain on the development of B16/F10 melanoma, compared to the control group, was characterized by several signs: early tumor onset, bifocal growth, early 100% metastasis, including that to atypical sites, and reduced survival.

Due to changes in the metabolic profile of the body, CNP stimulates the accumulation of growth factors, especially in perifocal tissues of melanoma, increasing its malignancy and provoking early metastasis.

Plasma kinin system in control of effectiveness of chemoradiation therapy for cutaneous melanoma

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The purpose was to evaluate possible control of the therapy and endogenous defense system effectiveness in patients with cutaneous melanoma using parameters of the kallikrein-kinin system (KKS) and blood inhibitors.

Blood plasma of patients with skin melanoma (T_2-$_3$NxM_o, 9 women, 6 men, 42–72 years) and 32 donors (N) was studied before surgery (b/s), on days 7 and 14 after surgery (a/s) and during standard chemoradiation treatment.

Activity of trypsin proteases (TP) and kallikrein (K) b/s exceeded N by 1.4 and 3.6 times, respectively; levels of prekallikrein (PK) were decreased by 1.5 times, activity of carboxypeptidase N (CPN) – by 7.3 times, α-2M by 4.6 times; activity of α-1PI was increased by 1.4 times. Day 7 a/s and treatment: CPN and α-2M activity increased by 1.9 and 1.3 times, PK level – by 1.3 times. Day 14: K decreased by 1.6 times, PK – increased by 1.3 times compared to b/s. CPN activity increased by 1.8 times compared to day 7 a/s, α-2M –by 1.6 times,

α-1PI – decreased to N levels. Ratios of TP/K and PK/K increased, not approaching N by 1.8 and 1.5 times, K/CPN 5.4 times lower than b/s. Ratios of TP/α-2M and K/α-2M were 1.2 and 2.6 times lower than b/s, while TP/α-1PI and K/α-1PI did not change. The results demonstrated an improvement in the balance of TP/K and K/α-2M, but increased K/α-1PI confirmed the insufficiency of inhibitors in the body affected by skin melanoma.

Pathological activation of plasma KKS and disturbed interaction of enzymes with inhibitors in patients with cutaneous melanoma b/s prevent protective functions of endogenous proteins. An improved balance of KKS and TP with α-2M a/s and treatment confirms positive impact of the therapy, even with α-1PI activity reduced to N. The TP/K and K/α-2M ratios can be used to control the effectiveness of chemoradiation therapy for cutaneous melanoma.

Specificity of markers CD44 and S100 for skin melanoma and nevi tissues

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Our aim was a comparative analysis of levels of tumor-specific proteins CD44 and S100 and protein composition in melanocytic lesions of the skin.

We studied 86 samples of cutaneous melanoma and nevus tissues, their perifocal tissues and resection line tissues obtained during tumor excision from 23 patients with cutaneous melanoma pT1-4N0-1M0 and 14 patients with nevi. Intact skin samples from non-cancer patients obtained during reconstructive plastic surgery were used as the comparison group. Levels of CD44 (BenderMedSystems, USA) and S100 (Fujirebio, Sweden) were determined by ELISA in 10% homogenates of all tissues; fractional composition of proteins were studied by turbidimetric method. Statistical processing of results was performed using the Statistika 6.0 program with Student's t-test for two independent groups.

Melanoma was characterized by a sharp increase in S100B levels, 28 and 7 times exceeding the levels in intact tissues and nevi. The level of CD44 in melanoma tissue was increased only by 2 times, in nevus tissue - by 48%. The ratio of albumin and gamma globulins in the tissue of melanoma and nevi was 79% and 29% lower than in healthy skin. A more than twofold increase in the gamma globulin fraction in the melanoma tumor tissue against a decrease in albumin and the absence of changes in other globulins, as well as a moderate but statistically significant increase in the gamma globulin fraction in nevi tissue, indicates that S100B and CD44 proteins belong to the gamma globulin fraction.

A highly specific increase of S100 levels and a less specific increase of CD44 levels in supernatant liquid of melanoma tissue homogenates, together with the predominance of the gamma globulin fraction, allow considering such factors as a prognostically unfavorable sign of tumor progression, which can be important when choosing a personalized treatment strategy.

Long-term survival of patients with stage III/IV melanoma receiving IMM-101

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IMM-101 is a suspension of heat-killed whole cell *Mycobacterium obuense* (NCTC 13365), which enhances the innate immune response and dendritic cell maturation. In animal models, it increases antigen specific responses and number of CD8 + CTL and CD4 + Th1 cells. In a randomised phase 2 study in combination with gemcitabine, it showed improvement in PFS and OS in metastatic pancreatic cancer patients (pts) compared to gemcitabine alone without additional toxicity (NCT01303172). Long term survival data are now available for pts with stage III/IV melanoma recruited into a Phase 1 placebo controlled, dose escalation study of IMM-101. Between Mar and Sep 2010, 18 pts with Stage III/IV melanoma completed a first-in-human (FIH) study to evaluate the safety and tolerability of IMM-101 (NCT01308762). Treatment with IMM-101 continued, initially on a compassionate use program (CUP) and then in an open label long term follow up (LTF) study, which closed in Dec 2018. Of the 18 pts (12 Stage IV, 3 Stage IIIc and 3 Stage IIIb) who completed the FIH study, 12 received subsequent treatment on a CUP and 10 (9 Stage IV, 1 Stage IIIc) were enrolled in the LTF study, which started in Feb 2012. At time of LTF study closure, 6 of the 10 LTF study pts were alive with 4 of them still receiving treatment with IMM-101. The median time on treatment from first dose in the FIH study was 5.2 years (range 2.7 to 7.95, n = 10). Three pts received immunotherapeutic agents other than IMM-101 whilst on study (2 pts IL-2 and 1 pt ipilimumab). Two pts (both with stage IIIB disease at start of FIH study) who did not enter the LTF were also still alive in Dec 2018. In conclusion, 8/18 (44%) pts with stage III/IV melanoma enrolled to the FIH study were alive over 8 years later in Dec 2018. IMM-101 was safe and well-tolerated and local reactions at the injection site were the most frequently reported adverse events.

Spatial distribution of intratumoral immune cells correlates with response to anti-PD-1 based therapies in metastatic melanoma patients

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Inhibitors targeting CTLA-4 and PD-1 receptors have greatly improved the survival and clinical outcomes of patients with advanced stage melanoma. However, the spatial distribution of immune and tumor cells and their association with response and outcome in patients treated with these therapies remains unknown. We investigated the spatial profiles of immune and melanoma cells in 61 pre-treatment tumor biopsies from metastatic melanoma patients treated with anti-PD-1 monotherapy (n = 18 responders; n = 9 non-responders) or combination anti-CTLA-4 and anti-PD-1 immunotherapy (n = 22 responders; n = 12 non-responders) via spatial analysis of multiplex immunofluorescence data including the markers CD8, FOXP3, PD-1, PD-L1 and SOX10. Patients were classified as responders (CR/PR/SD > 6 mo) or non-responders (SD ≤ 6 mo/PD) based on RECIST criteria. The densities of $CD8^+$ T cells and $PD\text{-}L1^+$ immune cells within a 20 μM distance from a melanoma cell were significantly higher in responders compared to non-responders in both anti-PD-1 monotherapy (CD8 p = 0.0024; PD-L1 p = 0.0005) and combination treated patients (CD8 p = 0.0096; PD-L1 p = 0.0134). Furthermore, the densities of these populations in proximity to tumor cells were associated with improved progression-free survival for both therapies in univariate analyses ($p < 0.05$). In multivariate analysis, the best model for 12-month progression-free survival for anti-PD-1 monotherapy included the density of $PD\text{-}L1^+$ cells within 20 μM of tumor cells and intratumoral $CD8^+$ density (AUC = 0.80). For combination therapy, the best model included the density of $CD8^+$ cells within 20 μM of tumor cells, intratumoral $PD\text{-}L1^+$ density and LDH (AUC = 0.85). These findings highlight the potential role of the spatial locations of immune cells in relation to tumor cells as predictors of response to anti-PD-1 based therapies.

Obesity related changes in AXL driven inflammatory signaling impact survival in melanoma.

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Introduction: The TYRO3, AXL and MERTK (TAM) receptor tyrosine kinase family has been associated oncogenesis and metastasis in melanoma. Recent evidence correlating obesity with a paradoxical improved response to immunotherapy in melanoma suggests both tumor microenvironment and clinical phenotype play a role in response. We sought to build a predictive model of response to therapy from biomarkers using TAM receptors in the normal weight and obese populations.

Methods: We used TCGA-SKCM melanoma tumor mRNA expression and clinical data for melanoma patients (n = 471). Biomarkers were defined as "high" or "low" expression in each patient. Differences in Kaplan-Meier survival curves based on level of expression were tested using G-rho family tests. Strength of relationships between biomarkers were measured using Pearson's correlation. All statistical analysis were performed using R package "survival".

Results: Normal weight and obese patients had markedly different biomarker profiles associated with survival. In the normal weight population, high CD8 (p = 0.0093), PD1 (p = 0.0093) and CD84 (p = 0.022) were associated with improved survival. In the obese population, high AXL expression was associated with improved survival (p = 0.004), while CD8 (p = 0.91) and PD1 (p = 0.89) demonstrated no association. In correlation analysis, AXL expression was most closely associated with macrophage markers CD163 (r = 0.52), CD84 (r = 0.56) and MS4A4A (r = 0.53) in the obese but not the normal weight population.

Conclusion: These data suggest that immunologic response in melanoma patients is driven by separate immune profiles for obese and non-obese populations. AXL appears to mediate response in the obese population by a macrophage-driven mechanism. The significant differences between obese and non-obese patients suggest potential clinical implications regarding targets for treatment based on clinical phenotype.

Updated Quality-of-life (QoL) of encorafenib + binimetinib (COMBO 450) versus (vs) vemurafenib (VEM) from COLUMBUS Part 1 phase 3 study in BRAF-mutant melanoma

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Combination of the BRAF inhibitor encorafenib + MEK inhibitor binimetinib (COMBO 450) showed favorable efficacy and safety versus VEM in patients (pts) with advanced BRAF V600-mutant melanoma. Results of QoL in COMBO 450 and VEM arms were updated.

Patient-reported QoL was assessed using 2 validated instruments, FACT-M and QLQ-C30 score with higher scores representing better QoL. Questionnaires were collected at baseline (BL), every 8 weeks after randomization for the first 24 months (mo) and every 12 weeks thereafter until progression of disease, end of treatment, or end of study. Time to definitive 10% deterioration for each scale was analyzed using the Kaplan-Meier method. A mixed-effect model for repeated measures (MMRM) was used to assess the impact of treatment on the change from BL for each scale.

A total of 192 and 191 patients were randomized to COMBO 450 and VEM, respectively. Median exposures were 51 and 26 weeks for combo 450 and VEM, respectively. Completion rate of questionnaires was high (> 85%) from BL to cycle 25 and mean BL scores were similar between arms.

Time to definitive 10% deterioration was longer in COMBO 450 arm vs VEM with FACT-M (HR, 0.40 [95% CI, 0.26–0.63]) and with QLQ-C30 (HR, 0.48 [95% CI, 0.33–0.68]). The MMRM analyses confirmed that the QoL was improved in COMBO 450 arm vs VEM across all cycles, with an estimated average 3.06 ($p < 0.0001$) and 5.63 ($p = 0.0021$) point improvement with FACT-M and QLQ-C30, respectively. The minimal clinically important difference was reached for both scales.

QoL is maintained longer in COMBO 450 arm compared to vemurafenib arm. The difference between arms was clinically relevant and consistent across the scales.

Dabrafenib plus Trametinib PKPD assessment in real-life patients with metastatic *BRAFV600^{mut}* melanoma.

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Background: Dabrafenib (D) plus trametinib (T) improves survival in *BRAFV600^{mut}* metastatic melanoma (MM) patients (P) with a 68% overall response rate (ORR) (COMBI-d). The aim of this retrospective study was to explore the impact of D and T pharmacokinetic (PK) on progression disease or related adverse events (RAE) occurrence in real-life P.

Methods: P with AJCC stage IIIc ($n = 4$) or IV ($n = 46$ including $n = 37$ M1c) *BRAFV600^{mut}* MM treated with D plus T were included after signed informed consent. Response evaluation, RAE (CTCAE v4.0) and LC-MS/MS quantification on plasma collections were performed monthly. D and T area under the curve 0–24 h (AUC), average (C_{avg}) and trough (C_{min}) concentrations were estimated using Bayesian approach. Median AUC, C_{avg} and C_{min} optimal cut-off to predict progression were determined with ROC curve analysis. Progression-free survival (PFS) was estimated using Kaplan Meier method and association with PK parameters (high *vs* low group) was assessed using log rank test. D and T AUC, C_{avg} and C_{min} association to all grades RAE was assessed by Wilcoxon test (R version 3.5.1).

Results: Fifty included P (1st line treatment $n = 26$) displayed a 10.6 [6.1-N/A] months median PFS and a 74% ORR. Median follow-up was 20.0 [8.4–70.9] months. All grades RAE occurred in 50% P (G 3 $n = 3$, G 4 $n = 0$). A median D C_{min} above 28.6 ng/mL was significantly associated with improved PFS ($p = 0.017$). Median trametinib PK parameters were not associated with PFS (NS). Unlike D (NS), T PK parameters were significantly higher in P experiencing all grades RAE (median C_{min} 13.1 [7.7–29.3] ng/mL) compared to P without treatment RAE (median C_{min} 11.7 [6.7–19.6] ng/mL) ($p = 0.0389$).

Conclusion: As low D exposure was associated to lower PFS, and high T exposure was correlated to AE occurrence, D and T therapeutic drug monitoring is of high interest to optimize therapeutic management.

Variants in *STAT3* gene in cutaneous melanoma susceptibility and functional analysis

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Background: The JAK/STAT signaling pathway supports the development, progression and metastatic potential of cutaneous melanoma (CM). In normal cells the activation of STAT3 is rapid and transient, but in abnormal melanocytes the activation of the protein occurs in a constitutive manner favoring tumor expansion. STAT3 is coded by the polymorphic gene in humans and thus it is possible that normal individuals show inherited differences in pathway functionality. The aim of the study was to evaluate whether *STAT3*c.*1671T>C and *STAT3*c.-1937C>G variants influence the risk of CM patients and its functional consequences.

Methods: We evaluated 248 MC patients and 274 controls. DNA was analyzed by real-time PCR for genotyping. Luciferase assay and gene expression in a genetically modified SK-MEL-28 melanoma cell line to present the *STAT3*c.-1937C>G ancestral and variant genotypes were realized in study. Differences between groups were assessed by the Fisher or chi-square test. Comparisons of luciferase and gene expression were performed using t-tests and ANOVA or Mann-Whitney and Kruskal-Wallis tests.

Results: Individuals with *STAT3*c.*1671TT and T allele, and *STAT3*c.-1937CC genotype had 1.76(95% CI: 1.08–2.85, $p = 0.02$), 1.42(95% CI: 1.03–1.96, $p = 0.02$) and 1.67(95% CI: 1.06–2.63, $p = 0.02$)-fold increased risks of CM than individuals with the other genotypes and allele, respectively. Individuals with TC haplotype of the *STAT3* variants were at 1.70(95% CI: 1.22–2.36, $p = 0.001$)-fold increased risk of CM than those with other haplotypes. In a genetically modified melanoma cell line to present the distinct genotypes of *STAT3*c.-1937C> G, we observed a 30% increase in the promoter activity of the gene and increase of mRNA in cells with the CC genotype.

Conclusions: The data present, for the first time, preliminary evidence that variants in *STAT3* gene alter the CM risk.

Combined inhibition of HMG-CoA Reductase and mitochondrial oxidative phosphorylation induces tumor regression of BRAF inhibitor-resistant Melanomas.

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BRAFV600-mutation-targeting drugs have dramatically improved clinical outcomes for melanoma patients with this mutation. However, primary and post-treatment secondary resistance are a significant problem, which in many instances are promoted by upregulation of mitochondrial oxidative phosphorylation (OxPhos). We recently showed that a novel OxPhos inhibitor, IACS-010759, induced tumor regression in high OxPhos, BRAF inhibitor (BRAFi)-resistant melanomas. BRAFi-resistant melanomas without high OxPhos were incompletely inhibited by IACS-010759 treatment, and combination with BRAFi did not provide additional benefit. However, there may be other molecular targets whose inhibition may synergize with IACS-010759 to induce complete tumor regression of these BRAFi-resistant melanomas.

To identify such targets, in this study, we performed a high-throughput combinatorial drug screen using FDA-approved small molecules. This *in vitro* drug screen identified hydroxymethylglutaryl-coenzyme A reductase inhibitors (HMGCRi), as among the most potent combinations with IACS-010759 that completely inhibited the growth of BRAFi-resistant melanomas. *In vivo* tumor xenograft growth studies revealed that combination of the HMGCRi, Atorvastatin, with IACS-010759 potently regressed the growth of these BRAFi-resistant melanomas. Molecular analysis showed that in addition to targeted inhibition of OxPhos and mevalonate pathways individually, the combination of IACS-010759 and Atorvastatin caused potent inhibition of the pro-growth and anti-apoptotic MAPK and PI3K-AKT pathways. As Atorvastatin and other clinically approved HMGCRi have well-known safety profiles, these could be promising combinatorial agents for improving the efficacy of OxPhos inhibitors in BRAFi-resistant melanomas.

Detecting plasma pembrolizumab concentrations in patients with melanoma using Liquid Chromatography/Mass Spectrometry

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Immunotherapy targets immune regulatory checkpoints and enhances the endogenous anti-tumour immune response. The main checkpoint inhibitor treatment options now available are anti-CTLA-4, anti-PD-1 and anti-PD-L1. In the clinical setting, 10% of patients receiving checkpoint immunotherapies will have a rapid disease progression with poor outcomes. To date, the plasma levels of immune checkpoint inhibitors has not been extensively investigated in relation to rapid disease progression whilst on treatment.

In our preliminary study, the plasma concentration of Pembrolizumab (pre-infusion) was measured by ELISA assay in a cohort of 16 metastatic melanoma patients on single agent Pembrolizumab. 2 patients

had rapid disease progression and consistently low or unquantifiable plasma concentrations of Pembrolizumab compared to the 14 patients who responded. This current study's aim was to develop a new approach to accurately quantify very low plasma Pembrolizumab levels.

ELISA assays are vulnerable to cross reactivity, lack linear dynamic range and require extensive validation as well as insufficient for measuring two immunotherapy agents simultaneously. Liquid chromatography-mass spectrometry (LC-MS/MS) combines the physical separation of liquid chromatography with two mass spectrometers. It offers analytical specificity, superior to that of ELISA and the ability to multiplex different analytes from the patient plasma. Here we have developed a LC-MS/MS to detect plasma levels of pembrolizumab that are too low for ELISA assays.

In conclusion, LC-MS/MS can more accurately detect low levels of Pembrolizumab in patient plasma than traditional ELISA assays. This approach can also be used to detect all monoclonal antibody checkpoint inhibitors, particularly when used in combination. In the future LC-MS/MS may be used to predict early progression on treatment.

Neoadjuvant Trial of Nivolumab in Combination with Canerpaturev (C-REV) Oncolytic Viral Therapy in Resectable Stage IIIB, IIIC, IVM1a Melanoma

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C-REV (formerly HF10) is a bioselected, replication-competent, neurovirulence attenuated oncolytic HSV-1 strain that has demonstrated antitumor activity in solid tumors as a single agent and in combination with checkpoint inhibitors. Herein we report interim clinical and correlative results from a single-arm, open label, Phase II study (NCT03259425) evaluating the safety and efficacy of neoadjuvant nivolumab and C-REV in resectable, advanced melanoma. Eligible patients had at least one injectable cutaneous, subcutaneous, or nodal metastasis. Nivolumab 240 mg IV was infused on Day 0, then q14 days until surgery on Day 90, followed by adjuvant 480 mg IV q28 days for up to one year. Single or multiple intratumoral injections of up to 5 mL of 1×10^7 TCID50/mL C-REV were delivered on Days 0, 7, 14, 21, 28, 42, 56, 70 and 84 in eligible tumors except for an untreated control lesion. Primary and secondary outcomes were pathological response rate at 90 days, and recurrence-free and overall survival, respectively. 7 patients were enrolled, and 6 received surgery and were evaluable for the primary outcome. Unexpected treatment-emergent adverse events included a red cell aplasia in 1 patient and secondary malignancies (squamous cell carcinoma) in 2 of 7 patients. 5 of 6 (83%) patients had major or complete pathologic response noted at surgery, with 4 (67%) having a pathologic complete response (pCR) in both injected/non-injected lesions resected. Radiographic partial response in both injected/non-injected target lesions was seen in 4 of 6 patients (67%) prior to surgery. 3 of 6 (50%) patients remained recurrence free with median follow up at 7.5 months. Cytokines and PBMCs from longitudinal blood samples along with on-treatment biopsies were evaluated in order to identify potential local and global biomarkers of response.

Melanoma circulating tumour cells: molecular heterogeneity and correlation with circulating tumour DNA

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Background: Circulating tumour cells (CTCs) can be assessed through a minimally invasive blood sample with potential utility as a predictive, prognostic and pharmacodynamic biomarker. The large heterogeneity of melanoma CTCs has hindered their detection and clinical application.

Methods: Here we compared three microfluidic devices for their recovery of circulating melanoma cells. The presence of CTCs in 43 blood samples from patients with metastatic melanoma was evaluated using a combination of immunocytochemistry and transcript analyses of 5 genes by RT-PCR and 19 genes by droplet digital PCR. Circulating tumour DNA (ctDNA) from the same patient blood sample, was assessed by droplet digital PCR (ddPCR) targeting tumour-specific mutations.

Results: Our analysis revealed an extraordinary heterogeneity amongst melanoma CTCs, with multiple non-overlapping subpopulations. CTC detection using our multimarker approach was associated with shorter overall and progression-free survival. Finally, we found that CTC scores correlated with plasma ctDNA concentrations, with similar pharmacodynamic changes upon treatment initiation.

Conclusions: Despite the high phenotypic and molecular heterogeneity of melanoma CTCs, multi-maker derived CTC scores could serve as viable tools for prognostication and treatment response monitoring in patients with metastatic melanoma.

Efficient BRAF^{V600E}-driven melanomagenesis requires the trafficking protein ARF6

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ARF6 is a small GTPase that controls the subcellular location of proteins to regulate invasion, proliferation and tumor microvesicle shedding. Aberrant ARF6 activation in cutaneous melanoma potentiates invasion and metastasis in BRAF^{V600E}-melanoma by controlling localization and activation of beta-catenin and PI3K/AKT. In uveal melanoma, ARF6 facilitates GNAQ^{Q209L} signaling to drive tumor cell growth by trafficking GNAQ to cytoplasmic vesicles. Although distinct diseases, pharmacologic inhibition of ARF6 reduces disease progression in both uveal and cutaneous melanoma models. These data underscore the importance of intracellular trafficking mechanisms within melanoma and highlight the potential clinical utility of targeting this machinery by inhibiting ARF6. In order to genetically dissect the role of ARF6 in BRAF and PI3K mediated melanomagenesis, we utilized RCAS-mediated delivery of Cre recombinase specifically to melanocytic cells to induce constitutive activation of BRAF^{V600E} (*Braf*^{CA}) and deletion of *Cdkn2a* (*Cdkn2a*$^{f/f}$) and *Arf6* (*Arf6*$^{f/f}$). Loss of *Arf6* significantly delays tumor onset and reduces tumor incidence and growth, while dramatically improving survival of *Braf*^{CA};*Cdkn2a*$^{f/f}$ mice. Activation of the PI3K pathway by deletion of *Pten*, with or without the addition of constitutively active AKT^{E17K}, accelerates tumorigenesis in *Braf*^{CA};*Cdkn2a*$^{f/f}$ mice. Despite the highly aggressive nature of these latter tumors, loss of *Arf6* nevertheless hinders melanomagenesis. Together these data are provocative, suggesting that ARF6 is crucial for BRAF^{V600E}-driven tumor initiation and growth and that oncogenic activation of the PI3K pathway is unable to fully restore tumorigenesis in the setting of ARF6 deletion. Our *in vivo* models provide a new paradigm for understanding the vulnerabilities of oncogenic BRAF and implicate intracellular trafficking mechanisms in melanoma development.

Allele-specific editing reveals that oncogenic NRAS modulates cell-intrinsic PD-L1 expression in NRAS-mutant melanoma

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NRAS mutations are highly prevalent drivers of melanomagenesis that constitute the second most common genetic subtype of cutaneous melanoma (~30%) after those with BRAF mutation. Unlike BRAF-mutant melanoma, NRAS-mutant tumors respond poorly to targeted therapies (such as MEK inhibitors) and oncogenic NRAS has proved challenging to target directly. Activating point mutations at codon 61 of NRAS (Q61K/R) account for the vast majority of these mutations. Thus, we investigated the consequences of selectively disrupting i) oncogenic NRAS^{Q61K} versus (ii) NRAS^{WT} or iii) both alleles regardless of codon 61 mutation status (pan-NRAS) using CRISPR-Cas systems in human melanoma cells. This allows us to dissect out the consequences of NRAS ablation in a genetically heterogeneous context. Guide RNAs targeting SNP-derived PAMs were screened using an exogenous reporter system and in a panel of melanoma cell lines following lentiviral delivery. One of two Q61K-directed gRNAs efficiently and selectively disrupted NRAS^{Q61K} genomic DNA, but not WT or Q61R. Targeted disruption of Q61K impaired NRAS^{Q61K} melanoma cell viability, elicited extensive apoptosis concurrent with p53 stimulation and suppressed colony formation. Notably, we found that PD-L1 expression was downregulated by NRAS ablation, raising the possibility that targeting NRAS may modulate anti-tumor immunity. We are further investigating the mechanism whereby oncogenic NRAS regulates PD-L1 expression and its contribution to immune response.

Bortezomib-induced immunogenic cell death enhances immune response in melanoma

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Neo-antigens derived from apoptotic tumor cells are sensed by the immune system and drive effective antitumor immunity. This 'immunogenic cell death' (ICD) can be elicited *in vivo* following bortezomib (26S proteasome inhibitor) treatment in some cancers. Here, we test the dynamics of bortezomib-induced ER stress and apoptosis in melanoma and whether this promotes ICD in the tumor microenvironment. We first confirmed that a clinically-relevant dose of bortezomib induced hallmarks of ICD *in vitro*. Necessary markers of ICD, including cell-surface expression of calreticulin, HSP70 and HSP90, were upregulated following bortezomib treatment in human and murine melanoma. Secretion of HMGB1 and ATP by apoptotic melanoma cells was also detected, further suggesting ICD initiation. We confirmed bortezomib-induced ER stress by real-time imaging of an ER stress biosensor in melanoma cells. We next asked whether bortezomib-treated melanoma cells could elicit anti-tumour immunity *in vivo*. Mice were primed subcutaneously with melanoma cells that were undergoing ICD. Mice were then challenged 10 days later on the opposite flank with matched live melanoma cells and tumor growth was monitored. We observed a delay in tumor onset and decreased tumor growth in mice that were primed by ICD compared to controls. Together these data demonstrate that melanoma cells undergo ICD following bortezomib treatment and this cellular stress

can be co-opted to enable effective anti-tumor immunity. We propose that targeting metastatic melanoma with an ICD-inducer could improve therapy outcomes, essentially immunizing the patient with their own personalised cancer vaccine in the form of ICD melanoma cells.

MITF-mediated extracellular matrix alteration reduces intratumoral heterogeneity in melanoma

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Tumor heterogeneity limits effectiveness of targeted melanoma therapies and promotes drug resistance. Understanding its underlying mechanisms is crucial to design novel or to further improve current therapies. We define dynamic heterogeneity as differential tumor cell cycle behavior in response to changing environmental conditions, which we have demonstrated in both melanoma xenograft tumors and 3D melanoma spheroids by real-time cell cycle imaging. This was characterized by the presence of defined clusters of proliferating cells and clusters of G1-arrested cells within the same tumor or spheroid. The location of the quiescent zones suggested oxygen and/or nutrient deprivation as the cause of cell cycle arrest, and the G1-arrested cells reversed to a normal cell cycle behavior when isolated and re-cultured under normoxia in 2D culture. We demonstrate that this dynamic heterogeneity is consistently decreased *in vitro* and *in vivo* by MITF, a transcription factor strongly associated with melanoma development, progression and therapy response. While this phenomenon was not associated with a reduced hypoxic core, we show that high MITF expression allows melanoma cell proliferation under hypoxia. In addition, using single-plane illumination microscopy, we show that modulation of MITF expression leads to changes of spheroid architecture, tensile stress and in extracellular matrix (ECM) and cell-ECM adhesion and crosstalk proteins. Furthermore, "ECM swapping" and inhibition of the Rho/ROCK signalling pathway, respectively, rescues and mimics the morphology and cell cycle effects of high MITF expression. These findings support a novel role of MITF in controlling dynamic intratumor melanoma heterogeneity through changes in the bilateral crosstalk between melanoma cells and the ECM of their tumor microenvironment.

Long-term outcomes in patients who achieved clinical benefit (SD, PR, CR) with pembrolizumab (pembro): pooled analysis of KEYNOTE-001 and KEYNOTE-006

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Pembro has robust antitumor activity and durable responses in pts with advanced melanoma. The prognostic value of an initial SD assessment with pembro treatment is unclear. This post hoc analysis of pts with melanoma treated with pembro in KEYNOTE-001 and KEYNOTE-006 evaluates long-term outcomes of pts with SD, CR, or PR at wk 12 (n = 284) and 24 (n = 241) from randomization. PFS and OS rates were estimated at 1 y, 2 y, and 3 y from the 24-wk assessment per Kaplan-Meier method. Of the 241 pts included in this analysis, 212 were treatment-naive; 29 received only BRAF±MEK inhibitors as prior therapy; 42 had CR, 160 had PR, and 39 had SD by central review at wk 24 (initial CR, PR, SD, respectively). At baseline, most pts, regardless of initial response, had ECOG PS of 0 and M1c disease. At data cutoff (Sep 1, 2017, KEYNOTE-001; Dec 4, 2017, KEYNOTE-006), pts with initial CR had 1-y PFS rate of 92.9%, 2-y PFS rate of 82.5%, 3-y PFS rate was not reached; pts with initial CR had 1-y OS rate of 100%; 2-y and 3-y OS rates were 97.6%. 32 (20%) pts with initial PR had CR as BOR; pts with initial PR had 1-y PFS rate of 79.2%, 2-y rate of 66.5%, 3-y rate of 44.4%; pts with initial PR had 1-y OS rate of 94.4%, 2-y rate of 87.5%, 3-y rate of 79.7%. Of pts with initial SD (n = 39), 1 (2.6%) had a BOR of CR, 13 (33.3%) had PR, and 25 (64.1%) remained SD. Pts with initial SD had 1-y PFS rate of 58.8% and 2-y and 3-y rates of 49.1%; pts with initial SD had 1-y OS rate of 97.4%, 2-y rate of 82.0%, 3-y rate of 73.5%. Of 241 pts, 21 (10.4%) had persistent SD. Similar analysis at wk 12 from randomization will be presented. Pembro treatment elicits a response, albeit delayed, in a substantial proportion of pts (35.9%) with initial SD. These results may inform treatment expectation for pts with initial SD with pembro and aid in future trial design.

Relationship between clinical efficacy and AEs of tebentafusp, a novel bispecific TCR–anti-CD3, in patients with advanced melanoma

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Bispecific antibodies have shown activity in hematologic but not solid tumors. Tebentafusp (TEBE; IMCgp100) is a first-in-class TCR–anti-CD3 bispecific ImmTAC molecule capable of redirecting polyclonal T cells against melanocyte-associated antigen gp100. In a Phase I/II study it showed activity in advanced melanoma; 1-yr OS of 65%. Treatment-related (TR) rash and cytokine-mediated AEs are hypothesized to be on-target effector mediated. We explored clinical and biologic pt characteristics associated with OS. 84 HLA-A2 + advanced melanoma pts (73% cutaneous, 23% uveal [UM] primaries; 51% LDH >ULN; 25% received prior anti-PD-[L]1) received TEBE in 13 dose escalation cohorts. Efficacy was assessed by KM survival and TRAEs by CTCAE v4.0. Serum samples evaluated changes in cytokines. A multivariate analysis investigated the relationship between efficacy and TRAEs. 83 (99%) pts had ≥ 1 TRAE with majority (56%) being Grade 1/2; mainly skin (rash 81%, pruritus 69%) or cytokine-mediated (pyrexia 57%) with tendency to decrease in frequency and severity post dose 3. The 2 most frequent Grade ≥ 3 TRAEs were rash (26%) and lymphopenia (13%). TEBE-induced transient increases in peripheral cytokines (peaking Day 1–2) attenuated with later doses; cytokine-mediated AEs had similar kinetics. Multivariate analysis associated longer OS with LDH ≤ULN (p = 0.002) and any-grade rash occurring within 21 days (p = 0.003); melanoma primary site and prior anti-PD-(L)1 did not significantly affect outcome. Longer OS trended with lower baseline serum IL-6 or TNFα (n = 45). TEBE demonstrated monotherapy activity in advanced melanoma, with generally manageable AEs consistent with the hypothesized MoA. We observed an association between TEBE efficacy and on-target TRAEs, as has been reported for bispecifics to heme lineage antigens. Pivotal studies in UM are ongoing.

BAP1 mutant uveal melanoma is stratified by metabolic phenotypes with distinct vulnerability to metabolic inhibition

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Targeting reprogrammed cancer cell metabolism is an encouraging therapeutic approach; however, a more precise understanding of metabolic subtypes is required to improve treatment efficacy. Mutations in BRCA1-associated protein 1 (BAP1) are highly prevalent in metastatic uveal melanoma (UM), which is the deadliest type of eye cancer in adults. Therefore, understanding of BAP1 functions in UM is important in order to improve overall survival. There is no study on the metabolic roles of BAP1 in UM. In this study, we observed that BAP1 altered samples were markedly separated from BAP1 unaltered samples in the expression of oxidative phosphorylation (OXPHOS) and glycolysis gene sets, which are the major parameters of cancer cell metabolism. Moreover, BAP1 mutant tumors were further divided into two subgroups that suggest BAP1 mutations result in heterogeneous metabolic modifications in UM. We re-expressed wild type BAP1 in two BAP1 mutant UM cell lines (MP46 and MP65) to investigate how BAP1 expression status modifies metabolism in the parental cells. We found two distinct metabolic dependencies by examining metabolomics data and key metabolic enzymes. MP65 cells relied on glucose utilization and nucleotide biosynthesis metabolic pathways whereas MP46 cells had increased dependency on fatty acid metabolism to maintain OXPHOS. Moreover, we observed that each metabolic subtype displayed specific sensitivities to unique classes of metabolic suppressors. In conclusion, our findings strongly indicate that targeting cancer cell metabolism can be a promising therapeutic option in BAP1 mutant UM and that tailored therapeutic approaches may be required to target BAP1 mutant UM based on their metabolic heterogeneity.

A small molecule that targets widespread dysregulation of the NRF2 pathway in melanoma

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The success in targeting lineage-specific oncogenes such as the estrogen receptor in breast cancer has offered the possibility that

targeting lineage-restricted survival factors can achieve both therapeutic efficacy and favorable tolerability. Guided by the dependency of the majority of melanomas to the melanocyte-specific transcription factor MITF, we have screened more than 330,000 compounds to identify inhibitors of MITF transcriptional activity. One candidate molecule, ML329, suppressed MITF activity at nanomolar concentrations and preferentially targeted the melanocyte lineage. However, in a panel of 489 cancer cell lines, we unexpectedly found that drug cytotoxicity was most strongly associated with the dysregulation of the KEAP1/NRF2 signaling pathway ($p < 10^{-21}$). The activation of the NRF2 pathway was sufficient to confer sensitivity to nanomolar concentrations of ML329, whereas its CRISPR-mediated depletion conferred resistance. Using mass spectrometry, we demonstrated that the activation of the NRF2 pathway converts ML329 to a bioactive form, whose direct target is the pan-essential kinase CK2. ML329 was found to bind to CK2alpha and CK2alpha prime (two subunits of the CK2 holoenzyme) *in vitro* and inhibited CK2 activity in an ATP-competitive manner. Thus, ML329 is an ATP-competitive inhibitor of the pan-essential kinase CK2 but requires metabolic activation that preferentially occurs in cells with activated NRF2 signaling. Collectively, this chemical-genetics approach has identified a unique and previously uncharacterized role of the NRF2 signaling pathway in melanoma. In light of recent data implicating the NRF2 pathway in BRAF inhibitor resistance, our studies establish the basis for the development of ML329 or its derivatives as an approach to target a clinically relevant pathway in melanoma.

The role of staging PETCT pT4b melanoma: a 5 year analysis

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In our UK tertiary referral centre patients diagnosed with pT4b cutaneous melanoma are offered PETCT for initial staging as a sensitive way to detect tumour metastasis. We assessed the value of PETCT in these patients in terms of positive findings from the scan, subsequent recurrence and survival. A 5 year retrospective analysis of all patients diagnosed histologically with pT4b melanoma who had staging PETCT in our hospital was carried out. Patients were identified using department records and cross-referenced with clinical coding. Patient demographics, final staging and results of PETCT were collected and recurrence and survival were monitored. Of the 60 patients identified over a 5 year period, 24 were females and 36 males. The median age was 74 (range 54–86). 13.3% had metastases identified on staging PETCT. 81.6% had wide local excision and 31.6% had sentinel lymph node biopsy. Over a median follow up period of 2.7 years, 58.3% had recurrence of their melanoma and 28.2% had died. Initial staging with PETCT may not be necessary for all patients with pT4b melanomas. Few patients had positive findings from the scan and it is a significant radiation dose which carries its own risks. Having the PETCT may also be delaying other interventions including wide local excision and sentinel lymph node biopsy. We can use these results to provide information to patients within our trust to assist them with making informed decisions regarding having PETCT. Development of national imaging guidelines for melanoma would be beneficial.

Modeling melanoma brain metastases with organotypic CNS slice co-cultures

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Melanoma is the deadliest form of skin cancer, and incidence is on the rise. Metastatic dissemination to the central nervous system (CNS) is responsible for the majority of melanoma associated deaths. In recent years, targeted and immunotherapies have had some success for patients with metastatic disease, however little progress has been made specifically treating melanoma brain metastases (MBM). Current pre-clinical models for studying MBM are laborious; and clinical advancements in managing brain metastases are slowed by the common practice of excluding the estimated 44% of melanoma patients with MBM from clinical trials. Here we have created an organotypic brain slice co-culture system to model MBM growth. Using this diagnostic platform we are able to grow both human melanoma and syngeneic mouse melanoma in thin slices of living C57BL/6 CNS tissue. We have successfully measured motility, invasiveness, and growth rates of ectopically seeded melanoma cells and spheroids. Importantly, melanoma cells cultured in the CNS slice induced perilesional reactive astrogliosis – a clinically relevant phenomena that may act to support tumor growth and is not observed with simple 2D cultures. Furthermore, we have used the organotypic brain slice co-culture to model high-throughput drug screening of MBM in its true microenvironment. Here as proof-of-concept, human melanoma cells expressing a luciferase based ERK1/2 reporter system showed reduced signal as the co-cultures were treated with increasing concentrations of inhibitors to the ERK1/2 pathway. Together these data suggest the CNS slice co-culture platform is a viable model system with broad utility for studying therapeutic applications against brain metastases.

Plasmid IL-12 gene electrotransfer combined with checkpoint inhibitors as an effective treatment for metastatic melanoma in a Murine model

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Interleukin-12 (IL-12) is a potent immunostimulator that has been shown to stimulate growth and survival of T cells as well as NK cells. Effective protocols to deliver plasmid DNA encoding IL-12 directly into tumors with reduced or no adverse events were developed utilizing gene electrotransfer (GET). Localized GET delivery of pIL-12 to the tumor microenvironment significantly delayed disease development and prolonged disease-free survival and induced long term immune memory protecting against challenge. The response was associated with a significant reduction in Treg and MDSC infiltration in B16F10 melanoma and increase of Granzyme B. Mice treated with pIL-12 GET that achieved protection against challenge were observed to have CD62LlowCD44high memory T cells. To enhance the systemic effect, we have evaluated combining PIL-12 GET with checkpoint inhibitors. In a metastatic model, in addition to a subcutaneous B16.F10 tumor, B16F10 melanoma cells expressing luciferase were injected via intraperitoneal route. A combination therapy consisted of pIL-12 GET delivered to the subcutaneous tumor together with an IP injection of anti-PD-1. Results on day 60 using an In Vivo Imaging System illuminated a lower flux signal in intraperitoneal metastases. Simultaneously, the primary subcutaneous tumors completely regressed in 80% of these mice. In mice that were partially treated (IL-12 or anti-PD-1 only) there was a reduced or no anti-tumor effect. The expression of $CD44^+$ $CD62L^-$ increased with time, which was detected on CD8 CTLs, suggesting that the memory response was still active at 120 days. The present findings have practical implications for the combination of IL-12 gene electrotransfer with anti-PD1. Other combinations are being explored as well as more comprehensive evaluation of the induced immune response.

Deconvoluting the 9p21 risk locus in melanoma

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~40% of familial melanoma cases have mutations in the *CDKN2A* gene on chromosome band 9p21, which encodes the tumor suppressors $p16^{INK4A}$ and $p14^{ARF}$. This region is also frequently lost, methylated or mutated somatically in both familial and sporadic melanomas, often affecting surrounding genes including *CDKN2B*, *MTAP*, *CDKN2B-AS1* and the Type I interferons. Here, we focus on the importance of common sequence variants at the 9p21 locus in predisposition to melanoma which have been firmly established by GWAS, including most recently a large GWAS meta-analysis (36,760 cases) completed by the Melanoma Meta-Analysis Consortium (lead SNP rs871024; $p = 3.32 \times 10^{-65}$, OR = 1.18). This region has a complex genetic architecture with conditional analyses of GWAS data indicating the presence of at least five additional independent causal variants marked by SNPs reaching genome-wide significance ($p < 5 \times 10^{-8}$; rs2027939, rs55797833, rs3217986, rs79356439, rs75883022) spanning from *MTAP* to *CDKN2B-AS1*. To identify likely causal variants in this region, we integrated a Bayesian-based fine-mapping approach with chromatin interaction data from promoter capture-C experiments in melanocytes, identifying physical interactions between risk variants and the promoters of *CDKN2A*, *MTAP* and Type I interferons. While *CDKN2A* is the strongest *a priori* gene candidate, analysis of primary melanocyte eQTL data suggests strong correlation between risk alleles and expression of *MTAP* ($p = 1.15 \times 10^{-7}$) and a poorly characterized pseudogene (RP11-149I2.5; $p = 9.10 \times 10^{-5}$). We are currently functionally testing our top credible causal variants via pooled and individual CRISPR and CRISPRi experiments in hTERT-immortalized dermal melanocytes. Local gene expression changes will be assessed by qPCR under melanoma-relevant conditions such as UVB exposure and $BRAF^{V600E}$ expression. These data will provide insights on how gene regulation alterations at 9p21 increase the risk for melanoma.

A novel pre-clinical model to study immune control of spontaneous melanoma metastases

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While melanoma is often diagnosed at an early stage, in some patients spread of tumour cells to the sentinel lymph nodes (SLN) will have already occurred at time of surgery. Although patients with cancer deposits in SLN have a higher risk of recurrence, the majority of patients will not relapse, which may indicate immune-mediated tumour cell control.

We have recently developed a murine model of cutaneous melanoma, that not only shows tumour formation with variable growth kinetics and penetrance but also spontaneously metastasises to draining lymph nodes. In order to better understand the mechanisms underlying immune control, we surgically excised primary tumours in mice, modelling curative-intend surgery in patients. Utilising melanoma cells genetically modified to express firefly luciferase, we were able to monitor metastasis progression in a longitudinal fashion by

bioluminescent in vivo imaging. We found that mice depleted of NK cells as well as mice genetically deficient for perforin but not TNF showed a profound defect in control of metastatic dissemination compared to control mice. Interestingly, some mice developed macroscopic metastases several weeks after surgery, suggesting that in these mice micro-metastatic deposits had been controlled over extended periods of time. Finally, in broadly immune-deficient mice but also in some wildtype mice metastases were not confined to draining lymph nodes but also spread to visceral organs. These results highlight the broad spectrum of disease outcomes in mice, ranging from immune control to metastatic disease, similar to what is seen in patients. Our novel epicutaneous melanoma and surgery model sets the stage for investigating the molecular mechanisms and identifying critical mediators in immune mediated melanoma control, with the ultimate goal of refining SLN assessment and preventing immune escape in the clinic.

A prospective observational study of Dabrafenib/Trametinib (D/T) Use in Austrian melanoma patients (Pts)

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Various baseline and treatment parameters impact outcomes in *BRAF* V600-mutated metastatic/unresectable melanoma pts in real-world. This was a prospective, non-interventional study (DATUM-NIS) of D/T use in Austrian pts with *BRAF* V600-mutated metastatic/unresectable melanoma. The aim of this study is to assess parameters identified in D/T clinical trials influencing the outcomes in clinical practice. Total 79 pts were included from 12 centers, with a median follow-up of 1.9 months (mo). Pts achieved a median PFS of 9.8 mo (6 mo PFS rate 78.5%) and a median OS of 15 mo. Overall, 62 pts received D/T as 1st-line, 16 as 2nd-line after PD-1 inhibition (OS 15.1 mo and 14.3 mo, respectively; $p = 0.96$) and 1 as 3rd-line. 53 D/T-treated pts (67.1%) had treatment-related adverse events (AEs); 95.1% of AEs were mild or moderate. Most pts had more than one metastatic site (79.8%, $n = 63$), with stage M1c being the most prevalent (74.7%, $n = 59$). Existence of brain as well as liver metastases had no significant impact on PFS (brain: 9.8 mo vs 10.5 mo, $p = 0.84$; liver: 7.3 mo vs 10.3 mo, $p = 0.19$). Elevated lactate dehydrogenase (LDH) levels ($n = 34$) had a detrimental effect on OS (≤ULN 17.9 mo, >ULN 9.7 mo, $p = 0.02$) but not on PFS (≤ULN 9.4 mo, >ULN 9.4 mo, $p = 0.64$). D/T treatment was interrupted in 49 pts (62%); however, the PFS rates were comparable (9.1 mo with interruption vs 10.5 mo without, $p = 0.09$). Major reasons for dose interruptions were pyrexia (34.5%) and abnormal laboratory results (24.1%). Pyrexia was noted in 40 pts (50.6%), with no impact on PFS (10.9 mo with pyrexia vs 9.4 mo without, $p = 0.34$). Dose reduction was recorded in 34 pts (43%), with no impact on PFS (9.8 mo with reduction vs 9.4 mo without, $p = 0.67$). The study confirms the efficacy of D/T treatment in real-world and describes the impact of prognostic factors, such as LDH, and AE-related therapy management.

Intracranial Anti-Tumor Activity with Encorafenib Plus Binimetinib in Patients with Melanoma Brain Metastases: A Case Series

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Background: Brain metastases in advanced melanoma is associated with poor prognosis. Phase III studies in melanoma usually exclude patients with untreated brain metastases, therefore identifying therapies with intracranial activity remains an area of unmet need.
Methods: We conducted a multicenter, retrospective case series investigation in consecutive *BRAF*- mutant patients with melanoma brain metastases (MBM) treated with the combination of BRAF inhibitor encorafenib plus MEK inhibitor binimetinib to evaluate the antitumor response. Assessments included intracranial, extracranial and global objective response rates (ORRs) (by modified RECIST v1.1); clinical benefit rate (CBR); time to response, duration of response, and safety.
Results: Twenty-four patients with stage IV *BRAF*-mutant MBM treated with encorafenib plus binimetinib in 3 centers in the United States were included. Patients had received a median of 2.5 prior lines of treatment and 88% had prior treatment with BRAF/MEK inhibitors. The intracranial ORR was 33% and CBR was 63%. Median time to response was 6 weeks and median duration of response was 22 weeks. Among the 21 MBM patients with prior BRAF/MEK inhibitor treatment, the intracranial ORR was 23% and CBR was 57%. Similar outcomes were observed for extracranial and global responses. The safety profile for encorafenib plus binimetinib was similar to that observed in patients with melanoma without brain metastases.
Conclusion: In summary, intracranial activity was observed for the combination of encorafenib plus binimetinib in patients *with BRAF*-mutant MBM, including in both BRAF/MEK inhibitor naïve and

pre-treated patients. Given these promising exploratory results and the limited options available for the treatment of brain metastases in *BRAF*-mutant metastatic melanoma, further prospective studies are warranted and ongoing.

Improved recurrence-free survival (RFS) from a randomized phase 2 study of neoadjuvant (neo) talimogene laherparepvec (T-VEC) plus surgery (surg) vs surg for resectable stage IIIB-IVM1a melanoma (MEL)

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We investigated the impact of neoadjuvant T-VEC in patients (pts) with resectable metastatic MEL. Here, we present the primary analysis of RFS at 2 years.

Pts with resectable stage IIIB/C/IVM1a MEL, ≥1 injectable cutaneous, subcutaneous, or nodal lesions ≥ 10 mm, and no systemic tx 3 mos prior were randomized 1:1 to 6 doses/12 wks of T-VEC followed by surg during wks 13–18 (Arm 1) vs surg during wks 1–6 (Arm 2). T-VEC was given as previously described (Dummer et al, ASCO 2019). The primary analysis estimated a between-group difference in 2-yr RFS on the ITT set. RFS events were defined as the first local, regional or distant recurrence or death due to any cause after surg. Per protocol, pts who withdrew prior to surg or had an R1 or R2 resection were considered an RFS event at randomization. An additional analysis calculated RFS from randomization to the date of first post-surg event regardless of surgical margin status.

150 pts were randomized (76 Arm 1, 74 Arm 2). Median (range) follow-up time was 31.2 (0.1, 49.9) mos. 75% in Arm 1 and 93% in Arm 2 had planned surg. In the per protocol analysis, 29.5% of pts in Arm 1 and 16.5% of pts in Arm 2 remained recurrence free (overall HR 0.75, $p = 0.07$). In the additional analysis, 50.5% of pts in Arm 1 and 30.2% in Arm 2 remain recurrence free (overall HR 0.66, $p = 0.038$). OS rates at 2 years were 88.9% in Arm 1 and 77.4% in Arm 2 (overall HR 0.49, $p = 0.050$). In Arm 1, T-VEC tx resulted in a 3x increase ($p < 0.001$) in intratumoral CD8 + cells. Mean CD8 + density and PD-L1 H-Score in arm 1 post-tx were higher than in arm 2 ($p < 0.001$).

Neo T-VEC improves 2-yr RFS and OS in resectable IIIB-IVM1a MEL. Increases in tumor inflammation after TVEC tx support a role for the adaptive immune system consistent with the mechanism of action.

Disparity in outcomes of melanoma adjuvant immunotherapy by demographic profile

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Demographic factors including age, sex, and race have been reported to impact melanoma survival. We obtained a melanoma Participant User File from the National Cancer Database to explore further the outcome disparities of melanoma patients by demographic profile. The file contained data on 523,492 patients treated for melanoma between 2004 and 2015. We focused on stage III patients since treatment options did not change substantially for this subset over the covered time period. After excluding patients with incomplete information, we analyzed data from 37,028 patients using SAS 9.4. Cox proportional hazard models were used to evaluate the covariates in relation to time to death separately and together in one model. Consistent with prior reports, we confirmed that age < or >= 65, sex, and race (white vs all other) correlated with survival in a highly significant manner ($p < 0.0001$ for all variables analyzed). Younger patients, females, and white patients all had superior outcome. Disease specific and treatment related variables of number of nodes (1 vs 2–3 vs 4 +), administration of adjuvant immunotherapy (yes vs no), and insurance status (private vs government) were also highly significant individually; fewer nodes, adjuvant treatment, and private insurance all provided a survival advantage. Combining all the variables into the Cox proportional hazards analysis, all variables except race remained as significant variables impacting survival: age >= 65 years HR = 1.50; females HR = 0.75; 2–3 vs 1 node HR = 1.51 and 4 + vs 1 node HR = 2.73; no immunotherapy HR = 1.41; Medicaid vs private HR = 1.65 and Medicare vs private HR = 1.42 (all p-values < 0.0001). Taken together, these findings indicate that disparities in melanoma survival due to biological differences based on age and gender exist, but access to timely diagnosis and multimodality treatment significantly contributes to outcome as well.

Improved survival outcomes of combination BRAF inhibitors and MEK inhibitors regimen in patients with advanced melanoma compared with BRAF inhibitor monotherapy

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BRAF inhibitor therapy has shown promising results in terms of survival in patients with BRAF mutated metastatic melanoma. However, emergence of the resistance against BRAF inhibitors is a concern, combination of BRAF INH and MEK INH has shown benefits in preclinical studies. This led to the trials on combination of BRAF INH and MEK INH. Our meta-analysis included three RCTs and we compared

long term survival outcomes of combination therapy against BRAF INH monotherapy. Comparison of Overall Survival rates at 1, 2 and 3 years was done. We conducted a systematic search of Medline (Pubmed) and Cochrane Central Register of Controlled Trials for abstracts and fully published studies. Data were abstracted by two independent reviewers. Using Peto Odds Ratio method, a Fixed effect model was used to calculate weighted Peto Odds Ratios. RevMan 5.3 was used for statistical analyses. Three randomized clinical trials & follow up analysis of same trials met criteria. Our analysis included 1279 patients. Using Peto Odds Ratio method, a fixed effect model was used to calculate the weighted peto odds ratios. Combination (Dabrafenib and Trametinib) therapy group showed significant increase in overall survival at 1 year compared to BRAF INH monotherapy group (Peto Odds ratio [peto], 0.77; 95% confidence interval, 0.60–0.98), $p = 0.03$). Combination therapy showed increasing trend in the overall survival at 2 (Peto Odds ratio [peto], 0.64; 95% confidence interval, 0.51–0.80), $p = 0.0001$), and at 3 years (Peto Odds ratio [peto], 0.60; 95% confidence interval, 0.47–0.76), $p = 0.0001$). Combination therapy (Dabrafenib and Trametinib) showed consistent improvement in the overall survival outcomes at 1, 2 and 3 years with increasing benefit at 2 and 3 years when compared with BRAF INH monotherapy in patients with advanced melanoma.

CDK13 mutations drive melanoma via accumulation of prematurely terminated transcripts

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Transcriptional Cyclin Dependent Kinases modulate RNA Polymerase II function to impact gene expression. Here, we show that *CDK13* is mutated in 4% of patient melanomas and mutation or downregulation is associated with poor overall survival. The amino acid changes produced by mutation in melanoma are identical to mutations recently implicated in a congenital syndrome, underscoring the critical function of these residues. Mutant CDK13 lacks kinase activity and overexpression in zebrafish leads to accelerated melanoma. CDK13 mutations function in a dominant negative manner and require CCNT1 for their oncogenic activity. Dominant negative mutant CDK13 localizes to chromatin and disrupts RNAPII indicating a transcriptional mechanism. CDK13 mutant fish and human melanomas accumulate prematurely terminated RNAs that are translated into truncated proteins. CDK13 binds to and regulates the phosphorylation of ZC3H14, a member of the PolyA eXosome Targeting (PAXT) RNA degradation complex. ZC3H14 phosphorylation recruits the PAXT complex to degrade prematurely terminated polyadenylated transcripts in the nucleus. In the presence of mutant CDK13, ZC3H14 phosphorylation is compromised and consequently fails to recruit the PAXT complex, leading to truncated transcript stabilization. This work establishes a role for CDK13 and the PAXT nuclear RNA degradation complex in cancer and has prognostic significance for melanoma patients with mutated or downregulated CDK13. Melanoma patients with mutant CDK13 would benefit from careful monitoring and possible adjuvant trial inclusion.

Implications of sarcopenia and obesity on patients undergoing checkpoint blockade for metastatic melanoma

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Background: The incidence of melanoma is one of the fastest rising of all cancers, and mortality in advanced cases approaches 80%. While numerous systemic therapy options exist, treatment responses vary and side effects can be severe. Thus, identification of factors affecting response is needed. Sarcopenia is associated with poor prognosis and outcomes across multiple malignancies. The predictive value of sarcopenia on survival and response to immune checkpoint blockade (ICB) in patients with metastatic melanoma (MM) remains unknown.

Methods: A retrospective review of patients with MM undergoing ICB between May 2011 and August 2017 was performed. Cross sectional imaging (CSI) acquired within 3 months before initiation of ICB was reviewed. The cross sectional area of the psoas muscle at the L3 vertebra was measured and normalized to height to obtain the skeletal muscle index (SMI), and muscle attenuation (MA) represented by Hounsfield units was noted. Body mass index (BMI) and response rates were recorded. Survival curves were analyzed.

Results: There were 93 patients with MM and CSI available before initiation of ICB. There was no survival difference based on gender, BMI, or MA. Patients with low SMI had shorter overall survival (OS) and progression free survival (PFS) than those with medium and high SMI ($p = 0.03$). Overweight patients had better response to therapy than those with normal or low BMI ($p < 0.05$). While there was no overall difference between response rates and SMI, there was a correlation with high SMI and better response to ICB in patients categorized as clinically obese ($p = 0.06$).

Conclusion: Sarcopenia is associated with decreased OS and PFS in patients receiving ICB for MM. While not statistically significant, there was an association between high SMI and better response to ICB in obese patients. SMI may predict response to ICB in obese patients with MM.

A device strategy for isolating melanoma Exosomes

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Melanoma-derived exosomes are 30–150 nm vesicles released from cancer cells that carry tumor cargo, prime the metastatic niche, suppress immunity, and transmit resistance to therapeutic drugs. There is an unmet need to leverage melanoma exosomes for disease monitoring and for strategies to ameliorate exosomes in a therapeutic context. This work evaluated Aethlon Medical's Hemopurifer device, a hollow fiber plasmapheresis cartridge with 200 nm average pore sizes that contains the lectin *Galanthus nivalis* agglutinin immobilized as an affinity agent to capture exosomes by binding high mannose glycoproteins on their surfaces. The data show that a benchtop Hemopurifier device can be used to isolate morphologically intact melanoma exosomes directly from recirculating plasma. Immunocapture and on-bead flow cytometry using the isolated exosomes revealed expression of typical exosomal markers, namely tetraspanins, and of a tumor-specific epitope of chondroitin sulfate proteoglycan 4. The isolated exosomes induced T cell apoptosis *in vitro*, indicative of their immunosuppressive functions. The benchtop Hemopurifier can thus be used to isolate melanoma exosomes for downstream profiling. The data also provide support for a therapeutic strategy using the clinical Hemopurifier, operated using hemodialysis infrastructure, for clearing circulating exosomes from melanoma patients.

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Cost-per-outcome analysis of nivolumab compared with dabrafenib + trametinib as adjuvant therapy for patients with stage IIIB/C *BRAF*-mutant cutaneous melanoma

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Nivolumab (NIVO) and dabrafenib + trametinib (DAB+TRAM) are approved as treatments in the adjuvant setting for patients with *BRAF*-mutant melanoma. This study compared the cost per recurrence-free survivor and cost per recurrence-free life-year (RFLY) of adjuvant NIVO and DAB+TRAM in patients with stage IIIB/C *BRAF*-mutant cutaneous melanoma.

Recurrence-free survival (RFS) rates and RFLYs were estimated using a matching-adjusted indirect comparison of NIVO and DAB+TRAM based on the CheckMate 238 and COMBI-AD trials, respectively. Total treatment costs at 12, 24, and 36 months (mo) were estimated based on package inserts, public data sources, and literature reviews. The cost per recurrence-free survivor and cost per RFLY were calculated by dividing the total treatment costs by RFS rates and RFLYs of treatment, respectively. All costs were presented in 2019 US dollars.

The RFS rates for NIVO and DAB+TRAM were 71% (95% CI, 64%–80%) and 86% (83%–90%) at 12 mo, 64% (56%–73%) and 63% (58%–69%) at 24 mo, and 59% (51%–69%) and 54% (49%–60%) at 36 mo, respectively. At 12 mo, the cost per recurrence-free survivor for NIVO ($228,906; 95% CI, $204,846–$255,791) was substantially lower than for DAB+TRAM ($282,236; $269,692–$292,437). This difference increased at 24 mo ($255,491 vs $385,274, respectively) and at 36 mo ($275,286 vs $449,487). Similarly, the cost per RFLY was substantially lower for NIVO ($196,430; 95% CI, $184,531–$209,970) than for DAB+TRAM ($255,129; $250,649–$259,771) at 12 mo. This difference was consistently lower at 24 mo ($108,740 vs $144,176, respectively) and at 36 mo ($77,037 vs $107,267).

With a substantially lower cost per recurrence-free survivor and cost per RFLY, NIVO was more cost-effective than DAB+TRAM as treatment in the adjuvant setting for patients with stage IIIB/C *BRAF*-mutant melanoma.

Real-world (RW) clinical characteristics, healthcare resource utilization (HCRU), and clinical outcomes in US patients (pts) with resected stage III/IV melanoma (MEL)

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Recurrence is high in pts with resected stage III/IV MEL. Although trial data suggest that adjuvant nivolumab (NIVO) or dabrafenib + trametinib (DAB+TRAM) may reduce recurrence risk, little is known about RW outcomes in these pts. This retrospective chart review assessed RW clinical characteristics, HCRU, and clinical outcomes in pts with resected stage III/IV MEL with no evidence of disease (NED) diagnosed in 59 US sites between 1/1/16 and 4/30/18. Pts aged < 18 years or with ocular MEL were excluded. Collected data were summarized descriptively. A total of 287 pts were identified; 123 received NIVO and 45 received DAB+TRAM (median follow-up, 12.6 and 13.0 months, respectively). Overall, the mean (SD) age was 60.5 (12.3) years, and most pts were male (56%) and Caucasian (92%). Stage IV MEL with NED was more common among DAB+TRAM-treated pts (22%) than among NIVO-treated pts (11%). Comorbidities at diagnosis were more frequent in NIVO-treated pts than DAB+TRAM-treated pts; these included diabetes (21% and

11%, respectively), chronic obstructive pulmonary disease (6% and 2%), heart failure (7% and 4%), and myocardial infarction (7% and 0%). Emergency department visits occurred in <1% (1/123) of NIVO-treated pts and 11% (5/45) of DAB+TRAM-treated pts; ≥1 hospitalizations occurred in 3% (4/123) of NIVO-treated pts and 7% (3/45) of DAB+TRAM-treated pts. Mean (SD) length of hospital stay was 4.8 (1.0) days for NIVO-treated pts and 5.3 (1.5) days for DAB+TRAM-treated pts. Among pts with at least 12 months of follow-up, 1-year recurrence rates were 11.6% (8/69) for NIVO-treated pts and 16.0% (4/25) for DAB+TRAM-treated pts. In this RW study, pts with resected stage III/IV MEL who received NIVO had several common comorbidities. Proportionally, NIVO-treated pts had lower HCRU, than those receiving DAB+TRAM.

Tumor-associated lymphoid aggregates determine T cell phenotype and are associated with melanoma outcome and response to immune checkpoint therapy

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Checkpoint blockade therapies that reactivate tumor-associated T cells can induce durable tumor control resulting in long-term survival of patients with advanced cancers. Currently, predictive biomarkers for therapy response include high intratumor immunological activity, high tumor mutational burden and specific characteristics of the gut microbiota. Although the role of T cells in antitumor responses has been thoroughly studied, other immune cells remain insufficiently explored. To investigate the role of B cells, we used clinical samples of metastatic melanomas (n = 177) and found that the concomitant presence of tumor-associated $CD8^+$ T cells and $CD20^+$ B cells was associated with improved survival independent of other clinical variables. Importantly, immunofluorescent staining of CXCR5 and CXCL13 in combination with CD20 supported the formation of lymphoid aggregates (LA) that have characteristics of tertiary lymphoid structures (TLS). To further understand the role of B cells in melanoma we employed Nanostring GeoMx™ data on 52 individual melanoma tumors and analysed B cell-, T cell and tumor populations separetely using 60 different immune proteins. We found that T cells in tumors without LAs had a dysfunctional or exhausted molecular phenotype. Together, these results indicate that tumor-associated LAs have a key role in the immune microenvironment conferring distinct T cell phenotypes in melanoma tumors. Therapeutic strategies aiming at inducing TLS formation should be explored to further improve clinical response to cancer immunotherapy.

MAPK-pathway inhibition driven changes in cellular thiol-redox state mediate drug resistance in metastatic melanoma

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Initial responses to MAPK-pathway inhibitors (MAPKih) led to a paradigm shift in treatment options for $BRAF^{V600E}$positive metastatic melanoma patients. However, the overall impact of MAPKih on patient survival has been limited by the acquisition of resistance to treatment that arises almost invariably (5-y survival < 25%). While ongoing research has identified potential cellular pathways that are implicated in the acquisition of resistance, the precise mechanism and clinical treatment options that could be applied to overcome resistance remain elusive. Recent studies have indicated a role of oncogenic BRAF in regulating cellular oxidative metabolism. Studies have identified autophagy and cellular metabolic adaptations as compensatory survival mechanisms mediating resistance to MAPKih in melanoma. In this study, we explore the contribution of cellular thiol-redox state in mediating adaptation of melanoma cells to MAPKih via autophagy. We show that acquisition of resistance to MAPKih in melanoma cells is accompanied by a steady increase in cellular autophagic flux, which coincides with a profound alteration in the cellular thiol-redox state indicated by a 68% depletion of reduced glutathione (GSH) and a 30-fold increase in oxidized glutathione (GSSG). Further, we establish thiol-redox state as an important contributor to the development of resistance by inhibiting the acquisition of resistance and autophagic flux in cells treated with MAPKih in combination with buthionine sulfoximine (BSO, GSH-synthesis inhibitor). Further, administration of vemurafenib in combination with BSO significantly improved the progression free survival (75% mice tumor-free) in mice bearing A375 xenografts. These findings suggest targeting cellular redox metabolism as a plausible treatment option to overcome the acquisition of resistance to MAPKih in metastatic melanoma patients.

Primary melanoma grow according to a hierarchical model

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Melanoma is notorious for its high degree of *heterogeneity and plasticity*, which are key drivers of metastatic spreading and therapy resistance. Studies addressing the contribution of the heterogeneous subpopulations to melanoma growth have so far remained limited to functional *in vitro* clonogenic assays and xenotransplantations and have led to conflicting conclusions. A key pending question in the field is whether melanoma follows the *stochastic model* of growth, where virtually all cells are *equipotent* in their ability to sustain tumor growth, or the *Cancer Stem Cell (CSC) model*, where only a small fraction of cells fuel tumor growth. To capture the dynamics of individual tumor growth capacity within their natural microenvironment, we performed multicolour lineage tracing in a spontaneous (NRAS-driven) mouse model of melanoma. Quantitative clonal analyses, dual pulse labelling proliferation assays, 3D tumor reconstruction and mathematical modeling revealed that the majority of labelled melanoma cells exhibited limited proliferative potential, whereas a small fraction of cells displayed the capacity to persist long term, giving rise to progenies that occupy a significant part of the tumour. These unbiased analyses uncovered the presence of two distinct proliferative cell states organized in a *hierarchical manner* indicating that the more persistent state has *stem cell-like* features, whereas the other gives rise to terminally differentiated tumour cells. This study highlights the existence of CSCs in primary melanoma lesions, an observation that have important therapeutic implications. Experiments aiming at isolating and characterizing those cells (i.e. single-cell -OMICS, spatial distribution by multiplex iamging) are ongoing.

A versatile ES cell-based platform to maximize melanoma mouse modeling

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The cumbersome and time-consuming process of generating new mouse strains and multi-allelic experimental animals often hinders the use of genetically engineered mouse models (GEMM) in cancer research. Here, we describe the development and validation of a melanoma embryonic stem cell (ESC)-GEMM platform. Our platform incorporates twelve clinically relevant genotypes composed of combinations of four driver alleles (LSL-BrafV600E, LSL-NrasQ61R, PtenFlox, Cdkn2aFlox) and regulatory alleles to spatiotemporally control the perturbation of genes-of-interest. Our ESCs produce high contribution chimeras, which develop melanoma phenotypes that are similar to conventionally bred mice. To validate our melanoma ESC-GEMM platform, we modulated Pten expression on a $Braf^{V600E}$ background for proof-of-principle experiments. We first demonstrate how the different efficiency and kinetics of CRISPR-Cas9, RNA interference (RNAi), and Cre/loxP impact melanoma formation, and highlight how inducible CRISPR-Cas9 can be utilized to study cancer gene function in vivo. Moreover, using inducible RNAi and cDNA expression constructs, we examine the effect of Pten restoration on melanoma prevention and maintenance. Finally, we show that chimera-derived melanoma cell lines retain regulatory allele competency and can be used in vitro and in syngeneic grafts in vivo to complement ESC-GEMM chimera experiments. Thus, when combined with modern genetic tools, our ESC-GEMM platform enables rapid, high-throughput, and versatile studies aimed at addressing outstanding questions in melanoma biology.

Identification and characterization of a fast migrating subpopulation that may drive organ-specific metastasis via interactions with extracellular matrix

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Late-stage melanoma is characterized by increased tumor invasion and poorer patient outcomes. While various studies have revealed that most commonly known metastasis markers are expressed in melanoma cells, not all melanoma cells expressing these markers are known to invade similarly, prompting us to question the differences between invasive cells at a single cell level. We selected a population of melanoma cells that invades through the transwell (invasive population) and cells that remain in the transwell (non-invasive population) from the bulk population of melanoma cells. We identified transcriptomic differences that explain the variable invasiveness of these melanoma cell populations and also observed differences in the growth rates of these different populations. We hypothesized that not only the melanoma cells but also their surrounding microenvironment, particularly the extracellular matrix, might also be involved in determining melanoma invasion. We tested this hypothesis by using fibroblasts isolated from multiple tissues to recreate ECM *in vitro* that closely resembles an *in vivo* matrix. The extracellular matrices from different tissues affect growth rates as well as migration frequencies of melanoma cells based on the tissue, which also matches with the observed rates of metastatic lesions in melanoma patients. We are now assessing these tissue matrices using proteomics to identify differences that may promote tumor migration and establishment of metastasis in these tissues. Further, we are characterizing the migrating melanoma cells at single cell resolution to

identify non-genetic differences within melanoma cells that allow some melanoma cells to escape the primary tumor and establish metastases. Our work has important implications towards the development of rational therapies for metastatic melanomas.

Gut microbial signatures predict response to immune checkpoint blockade (ICB) in melanoma

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The gut microbiome is recognized as an important modulator of immunity and response to cancer therapy. We recently identified a gut microbial signature ('Type-I' signature) that was highly associated with response, and was dominated by enrichment of Ruminococcacceae. To validate this, we performed analysis in an expanded cohort of melanoma patients treated with ICB (anti-PD-1 therapy). We characterized the gut microbiome using 16S rRNA v4 (n = 114) and shotgun metagenome sequencing (n = 74). We constructed microbial networks by using pairs of operational taxonomic units (OTUs) that met $-0.80 \geq \text{rho} \geq 0.80$, $p < 0.01$. Consistent with our prior findings, alpha-diversity and Firmicutes, notably Ruminococcaceae, *Faecalibacterium*, *F. prausnitzii* were significantly higher in responders at baseline. Network analysis revealead that topological features were not conserved between responders and non-responders, suggesting an alteration of co-occurrence patterns of OTUs. Lastly, functional metagenomics demonstrated differential metabolic capabilities in responders versus non-responders, and selective enrichment of pathways within each group. Our results point towards a continued utility of the Type-I signature and exploits differences in physiological and ecological traits of microbial communities between the response groups. Further analysis is currently underway to better understand the mechanisms through which these microbes facilitate immunotherapy response.

Clinico-pathological characteristics of cutaneous malignant melanoma: 20-year experience from a lower middle income country

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Background: The worldwide incidence of cutaneous melanoma (CM) has been on the rise over the past few decades. Primary prevention and early treatment remain the focus of management to reduce disease burden. This entails identification of risk factors to prompt early diagnosis. In Pakistan, there is a scarcity of clinico-pathological data relating to cutaneous malignant melanoma.

Objective: To analyze clinico-pathological characteristics of patients with cutaneous malignant melanoma in Pakistan, and to compare the results with other studies.

Method: All patients presenting to Shaukat Khanum Memorial Cancer Hospital with a diagnosis of cutaneous malignant melanoma were included in the study. Patient demographics, tumor type and tumor stage were recorded. Data was analyzed in SPSS. For variables such as age mean values were calculated. For categorical variables percentages were recorded. Chi Square test was used to measure associations.

Results: Between 1997 and 2017, a total of 169 cutaneous melanoma patients were registered at Shaukat Khanum. Mean age was 47.5 years. The highest incidence of melanoma was seen in the age group 40–59 years (n = 69, 40.8%). Most commonly reported clinical subtype was unspecified melanoma (n = 154, 91%). Amongst those in which it was reported, the most frequently observed T-stage at presentation was T4 (n = 23, 13.6%). In this study, CM developed most frequently in the lower limbs. The yearly incidence of melanoma has remained stable from 2007 to 2017.

Conclusion: Cutaneous malignant melanoma is a rare disease in Pakistan. However, patients tend to present at more advanced stage as compared to patients in developed countries. Identification of risk factors and tumor characteristics is therefore of paramount importance in dealing with these patients.

Keywords:
cutaneous malignant melanoma, Pakistan, lower middle income country

PRMT5 control of antitumor immunity in melanoma

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Protein arginine methyltransferase 5 (PRMT5) controls diverse cellular processes implicated in cancer development and progression. Here, we identify an additional role in controlling antitumor immunity. Gene expression analysis indicated an inverse correlation between immune-associated gene signature and PRMT5 activity/expression in melanoma. Notably, the loss of PRMT5 expression/activity reduced, while the gain of PRMT5 expression/activity increased, melanoma growth in immunocompetent but not immunocompromised mice, which was supported by the abundance of TILs (tumor infiltrating lymphocytes) in tumors. Mechanistically, PRMT5 attenuated cytosolic-DNA-induced interferon and chemokine production by methylation of IFI16/IFI204, part of the cGAS/STING complex. Furthermore, PRMT5 inhibited NLRC5 transcription, a master regulator of genes involved in MHCI antigen presentation. Indeed,

PRMT5 knockdown augmented interferon and chemokine production, and MHCI expression. Correspondingly, elevated expression of IFI16/IFI204 and NLRC5 was associated with growth inhibition of murine melanoma and with prolonged survival of melanoma patients. Importantly, PRMT5 inhibition significantly enhanced the efficacy of immune checkpoint therapy *in vivo*. Our study defines antitumor immunity based on tumor intrinsic PRMT5 activity, providing a rationale to include PRMT5 inhibitors in immunotherapy-based clinical trials.

The necessity of restaging in korean melanoma patients according to 8th Edition of the AJCC cancer staging manual

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American Joint Committee on Cancer (AJCC) has recently revised the melanoma staging manual from 7th to 8th edition, including key changes in Tumor, Nodes, Metastasis (TNM) classification and prognostic stage groups. However, it is not recommended to restage all previously diagnosed melanomas. Therefore, we aimed to investigate the necessity of restaging and additional tests or treatments in Korean melanoma patients staged by 7th edition of AJCC cancer staging manual (7th AJCC CSM). The 7th and 8th AJCC CSMs were meticulously compared and analyzed. The final pathological prognostic staging (PPS) of 275 melanoma patients from 2011 to 2018 were classified using both 7th and 8th AJCC CSMs, and their differences were analyzed.

Among the total 276 primary melanomas from 275 patients, 64 cases (23.2%) showed differences in staging between 7th and 8th AJCC CSMs. The PPS changed in 35 cases (12.7%), and 29 cases (10.5%) had altered only TNM classification in 8th edition with the same final PPS. Main causes of the changes in the PPS include: 1) 7 cases (20.0%) was restaged from stage IB to IA. In which, revision of stage IA to include T1bN0M0 caused 5 cases, and redefinition of T1a and T1b caused 2 cases; 2) final stages of 27 cases (77.1%) in stage III group by 7th edition, increased to stage IIIB ~ IIID by 8th edition through changes of T, N categories. There was no patient in our cases who needed additional sentinel lymph node biopsy (SLNB) or systemic treatment. However, complementary counseling was needed for stage III patients, because melanoma-specific survival rate for stage III melanomas increased in 8th edition. Although careful attention should be needed for the patients at changing points for additional SLNB, systemic treatment, and disease prognosis, restaging of previously diagnosed melanomas was not significantly needed in our patients.

Clinical efficacy of a combination of nivolumab and ipilimumab (Nivo/Ipi) in patients with metastatic melanoma (MM) with low tumor mutation burden (TMB)

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TMB is positively correlated with clinical response to checkpoint inhibitors (CPI). It is essential to identify negative predictors for CPI to select appropriate patients (pts) for the Nivo/Ipi regimen, which is associated with severe toxicity. We conducted a retrospective study to evaluate clinical activity of Nivo/Ipi in pts with MM with low TMB.

Melanoma databases from 2 institutions were utilized to identify pts who were treated with Nivo/Ipi. Eligibility criteria included metastatic or unresectable melanoma with evaluable lesion(s) for clinical response and adequate survival follow up. Low TMB was defined as ≤ 8 mutations/Mb and was assessed on 0.8–1.1 Mb of DNA using the FoundationOne assay.

We identified 34 pts who met the eligibility criteria: median age 62 years; male 50%; AJCC v7 stage IV(M1c) 68%; mucosal origin 12%, uveal origin, 24%; *V600 BRAF* 32%; high LDH 50%; median number of metastatic organ sites 2; median TMB 3.5 mutations/Mb; previous PD-1 therapy 12%; previous BRAF inhibitor therapy 3%. The overall response rate for Nivo/Ipi was 18% (1 CR; 5 PR); 68% of pts had early disease progression. Median PFS was 3.0 months, and median OS was 9.6 + months. Among 26 pts with visceral organ (including lung) metastases, 2 (8%) had a PR, and 20 (77%) had PD. Among 18 pts with non-uveal melanoma with visceral organ metastases, 2 (11%) had a PR and 15 (83%) had PD. Characteristics of the responders (n = 6): median age 63; median TMB 4 mutations/Mb; *V600 BRAF*, 50%; high LDH, 66%, metastatic sites in skin/LN/Soft tissue only, 66%.

The clinical activity of the Nivo/Ipi regimen in MM pts with a low TMB is modest, with most pts experiencing early disease progression within 3 months. This population is an ideal target for clinical trials to improve clinical outcomes.

Intrinsic microsomal PGE2 synthase-1 regulates immune suppression via COX-2-independent pathway in human and mouse melanoma models

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We has identified microsomal prostaglandin E2 synthase 1 (mPGES1) as an important downstream effector of the cyclooxygenase 2

(COX2) pathway often expressed by melanoma cells, and largely responsible for tumor-mediated immunosuppression. We found strong correlations in expression and co-localization of COX2 and mPGES1, for which both are associated with increased expression of immunosuppressive markers in human melanoma. In a syngeneic melanoma mouse model, mPGES1 knockout (KO) significantly suppressed tumor growth (around 70%) as it did in COX-2 KO. However, RNAseq analysis demonstrated that the gene expression changed by mPGES1 or COX2 KO showed distinct profiles, suggesting that mPGES1 could have additional functions. In addition, we found that mPGES1 KO dramatically increased PGD2, which has been known as a tumor suppressor, whereas it was not detectable in control and COX-2 KO cells. Therefore, mPGES1 inhibition might be more efficient to suppress tumor growth than COX2 inhibition through suppressing pro-tumorigenic PGE2 and enhancing tumor-suppressive PGD2. mPGES1 deletion enhanced infiltration of CD8a+ T cells (around 8 fold) into tumors and durable tumor regression was observed in mice bearing mPGES1 KO tumors that were given anti-PD-1 treatment in a syngeneic melanoma mouse model. Research in the development of mPGES1 inhibitors is needed to validate their use as safe treatment options, but appears attractive and feasible based on data to date.

AKT1^{E17K} activates focal adhesion kinase and promotes melanoma brain metastasis

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Hyper-activation of the PI3K/AKT signaling pathway occurs in most metastatic melanomas and increased PI3K/AKT pathway activity correlates with disease progression. The serine/threonine kinase, AKT, represents a major signaling hub within the pathway and consists of three highly conserved paralogs that have both distinct and overlapping functions. Activating mutations of AKT1 and AKT3 occur in human melanoma but their role in melanoma formation and metastasis remains unclear. Using an established melanoma mouse model, we evaluated the ability of constitutively active E17K, E40K, or Q79K mutants of each AKT paralog to promote tumor progression and metastasis in the context of BRAF^{V600E} expression and loss of *Cdkn2a* and *Pten*. Expression of AKT1^{E17K} promoted highly aggressive melanomas that metastasized to the lungs and brain. This metastatic phenotype was not significantly observed in the case of other mutant AKT-positive tumors, suggesting that the AKT paralogs have distinct, non-overlapping roles in the development of melanoma metastases. AKT1^{E17K}-positive tumors showed AKT1^{E17K}-dependent up-regulation of multiple focal adhesion (FA) factors, which are key components of focal adhesions and established stimulators of cell motility, as well as phosphorylation of focal adhesion kinase (FAK). Ectopic expression of AKT1^{E17K} in non-metastatic melanoma cells increased cell invasion, a phenotype abrogated by pharmacological inhibition of AKT or FAK. These findings strongly suggest that one mechanism by which AKT1 promotes melanoma metastasis is through regulation and activation of proteins involved in focal adhesions. This has important implications for the development of therapeutic strategies aimed at preventing or treating disseminated disease.

Melanoma-secreted amyloid beta supresses neuroinflammation and promotes brain metastasis

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Melanoma brain metastasis is the largest cause of melanoma morbidity and mortality, and melanoma has the highest rate of brain metastasis of any cancer. The mechanisms that mediate melanoma brain metastasis remain poorly understood.

We characterized patient-derived Short-Term Cultures (STCs) as a novel model system for the study of melanoma brain metastasis. Unbiased proteomics analysis of STCs revealed striking alterations in brain metastasis vs non-brain metastasis derived STCs in proteins related to neurodegeneration. Through in-vivo assays, we show that loss of Amyloid Precursor Protein (APP) in melanoma cells dramatically inhibits melanoma brain metastasis formation without affecting metastasis to other organs and that amyloid beta is the form of APP critically required for melanoma brain metastasis. Additionally, we demonstrate that APP is required for late growth and survival of melanoma cells in the brain parenchyma. Furthermore, we demonstrate that melanoma-derived amyloid beta polarizes astrocytes to an anti-inflammatory secretory phenotype that inhibits microglial phagocytosis of melanoma cells. Finally, we show that treatment of mice with a beta secretase inhibitor (LY2886721), which prevents amyloid beta production, decreases brain metastatic burden.

Our results demonstrate a critical role for amyloid beta in melanoma brain metastasis, establish a novel connection between brain metastasis and neurodegenerative pathologies, and show that amyloid beta is a promising therapeutic target for brain metastasis treatment. Studies to further characterize how amyloid beta acts in the melanoma brain metastasis microenvironment are currently underway.

IL-17 and Th17 in melanoma outcome

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The role of IL-17 and Th17 in cancer is controversial, and IL-1β is a key regulator. T-cell polarization (intracellular flow cytometry) was assayed in 111 melanoma patients. CD4 + T-helper (CCR6 + and CCR6-) subtypes (stimulation with or without IL-1β culture) and myeloid-derived suppressor cells (CD33 + HLADR- total MDSC) and subsets (CD15 + CD14- polymorphonuclear PMN-MDSC, CD15-CD14 + monocytic M-MDSC, and CD15-CD14- uncommitted UC-MDSC) were compared to sentinel lymph node status, progression free survival (PFS), overall survival (OS) and disease specific survival (DSS). MDSC increased with increasing disease stage when isolated out of whole blood (total MDSC $p = 0.006$, PMN-MDSC $p = 0.049$, and M-MDSC $p = 0.036$) or PBMC (total MDSC $p = 0.020$, PMN-MDSC $p = 0.022$). M-MDSC were associated with worse PFS (whole blood $p = 0.011$, PBMC $p = 0.003$), OS ($p = 0.007$ and 0.003) and DSS ($p = 0.008$ and 0.006). Expression of IL-10 among CCR6 + cells (IL-10 + IL-17- Tregs) in the presence of IL-1β was associated with worse PFS ($p = 0.024$) and OS ($p = 0.006$). IL-17 expression in CCR6- helper T-cells alone was predictive of SLNBx positivity ($p = 0.046$). Co-expression of IL-17 with IFNγ among CCR6- cells was associated with worse OS (0.038) and DSS (0.021). IL-17 + IL10 + cells were associated with worse PFS ($p = 0.021$), OS ($p = 0.012$) and DSS (0.005). In contrast, so called "ex-Th17" IFNγ+ CCR6 + helper T-cells were associated with improved PFS ($p = 0.03$), OS ($p = 0.026$) and DSS ($p = 0.026$). A deleterious role of M-MDSC and Treg in melanoma is consistent with the literature. Expression of the IL-17 cytokine appears to predict worse outcome among melanoma patients, while CCR6 + Th17-cells, when expressing IFNγ alone, predict improved outcome. Our studies affirm a difference between IL-17 expression and Th17 cells in melanoma patients. The high degree of plasticity, known to occur in Th17 cells, is likely an important factor, and may be exploited for synergy with immunotherapy.

Selective killing of senescent naevus and melanoma cells: implications for tumour prevention and therapy

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Excessive oncogenic signalling can induce a state of irreversible cell cycle arrest, termed 'cellular senescence'. A well-known example *in vivo* occurs when melanocytes gain a *BRAFV600E* mutation, resulting in formation of a clonal mass of senescent melanocytes and establishment of a naevus. However loss of function of genes associated with senescence such as *p16* or *PTEN*, results in cell cycle re-entry and malignant melanoma development. Melanomas can be programmed to re-enter senescence via chemotherapy, but subsequent loss of the senescent phenotype induces cancer relapse.

Senescent cells maintain viability by upregulating the BCL-2 family of anti-apoptotic proteins and therefore drugs which inhibit them, such as ABT-263, may be an option to kill senescent melanocytic cells. Interestingly, we find that senescent melanocytes are highly resistant to ABT-263 despite upregulation of certain BCL-2 members. Resistance occurs due to upregulation of anti-apoptotic MCL-1 at the protein, but not transcript level, upon exposure to ABT-263. Genetic or pharmacological silencing of MCL-1 co-operates with ABT-263 and subsequently induces caspase-dependent intrinsic apoptosis, predominately in senescent melanocytes.

We also find that mouse and human melanoma cells can be induced to senescence *in vitro* via the chemotherapeutic temozolomide. Senescent melanoma cells are also resistant to ABT-263 and require co-operative inhibition of MCL-1 to induce death. We are currently analysing whether this dual inhibition can induce death of naevus and senescent melanoma cells *in vivo*.

Targeting cancer-associated fibroblasts synergizes with anti-PD-1 immunotherapy in advanced acral melanoma

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Anti-PD-1/PD-L1 antibodies have improved outcomes in non-acral cutaneous melanoma. However, the overall ORR (objective response rate) of a cohort of acral and mucosal melanoma was 20.7% overall, which is lower than that of the Caucasian population and the mechanism remains unclear. Cancer-associated fibroblasts (CAFs) was proved to promote tumor progression and mediate therapy resistance. In this study, we performed RNA sequencing (RNA-Seq) analyses of 15 Chinese melanoma cases (including 9 acral melanomas, 3 cutaneous melanomas and 3 primary unknown melanomas), which were enrolled in anti-PD-1 therapy clinical trial. Then, a total of 50 cases were analyzed for CAFs by immunohistochemical (IHC) staining for alpha-smooth muscle actin (a-SMA). The effects of FAK inhibitor (Defactinib), anti-PD-1 antibody and the combination of both were evaluated in PD-1 humanized mouse (C57BL/6-hPD-1). RNA-Seq revealed CAFs were negatively correlated with response of anti-PD-1 therapy in acral melanoma cases. The association between CAFs higher infiltration and innate resistance to anti-PD-1 therapy was validated in 50 acral melanoma patients by IHC

($p < 0.05$). Additionally, FAK inhibitor treatment enhanced efficacy of anti-PD -1 antibody ($p < 0.05$)in the C57BL/6-hPD-1 melanoma. Furthmore, different expression of CAF markers maybe involved in the reaction of acral melanoma to anti-PD-1 therapy. In conclusion, higher infiltration of CAFs and different expression of CAF markers are associated with innate resistance to anti-PD-1 therapy in acral melanoma patients. Our study provides an evidence for combining FAK inhibitor with anti-PD-1 antibody for the treatment of advanced acral melanomas.

The landscape of progressive and fatal melanoma in adolescents and young adults

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Adolescent/young adult (AYA) melanoma often presents atypically and leads to diagnostic and management uncertainty, leading to suboptimal outcomes. There has been a trend toward less aggressive management for this entity, although specific features predicting progression or mortality are not clearly defined. Here, we present data from a single institution study of 71 AYA melanomas at the University of Pittsburgh Medical Center. Age at diagnosis ranged from 8 to 25 years (mean, 19.5), 43 patients (61%) were female. Overall, 27 patients (38%) had a melanoma recurrence and 18 (25%) died of disease. Mean time to recurrence was 3.4 years and to death 6.2 years. Only 1% of patients initially presented with distant metastatic disease. Half (50%) had local disease at diagnosis. Presence of ulceration was significantly associated with shorter recurrence-free survival (RFS) (HR 4.59, [1.87, 11.27], $p = 0.0009$) and overall survival (OS) (HR 11.50, [2.51, 52.67], $p = 0.0017$); as was tumor depth > 4 mm with shorter RFS (HR 3.19, [1.03, 9.91], $p = 0.0452$) and OS (HR 6.53, [1.27, 33.68], $p = 0.0250$). Increasing age at diagnosis was significantly associated with shorter RFS (HR 1.11 [1.01, 1.22], $p = 0.0312$). Spitzoid pattern was present in 16% of cases and was not significantly associated with a longer RFS (HR 0.430, [0.10, 1.82], $p = 0.2507$) or greater OS (HR 0.866, [0.198 3.797], $p = 0.8488$). In summary, AYA melanoma can be an aggressive disease with a high rate of recurrence and mortality, despite local disease at diagnosis. Increasing age at diagnosis, tumor ulceration, and deep Breslow are significantly associated with adverse outcomes. Spitzoid histologic pattern is not predictive of improved overall or recurrence-free survival. Our data refutes the tendency for less aggressive management of melanoma in younger patients. Additional studies are necessary to formulate standardized management guidelines.

TERT promoter mutations in melanoma

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TERT promoter mutations are the most frequent somatic alterations in melanoma and many other cancers. The initial discovery came through identification of the A>C germline mutation at the -57 bp position from ATG start site of the *TERT* gene in a multigenerational melanoma family. Follow-up screening of melanoma cell lines from metastasized tumors from unrelated patients led to the identification of recurrent and mutually exclusive somatic C>T mutations at -124 and -146 bp positions from the ATG start site of the *TERT* gene. With overall frequency of 74 percent, the *TERT* promoter mutations including CC>TT tandem alterations at -124/-124 and -138/-139 bp positions tend to occur together with *BRAF* mutations and *CDKN2A* alterations. The presence of the *TERT* promoter mutations in primary melanomas associated with increased patient age and Breslow thickness, tumor ulceration and increased growth rate. In combination with *BRAF/NRAS* alterations, the promoter mutations, through modulation by a common polymorphism within the *TERT* promoter, in primary tumors associated with decreased disease-free and melanoma specific survival. Further, data showed that the worst effect on the disease-free and melanoma specific survival was due to the -138/-139 CC>TT tandem TERT promoter mutation. In particular, in combination with BRAF/NRAS mutations, the -138/-139 CC>TT TERT promoter mutation associated with statistically significant poor disease-free and melanoma-specific survival with hazard ratios of 6.04 (95% CI 2.03–17.94, $p = 0.001$) and 12.59 (95% CI 2.18–72.70, $p = 0.005$), respectively. Thus, TERT promoter mutations are potentially strong biomarkers of the prognosis and outcome.

Mutation hot spots and survival analysis of melanoma patients in Northeast China

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Objective: To investigate the frequency of mutation hot spots in melanoma patients in Northeast China, and find out the potential clinical significance.

Methods: This study included 59 patients who underwent surgical resection and pathological diagnosis of melanoma from June 2013 to June 2018. The last follow-up time was January 1, 2019. Clinical data of the patients and genetic mutation data obtained by performing gene detection sequencing of 11 cancer-related gene exons were collected. The data was sorted, screened and statistically analyzed with SPSS20.0 software. The log-rank test was used to compare the difference between the general data and the clinical

data, and the meaningful variables were included according to the log-rank test results.

Results: There are 11 oncogene exons in this study, including B-RAF15, N-RAS2, N-RAS3, c-kit9, c-kit11, c-kit13, c-kit17, c-kit18, PDGFR12, PDGFR14, PDGFR18. There were 7 mutations found in 59 cases of MM, including 12 cases of B-RAF15 mutation, 2 cases of N-RAS3 mutation, 2 cases of c-kit13 mutation, 2 cases of c-kit18 mutation, 1 case of c-kit11 mutation, c- There was 1 case of kit17 mutation and 1 case of PDGFR18 mutation.

Kaplan-Meier single-factor survival analysis (Log-rank test) found that NRAS, cKIT gene mutation, gender, age and MM patients were associated with overall survival (OS). However, there were no significant correlations between clinical features such as tumor thickness, tumor location, presence or absence of ulcers.

Multivariate analysis using COX proportional hazards regression model showed that NRAS and cKIT gene mutations were independent influencing factors of MM overall survival.

Concusions: Melanoma patients with gender, age, NRAS, cKIT gene mutation and OS have correlation, tumor thickness, tumor location, presence or absence of ulcer have no significant correlation with OS.

Prospective study of clinical characteristics of melanoma patients with retinopathy caused by a high dose interferon α-2b

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Objective: Retinopathy is a rare side effect of interferon α-2b treatment. The goal of this study was to prospectively investigate the clinical characteristics of Chinese patients with melanomas who developed retinopathy following high doses of interferon α-2b (HD-IFN) therapy.

Methods: This study included 43 melanoma patients with HD-IFN-induced retinopathies and 13 patients without retinopathies (controls) who were observed using visual acuity, visual field, and fundus color photography before and after HD-IFN treatment.

Results: Forty-three melanoma patients (76%) developed retinopathy after being treated with HD-IFN. Among these patients, 49% had cotton-wool spots, 19% had retinal hemorrhage, and 30% had retinal hemorrhage. The median time of occurrence of retinopathy was 4 weeks [2 weeks (25%), 6 weeks (75%)]. No patient showed symptoms except one who had blurred vision. A comparison of clinical characteristics (age, gender, primary site, stage, and ulceration) and laboratory examinations (white blood cell and platelet counts, hemoglobin, serum lactate dehydrogenase, alanine transaminase, aspartate aminotransferase, triiodothyronine, thyroxine, thyroid stimulating hormone, and lipid) between the HD-IFN-induced retinopathy patients and non-retinopathy patients did not show any significant differences. Pathological complications (diabetes and/or hypertension) were not associated with any significant differences between the patient groups.

Conclusion: HD-IFN therapy in patients with melanomas may induce mild retinopathy. Out results, however, do not necessarily indicate discontinued HD-IFN treatment because retinopathy is a reversible disorder.

IMP dehydrogenase rods/rings structures in Acral Melanomas.

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Acral lentiginous melanoma (ALM) is a rare subtype of melanoma and has an aggressive behavior. IMPDH enzyme is involved with de novo GTP biosynthesis pathway, catalyzing the rate-limiting step of converting the substrate IMP to XMP. Several groups have reported that IMPDH will assemble into large filamentary structures called rods and rings (RR), or cytoophidium (cellular snakes). Our group have recently described that IMPDH assembling into RR induces a hyperactive state, usually to supply a high consumption of GTP nucleotides, such as in highly proliferative cells. Since IMPDH RR is present in cells with a highly proliferative state, our goal is to study if melanoma cells present IMPDH RR structures. Methods:Samples were organized in a TMA containing forty-five samples of ALM and 57 melanocytic nevi. Paraffin-embedded tissue samples were probed with an anti-IMPDH2 antibody. Immunohistochemistry data were also obtained for ALM samples.

Results: In ALM samples, 42% were RR positive, while in nevi samples only 14% were RR positive (p = 0.004). Both the rod and ring-shaped structures could be observed. In the 19 ALM RR positive samples, RR ranged from ~15% up to ~80%, with an average of 39%. In the RR positive group, 76% of the samples present Breslow thickness > 4.0 mm, compared to only 30% in the RR negative group (p = 0.04). Other variables such as ulceration and mitotic index > 3 per mm^2were similar in RR positive and RR negative groups. RR positive was evenly distributed for the markers MYC, SCF, PTEN, KIT, BRAF and CYCLIN D1, screened by immunohistochemistry in ALM, with no distinction for any marker. However, from 17 RR positive samples, 15 were also MYC positive. In conclusion, screening for RR structures in melanomas may be a valuable additional tool to help differentiate ALM from nevi, and access malignancy of the tumor.

The ciliation index is an informative molecular diagnostic tool across a spectrum of melanocytic neoplasms

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Classifying melanocytic neoplasms can present a histopathological challenge and validation of alternate ancillary tests would be diagnostically useful. Primary cilia are cell surface organelles that have been shown to reliably differentiate melanoma, which fail to ciliate, from melanocytic nevi, which retain primary cilia. This study demonstrates the utility of this molecular test in a range of histopathologically challenging melanocytic neoplasms in which the ciliation index has yet to be investigated. We collected 80 cases for which array comparative genomic hybridization (aCGH) was performed as part of the diagnostic evaluation by expert consultation. These were further divided into either benign (n = 12), atypical (n = 48) or malignant (n = 20) diagnoses. This cohort included spitzoid, blue nevus-like, BAP1-loss, ALK-fusion, NTRK-fusion, deep penetrating nevus-like, and conventional melanocytic neoplasms. We performed immunofluorescence labeling for melanocytes with SOX-10, gamma-Tubulin for centrioles and acetylated alpha-Tubulin to highlight the ciliary axoneme. The average ciliation index for the benign group was significantly higher than the malignant group (p < 0.0001); 78% versus 15%, respectively. Interestingly, lesions in the atypical group demonstrated a bimodal distribution of ciliation with 19 cases showing less than 20% and 22 cases having between 73–100% ciliation. Upon histopathologic consensus re-examination of the atypical group, only 5 of the cases were favored to be severely atypical with an average ciliation index of 6% and 13 cases were found to have minimal atypia with an average ciliation index of 88%. These results demonstrate that the ciliation index can serve as a biomarker for benign versus malignant melanocytic neoplasms across a spectrum of histopathologically challenging cases.

A single institution analysis of BRAF V600 mutant melanoma brain metastases treated with Dabrafenib and Trametinib

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Background: Brain metastases (BM) commonly cause mortality in patients (pts) with metastatic melanoma (MM). Optimal treatment sequencing of BRAF V600 mutant (BRAFm) BM is unknown, with options including targeted therapy (TT), immunotherapy (IT), radiotherapy (RT) and surgery. Whilst TT can provide rapid symptomatic improvement, intracranial responses are seldom durable. We investigated the overall survival (OS) of BRAFm MM pts treated with 1st line dabrafenib and trametinib (DT), examining pts with BM at the start of DT (de-novo BM, DNBM) and those who developed BM on DT (acquired BM, ABM).

Methods: Institutional medical records from June 2015 to November 2018 were retrospectively analysed. Inclusion criteria were pts with BRAFm MM treated with 1st line DT and MRI-brain staging throughout. Pt demographics, intracranial/extracranial response, local and systemic therapy were recorded. Intracranial response rate (IRR) will be assessed using modified RECIST criteria.

Results: 55 pts were included; 39 with DNBM and 16 with ABM. Median age was 61 years. ECOG was 0–1 in 89% (49/55). BRAF V600E, K and R mutations were identified in 69%, 29% and 2% respectively. 67% (26/39) of DNBM pts were symptomatic at diagnosis. Median follow up of DNBM and ABM groups was 350 and 458.5 days respectively. 36 pts progressed intracranially on 1st line DT, of which 4 had surgery, 13 had RT and 2 had both. 2ndline treatment for pts with DNBM versus ABM was: 24 vs 12 pts IT, 7 vs 2 pts TT and 2 vs 0 pts combination therapy. Median time to BM development in the ABM group was 467 days. Median OS from initiation of DT was 66 and 81 weeks for DNMB and ABM groups respectively (p = 0.69).

Conclusion: Melanoma pts with DNBM commenced on 1stline TT demonstrated OS similar to that of the COMBI-MB trial. OS was similar in both DNBM and ABM pts. Data on IRR and progression free survival will be presented.

Second line ipilimumab-nivolumab for melanoma brain metastases following progression on BRAF-MEK inhibitors

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Introduction: Recent trials have established ipilimumab-nivolumab (I-N) as a robust first line (IL) treatment for patients (pts) with asymptomatic melanoma brain metastases (MBM). However, the efficacy of I-N for symptomatic MBM or after intracranial progression on either a BRAF-MEK inhibitor (BRAF-MEKi) or anti-PD1 is unknown. We performed a single site retrospective analysis of pts treated with I-N for MBM to investigate these issues.

Methods: All pts treated with I-N as 1L or second/third line (2-3L) for MBM from 1/3/2015 to 1/8/2018 were identified. Patient demographics, tumor characteristics and therapies were recorded. Gadolinium enhanced MRI was used to stage MBM and assess response using modified intracranial RECIST. Non measurable

MBM < 5 mm, excised MBM and leptomeningeal disease were included in PFS analysis.

Results: Sixty patients with MBM received I-N: 25 in the 1L and 35 in 2-3L. Median age was 64 and 55 years for 1L and 2-3L I-N, respectively. BRAF V600 mutant (BRAFm) MBM comprised 8% (2/25) of 1L and 85.7% (30/35) of 2-3L groups. Prior to 2-3L I-N, all BRAFm MBM had progressed on 1L BRAF-MEKi and 5 BRAF wildtype patients had progressed on anti-PD1 monotherapy. Symptomatic MBM prior to I-N in 1L was 44% (11/25) and 34.3% (12/35) in 2-3L. Median baseline MBM diameter was 13 mm in 1L & 18 mm in 2-3L I-N groups. Intracranial response rate (IRR) of 1L I-N was 66.7% (16/24) and 2-3L was 11.5% (3/26). IRR of BRAFm MBM treated with 2-3L I-N was 4.8% (1/21) & an additional 2 out of 6 BRAFm pts treated with radiation up to 12 weeks prior to 2-3L I-N responded. Median intracranial PFS for 1L was not reached and was 5.6 weeks for 2-3L I-N.

Conclusion: In our study, IRR to 1L I-N including pts with symptomatic MBM were broadly consistent with Phase II trial data. IRR and PFS to 2-3L I-N in BRAFm MBM was poor highlighting the potential benefit of 1L I-N in this group.

Comparison of methodologies to detect *BRAF* mutation in plasma of melanoma patients

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Melanoma is the most aggressive skin cancer and the detection of *BRAF* V600 mutation became essential to drive the management of advanced disease. Its detection in liquid biopsy samples could identify patients eligible for therapy without an invasive examination. The aim of this study was to compare two different methodologies to access *BRAF* V600 mutation in plasma from melanoma patients. Eighteen, patients diagnosed with melanoma attended from Barretos Cancer Hospital were included., *BRAF* V600 mutations were analyzed in both tumor tissue and plasma from the same patients prior to any systemic treatment. In tumor tissue *BRAF* mutation was done by Cobas or MiSeq System, whereas in plasma, droplet digital PCR (ddPCR) and Idylla System were used. For ddPCR, DNA was isolated from 2 mL of plasma using commercial kit (Bio Rad) in QX200 Droplet Digital PCR System (Bio Rad). The Idylla System used 1 mL of plasma and Idylla ctNRAS-BRAF Mutation Test (Biocartis). From the 18 cases, 15 showed *BRAF* V600 mutated (83%) in the tissue. Among the 15 mutated patients, plasma *BRAF* V600 mutation was not identified in 8 cases (53%) with both ddPCR and Idylla assays. Using ddPCR, the mutation was detected in 6 cases (40%) and using Idylla also 6 patients (40%) showed positivity. However, 2 cases were discrepant, being one case mutated in ddPCR and wild type in Idylla, and other case, wild type in ddPCR and mutated in Idylla (5.5%). In the 3 *BRAF* V600 wild type cases, the mutation was also not detected in plasma in both methods. We determined that sensitivity and specificity of both methods to detect *BRAF* V600 mutation in plasma were 40% and 100%, respectively. We suggest that patients with a baseline mutation detected in plasma could be treated with target therapy, since the specificity was 100%. In wild type cases, the mutation should be tested on tumor tissue.

SPANX control of nuclear architecture promotes melanoma cell growth

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SPANX is part of a 5 members cancer-testis antigen family. While under normal physiological condition these genes are expressed in the testis, elevated expression is often found in number of tumors, including melanoma. SPANX expression in cancer cells has been implicated in cell invasion of breast and lung cancer. Here we report that elevated SPANX expression in melanoma affects nuclear envelope organization, culminating in melanoma growth and invasion. Loss of SPANX induces melanoma growth arrest with a concomitant cell death. Conversely, SPANX overexpression promotes melanoma cell growth in 3D, but not in 2D cultures. Mapping of SPANXA interactome identified type A lamins, which were confirmed as SPANX interacting proteins. Imaging studies revealed that SPANX presents a filament-like structure, which co-localizes with lamin A/C. The impact of SPANX on lamin is best noted upon its loss, which results in altered lamin A/C structure and disorganization of the nuclear architecture. The latter coincides with increased lamin A/C interaction with BANF1, sequestering its function in nuclear envelope organization and the maintenance of nuclear integrity. The homology shared between SPANX and BANF1 may underlie the competition for their interaction with lamin A/C which impacts nuclear architecture, seen in tumors overexpressing SPANX. Initial assessment of melanoma specimens identified elevated SPANX expression in metastatic melanoma as in BRAFi and MEKi resistant tumors. The implications of SPANX-controlled nuclear architecture to melanomagenesis will be discussed.

Hyperprogression in 'real world' advanced melanoma patients treated by anti-PD1

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Anti-PD1 (APD) are approved in many cancer, pointing out different outcome: response, (pseudo-)progression, stabilization and progression, and a particular group with unexpected rapid disease progression (PD): hyperprogression (HP), characterized by rapid increase in tumor volume with sudden death. HP is described in about 10% of patients in various cancer types (lung, bladder, head and neck) but has not been established in advanced melanoma (AM) (unresectable stage III or metastatic stage IV).

Patients included in this study were identified in MelBase, a French multicenter clinical biobank dedicated to the prospective follow-up (FU) of adult AM patients (NCT02828202). Among 793 patients treated by APD (nivolumab, pembrolizumab) from 2014 to 2018, HP patients (n = 82) were selected by the combination of the following items: i/ECOG 0-1 and normal LDH at treatment initiation (TI), ii/PD or death in the first 3 months as best response, iii/ECOG 3-4 or high LDH within 3 months after TI.

Characteristics at baseline of HP were: median age 67 years (Q1-Q3 59–75), 39% BRAF mutated, 100% ECOG 0–1, 0% elevated LDH, 78% M1c (brain and liver metastases 22 and 52% resp.), 48% >3 metastatic organs and 45% treated 1st line. With a median FU of 11.3 months (Q1-Q3 4.8–23.6), 41% of HP patients died in the first 3 months after TI. Among the remaining 59%, 89% died within 1 year after TI. In comparison, in the non HP group, 23% died at 1st PD; among the remaining 77%, 15% were treated beyond by APD, with 26.3% (IC95% 7.8–13.6) still alive 1 year after 1st PD.

Overall survival estimation in HP was 33.9% (IC95% 25.0–45.9) at 6 months and 11.5% at 12 (IC95% 7.2–21.1), compared to non HP 79.8% (IC95% 76.9–82.9) and 64.8% (IC95% 61.2–68.6) respectively.

This study shows that HP can occur in AM treated by APD and are mostly found in M1c patients with liver metastases. However, HP molecular phenomenon is largely unknown.

Campylobacter infection in Patients treated for Immune Checkpoint inhibitor-induced Colitis

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Background: Emerging data that gut microbiome diversity at baseline may influence response and toxicity of checkpoint inhibitor immunotherapy (CPI) are of great interest.

Methods: We report 3 patients receiving CPI under our care over a 6 month period who developed Campylobacter-associated diarrhoea. All had received treatment for immunotherapy-induced colitis in the period preceding campylobacter infection.

Results: **Case 1**: An 81-year old man receiving pembrolizumab for stage IV melanoma developed colitis after 1 cycle. High dose IV steroids were commenced. Stool cultures were negative. The cellulitis settled but he remained an inpatient with initially improving colitic symptoms for a further 10 days prior to deterioration and commencement of infliximab. Campylobacter was subsequently cultured from stool.

Case 2: A 72-year old female with metastatic renal carcinoma receiving nivolumab developed a blistering rash and colitis 10 months after starting treatment. Her treatment was stopped and high dose steroids commenced. 3 months later, while still on a weaning dose of oral steroids, she presented with Campylobacter confirmed diarrhoea.

Case 3: A 60-year old man receiving adjuvant nivolumab for resected stage III melanoma developed colitis after 6 cycles. Stool cultures were negative and high dose steroids were commenced. Weeks later, his bowel related symptoms flared and motions became bloody. Campylobacter was cultured from stools.

Conclusions: All patients developed proven campylobacter infection following treatment for CPI toxicity. Infection was excluded as the cause of initial symptoms. CPI induced toxicity and the immunosuppression required to treat it may lead to an increased risk of outgrowth of pathogenic organisms. Clinicians should be aware that worsening of colitic symptoms in patients with a history of treatment for CPI induced colitis may be due to an outgrowth of pathogenic organisms.

Role of fucosylation in CD4+ T cell-mediated melanoma suppression

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Significant proportions of melanoma patients exhibit poor responsiveness to immunotherapies. Understanding immunoregulatory mechanisms is needed to improve immunotherapies. We previously discovered that increasing fucosylation in melanomas reduces tumor growth and metastasis (Lau et al., 2015). To identify tumor-infiltrating lymphocytes (TILs) affected by L-fucose, we profiled lymphocytes from syngeneic tumors of control- or fucose-fed mice tumors. Dietary L-fucose increased TILs by ~10–50-fold. Of total TILs, CD3+ T cells (including CD4+ and CD8+ T cells) doubled. L-fucose did not trigger tumor suppression in immune-deficient mouse melanoma models, indicating the immune system is required for L-fucose-triggered suppression. Immunodepletion of CD4+ or CD8+ T cells during L-fucose treatment revealed that CD4+ T cells are central for tumor suppression. CD4+ T cell depletion abrogated tumor infiltration of NK, dendritic, and CD8+ T cells, implicating these populations as downstream effectors of fucosylation- and CD4+ T cell-triggered tumor suppression. To identify melanoma proteins contributing to fucosylation-triggered immune responses, we performed mass spectrometric analysis of all fucosylated proteins in melanoma and identified 2 MHC proteins, HLA-A and HLA-DRB1, as fucosylated. *In vivo* knockdown of HLA-A and HLA-DRB1 revealed that HLA-DRB1 is required for L-fucose-mediated tumor suppression, and importantly, for the recruitment of CD4+ T cells.

We further studied how fucosylation affects CD4+ T cell biology using CD4+ T cells isolated from healthy donors, which we pharmacologically modulated fucosylation. The roles of fucosylation on tumor vs. CD4+ T cells and implications/clinical utility for melanoma patients will be discussed. Our data shows how increased fucosylation drives tumor suppression through CD4+ T cells, and supports the potential of dietary L-fucose to boost anti-melanoma immunity.

The UV-protecting and tumor suppressor role of UVRAG in melanoma

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Ultraviolet radiation (UVR) from sunlight has been epidemiologically identified as a leading risk factor for melanoma development. However, the mechanistic details of how sunlight UVR causes melanoma are still being elucidated. Recent studies revealed tremendous amounts of UV-induced genetic mutations in melanoma genomes compared to most other types of tumors. Furthermore, UV-induced mutagenesis accelerates melanoma progression and recurrence. These studies highlighted the need to better understand the molecular mechanisms protecting against environmentally UVR-induced mutagenesis, and to delineate why they fail to work in melanoma, providing answers that could pave the way for personalized prevention and treatment of this often-fatal illness. Our recent discovery of an autophagy modulator as a bona fide UV protector through distinct mechanisms and its strong correlation with reduced melanoma risk suggest that reduced capacity of UV-induced photolesion repair and adaptive skin pigmentation represents the main reasons of genetic instability of melanoma cells and is responsible for melanoma predisposition. Using cell-based models and genetic models, we investigated the molecular mechanism by which UV-induced photolesion is repaired in melanocytes to confer UV resistance; we also found that the mechanism governing UVinduced melanosome biogenesis and pigmentation plays an equally important role in preventing UV penetration. We are now using genetically engineered mouse carrying BRAF(V600E) mutation in combination with PTEN deletion to study the impact of the UVprotecting mechanisms of UVRAG in melanoma. We believe that our studies will identify new UV-protecting mechanisms that regulate melanoma disease penetrance and provide compelling in vivo validation of a novel prognostic and predictive biomarker in melanoma.

RAC1^{P29S} induces a mesenchymal phenotypic switch via SRF to promote melanoma development and therapy resistance

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RAC1 P29S is the third most common mutation in human cutaneous melanoma, after *BRAF* V600E and *NRAS* codon 61 mutations. Here, we report on the role of RAC1^{P29S} in melanoma development and therapy resistance. Signaling studies and functional screening for effectors reveals that endogenous RAC1^{P29S} activates PAK and AKT, while not affecting the MAPK pathway. In addition, RAC1^{P29S} induces actin polymerization to initiate a gene expression program via the SRF/MRTF transcriptional pathway. This results in a melanocytic to mesenchymal phenotypic switch characterized by suppression of apoptosis and enhanced survival when cells are deprived of anchorage or growth factors. Using genetically engineered mouse models,

we show that germline expression of RAC1^{P29S} from the endogenous locus is embryonically lethal. When expressed in the whole body of adult mice, RAC1^{P29S} induces late-onset B cell lymphoma. When expressed only in melanocytes of adult mice, RAC1^{P29S} cooperates with oncogenic BRAF or with NF1-loss to promote tumorigenesis. Using oncogenic BRAF-driven;p53-null and PTEN-null models, we find that spontaneous lung metastasis is not enhanced by RAC1^{P29S}. However, RAC1^{P29S} drives resistance to BRAF inhibitors, which is reversed by SRF/MRTF inhibitors. In conclusion, these findings establish RAC1^{P29S} as a promoter of melanoma initiation and mediator of therapy resistance, while identifying SRF/MRTF as a downstream effector with potential for therapeutic targeting.

The genomic landscape of clear cell sarcoma of soft tissue

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Clear cell sarcoma (CCS) of soft tissue is a rare and distinct subtype of sarcoma which always harbors EWSR1-ATF1 or EWSR1-CREB1 gene-fusions, but the genomics mutation patterns have not been characterized yet. Herein, we reported the mutational landscape of CCS using whole exome sequencing (WES) from 20 tumor-normal paired samples. We identified a low degree of tumor mutation burden (TMB), with a median of 0.95 mutation per million bases. Several cancer-related genes were found to recurrently mutated in CCS, such as ARNT, FGFR2 and PALB2. On the other hand, widespread of copy number variations were detected across samples. The chromosomes 7, 8 and 17p were frequently amplified in CCS, which was consistent with malignant melanoma. Frequent losses were observed on chromosomes 16, 17, 19, 20, 21q, 22q. Notably, frequent loss of interferon alpha (IFNA) genes in 9p21.3, which are involved in the immune response IFN alpha/beta signaling pathway, suggesting that they may not be effective against PD1/PD-L1 blockade immunotherapy. Gene Set Enrichment Analysis (GSEA) shows enrichment of genetic lesions affecting several critical pathways, including JAK/STAT, Regulation of autophagy, MAPK signaling pathway and p53 signaling pathway. Overall, our study illustrates a comprehensive genomic landscape of CCS and provides a road map to facilitate genome-guided personalized therapy.

Antitumor activity of ipilimumab or BRAF±MEK inhibition after pembrolizumab in patients with advanced melanoma in KEYNOTE-006

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In the KEYNOTE-006 (NCT01866319) study, pembrolizumab (10 mg/kg Q2W or Q3W) had superior OS vs ipilimumab (3 mg/kg, Q3W 4 doses) in patients with advanced melanoma who had ≤1 prior therapy. At data cutoff (Dec 4, 2017) with a median follow-up of 46.9 months, of 555 patients treated with pembrolizumab, first subsequent therapy was ipilimumab in 103 patients; and BRAFi±MEKi in 59 patients (33 received BRAFi+MEKi, 26 received BRAFi alone). At the start of subsequent ipilimumab therapy, 73.8% had ECOG PS of 0 or 1; 35.0% had elevated LDH. At the start of subsequent BRAFi±MEKi therapy, 76.3% had ECOG PS of 0 or 1; 35.6% had elevated LDH; 37% had received BRAFi±MEKi before study enrollment. In the subsequent ipilimumab group, ORR with pembrolizumab was 17.5% (1 CR; 17 PR); median ipilimumab treatment duration was 1.7 months (range, 1 day–6.9 months); ORR with ipilimumab was 15.5%; 11 (8 CR, 3 PR) of 16 responses were ongoing; median OS from ipilimumab start was 9.8 months (95% CI, 7.7–16.4). In the BRAFi±MEKi group, ORR with pembrolizumab was 13.5% (8 PR); median BRAFi±MEKi duration was 7.2 months (range, 0.4–44.4); ORR with BRAFi±MEKi was 30.5%; 7 (4 CR, 3 PR) of 18 responses were ongoing; median OS from BRAFi±MEKi start was 12.9 months (95% CI, 9.9–20.8). Of 22 patients in the BRAFi±MEKi group who received prior BRAFi±MEKi, ORR was 9.1%; 1 responder (CR) had ongoing response. Of 37 patients in the BRAFi±MEKi group who received no prior BRAFi±MEKi, ORR was 43.2%; 6 responders (3 CR) had ongoing response. In conclusion, ipilimumab and BRAFi±MEKi both have antitumor activity as the first subsequent therapy after pembrolizumab in patients with advanced melanoma.

AMBLor: A stratifying biomarker for adjuvant immunotherapy of AJCC stage II melanoma

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The success of adjuvant therapy for patients with AJCC stage III melanomas coupled with the increased risk of mortality for patients with AJCC stage IIb or c compared to patients with IIIa tumours has paved the way to adjuvant immunotherapy trials for patients with SLNB –ve AJCC stage II disease. However, the lack of biomarkers able to identify high-risk tumour subsets limits patient recruitment; particularly for those with AJCC stage IIb melanomas. We have recently identified the combined immunohistochemical expression of epidermal AMBRA1 and Loricrin overlying AJCC stage I melanomas as a robust prognostic biomarker and valuable pre SLNB test. In the present multicentre study, retrospective analysis of AJCC stage II melanomas for epidermal AMBRA1/Loricrin (AMBLor) expression using a clinically developed /commercial automated IHC assay revealed loss of AMBLor was associated with a significant reduction in disease free survival (DFS) compared to patients with AJCC stage II melanomas in which AMBLor was maintained. With a positive predictive value of 62%, these studies highlight AMBLor as stronger prognostic indicator than SLNB alone. Furthermore, sub-cohort multivariate analysis of SLNB negative stage IIb tumours also revealed significantly reduced DFS in AMBLor high compared to low risk cases, identifying those genuinely high-risk patients most likely to benefit from adjuvant therapy. Collectively these data suggest AMBLor as a standalone stratifying biomarker for adjuvant immunotherapy, the use of which will increase clinician confidence for patient recruitment as well as reduce patient morbidity.

Melanoma TGFβ2 secretion mediates loss of AMBRA1 and epidermal integrity facilitating tumour ulceration

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Ulceration is an adverse prognostic factor in melanoma for which the underlying cause remains enigmatic. We recently reported the combined loss of AMBRA1 and Loricrin (AMLo) expression in the epidermis overlying primary early stage melanomas as a prognostic biomarker, with loss of AMBRA1 mediated by a tumour secretory mechanism. Bioinformatic data revealing TGFβ responsive elements in the AMBRA1 promotor and the association of increased TGFβ2 with a metastatic phenotype, further led to the current hypothesis that increased melanoma TGFβ2 secretion results in loss of AMBRA1, epidermal differentiation/integrity and tumour ulceration.

IHC analysis revealed a significant correlation between increased tumoural TGFβ2 and epidermal AMBRA loss in a cohort of all AJCC stage melanomas, with increased TGFβ2 secretion by AJCC stage I tumours correlating with metastasis and high-risk AMLo status. qPCR analysis revealed ALK5 but not ALK1 mRNA expression by primary keratinocytes, while treatment of calcium-induced differentiated keratinocytes with recombinant TGF-β2 resulted in AMBRA1 and Claudin1 downregulation and increased psmad 2/3. Together these data suggest melanoma TGFβ2 secretion-mediated AMBRA1 and tight junctional protein downregulation results from canonical TGFβ2 signalling. Incorporation of keratinocytes into epidermal skin equivalents following siAMBRA1 knockdown also resulted in the formation of a thicker hyper-proliferative epidermis with impaired differentiation. Finally IHC analysis of AMLo high risk AJCC stage I melanomas revealed loss of AMBRA1 was associated with reduced Claudin 1 expression and histological features of pre ulceration, cleft formation and loss of rete ridges.

Collectively these data suggest secretion of TGFβ2 by high-risk melanomas results in canonical mediated-down regulation of AMBRA1, loss of epidermal integrity and tumour ulceration.

Intrinsic adaptive resistance development to a cytostatic dose of vemurafenib in melanoma cell lines

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Adaptive resistance mechanisms to BRAF inhibitor treatment may develop both intrinsically and extrinsically. As one extrinsic example, stromal cells have been shown to support BRAF inhibitor resistance through secretion of hepatocyte growth factor (HGF), which activates MAPK/ERK signaling through the MET receptor tyrosine kinase. I hypothesized that melanoma cell lines would develop intrinsic adaptive resistance to an initially cytostatic dose of vemurafenib independently of stromal cell mediated activation. Single-cell clones were established from melanoma cell lines (SKMEL28, SKMEL5, and HS695T) harboring BRAF V600E mutations. The dose-dependent response of each clone to vemurafenib was established through a 96-well live/dead cell counting assay on an Operetta High Content Screening System (Perkin Elmer) after 72 h of treatment with vemurafenib or DMSO vehicle as a negative control. Relative growth rates were calculated using the GRmetrics package in R 3.4.3. The dose-response curves were used to calculate the concentration of vemurafenib yielding a cytostatic response in each clone. Each clone was continuously treated with a cytostatic dose of vemurafenib until they achieved equivalent vemurafenib sensitivity to drug naive cells co-treated with 2 ng/mL recombinant HGF as a positive control. Single-cell clones from melanoma cell lines developed resistance to vemurafenib independently of stromal-mediated HGF secretion with equivalent decreases in BRAF inhibitor sensitivity. Resistant cells also developed morphological changes and a depressed growth rate relative to pretreatment cells. SKMEL28 and SKMEL5 cells developed an elongated stellate morphology, but HS695T cells developed a round spreading morphology. Resistant clones that initially developed increased pigmentation gradually lost their pigmentation after serial passaging.

Adjuvant pembrolizumab (pembro) in patients (pts) with resected high-risk stage II melanoma: phase 3 KEYNOTE-716 with crossover or rechallenge

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Adjuvant pembro (vs placebo) conferred RFS benefit in pts with resected stage III melanoma in the phase 3 KEYNOTE-054 trial. Pts with stage II melanoma are at high risk for recurrence after standard-of-care complete surgical resection. The 2-part, multicenter phase 3 KEYNOTE-716 study (NCT03553836) will compare adjuvant pembro vs placebo in pts with surgically resected high-risk stage II melanoma. Pts ≥12 years with newly diagnosed, completely resected (≤12 weeks since final resection at randomization) and stage IIB/IIC cutaneous melanoma (per *AJCC Cancer Staging Manual*, 8th ed, including negative sentinel lymph node biopsy and no evidence of distant metastasis); ECOG PS 0/1 (LPS score ≥50 if age ≤16 years; KPS score ≥50 if age >16 and <18 years) are eligible. Pts with a history of mucosal/uveal melanoma or prior treatment for melanoma beyond primary disease resection are excluded. In part 1 (double-blind), ~954 pts will be randomized 1:1 to pembro (200 mg for ≥18 years or 2 mg/kg for ≥12 to <18 years, to a maximum dose of 200 mg) or placebo Q3W for up to 17 cycles. In part 2 (unblinded), pts with confirmed first recurrence after 17 cycles of pembro with no delays ≥12 weeks and >6 months from last dose may be rechallenged; pts treated with placebo may cross over to pembro. Treatment will continue for an additional 17 (resected local or distant recurrence) or 35 cycles (unresectable disease). Primary end point: RFS; secondary end points: DMFS, OS, and safety. Key exploratory analyses: HRQOL, time to subsequent surgery/therapy, biomarkers. Tumor response in part 1 will be assessed Q24W during treatment, at treatment end, Q24W from years 2 to 4, and annually in year 5 or until recurrence. In part

2, tumor response will be assessed Q12W during treatment. AEs graded per NCI CTCAE v 4.0 will be recorded until 30 d after treatment end (90 d for serious AEs).

Combination ipilimumab/nivolumab versus single agent PD-1 inhibitor in metastatic melanoma patients with brain metastases

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Brain metastases is a frequent cause of disabling neurologic morbidity and mortality in patients with metastatic melanoma. Recent studies have shown that combination ipilimumab/nivolumab (I/N) for melanoma brain metastases (MBM) have produced intracranial responses comparable to that seen in the body. Data on single agent PD-1 inhibitor therapy for MBM is limited. We performed a retrospective analysis on contemporaneously treated patients with MBM to better assess clinical benefit of both approaches. 253 patients treated with checkpoint inhibitor therapy between 2014 and 2019 from the University of Michigan were identified. 105 patients were treated with I/N and 148 patients were treated with single agent PD-1 inhibitor, nivolumab (N) or pembrolizumab (P). 185 patients had no brain metastases and 68 had brain metastases. Progression free survival (PFS) for patients without MBM was superior for I/N compared to N or P (HR = 0.61 95% CI [0.38–0.99], $p = 0.045$). There was no difference in PFS for either treatment group amongst patients with MBM (HR = 1.08, 95% CI [0.58–2.0], $p = 0.805$). No overall survival benefit was seen with I/N in patients without MBM (HR = 0.69, 95% CI [0.35–1.4], $p = 0.188$) or patients with MBM (HR = 0.66, 95% CI [0.29–1.5], $p = 0.419$). Treatment discontinuation due to adverse events was significantly higher in the I/N group compared to N or P (44.8% vs 16.2% respectively, $p < 0.0001$). The use of I/N appears to have more clinical benefit in metastatic melanoma patients without brain metastases compared to N or P. Patients with MBM have no statistical benefit with I/N compared to N or P. Due to generally higher levels of toxicity associated with I/N, the use of single agent PD-1 inhibitor therapy may be a preferred approach for some patients.

A novel wild-type *BRAF* p61 isoform cooperates with *GNAQ* Q209P to activate MAPK and PI3K/AKT pathways in uveal melanoma

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Uveal melanoma is a rare subtype of melanoma with only about 5 cases per million per year. Nearly 50% of these patients develop metastases with dismal outcomes due to lack of effective therapies. Through a targeted next-gen sequencing panel for cancer genes, we identified a uveal melanoma patient whose tumor had both a *GNAQ* Q209P mutation and a novel p61 *BRAF* splice isoform of wild type *BRAF*, which has never before reported in uveal melanoma or any other cancer. *GNAQ* Q209P mutations are quite common in uveal melanomas, occurring in approximately 80% of cases. However, *BRAF* is rarely mutated in uveal melanomas. The p61 *BRAF* splice isoform lacks exons including the inhibitory RAS binding domain. This isoform was previously found as a RAF inhibitor resistance mechanism in *BRAF* V600E mutated cutaneous melanomas. Expression of the p61 *BRAF* splice isoform in HEK293T cells increased phosphorylated ERK levels and activated the MAPK pathway, whereas expression of the *GNAQ* Q209P mutation had no effect on MAPK or PI3K/AKT pathways. Interestingly, co-expression of the p61 *BRAF* splice isoform and *GNAQ* Q209P led to enhanced activation of the MAPK pathway as well as activation of the PI3K/AKT pathway. Based on this, we treated cells expressing the splice isoform and *GNAQ* mutation with cobimetinib (MEKi), LY3009120 (panRAFi), or pictilisib (PI3Ki), individually and in combination. Western blot analysis showed marked decrease in phosphorylated AKT with pictilisib alone and a decrease in phosphorylated ERK with LY3009120 or cobimetinib alone. With the combination of pictilisib, LY3009120, and cobimetinib, we saw significant inhibition of phosphorylated AKT and ERK. These findings provide a hopeful outlook for the future treatment and further understanding of uveal melanoma.

Adaptive counter strike mechanism of the immune system

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Baseline T cell location and density in metastatic melanomas have predictive value in the treatment outcome of patients receiving Immunotherapy. Using a modified Boyden chambers, here we show that the melanoma–T cell interactions lead to an increase in the number of T cells that migrate toward melanoma cells. This interaction is both HLA I and IFNγ dependent as was demonstrated using β2M KD, JAK1 inhibitors and IFNgR1 blocking antibodies. Indeed, all three experiments eliminated the migratory effect. Co-cultures using T cells and melanoma cells demonstrate an induction of the inducible isoform of ADAR1. This was visualized in-situ using dynamic cell blocks as well as observed with western blots. Moreover, experimental silencing or overexpression of ADAR1 decreases or increases, respectively, specific T cell migration toward melanoma cells. Indeed, chemokine arrays demonstrate

that ADAR1 significantly alters the secretion levels of multiple chemokines from melanoma cells. Collectively, ADAR1 downregulation facilitates a pro-tumor environment by limiting the amount of T-cells that migrate to the area hence creating a cold tumor. On the other hand, antigen-specific T-cells forces an adaptive counter attack by eliciting a positive feedback loop through ADAR1 induction in the surviving tumor cells, which in turn induces the expression of several chemokines and thereby increasing the chemotactic potential and influx of new T cells.

Identification and characterization of melanoma metastatic initiating cells

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An incomplete view of the mechanisms that drive metastasis, the primary cause of melanoma-related death, has been a major barrier to development of effective therapeutics and prognostic diagnostics. Increasing evidence indicates that the interplay between microenvironment, genetic lesions and cellular plasticity drives the metastatic cascade. We performed a longitudinal and exhaustive analysis of the diversity and trajectories of melanoma cell states during metastatic dissemination by combining single-cell profiling techniques with lineage tracing in clinically-relevant mouse models of melanoma. Among others, we identified one specific melanoma cell state, which is present in both mouse and human primary tumors, that drives the entire metastatic process. We deciphered the gene regulatory network underlying this particular state and developed therapeutic modalities that prevent phenotype switching into this state.This study highlights how understanding the magnitude and dynamics of non-genetic reprogramming in space and time at single-cell resolution can be exploited to develop therapeutic strategies that capitalize on non-genetic tumor evolution.

Age-dependent loss of HAPLN1 affects vascular integrity and metastasis in melanoma

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Older melanoma patients (>55 years) have worse prognoses than younger patients (<45 years) independent of other clinical prognostic factors. Further, while younger patients have more lymph node metastases, older patients have more distal metastases which significantly impacts their survival. We have shown that age-dependent loss of a secreted factor from dermal fibroblasts, HAPLN1, contributes to increased metastasis *in vivo* via ECM reorganization. Previous work from our lab has shown that age-dependent loss of HAPLN1 confers fewer lymphatic metastases but more lung metastases, consistent with clinical observations. We have demonstrated that this is due to ECM structural degradation and loss of lymphatic vasculature integrity. However, the biological mechanisms by which dermal ECM structure influences metastatic spread and localization are still largely unknown. Here we report that age-dependent loss of HAPLN1 contributes to increased intratumoral blood vessel formation and permeability. Given the pivotal role of intratumoral vasculature in metastasis, we hypothesize that these changes contribute to the increased incidence of lung metastasis in older patients. Our data indicate that aged mice have aberrant collagen structure surrounding intratumoral vessels, but that treatment with recombinant HAPLN1 is sufficient to restore a collagen pattern consistent with that in ECM from young mice. Given that dermal ECM structural complexity is essential for maintaining vascular integrity, loss of HAPLN1 may contribute to leakier vasculature. Additionally, tumors from aged mice treated with recombinant HAPLN1 have significantly fewer neoangiogenic blood vessels than untreated aged mice, suggesting HAPLN1 may also signal to inhibit neoangiogenesis. These data may provide clues as to why young and aged patients exhibit differential routes of metastases via lymphatic vs. hematogenous dissemination.

Prognostic & predictive implications of absolute lymphocytes count 7 days after adoptive cell therapy with tumor infiltrating lymphocytes

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Metastatic melanoma (MM) treatment has undergone a revolution in recent years. Adoptive cell therapy (ACT) with tumor infiltrating lymphocytes (TIL) transfusion therapy is an advanced treatment for MM which has been under clinical research in recent years with response rates ranging ~30% and 3y OS of 60% in responding patients. Markers for predicting response to therapy and durability of response are lacking. Serological markes may have prognostic value in relation to immunotherapy. Here we sought the assess the utility of day 7 post-transplant absolute lymphocytes count (ALC) as a prognostic and predictive marker to ACT with TILs.

MM patients were screened for ACT therapy between the years 2006–2016 at the Ella Lemelbaum Institute for Immuno-oncology & melanoma at Sheba Medical Center in Israel as part of a phase II clinical trial. Records for consecutive patients who were treated with ACT were analyzed.

105 patients initiated ACT with TILs. All patients failed approved treatment lines.Median follow-up for all patients was 11 months (range 1–128 months).Overall response rate was 27.6%, complete

response (CR) rate was 6.6%. Median D7 ALC for responding patients was 750/μl, 250/μl for non-responding patients, $p = 0.0051$. Response rate(RR) for a D7 ALC of >500/μl was 43.4% vs 8.5% for ALC < 500/μl (OR for response 4.2, $p = 0.002$). RR for D7 ALC of >1,000/μl was 53.8% vs 18.9% for ALC < 1,000/μl (OR 4.9, $p = 0.001$). Median overall survival (OS) for all patients was 11.9 months. OS for patients with D7 ALC > 500/μl was 25.7 vs 8 months for ALC < 500/μl (HR for death 0.35, $p = 0.000$). Median OS for patients with D7 ALC > 1,000/μl was 49.1 months vs 8.8 months for D7 ALC < 1,000/μl (HR for death 0.27, $p = 0.000$).

Day 7 post TIL infusion absolute lymphocyte count may serve as a prognostic and predictive marker for response and durability of response to therapy.

The role of Myc in interferon-γ signaling inhibition and immunotherapy resistance in advanced melanoma patients

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Novel immunotherapies have revolutionized the treatment of advanced melanoma. As the pathways mediating resistance to immunotherapy are largely unknown, we conducted transcriptome profiling of pre-immunotherapy tumor biopsies from melanoma patients that received either PD-1 blockade ($n = 36$) or adoptive cell therapy with tumor infiltrating lymphocytes ($n = 37$).

We identified 1952 differentially expressed genes (FDR < 0.05) between responders and non-responders that segregated into 3 distinct clusters, which were enriched for leukocyte markers, response to interferon-γ ($IFN\gamma^+$) and oncogenic pathways ($Onco^+$) such as dedifferentiation and mitotic cell cycle. The $IFN\gamma^+$ and $Onco^+$ gene clusters were inversely correlated in melanoma cells and significantly associated with good and poor immunotherapy outcome in two external melanoma immunotherapy cohorts (GSE91061 and GSE100797), thus suggesting two opposing gene expression programs that govern the response to immunotherapy. IPA transcription factor analysis identified Myc as the main upstream regulator associated with the $IFN\gamma^-$ $Onco^+$ phenotype. Indeed, immunohistochemistry showed increased nuclear Myc staining among non-responding patients ($n = 33$, $p = 0.05$).

Myc and Mock stably transfected cells from patient-derived melanoma cell cultures were exposed for 48 hours to IFNγ. Remarkably, Myc overexpression rendered cells from non-responders resistant to IFNγ, as it hindered the induction of 6 IFNγ-responsive genes and MHC class I and PD-L1 protein upregulation, compared to Mock-transfected cells. In contrast, Myc did not confer IFNγ-resistance in melanoma cells from responders. This suggests that Myc-responsive cells can utilize Myc to oppose IFNγ signaling and thereby become immuno-resistant.

In summary, we suggest a potential major mechanistic role for Myc in resistance to immunotherapy by impairing the tumoral response to IFNγ.

BRAF V600E/K mutated Circulating Tumour DNA (ctDNA) kinetics represent a promising tool for resistance of targeted therapy in metastatic melanoma patients

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Backckground: Metastatic melanoma patients with a BRAF V600E or V600K mutation are commonly treated with BRAF plus MEK inhibitors as first line therapy with ~1/3 of patients achieving long-term benefit. Targeted therapy and immune checkpoint inhibitor treatment is an open clinical question, with several trials evaluating the most effective sequencing of treatment. However, no biomarkers are available to determine which patients are likely to progress on BRAF inhibitors. Early identification of non-responder patients would aid clinicians in determining an optimal time to discontinue targeted therapy and introduce immune checkpoint inhibitors.

Patients and Methods: Prospective analysis of repeated measurements of BRAF V600E/V600K mutant ctDNA in 68 metastatic melanoma patients who received dabrafenib and trametinib as first line therapy explored the potential of determining patients likely to develop resistance to BRAF inhibitors.

Results: Mutant BRAF ctDNA was present in 86.7% ($n = 59$) of patients prior to starting therapy. Undetectable ctDNA at baseline and/or a significant decrease in ctDNA levels during the first 12 weeks of treatment was associated with longer PFS ($p < 0.0001$). CtDNA was detectable between 2 to 12 weeks of starting treatment in 24 of 39 who developed extracranial progressive disease. Increasing levels of ctDNA were seen in 22 of 39 (56%) patients experiencing PD during BRAF therapy: either before radiologically confirmed PD (N = 10) or at the time of progression (N = 12). CtDNA was not detected in any repeat blood samples of patients who have had long-term durable response to treatment (mean 21 months) (N = 20).

Conclusion: Mutant BRAF ctDNA kinetics may provide some insight into which patients will develop resistance to BRAF inhibitors and may provide earlier evidence of disease progression than radiological scans.

The effect of interferon gamma on melanoma progression under MAPK inhibitor therapy

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Half of all metastatic cutaneous melanoma harbor a mutation at the V600 position of the BRAF protein, causing a constitutive activation of the MAPK pathway. Patients with BRAF mutant melanoma are treated with MAPK pathway inhibitors (MAPKi) targeting the mutant BRAF and its downstream MEK protein. Over time, their tumors will acquire resistance to this treatment. Many studies have demonstrated that MAPKi-resistant melanoma acquires a dedifferentiation phenotype, characterized by a downregulation in melanocytic master regulator, MITF, and an upregulation of AXL. However, these studies were based on in vitro and immunodeficient mouse models. In our previous publication, melanoma biopsies from patients responding to MAPKi treatment showed a substantial increase in T cell infiltration and antitumor inflammatory response. One of the dominant immune factors produced by infiltrating T cells is interferon gamma (IFNg). Interestingly, IFNg treatment can upregulate MITF expression in melanoma in vitro, posing the question of the effect of IFNg on the development of a dedifferentiation phenotype under MAPKi treatment. Here, we characterize five patient-derived melanoma models treated under MAPKi, with or without IFNg. It was confirmed that melanoma treated with MAPKi and IFNg express significantly higher levels of MITF compared to treatment with MAPKi alone. Despite previous reports suggesting an anticorrelation between MITF and AXL expression levels, the protein levels of AXL remain the same in the presence of MAPKi with or without IFNg. To further study this MITF/AXL disconnect, we performed a paired RNA sequencing analysis on the resistant sublines resulting from either MAPKi treatment alone or MAPKi with IFNg. By considering the presence of IFNg in the tumor microenvironment, we argue that our proposed resistance mechanisms to MAPKi will be more clinically relevant.

microRNAs restrain proliferation during $BRAF^{V600E}$-driven melanocytic nevus formation

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Melanocytes that acquire a $BRAF^{V600E}$ mutation undergo a period of rapid proliferation before growth-arresting and forming benign melanocytic nevi. Constitutive activation of the MAPKinase pathway downstream of BRAF drives the initial proliferative phenotype. However, the factors that establish and maintain growth-arrest in nevi remain elusive. Additional acquired genetic alterations do not distinguish proliferating $BRAF^{V600E}$ melanocytes from their growth-arrested nevus counterparts, suggesting a role for regulatory elements. We investigated the role of microRNAs in the initiation and maintenance of nevus growth-arrest. Using primary human melanocytes, melanocytic nevi, and adjacent melanoma, we show that miR-211-5p and miR-328-3p are enriched in nevi compared to normal melanocytes, then subsequently downregulated in adjacent melanoma. In primary human melanocytes, expression of miR-211-5p and miR-328-3p results in a growth-arrest phenotype similar to that induced by $BRAF^{V600E}$. We further identified 15 direct targets of these microRNAs which, when suppressed, phenocopy $BRAF^{V600E}$-induced growth arrest. These networks converge on inhibition of GPR3 and AURKB signaling to block cell cycle progression in primary human melanocytes. We show that GPR3 and AURKB overexpression each partially rescues growth arrest induced by either the microRNAs or $BRAF^{V600E}$. Finally, we demonstrate that each of these direct and indirect miRNA targets are more highly expressed in human primary melanomas, as compared to adjacent benign nevi. This study further elucidates the cell intrinsic mechanisms that are essential for both nevus formation and preventing malignant transformation in melanocytes harboring oncogenes. Our results can contribute to biomarker strategies to improve differentiation between nevi and melanoma.

Single institution real world data of pembrolizumab therapy in metastatic melanoma patients and pharmacoeconomic analysis

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The use of immune checkpoint inhibitors in treating patients with metastatic melanoma has greatly improved survival rates. Pembrolizumab is reported to produce a complete response in approximately 15% of metastatic melanoma patients with a median progression free survival (PFS) of 8.3 months. The cost of cancer immunotherapy is a significant factor in the design of treatment plans and remains a burden for providers. We did a retrospective analysis of 84 metastatic melanoma patients who received pembrolizumab between June 2015 and April 2019. We found that 22 (26%) patients had a complete response (CR) to pembrolizumab. 15 (19%) patients achieved partial responses or stable disease using pembrolizumab. 29 (35%) patients had disease progression and 18 (22%) patients had non-evaluable responses to pembrolizumab. Out of the 29 patients who progressed on pembrolizumab, 10 (34%) patients responded to subsequent lines of treatments and 3 of those 10 patients (30%) had a CR. The median PFS of pembrolizumab responders is 14 months. A pharmacoeconomic analysis of the cohort found that the mean cost of pembrolizumab per patient was $65 482. The average number of

cycles for the 22 CR patients was 24. Out of the 22 CR patients, 11 were on clinical trials or access programs with average of 30 cycles and the other 11 patients whose treatment was paid by the healthcare system received an average of 17 cycles. Real world data results of pembrolizumab showed a higher complete response rate and median PFS than in clinical trials. Patients who progressed on pembrolizumab could be rescued with subsequent therapies. Our data shows that the majority of patients did not require 2 years of pembrolizumab to achieve effective disease control making the treatment much more cost effective.

An in-depth map of the melanoma immune microenvironment and correlates of immunotherapy response by imaging mass cytometry

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Melanoma is one of the most immunogenic malignancies as it harbors one of the highest mutation burdens of all human solid tumors. As a result, abundant neoantigens are generated and can be recognized by tumor infiltrating lymphocytes (TILs) within the tumor microenvironment. However, the complex interplay between the various populations of TILs and spatial dynamics within the tumor environment remain largely understudied in human melanoma samples due to methodological limitations. Here, we used CyTOF imaging mass cytometry to simultaneously quantify the expression of 35 proteins within the microenvironment of 67 melanomas and 5 benign nevi. Assisted by machine learning algorithms, we segmented over 200,000 individual cells and clustered them into immune, malignant, or stromal subtypes based on the expression of phenotypic markers. We highlight the inter-patient variability in immune infiltration and explore the relationship between infiltrating immune populations and response to immune checkpoint inhibitors. The spatial organization of tumors revealed a continuum across degrees of infiltration and distribution of immune cells ranging from absent (cold) to mixed and compartmentalized tumors. The expression of immune checkpoints (PD-L1, LAG3, TIM3, and VISTA), proliferation markers (Ki67), lineage markers, as well as MAPK signaling proteins revealed new potential correlates of immunotherapy response. In summary, imaging mass cytometry allows a detailed characterization of the melanoma immune microenvironment, revealing patterns of spatial arrangement, signaling, and immunomodulatory marker expression that have important clinical implications.

Safety and feasibility of cryoablation (cryo) to a site of progressive disease in patients (pts) receiving immune-checkpoint inhibition (ICI)

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Background: Image-guided percutaneous cryo is a minimally invasive oncologic treatment. Through direct modulation of the tumor, it is theorized that this local therapy may modify the immune microenvironment. We hypothesize that it can augment an anti-tumor response when used concurrently with ICI.

Methods: A planned analysis to assess safety and feasibility of a phase II single-arm study was performed. 10 pts with advanced melanoma progressing on ICI underwent cryo of an enlarging lesion and ICI was continued for a minimum 2 additional cycles. Computed tomography was performed at 4–6 weeks following cryo to determine tumor response in non-ablated lesions per RECIST1.1, with confirmatory scans at 8–10 weeks. The primary endpoint was safety and feasibility. Secondary endpoints were overall response rate (ORR) and disease control rate (DCR) with DCR defined as the percentage of pts who achieve complete response (CR), partial response (PR), and stable disease (SD). PR was based on response in untreated lesions, consistent with abscopal effect.

Results: Ten pts in the safety cohort received anti-PD-1 monotherapy. Median duration of ICI prior to cryo was 14.5 weeks (8.5–60.8). Treated lesions were in lung (n = 3), bone/soft tissue (n = 3), lymph nodes (n = 2), liver (n = 1) and adrenal gland (n = 1). No immune-related adverse events post-cryo occurred. There was one treatment-related CTCAE grade 3 event (osteomyelitis); no grade 4/5 events. In the 10 pts, 2 had PR, 3 SD, 3 PD, and 2 are non-evaluable due to lack of confirmatory imaging.

Conclusions: During this scheduled safety analysis, cryo following progression on ICI showed that combination therapy is feasible and has an acceptable side effect profile. Early efficacy data of this potentially synergistic approach in the metastatic melanoma is encouraging but warrants further investigation.

Comparative efficacy of nivolumab as adjuvant treatment of melanoma: a network meta-analysis (NMA) and matching-adjusted indirect comparison (MAIC)

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The objective of this study was to compare the efficacy of the anti-PD-1 agent nivolumab (NIVO) with other adjuvant treatments for resected melanoma using an NMA and MAIC that incorporated 3-year data from the CheckMate 238 trial. Relevant comparator treatments included in this study were combination dabrafenib and trametinib (DAB+TRAM) and another anti-PD-1 agent, pembrolizumab (PEM). A systematic literature review was used to identify trials that evaluated the treatments of interest. One trial was identified for each treatment: CheckMate 238 (NIVO vs ipilimumab [IPI]), COMBI-AD (DAB+TRAM vs placebo), and KEYNOTE-054 (PEM vs placebo). To form a single connected network, the NMA also included the EORTC 18071 trial (IPI vs placebo). A Bayesian NMA was conducted using fractional polynomials to estimate time-varying HRs with corresponding 95% credible intervals (CrI) for recurrence-free survival, as reported by the most recently published Kaplan-Meier curves from each trial. Comparisons between anti-PD-1 agents showed no differences in the risk of recurrence at all time points. For the longitudinal comparison of NIVO with DAB+TRAM, the HR decreased steadily over time, reaching statistical significance at 18 months (HR, 0.64; 95% CrI, 0.42–0.99), with sustained improvements at 36 months (HR, 0.28; 95% CrI, 0.14–0.59) and beyond. NMA results for NIVO vs DAB+TRAM were validated using an MAIC in the *BRAF*-mutant population. MAIC analysis results for NIVO vs DAB+TRAM were consistent with NMA results, with HRs of 0.50 (95% CrI, 0.32–0.77) at 18 months and 0.19 (95% CrI, 0.09–0.38) at 36 months. Overall, in this study using an NMA and MAIC, no differences were found between the anti-PD-1 agents, whereas NIVO was associated with a sustained reduction in the risk of recurrence at and after 18 months when compared with DAB+TRAM.

BH3 mimetics as promising treatment options for melanoma

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Discovering effective therapies for melanoma is a pressing issue, especially for patients who do not respond or relapse from treatments. BH3 mimetics are a clinically attractive class of therapies that may provide alternative approaches. BH3 mimetics are small molecule inhibitors of the anti-apoptotic members of the BCL2 family, and we explored their therapeutic potential in melanoma. We used both genetic (shRNA or CRISPR/Cas9) and chemical (BH3 mimetics) approaches to determine the contribution of anti-apoptotic BCL2 members to melanoma survival, and identified MCL1 and BCLXL as crucial. We used the following clinical-trial-ready BH3-mimetics: S63845 (MCL1-i), A1331852 (BCLXL-i) and ABT263 (BCL2/BCLXL/BCLW-i). Results suggest targeting both MCL1 and BCLXL, with S63845 plus ABT263 or A1331852, has therapeutic potential. We examined their effects on de-bulking the tumors and killing the melanoma-initiating cell (MICs), *in vitro* and *in vivo*. We used multiple assays including cell viability, immunoblot, and sphere formation. To increase clinical relevance, we used cells derived from patients, including those relapsed from targeted and/or anti-PD-1 immunotherapy. These lines have diverse mutation status and include various types (cutaneous, acral, and mucosal). Excitingly, both combinations efficiently de-bulks and kills MICs within the nM range, significantly better than single drugs ($p < 0.05$). Furthermore, the combinations down-regulate stemness markers OCT-4 and SOX-2. Lastly, in a mouse xenograft model, both combinations significantly inhibited tumor growth compared to single drugs ($p < 0.01$). In tumor cells isolated from these mice, the combination treatments had significantly lower sphere forming capacity compared to single drugs ($p < 0.05$), supporting the idea that the combinations kill MICs. In summary, data strongly suggest combinations targeting MCL1 and BCLXL is an alternate treatment option for melanoma.

Secondary intention healing after functional surgery for in situ or minimally invasive nail melanoma: an analysis of healing time and outcomes

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Background: Functional surgery (FS) has an excellent oncologic, functional, and cosmetic outcome for in situ or minimally invasive nail melanoma. Secondary intention healing (SIH) can be a useful reconstructive method for the defect. However, the healing process and outcomes have not been sufficiently reported in the literature.

Objective: To investigate the course of SIH after conservative surgery for nail melanoma in situ (NMIS) or minimally invasive nail melanoma (MINM).

Methods: Patients with pathologically confirmed NMIS or MINM who were treated by FS and SIH in our hospital from September 2015 to December 2018 were enrolled.

Results: In total, 12 patients were evaluated. The median age was 51 (range: 28–69) years old. Eight patients were female. Nine patients had fingernail lesion. Granulation tissue coverage over phalangeal bone was achieved at a mean 4.2 ± 2.3 weeks. Re-epithelialization was completed at mean 10.6 ± 2.8 weeks. All lesions healed without serious complications. Patients' median subjective global satisfaction was 8 (range: 7–10).

Conclusions: This study suggests that SIH is a good reconstructive method handling the defect after FS for NMIS or MINM.

Combined use of topical imiquimod and 5-fluorouracil in the management of in-transit cutaneous melanoma: A Case Report and Literature Review

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Statement of the Problem: While there are numerous therapeutic options available for in-transit cutaneous melanoma, the treatments are often unsuccessful due to suboptimal response rates. This motivates the use of combination therapies such as imiquimod and 5-fluorouracil to increase therapeutic efficacy. Imiquimod and 5-fluorouracil have distinct mechanisms of action, with imiquimod binding to toll-like receptor 7 and 8 on leukocytes and 5-FU inhibiting thymidylate synthase and inhibiting DNA synthesis. The purpose of this case report is to describe the course of an 87-year-old female with an 8-year history of cutaneous melanoma. Findings: The patient was initially managed with a wide local excision of the right shin lesion, as well as a split-thickness graft. She was further treated with a combination of 5% topical imiquimod cream and 5% 5-fluorouracil cream. There was complete clearance within 23 months, and the patient has been disease-free since. Conclusion & Significance: Given the patient's complete clearance and disease-free state since treatment, we postulate that imiquimod and 5-FU's synergistic mechanisms of action provide a valuable potential for targeting in-transit cutaneous melanoma in patients.

Actionable genetic alterations detected by next generation sequencing over a conventional BRAF testing in patients with advanced melanoma

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Next generation sequencing (NGS) can detect several genetic alterations which cannot be identified by a conventional companion diagnostics (CDx). However, most of the previous studies comparing different sequencing methods examined the different pathological specimen thus cannot exclude a limitation of intra-patient heterogeneity. This study aimed to assess the actionable genetic alterations detected by NGS over a conventional BRAF testing by using exactly the same sample. We examined the residual DNA extractions from the specimens previously submitted outside for CDx and returned to our hospital thereafter using the primer designed in our own facility. We identified eligible 116 samples from 115 patients with advanced melanoma treated in the National Cancer Center Hospital in Tokyo, Japan from December 2017 to March 2019. The patients consisted of 53 men and 62 women, the median age at the CDx was 62 years, and the median follow-up period was 10 months. Subtypes were non-acral cutaneous melanoma in 51, mucosal in 28, acral in 23, unknown primary in 5, conjunctival in 4, and uveal in 4 patients. The THxID BRAF kit detected BRAF mutations in 35 (30%, V600E in 31 and V600K in 4), while NGS detected in 36 (31%, V600E in 28, V600K in 4, other V600 in 2, and non-V600 in 2) out of 116 samples. Of these, 3 BRAF-mutant melanomas were detected only by the THxID BRAF kit, while 4 were detected only by the NGS. NGS detected any kind of mutations in 60 (52%) samples; BRAF in 36, NRAS in 10, KIT in 8, KRAS in 4, MAP2K1 in 3, GNA11 in 2, and GNAQ in 1, including 4 samples with co-existing 2 different mutations. NGS can detect additional potentially actionable genetic alterations over a conventional BRAF testing, therefore, genetic testing with NGS should be considered in the coming era of precision medicine.

BRN2 promotes anoikis resistance in melanoma

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Melanoma tumours are highly heterogeneous, comprising of many cell populations that vary in their potential for growth and invasion. Differential transcription factor expression contributes to these phenotypic traits. Previous studies have shown mutually-exclusive expression of key transcription factors MITF and BRN2 in melanoma tumours. MITF expressing cells are thought proliferative, whereas BRN2 expressing cells considered to be the invasive population. We have previously shown that metastatic dissemination and subsequent growth of melanoma requires the expression of both transcription factors.

We have investigated the contribution of BRN2 to the metastatic process using a doxycycline-inducible system to express BRN2 in metastatic melanoma cells with no or low constitutive expression of the transcription factor. Whole genome profiling analysis revealed signalling pathway changes related to resistance to anoikis (cell death induced by detachment from the extracellular matrix) in

response to BRN2 expression. Further investigation of growth in non-adherent conditions confirmed increased survival of BRN2 expressing cell lines.

Expression of BRN2 imparted a slow growth phenotype, and these cells that were partially resistant to drugs targeting mutant BRAF. Functionally, expression of BRN2 promoted induction of c-MET levels as well as increased phosphorylation of STAT3, both known to be activated to induce resistance to anoikis. These results highlight the importance of a largely overlooked transcription factor in the progression of melanoma to one of a potentially drug resistant phenotype resistant to cell death under non-adherent conditions.

Survival and recurrence patterns of stage II and III cutaneous melanoma by AJCC 8th edition staging

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Under the new 8th edition AJCC staging system, survival outcomes for melanoma patients are greater across stages compared to previous editions. However, lack of data on patterns of recurrence makes it difficult to establish clear and rational surveillance strategies. We perform a retrospective cohort analysis of stage II and III melanoma patients diagnosed between 2000 to 2012 using AJCC 8th staging. Overall survivals (OS) from time of diagnosis and relapse were estimated using Kaplan-Meier and Cox proportional hazard models. Of 169 evaluable patients, median age was 63.2 (range 14–93) at initial diagnosis; 62.7% (106) were male and 47.9% (81) stage II. Stage II patients had greater median age (69.3 vs 58.1; $p \leq 0.001$) compared to stage III. Five year relapse-free survival from diagnosis was 72.0% for stage II and 57.3% for stage III ($p = 0.016$). In this cohort, 58 (34.3%) relapsed. OS from time of relapse to death was no different between those with initial stage II or III melanoma ($p = 0.57$). Of those whose relapse was diagnosed with imaging, 67.6% (25) were stage III, with 45.9% (17) from routine surveillance imaging. When relapse was identified by patients, 57.1% (12) had initial stage II disease. OS after relapse was worse if multiple lesions (≥ 10) were present on relapse compared to those with just 1 lesion (Median OS 0.6 vs 4.2 years, $p < 0.001$) or >1 organ systems were involved compared to a single organ (Median OS 0.7 vs 1.8 years, $p = 0.009$). Disease burden at time of relapse, as determined by number of lesions and involved organs, regardless of initial diagnostic staging may help predict OS in relapsed disease. More data is needed to determine if more frequent surveillance could diagnose relapse at a lower disease burden and improve survival, particularly for patients treated currently in the age of immunotherapy.

Stress granules as a resistance marker and therapeutic target in MEK inhibitor-resistant *NRAS* mutant melanoma

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NRAS mutations occur in 25–30% of melanoma tumors but *NRAS* mutant melanoma patients have limited therapeutic options, poor disease prognosis, and no FDA-approved targeted therapies. MEK inhibitors, among the most promising targeted therapy options, have variable effects and lead to resistance after only a few months. There has been evidence linking RAS-MEK-ERK signaling to stress adaptation processes and the lack of drug response. *KRAS* mutant pancreatic cancer cells enhance tumor fitness by upregulating stress granules (SGs), non-membranous cytosolic structures consisting of mRNAs and proteins that form upon cellular exposure to a variety of stress stimuli. Our preliminary data showed that SGs were also found in treatment-naïve patient melanoma tumor samples. We showed that melanoma cell lines produced SGs *in vitro* in response to oxidative stress and that pre-treatment with the MEK inhibitor trametinib completely eliminated the SG response in some cell lines but not others. In cell lines with SG elimination upon trametinib treatment, once they acquired resistance to trametinib, they re-gained the ability to make SGs in response to SA. Our data also suggested a potential connection between the maintenance of SG response and the reactivation of the p38/JNK pathway. Therefore, we propose that SGs might play a role in tumor fitness prior to drug treatment and might act as a marker and potential target in the context of resistance to targeted therapy.

Somatic mutation profiles within cutaneous melanomas with germline cancer predisposition mutations

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Germline mutations, which are usually present at birth and found consistently in all cells, and somatic mutations, which are acquired during tumor development, have classically been thought of as separate entities with different consequences for patient care. However, the recent approvals of immunotherapy in Lynch-associated cancers and PARP inhibitors in BRCA-mutated cancers demonstrate the interplay between germline variations and acquired somatic alterations in cancer development. The somatic-germline interactions within cutaneous melanoma are largely unexplored. Here we assessed genome-wide somatic mutations in melanomas carrying germline pathogenic mutations from The Cancer Genome Atlas (TCGA). We analyzed germline variants in 81 known tumor predisposition genes based on a strict definition of pathogenicity. Tumor mutational

burden was calculated, and classically melanoma-associated somatic gene mutation rates were assessed. Among 438 patients with cutaneous melanoma, 40 (9%) carry germline mutations in 12 genes including *ATM*, *BRCA2*, *BRIP1*, *CDKN2A*, *CHEK2*, *FH*, *MUTYH*, *NBN*, *RAD51C*, *RECQL4*, *SDHA*, and *WRN*. Overall, patients with germline mutations carry a similar overall tumor mutational burden ($p = 0.51$). For frequently mutated somatic genes (*BRAF*, *NRAS*, and *TP53*), patients with germline mutations did not demonstrate differences in somatic mutation prevalence. In conclusion, while approximately 9% of patients within TCGA harbored a pathogenic germline alteration in a known cancer predisposition gene, these inherited mutations were not associated with differences in specific somatic mutations or overall mutational burden.

The efficacy of anti-PD-1 in metastatic acral and mucosal melanoma patients (pts)

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Anti-PD-1 antibodies are standard therapy in metastatic melanoma pts. However, the efficacy of anti-PD-1 for acral (AM) and mucosal melanoma (MM) has not been well described since these subtypes are relatively rare compared to cutaneous melanoma (CM). As the epidemiology and biology of AM and MM are distinct from CM, it is possible that the response to anti PD-1 therapy also differs from CM. A single-institutional, retrospective cohort analysis identified pts with advanced AM and MM who received anti-PD-1 for metastatic melanoma at any point in their treatment course between 2012 and 2018. Objective responses were determined using investigator-assessed Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1. Progression free survival (PFS) and overall survival (OS) was assessed using the Kaplan-Meier method. Cox regression analysis was performed to identify factors associated with outcomes.

97 pts were identified, including 38 (39%) AM and 59 (61%) MM. 75 (77%) received pembrolizumab and 22 (23%) received nivolumab. 77 patients (79%) had received previous systemic therapy, including 44 (45%) with prior ipilimumab. The objective response rate (ORR) was 21.6% in AM and 16.7% MM pts. With a median follow-up of 15.6 months (AM) and 16.5 months (MM), the median PFS and OS for AM were 3.3 months and 25.7, and for MM were 3.0 and 20.1 months, respectively. On multivariable analysis, elevated serum LDH [$p = 0.00014$, HR: 3.31 (1.79–6.13)] and brain metastasis [$p = 0.05$, HR: 2.01 (1.00–4.05)] were significantly associated with OS; AM/MM subtypes, prior treatment and number of metastases were not significant.

The ORR, PFS and OS with anti-PD-1 appeared worse in AM and MM than in CM. But there are no significant difference OS and PFS between AM and MM. LDH level and presence of brain metastasis significantly correlated with worse OS.

Immunomodulatory and therapeutic properties of dsRNA nanoplexes identified by live imaging of pre-metastatic niches in MetAlert mice

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Melanoma is a paradigm of cancers with a high potential for colonization of lymph nodes, a process usually preceded by neo-lymphangiogenesis. However, removal of sentinel lymph nodes does not necessarily increase patient survival. Therefore, a key pending question in the field is the specific contribution of tumor-induced lymphangiogenesis to immune-evasion and the generation of visceral metastases. Moreover, anticancer agents able to reprogram the immune system against the tumor, blunt melanoma-associated lymphangiogenesis and metastasis in a sustained manner and without secondary effects to the normal vasculature have yet to be identified. We have generated melanoma "Met Alert" mouse models that allow for whole-body imaging of lymphovascular pre-metastatic niches in vivo. Pharmacological studies in these mice identified synthetic long dsRNA nanoplexes as potent inhibitors of melanoma metastasis. Mechanistically, the therapeutic effect of these dsRNA particles was found associated to the re-programing of the immune system towards an anti-tumoral phenotype and the repression of lymphovascular pre-metastatic niches. Anti-tumoral effects included the transcriptional blockade of MIDKINE and the lymphangiogenic factor VEGFR3, features we demonstrated driven by type I interferon responses, and the induction of immunogenic cell death. Ultimately, these compounds inhibited melanoma progression and metastatic relapse after surgery. Importantly, dsRNA nanoplexes showed synergy with immunotherapy treatments (PD-L1). These results are particularly relevant since derivatives of these dsRNA nanoplexes are now being tested in phase I clinical trials.

Targeting nucleotide exchange to inhibit Gq/11 driver mutations in uveal melanoma

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Constitutively activating mutations in the G-protein alpha subunits *GNAQ*(Gαq) and *GNA11*(Gα11) act as oncogenic drivers in over 90%

of uveal (eye) melanoma (UM) tumors. We show that constitutively active Gαq and Gα11 can be targeted in UM cells by the cyclic depsipeptide FR900359 (FR). FR inhibits GDP/GTP guanine nucleotide exchange allosterically to trap constitutively active Gαq/11 in inactive GDP-bound Gαβγ heterotrimers. FR inhibits second messenger signaling, arrests proliferation and reinstates melanocytic differentiation in UM cells driven by constitutively active Gαq or Gα11. At higher doses, FR also induces apoptosis. The re-differentiation and anti-proliferative effects of FR are not seen in UM cells that lack mutations in Gαq or Gα11. FR promotes UM cell differentiation by reactivating polycomb repressive complex 2 (PRC2)-mediated gene silencing, and this re-differentiation can be blocked with an EZH2 inhibitor. The effector system regulating PRC2 downstream of constitutively active Gαq/11 in UM is currently under investigation. Preliminary data from human primary tumor samples suggest that targeting constitutively active Gαq/11 with FR could provide an important therapeutic approach for UM.

Clinical Predictors of outcome in metastatic melanoma (MM) patients (pts) with brain metastases (BM) treated with combination IPI+PD-1 immunotherapy

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Combination IPI+PD-1 has become standard of care in treating BM pts with response rates in clinical trials of >50%. However, predicting response to prospectively inform treatment sequencing decisions remains challenging and requires further investigation. We examined baseline characteristics of BM pts treated with IPI+PD-1 to identify factors associated with good outcomes. IPI+PD-1 treated BM pts at 2 Australian centres were included. Baseline demographic and disease characteristics were correlated with outcome, including response rate (RR), progression free survival (PFS) and overall survival (OS). Of 85 pts identified, 59 (69%) were male, 81 (95%) ECOG PS 0–1, 39 (46%) elevated baseline LDH, 50 (59%) had BRAF mutation and med age was 59 years. 29 (34%) pts received local BM treatment prior to commencing IPI+PD-1 and 41 (48%) received IPI+PD-1 as 1st systemic therapy (27 BRAF wild-type/14 BRAF mutant). 10/85 (11%) required corticosteroids at time of commencing treatment for neurological symptoms, of whom 2/10 (20%) responded to IPI+PD-1. Overall RR was 32/85 (38%); 21/50 (42%) in BRAF mutant pts, 11/35 (31%) in BRAF wild-type pts. Pts treated with 1st line IPI+PD-1 had RR of 51%, med PFS 5.6 months and med OS 25.6 months compared to 26%, 2.6 months (p = 0.052) and 13.8 months (p = 0.8) when treated in 2nd or subsequent line. Pts with a BM ≥ 10 mm (n = 36) or ≥ 3 BM (n = 37) had med OS of 10.4 months and 13.8 months compared with 25.6 months (p = 0.1) and 25.6 months (p = 0.11) in pts with BM < 10 mm or <3 BM respectively. In pts with normal LDH at baseline (n = 46), med PFS was 5.9 m and med OS was not reached compared with 2.1 months (p = 0.007) and 5.6 months (p < 0.0001) with elevated LDH. Baseline LDH, BM burden and prior treatment may be important determinants in predicting outcomes to treatment with IPI+PD-1 in BM pts. Further pt accrual and multi-variate analysis are on-going.

UVB induce appetite changes in males

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It is commonly believed that ultraviolet (UV) exposure affects appetite. However, a clear understanding of UV exposure systemic effect and the mechanism which affect appetite, is unknown. Here we show that, UVB exposure effects of appetite is hormonal dependent as it induces appetite in males and not in females, both in mice and humans. We have established an animal model system to study the appetite related behavioral changes upon chronic UVB exposure, including Open-field and Staircase tests. Our preliminary data unveiled significant increase in weight, body mass index (BMI) and eating behavior in male mice upon UV exposure, unlike females. We examined similar results by providing questionnaire to phototherapy clinic patients regarding their appetite modification post treatment. We have further analyzed the plasma proteome of mice upon chronic UVB, in order to understand the mechanism that allows increase in appetite upon UVB exposure. Our preliminary data provides promising direction regarding the mechanism behind the appetite changes observed in males and not in females, suggests the potential of revealing enigmas related to the systemic effect of UVB exposure and leads the understanding of gender specific UV induced appetite changes.

The role of PI3K lipid effectors in melanoma

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Metastatic melanoma accounts for the highest number of skin cancer deaths, and while current treatments for this disease have progressed significantly in recent years, stage IV melanoma currently has only a 15–20% five-year survival rate. This highlights the critical need for new therapeutics to treat this complex disease. The PI3K/AKT pathway and RAS-RAF-MEK-ERK pathway play a major role in melanoma initiation and progression. Mounting reports indicate that these pathways can be concurrently activated, which complicates the prospect of treating with targeted

therapies. Dysregulated PI3K signaling is characteristic of a large portion of melanomas, yet AKT pharmacological inhibitors have been largely ineffective in clinical trials as single agents. Despite this, studies in our lab have shown that targeted genetic knockdown of AKT leads to complete lethality in multiple melanoma cell lines. In addition, we found that simultaneous transfection of siRNAs targeting all three AKT paralogs along with overexpression of each of the AKT proteins was able to significantly rescue this deleterious effect. Thus, there is an underlying discrepancy between lethal triple AKT paralog genetic knockdown in melanoma cells and the observed clinical and *in vitro* failure of AKT pharmacological inhibitors. Dysregulation of the PI3K lipid signaling cascade in melanoma is conventionally believed to occur through activation of AKT, and we have previously shown that either loss of PTEN or mutationally activated PI3K cooperates with mutant BRAF to promote melanoma formation. However, it has not been shown if constitutively active AKT alone is able to substitute for this effect. Further research will elucidate the role of AKT in the initiating stages of melanoma and provide a more thorough understanding of the PI3K/AKT signaling pathway in promoting melanomagnesis.

Risk tolerance of melanoma treatments in the adjuvant setting: a patient perspective

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There are more systemic therapies in the adjuvant setting available to lower the chance of recurrence in patients with operable high-risk melanoma. However, risk of toxicities may contribute to lower than expected uptake. This study assessed the risk tolerance for pyrexia of patients with advanced melanoma in the adjuvant setting using the threshold technique (TT), a survey-based patient-preference method administered online to a sample of 50 adults with self-reported stage III melanoma in the US. Respondents evaluated two adjuvant treatment options—a reference treatment representing the standard of care with a 44% chance of recurrence after 1 year of systemic therapy and no treatment-related risk of pyrexia and an alternative treatment with a 63% risk of pyrexia (described as fever of 100–104+ that might recur). In the first series of TT questions, the alternative treatment had unknown efficacy, representing the context of a clinical trial. In a second series of TT questions, respondents were told the chance of recurrence for the alternative treatment was 12% after 1 year. The risk of pyrexia for the alternative treatment was varied systematically to define the range of pyrexia risk acceptable to the respondent. Data were analyzed with an interval regression to estimate the maximum acceptable risk (MAR) of pyrexia to take the alternative treatment. With a 63% risk of pyrexia, 26% of stage III patients would take the alternative treatment over the reference when efficacy was unknown, compared with 88% when the chance of recurrence was 12%. The mean MAR of pyrexia when efficacy was unknown was 34% compared with 85% when it was known, indicating that respondents were willing to accept a higher risk of pyrexia when they knew the estimated efficacy was better. When patients are informed, they may choose to accept higher adverse event risks for a better chance of recurrence.

Metastatic risk of GEP 1A classified uveal melanoma patients: a retrospective cohort analysis

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Background: In uveal melanoma (UM), gene expression profiling (GEP) is commonly used to classify metastatic risk into 3 groups. Class 1A patients have a low metastatic risk of 2% at 5 years. We aimed to describe clinical features associated with the development of metastasis in this low-risk group.

Methods: We performed a single-center IRB-approved retrospective case series. All UM patients who were GEP 1A classified between February 2009 and May 2019 were included. Patient age at diagnosis, gender, tumor location, apical thickness, largest basal diameter (LBD), COMS size, mutation status, initial therapy, time to recurrence/metastasis, site of metastasis and overall survival were extracted from electronic medical records. Statistical analysis was used to identify factors associated with metastasis development and to evaluate their collective utility for identifying high risk patients.

Results: A total of 81 UM patients with Class 1A GEP were included, of which 14 (17%) experienced local primary recurrence (5 patients, 6%) or distant metastatic disease (11 patients, 14%). 2 patients (2%) experienced both recurrence and metastasis. Factors associated with metastases included LBD (HR = 1.25; 95% CI = 1.05–1.49; p = 0.0109), large COMS size (HR = 5.05; 95% CI = 1.51–16.95; p = 0.0087) and primary tumor in the iris (HR = 14.13; 95% CI = 2.25–88.70; p = 0.0047). Multivariate logistic regression modelling yielded an area under the curve of 0.887, suggesting good operating characteristics for predicting risk.

Conclusions: Combined clinical decision-making utilizing factors such as GEP class, LBD, COMS size, and primary tumor site could have substantial clinical impact by improving risk stratification and augmenting follow-up intervals in UM GEP 1A patients. The impact of mutation status in this population remains unknown due to the infrequency of testing in this cohort.

Real-world effectiveness of first-line combination treatment with immunotherapy versus targeted therapy in patients with *BRAF*-mutant advanced melanoma

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Nivolumab plus ipilimumab (NIVO+IPI) and BRAF+MEK inhibitors (BRAFi+MEKi) are approved as first-line (1L) combination treatments for patients with *BRAF*-mutant advanced melanoma. However, no head-to-head clinical trial data exist. Here, we report overall survival (OS) and progression-free survival (PFS) in adult patients with advanced melanoma using the real-world US Flatiron Health electronic record database. Of the treatment-naive patients who received any 1L systemic therapy (n = 1,198) between 2015 and 2018, those with *BRAF*-mutant advanced melanoma (n = 496) were identified. Among these, 92 received NIVO+IPI and 199 received BRAFi+MEKi. In the NIVO+IPI and BRAFi+MEKi groups, the mean age was 57 and 59 years, the proportion of patients with ECOG performance status 0/1 was 72% and 64%, the proportion with elevated lactate dehydrogenase was 23% and 28%, and the mean duration of follow-up was 13.4 and 14.3 months, respectively. The 1-year OS rate was 69% in the NIVO+IPI and 61% in the BRAFi+MEKi groups (HR, 0.64; 95% CI, 0.43–0.97; p = 0.03). The 1-year PFS rate was 40% in the NIVO+IPI and 31% in the BRAFi+MEKi groups (HR, 0.75; 95% CI, 0.54–1.03; p = 0.07). A similar proportion of patients received subsequent therapy in the NIVO+IPI (35%) and BRAFi+MEKi (38%) groups. Regardless of whether patients received subsequent therapy, the overall observed death rates were lower in patients who received 1L NIVO+IPI (32%) versus BRAFi+MEKi (52%). To conclude, in this retrospective real-world study, patients with *BRAF*-mutant advanced melanoma who started with 1L NIVO+IPI had significantly longer OS compared with those treated with BRAFi+MEKi. More patients remained alive when treated with NIVO+IPI 1L compared with BRAFi+MEKi, regardless of whether or not subsequent therapy was initiated.

CNS metastases in uveal melanoma

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Little is known about uveal melanoma (UM) patients with CNS metastases (CNSM). We describe a cohort of UM patients with CNSM identified from our institutional database.

Of 385 UM cases diagnosed from 1981–2018, 28 had CNSM. Median age at UM diagnosis was 50.5 years, and median age at CNSM diagnosis was 56.5 years. 1 patient (4%) had CNSM and liver involvement. 8 patients (29%) had only non-hepatic metastases and CNSM. 18 patients (64%) had liver and non-hepatic metastases and CNSM. Frequent non-hepatic metastatic sites were lung, bone, soft tissue, and adrenal gland. Only 1 patient (4%) had isolated CNSM.

The median time from UM diagnosis to CNSM diagnosis was 60.5 months. The median time from initial metastatic diagnosis to CNSM diagnosis was 15.5 months. The median number of systemic therapies received prior to CNSM diagnosis was 2. Treatments prior to CNSM diagnosis were: chemotherapy/biochemotherapy in 8 patients, immunotherapy in 12, targeted therapy in 11, glembatumumab vedotin in 4, and IMc GP 100 in 1.

9 patients had neurologic symptoms at CNSM diagnosis. 21 patients had intraparenchymal metastases, with a median of 7 lesions. 4 patients had leptomeningeal disease. 3 patients had dural based metastases.

CNSM directed therapy was as follows: stereotactic radiosurgery (SRS) in 8 patients, SRS with whole brain radiation therapy (WBRT) in 4 (with temozolamide in 1 patient), craniotomy and resection in 6 (with subsequent SRS in 4 and WBRT in 1), biochemotherapy in 1, and craniospinal radiotherapy in 1. 1 patient with LMD received intrathecal IL-2. 7 patients received no treatment. After CNSM diagnosis, 15 patients received at least 1 additional systemic therapy.

The median overall survival from UM diagnosis, from metastatic UM diagnosis, and from CNSM diagnosis in this cohort was 93 months, 29 months, and 7.5 months, respectively.

To our knowledge this is the largest cohort of UM patients with CNSM described.

Multi-model preclinical platform correlates melanoma multipotency and differentiation status with clinical response to immunotherapy

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Immunotherapy has revolutionized cancer treatment but still only a subset of patients has demonstrated durable responses. Despite the intensive efforts to enhance immune checkpoint blockade (ICB)

efficacy, the definitive predictive biomarkers and targets to overcome resistance remain unidentified, underscoring the urgency to develop reliable immunocompetent models for mechanistic assessment. Here we characterize a panel of four syngeneic mouse models representing the main molecular and phenotypic subtypes of human melanomas. Our models exhibited a broad range of responses to anti-CTLA-4; from fully resistant models to 30–60% response rates in sensitive models, mirroring the clinical situation. Comparative analyses of genomic, transcriptomic and intratumoral immune cell profiles demonstrated alignment with clinical associations of tumor mutation burden, tumor-infiltrating lymphocytes (TILs) densities and the phenotype of specific immune populations. Moreover, our study validated the correlation of T cell dysfunction and exclusion programs with resistance to ICB, while MHC-I antigen presentation function was intact in the four models. Notably, genome-wide expression analysis uncovered a melanocytic plasticity signature (MPS) predictive of patient outcome in response to anti-CTLA-4 or Anti-PD-1, suggesting that the multipotency and differentiation status of melanoma can determine ICB benefit. Importantly, the combination of MPS with recent TIDE method (Tumor Immune Dysfunction and Exclusion) improved the predictive performance, highlighting the complexity of ICB responses. Our comparative preclinical platform recapitulates melanoma clinical behavior and has provided insight into both melanoma cell extrinsic and intrinsic determinants of ICB efficacy that can serve as novel targets of immunotherapy.

Ultraviolet light-induced senescence promotes melanomagenesis in mice

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Exposure to ultraviolet radiation (UVR) is recognized as the major risk factor for the development of melanoma through the induction of DNA damage and an immune tolerogenic microenvironment. However, UVR following melanoma initiation does not increase tumor burden, but instead enhances invasiveness and metastasis. UVR induces senescence and the senescence-associated secretory phenotype (SASP) in melanocytes, keratinocytes, and fibroblasts in the skin. The SASP includes several factors that modify the tissue microenvironment by inducing deleterious chronic inflammation, angiogenesis, epithelial-to-mesenchymal transition (EMT), and stimulating a malignant phenotype of nearby tumor cells. We postulated that UVR exposure during tumor development results in the accumulation of non-tumor senescent cells that express the SASP, leading to a protumorigenic microenvironment. Here, we induced senescence in C57BL/6 cultured dermal fibroblasts via UVR. *In vitro*exposure of B16F10 mouse melanoma cells to the conditioned media from these senescent fibroblasts resulted in increased pigmentation and cell proliferation *in vitro*. To model the protumorigenic effects of accumulation of senescent fibroblasts in the tumor microenvironment, we prepared a mixture of B16F10 melanoma cells and UVR- or naïve dermal fibroblasts and grafted them subcutaneously onto C57BL/6 mice. We found that tumors containing UVR-fibroblasts showed outgrowths earlier and larger compared to non-irradiated controls. Moreover, in the B16 mouse model of subcutaneous melanoma, we found that *in vitro* pretreatment of B16F10 cells with the senolytic small molecule BH3 mimetic ABT-737 significantly delayed the onset and reduced the growth of subcutaneous melanomas in C57BL/6 mice. This suggests that senescent cells, which are sensitive to senolytic drugs, are involved in the development of mouse melanomas, opening a potential avenue of new treatments.

Mitfa loss accelerates tumor formation in a GNAQ-driven uveal melanoma Zebrafish model

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Uveal melanoma (UM) is the most common primary malignancy of the eye in adults. UM arises from transformed melanocytes in the uvea of the eye, which consists of the iris, ciliary body, and choroid. The primary tumor is treatable, however no approved therapeutics currently exist for metastatic UM patients. Activating mutations in GNAQ or GNA11 (heterotrimeric G-protein coupled receptor alpha subunits) are found in 83% of human UMs, specifically at codons Q209 or R183. Patients lacking GNAQ/11 mutation instead have putative activating mutations in CYSLTR2 or PLCB4. Additionally, GNAQ/11-driven UM patient samples have increased YAP nuclear localization. Our lab has developed a transgenic zebrafish UM model expressing activated human GNAQ under the control of the melanocyte lineage transcription factor Mitfa promoter. In combination with $p53^{-/-}$ mutation, GNAQ zebrafish readily develop melanomas in the eye, skin, and abdomen. Strikingly, we have found that GNAQ $p53^{-/-}$ zebrafish cooperate with Mitfa loss to accelerate tumorigenesis, suggesting Mitfa serves a tumor-suppressive role in the context of UM. This is in stark contrast to BRAF-driven cutaneous melanoma (CM), where loss of Mitfa has been shown to be incompatible with CM tumor development or maintenance. Additionally, pathway analysis in zebrafish shows that the YAP signaling arm is the key pathway in UM formation, instead of PLCB4-driven MAPK pathway signaling. Moreover, we have found GNAQ $mitfa^{-/-}$ $p53^{-/-}$ tumors are negative for phosphorylated ERK, suggesting decreasing MAPK pathway signaling may be beneficial for GNAQ-driven tumors in zebrafish. We are currently studying the role of MITF in mammalian UM.

Real-world (RW) recurrence rates and economic impact in patients with resected early-stage melanoma

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Limited data are available on RW recurrence rates and economic impact in patients with resected early-stage melanoma. Here, we report RW recurrence rates, risk factors, and economic impact in patients with resected stage IIB/C or IIIA melanoma.

A retrospective analysis of SEER-Medicare data (2003–2014) was conducted. Patients diagnosed with stage IIB/C or IIIA (AJCC 7th edition) melanoma who had resection (index date) within 4 months were included (2010–2013). Patients with non-melanoma malignancies pre-index were excluded. Recurrence was identified by metastases, end-of-life care, death, or cancer treatment (chemotherapy, radiotherapy, or surgery) post-index, following a 3-month treatment-free interval after the primary treatment. Sensitivity analysis was conducted by excluding death as indicator of recurrence. Multivariate regression models assessed recurrence risk factors. All-cause healthcare costs, annualized and inflated to 2018 USD, were assessed post-recurrence.

1174 stage IIB/C and 142 patients with stage IIIA melanoma were identified. The 1-yr post-index recurrence rates for IIB, IIC, and IIIA were 12.5%, 21.8%, and 28.2%, respectively; 2-yr rates were 29.3%, 43.5%, and 46.5%, respectively. In the sensitivity analysis, 1-yr rates were 10.3%, 16.5%, and 27.7%, respectively; 2-yr rates were 24.3%, 37.7%, 45.3%, respectively. Risk factors for recurrence in IIB/C were age ≥75 years, ulceration, and higher Charlson Comorbidity Index; T3 had a lower risk of recurrence than T4. In IIIA, superficial subtype had a lower risk of recurrence than nodular. Mean (SD) healthcare costs at 1-yr were $31,870 (49,147) in IIB/C and $29,224 (48,837) in IIIA.

Over one-third of IIB/C and nearly half of IIIA patients experienced recurrence in the 2-yr after resection. As recurrence incurs substantial economic burden, newer adjuvant therapies may reduce recurrence and associated costs.

A novel *in vivo* model assessing small caliber nerve perineural invasion by ear melanoma

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Desmoplastic and neurotropic melanomas invade along nerves. This process, termed perineural invasion (PNI), is associated with local recurrence and adverse clinical outcomes. Current *in vivo* experimental models of PNI are artificial and rely on large caliber nerves. A more accurate model would examine small caliber nerves that innervate the skin. We created a novel murine model of ear melanoma to study small caliber PNI.

Transgenic mice were engineered with myelinating Schwann cells expressing green fluorescent protein (GFP). B16-F10 melanoma cells in Matrigel were injected into the ear skin. The GFP expression and the ear thinness together facilitated high-resolution visualization of tiny nerves not detectable with other techniques. Ears were stained with melanoma cell adhesion molecule (MCAM) antibody, and underwent confocal microscopy at varying time points. Images were analyzed with Imaris and ImageJ.

High-resolution images allowed the detection of tiny, individual GFP nerve fibers and Schwann cells. At week 4 post injection, small caliber nerves were detected in direct association with MCAM positive cells, mostly at the tumor periphery. Larger caliber nerves were not associated with melanoma. A mean of 3 distinct nerve invasion foci were observed associated with melanomas. Interestingly, small caliber nerves in association with MCAM positive cells form a markedly disorganized pattern with widely varying branch angles. In contrast, nerves away from the melanoma form a consistent and uniform arborization pattern.

This novel model allows for high-resolution visualization of small caliber nerves in relationship with a developing cutaneous melanoma. This model demonstrates the role that small caliber cutaneous nerves play in the early genesis of PNI, which may be a precursor to larger nerve invasion, and enables further study into this poorly understood form of melanoma progression.

Murine synchronous melanoma model generates distinct tumor microenvironments via PD-1/ PD-L1 axis

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Despite recent advances in immunotherapy, metastatic melanoma still carries a poor prognosis as only a minority of patients obtain durable responses. Genetic heterogeneity and resulting tumor microenvironmental differences between metastases often lead to unpredictable, lesion-specific responses that have yet to be studied. We generated a murine synchronous melanoma model using the parental YUMM 1.7 cell line, containing $Braf^{V600E}/Pten^{-/-}/Cdkn2^{-/-}$ driver mutations, and UVB-irradiated derivative, YUMMER 1.7 to

recapitulate human disease. When synchronously into the same mouse, the two cell lines generate distinct tumor microenvironments varying in immune infiltration, stromal response, and MHC Class I induction. Interestingly, YUMMER tumors stimulate a greater infiltration of CD8+ effector T cells, however compensatory inhibitory mechanisms such as an upregulation of surface PD-L1 expression on stoma and increased PD1 expression on intratumoral CD8 cells ultimately dampens the immune response. In contrast, although YUMM tumors facilitate synchronous YUMMER tumor growth, they remain immunologically "cold" regardless of the presence of another immunologically "hot" but genetically different tumor. These results suggest that the type of tumor microenvironment created depends on tumor cell genomics and varies between lesions. Lastly, this synchronous murine metastatic melanoma model not only allows further investigation of tumor heterogeneity on lesion-specific immune responses, but it also suggest that individual tumor microenvironment influence immunotherapeutic response.

Biomolecular and biological profile of melanoma subtypes selected by reflectance confocal microscopy

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Heterogeneity renders melanoma one the most complex type of cancer and makes it difficult to find individualized therapy. Reflectance confocal microscopy (RCM) analyses features of the tumors at nearly histological resolution. RCM allows the identification of four primary-melanoma-subtypes: dendritic-cell (DC), round cell (RN), dermal-nest (DN) and combined-type (CT). This work aims to characterize the RCM melanoma-subtypes in terms of biomarkers, gene expression, aggressiveness and response to therapy. Clinical and immunohistochemical evaluation suggest that CT and DN are more aggressive than DC and RC types. NanoString technology was performed on 770 genes in the different melanoma subtypes. Significant differences were found in terms of genes involved in chemotaxis, inflammation, cell-cell adhesion, cell motility and angiogenesis. While cells from CT and DN biopsies are able to generate spheroids, DC and RC did not form compact spheroids, probably because of their less proliferative capacity. Moreover, the invasion assay reveals that DN has higher invasive capacity than CT. In skin reconstructs, CT cells are sited in the dermal-epidermal junction and penetrate into the dermis after 14 days of culture, while DN cells grow widely and deeply in the dermis, according to their biological behavior. Additionally, subtypes responded differently to chemotherapy and target-therapy, according to their biomolecular and biological profile. Vemurafenib inhibits metastasis in zebrafish injected with DN cells. Taken together, these results show a progressive increase of aggressiveness from DC to CT melanoma-subtypes, while DN seems to have a unique behaviour. This study represents a first step to the creation of an integrated clinical-biomolecular model for melanoma classification for reaching a more accurate patient/tumor tailored therapeutic approach.

Delineating the co-operativity of *NF1* loss-of-function and non-p.V600 *BRAF* mutations in cutaneous melanoma

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Genetic studies of cutaneous melanoma have uncovered activating, hotspot mutations in *BRAF* (p.V600), *N-RAS* (p.G12, G13, Q61) and loss-of-function mutations of *NF1* in approximately 50%, 20% and 15% of patients respectively. Past studies have characterized a class of BRAF non-p.V600 mutants (Class III) that require cellular dysfunctions that increase RAS to activate the MAPK pathway. These mutants co-operate with oncogenic RAS by forming BRAF mutant/ wild-type CRAF heterodimers driving MAPK pathway activation (Yao et al., 2017). The TCGA and previous studies have reported that NF1 loss-of-function mutations are anti-correlated with BRAF p.V600 mutations but co-occur with Class III, non-p.V600 BRAF mutations in melanoma. However, the mechanism underlying the co-operation of NF1 loss with Class III BRAF mutants compared to oncogenic NRAS is not well-known. Such knowledge is critical to advance therapeutic strategies to treat patients with these genetic aberrations. Our *in vitro* cell signaling studies demonstrated that the Class III BRAF mutant, p.D594N, found to co-occur with *NF1* loss in melanoma patients, led to MAPK pathway activation upon NF1 knockdown. Interestingly, MAPK pathway activation of the BRAF p. D594N mutant within an NF1 null context was not the result of increased RAF-RAF dimer formation nor the binding to other MAPK scaffold proteins. We speculate from our preliminary data that the co-operativity of Class III BRAF mutants and NF1 loss may be due to an alternative mechanism not mediated by increased BRAF mutant/wild-type CRAF heterodimerization, in contrast to what is observed within an oncogenic NRAS context. Future studies will investigate this mechanism and test optimal MAPK inhibitor therapies to target melanomas with co-occurring NF1 loss-of-function and non-p.V600 BRAF mutations.

Real-world (RW) outcomes and quality of life (QoL) assessment with immuno-oncology (IO) therapies in advanced melanoma (advMEL)

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OPTIMIzE (NCT02780089) is a multisite, prospective, community-based US study assessing RW outcomes in advMEL patients (pts) receiving IO agents. Pts received first-line nivolumab (NIVO) + ipilimumab (IPI) or anti-PD-1 therapy (NIVO or pembrolizumab) from 2015 to 2018 and had a follow-up of ≥2 years. Data for baseline characteristics, disease control rate (DCR) at first assessment, overall survival (OS), and treatment-related adverse events (TRAEs) were collected. QoL measures included the Functional Assessment of Cancer Therapy-Melanoma (FACT-M) and the EQ-5D index and VAS; minimally important difference (MID) estimates determined clinical meaningfulness. A total of 81 NIVO+IPI-treated and 147 anti-PD-1-treated pts were eligible and included. Compared with the anti-PD-1 group, the NIVO+IPI group was younger (median age, 61 vs 69 years) and had more M1c stage disease (48% vs 35%), elevated LDH (52% vs 37%), and *BRAF*-mutant tumors (48% vs 38%). DCR was higher with NIVO+IPI than with anti-PD-1 (71% vs 56%; $p < 0.05$). Unadjusted 1-y and 2-y OS rates were 78% and 64% for NIVO+IPI and 73% and 59% for anti-PD-1. In a multivariate Cox model, the adjusted OS HR for NIVO+IPI vs anti-PD-1 was 0.78 (95% CI, 0.49–1.25; $p = 0.30$). Grade 3/4 TRAEs occurred in 53% of NIVO+IPI-treated pts and 22% of anti-PD-1-treated pts ($p < 0.05$). Clinically meaningful improvement from baseline in the FACT-M (MID, 4–7) was noted for NIVO+IPI (1y, +6.8; 2y, +5.0), but not for anti-PD-1 (1y, −4.2; 2y, −8.7). QoL was maintained in both groups based on other QoL measures. In this prospective RW study of treatment-naive pts with advMEL receiving IO agents, OS outcomes were consistent with those reported from clinical trials with these agents. QoL was generally stable over follow-up; NIVO+IPI-treated pts appeared to experience clinically meaningful improvement in disease-specific QoL.

Dissecting RAF inhibitor resistance by structure-based modeling reveals ways to overcome oncogenic RAS signaling in malignant melanoma

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Clinically used RAF inhibitors are ineffective in RAS mutant melanomas because they enhance homo- and heterodimerization of RAF kinases, leading to paradoxical activation of ERK signalling. Overcoming RAF dimerization and resistance is a challenge for drug design. Combining multiple inhibitors could be more effective, but it is unclear how the best combinations can be chosen.

Here, we present a mechanistic ERK pathway model that integrates the structural, thermodynamic, and kinetic analyses of RAF kinases, inhibitors, and their interactions with pathway biochemical data to predict RAF inhibitor responses at the network level. This comprehensive model is based on RAF kinase regulation by phosphosites, dimerization, and detailed RAF inhibitor action. Our model predicts unknown, hidden properties of network responses to different types of RAF inhibitors and makes wide strides in understanding resistance to these drugs. The model suggests that synergy can emerge between Type I and Type II, as well as between Type I1/2 and Type II inhibitors and predicts ways of overcoming RAF inhibitor resistance in RAS mutant melanomas.

Our experimental results on responses of ERK signalling to different RAF inhibitors and combinations in melanomas bearing oncogenic RAS or BRAFV600E mutations support the model predictions. Inhibition of oncogenic RAS signalling in melanomas is associated with reduced cell proliferation and colony formation. Furthermore, our data suggest additional targets including scaffolds and we developed small molecule inhibitors to disrupt the KSR1 signalling complex in resistant melanoma.

Our results highlight the role of scaffolds in targeted therapies in malignant melanoma and suggest an alternative principle of targeting the same kinase with two structurally different inhibitors that bind to different kinase conformations.

Alpha-1 antitrypsin suppresses melanoma progression by dampening inflammation and enhancing T cell function

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Abstract: Self-sustaining chronic Inflammation plays a vital role in disease progression and immune suppression in cancer. Alpha-1 antitrypsin (AAT) is a serine protein inhibitor belonging to the serpin family and is well known for its anti-inflammatory function. We hypothesized that AAT could reverse the immunosuppressive tumor microenvironment induced by tumor-mediated inflammation. We tested our hypothesis using C57BL/6 transgenic (TG) mice expressing hAAT by subcutaneously injecting B16F10 melanoma cells. The hAAT TG mice displayed a reduction in tumor growth compared to wild-type mice. The histology of hAAT TG mouse tumors showed a reduction in tumor cell mitosis and increase in tumor cell apoptosis, melanin pigmentation, and CD3+ T-cell infiltration To understand immunomodulatory effects of AAT in tumor cells, nanostring PanCancer Immune Profiling Panel was used on a focused panel of human melanoma cell lines treated with AAT. We identified downregulated pathways such as complement and TNF superfamily as well as upregulated leukocyte function pathway. We confirmed AAT-mediated downregulation of immune suppressive genes such as SAA1 and ST2 as well as chemokines such as CCL2 by qRT-PCR.. Furthermore, we found downregulation of PD-L1 in B16F10 cells and PD-1 in mouse T-cells after AAT treatment. Consistent with these findings, functional studies showed that AAT enhanced lysis of B16F10 cells by activated mouse T cells *in vitro*. Finally, *in vivo* T-cell depletion experiments revealed a suppressive role of CD8 T-cells in tumor growth of AAT mice, while CD4 T-cells revealed a tumor promoting role. Thus, our data demonstrate AAT suppresses melanoma progression by inhibiting inflammation, enhancing T-cell cytotoxicity and dampening immune checkpoint responses, making AAT an interesting candidate for immune modulation of cancer.

Drug repurposing screen using BRAFi/MEKi- and immunotherapy-resistant PDX identifies salvage strategies

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Resistance to BRAFi/MEKi arises in nearly all patients with *BRAF*-MT melanoma despite initial response. Achieving cures in this expanding BRAFi/MEKi-resistant cohort represents a great challenge to the field; few experience durable benefit from immunotherapy and no alternative therapies exist. Multiple RTKs (i.e., PDGFRb, cMET, AXL) and non-RTKs (i.e., SRC) are implicated in driving targeted- and immunotherapy-resistance via MAPK and PI3K-mTOR pathway hyperactivation, however no clinical strategies targeting these mechanisms have been realized. To facilitate further advancements through preclinical *in vivo* modeling of therapy resistance, we have established and characterized 25 PDX derived from heavily pretreated patients with resistance to BRAFi/MEKi and/or immunotherapy (R-PDX), representing a spectrum of biological, mutational, and clinical heterogeneity of therapy resistant human melanoma. We screened R-PDX against triple combinations of BRAFi/MEKi and a third agent from an NCI portfolio of approved agents with pan-RTK, non-RTK and/or PI3K-mTOR specificity. Of 11 triple combinations, 5 (of 7) pan-RTK- and 3 (of 4) PI3K-mTOR-inhibitor cocktails tested elicit transient activity in R-PDX models. Only dasatinib (in combination with BRAFi/MEKi) most durably elicits antitumor activity and increases overall survival in 70% R-PDX models. Notably, dasatinib also increases the antitumor efficacy of MAPKi in rare melanomas (uveal and acral). RPPA analysis identified higher pSRC-Y527 levels in R-PDX sensitive to dasatinib/MAPKi relative to those that were resistant. We hypothesize combination dasatinib/MAPKi will improve the overall survival of at-risk melanoma patient cohorts using pSRC as a predictive biomarker. Furthermore, we propose performing preclinical trials with R-PDX will be critical to develop future efficacious therapies.

Inhibition of BET and MEK decreases cholesterol content and promotes cell death in melanoma

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Melanoma is the deadliest form of skin cancer. Close to 30% of tumors harbor mutations in NRAS. Additionally, around 20% of melanomas that are resistant to BRAF inhibitors are driven by secondary mutations in NRAS. Currently, there are no targeted

therapies approved for patients whose tumors carry mutations in NRAS. In this study we report that concurrently targeting BET proteins and MEK inhibited cell proliferation and increased cell death in NRAS mutant melanoma, as well as tumors resistant to BRAF/MEK-inhibitors and/or CTLA4 and anti-PD1. Furthermore, we found that the combination of BET and MEK inhibitors decreased the activation of the LXR/RXR pathway and the synthesis of cholesterol *in vitro*and *in vivo*. We also show that reducing cholesterol levels, by treating melanoma cells with statins, further potentiated the effect of BET and MEK treatment, leading to increased caspase-3 cleavage and significantly enhancing melanoma cell death. In contrast, supplementing cell cultures with lipids such as cholesterol or Geranylgeranyl-pyrophosphate (GGPP), decreased sensitivity to BET and MEK inhibition, substantially attenuating cell death. Our study reveals an important contribution of lipids protecting melanomas from cell death induced by co-targeting BET and MEK. Furthermore, our study provides a new therapeutic option for NRAS-mutant melanoma and possibly other types of melanomas by combining BET and MEK inhibitors with statins.

Impact of surgery on outcomes in melanoma patients with brain metastases in the era of immunotherapy

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Melanoma has a high propensity to migrate to the brain, which is associated with a poor prognosis. Despite recent advances in the treatment of advanced melanoma, brain metastases remain a significant source of morbidity and mortality. Here we evaluated the role of surgery as compared to other treatment modalities and the impact on survival in the era of molecular targeted and immunotherapies.

Data from patients with melanoma brain metastases treated at Holden Comprehensive Cancer Center, University of Iowa, from 1/1/2008 to 1/1/2019 were reviewed (IRB# 201902732). Melanoma patients with brain metastases (165 total) had a median age of 63 (range 22—93) years, male predominance [114 male, 51 female]. 21 (12.7%) patients had melanoma of unknown primary. Brain metastases as first evidence of metastatic disease was found in 92 (55.8%) patients and was the presenting symptom of melanoma in 39 (23.6%). The majority of patients (111/165) had mutational analysis performed and 60.4% (67/111) of patients had a mutation in BRAF. Initial treatment after diagnosis of brain metastasis included chemotherapy in 29 (17.6%) cases, BRAF +/- MEK inhibitors in 18 (10.9%) and immune checkpoint inhibitors in 48 (29.1%). 67 (40.6%) patients underwent surgical intervention, 88 (53.3%) underwent stereotactic radiosurgery and 87 (52.7%) received whole brain radiation. Median survival was 4.9 months after identification of brain metastasis. Surgery was associated with increased median overall survival when compared to no surgical intervention (7.6 vs 2.9 months; $p < 0.01$). The survival advantage persisted when controlled for number of brain metastases.

Results reveal that surgical resection in patients with melanoma brain metastases is associated with enhanced survival when compared to patients without surgical intervention. Future studies are planned to identify prognostic and predictive biomarkers.

Uncovering essential mediators of mutant GNAQ/11-driven oncogenesis in uveal melanoma

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Uveal melanoma (UM) is the most common intraocular tumor in adults. Current treatments can eliminate primary tumors, but they fail to prevent metastasis, which occurs in half of patients and is associated with a 14-month median survival. Genetic drivers of transformation are known for >95% of tumors and are relatively few, compared to other cancers. One issue preventing translation of this information to clinical success is the absence of an approved drug to inhibit constitutively-active mutant GNAQ and GNA11 proteins, which initiate tumorigenesis in ~90% of UM cases. Genetic ablation experiments in pre-clinical models have shown that UM cells depend on these oncogenic proteins for their viability. Although several signaling pathways activated downstream of mutant GNAQ/11 have been characterized (e.g. MAPK, Hippo, and AKT), dozens of clinical trials with targeted inhibitors have so far failed to provide any survival benefit for UM patients, indicating an incomplete understanding of the critical mediators of transformation.

We sought to discover essential mechanistic drivers of oncogenesis using a Sleeping Beauty (SB) transposon forward mutagenesis screen to identify genes whose activation or disruption confers resistance to FR900359, a compound recently demonstrated to shut down oncogenic GNAQ/11 signaling in UM cells and induce growth arrest or cell death. Pilot screens in two GNAQ-mutant UM cell lines confirmed our ability to drive FR900359 resistance with SB. We identified genes previously linked to UM metastasis and GNAQ/11 signaling, along with potential novel drivers. Recently completed full screens are expected to identify several more candidates. We hypothesize that genetic mechanisms which restore cell viability after GNAQ/11 inhibition will reveal the genes and pathways crucial to promoting UM formation and progression, thus constituting promising therapeutic targets.

Characteristics of patients (pts) with a complete response (CR) treated with dabrafenib plus trametinib (D+T) combination therapy: findings from COMBI-d and COMBI-v 5-year analysis

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A recent 5-y analysis of D+T in *BRAF* V600E/K–mutant unresectable or metastatic melanoma using data from 2 large phase 3 studies (COMBI-d [NCT01584648]; COMBI-v [NCT01597908]) was conducted. Pts with confirmed CR (*n* = 109 [19%]) had favorable outcomes (5-y PFS, 49%; 5-y OS, 71%) vs the overall population (*n* = 563; 5-y PFS, 19%; 5-y OS, 34%). Median duration of CR was 36.7 months (95% CI, 24.1-NR); 55 of 109 pts with CR (50%) had ongoing CR at the data cutoff (COMBI-d, 10 Dec 2018; COMBI-v, 8 Oct 2018; median follow-up, 22.0 months) or at study withdrawal. Median PFS (54.3 vs 11.1 months) and OS (NR vs 25.9 months) were higher in pts with CR vs the overall population. To further characterize pts most likely to benefit from D+T, we analyzed outcomes and clinical features of confirmed pts with CR in this data set. Pts with CR had favorable baseline prognostic factors compared with the overall population, including normal lactate dehydrogenase levels (90% vs 65%), ECOG performance status of 0 (86% vs 72%), < 3 organ sites with metastases (84% vs 51%), and a lower median sum of target lesions (34 vs 57 mm). Baseline factors such as median age and patient sex were similar between both groups. New lesions in pts with CR who progressed (*n* = 48) were most commonly reported in the CNS (54%), lung (17%), and lymph nodes (17%), which were similar to sites of progression in the overall population. Subsequent therapy was received by 43 pts with CR (39%) and most commonly included immunotherapy (*n* = 28, 26% [anti–PD-1, *n* = 21; anti–CTLA-4, *n* = 17]) and BRAF targeted therapy (*n* = 25, 23%). These results suggest that baseline characteristics may be useful for selecting pts with advanced *BRAF* V600E/K–mutant melanoma who may derive the greatest clinical benefit from first-line D+T combination therapy. Further validation is warranted.

BRAF inhibition in melanoma is associated with progressive loss of histone H3 methylation

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The development of mutant BRAF inhibitors vemurafenib and dabrafenib has improved the outcome for melanoma patients with BRAFV600E mutations. Although the initial response to these inhibitors can be dramatic, sometimes resulting in complete tumor regression, the majority of melanomas become resistant. To study resistance to BRAF inhibition, we developed a novel mouse model of melanoma that permits control of mutant BRAF expression using doxycycline. Treatment with doxycycline leads to loss of mutant BRAF expression and tumor regression, but tumors reoccur after a prolonged period of dormancy. Vemurafenib induces cell cycle arrest and apoptosis in BRAF melanoma cell lines; however, a residual population of dormant cells survives. Comparing gene expression in human cell lines and mouse tumors allows us to strip away experimental artifacts. Accordingly, we conducted RNA sequencing analysis on untreated, and doxycycline-treated dormant mouse melanomas and human BRAF melanoma cell lines treated with vemurafenib for 20 days. We found conserved expression changes in histone methyltransferase genes EZH2, SUV39H1, SUV39H2, ASH2L, SYMD2, PRMT5, SNAI1, and SET8. Analysis of TCGA melanoma expression data determined a strong correlation between the expression of these genes. Immunoblotting protein from melanoma cell lines (A375s, M14s, M238s, M229s and Yumm2.1s) treated with vemurafenib or dabrafenib for 20 days confirmed a reduction in methyltransferase protein expression and reveled the progressive de-trimethylation of histone H3 lysine residues. Entinostat, a histone deacetylation inhibitor that indirectly prevents histone demethylation, synergized with vemurafenib to reduce the survival of dormant melanoma cells. In conclusion, preventing H3 demethylation reduces resistance to BRAF inhibition and warrants further investigation.

T-type calcium channel (TTCC) blockers impair tumor cell proliferation of BRAF^{V600E} Vemurafenib resistant melanoma cells due to autophagic blockade.

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Melanoma is a malignant neoplasia derived from melanocytes that once disseminated becomes highly resistant to chemotherapy and associates with poor prognosis. Around 50% of melanomas carry the BRAF^{V600E} mutation. Vemurafenib, a BRAF^{V600E} inhibitor, reduces metastatic melanoma tumor burden. However, development of Vemurafenib resistance is frequent. PTEN is a tumor suppressor that is non-functional in more than 30% of melanomas. Loss of PTEN coexists with BRAF mutation and contributes to acquired resistance to BRAF inhibitors (BRAFi) in a dependent mechanism.

The aim of this study was to describe the role of T-type calcium channel (TTCC) blockers in Vemurafenib resistant (Vem-R) melanomas.

Six human BRAF^{V600E} melanoma cell lines sensitive (Vem-S) and Vem-R pairs were used. *In vitro* and *in vivo* assays were performed to evaluate apoptosis, migration and invasion under a TTCC blocker (Mibefradil, Mib).

We report that Cav3.1 TTCC isoform and autophagic biomarkers were highly expressed in all Vem-R lines compared to their parental cells. Encouraged by our findings, Mib treatment induces apoptosis and impaired migration and invasion due to autophagic blockade in Vem-R melanoma cells *in vitro* and *in vivo*. Combination therapies reduced cell viability and migration in all Vem-S and Vem-R cells when PTEN is functional. However, when PTEN is lost or mutated, Cav3.1 mRNA levels were increased and combination therapies rescue viability and motility in Vem-R cells. Finally, Mib inhibited the acquisition of Vemurafenib resistance in BRAF^{V600E} melanoma cells.

Our data reveal an unknown link between Cav3.1 expression depending on PTEN status, suggesting a new mechanism of acquired resistance in melanoma. Our results could suggest that TTCC blockers could be useful in disseminated melanomas even after the development of BRAFi resistance.

Post translational modifications modulate anti-proliferative effects of Peroxisome Proliferator Activated Receptor Gamma (PPARy) within Melamoma Cells

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Peroxisome proliferator-activated receptor gamma (PPARy) is a nuclear hormone receptor (NHR) with an established transcriptional role facilitating fatty acid metabolism and adipocyte differentiation. There is accumulating evidence that PPARy and its cognate ligands that include thiazolidinediones (TZDs) may have utility for the treatment of certain cancers that include melanoma. We wished to evaluate the relevance of PPARy in limiting growth of a panel of melanoma cell lines and how such effects may be influenced through post-translational events, possibly imposed through constitutive MAPK activity associated with BRAF^{V600E} expressing melanoma cells. Our data reveal that melanoma cells that express endogenous PPARy exhibit markedly differential responses to TZD agonists. For example, troglitazone achieves a dose-dependent inhibition of A375 cell proliferation that approximates the activation profile of the endogenous receptor, while ciglitazone can exert inhibition only at high concentrations and via mechanisms likely independent of PPARy-mediated genomic activity. The responses of PPARy to troglitazone are relatively unaffected when applied in combination with vemurfenib, indicating that phosphorylation appears to be not a significant factor impacting on PPARy activity within BRAF^{V600E} cells. In contrast, we report that co-expression of the SUMO-specific protease SENP1, elicits a dramatically elevated transcriptional and biological response to troglitazone, an effect we note to be specific for PPARy when tested among a set of related NHRs. Our ongoing studies intimate PPARy signaling within BRAFV600E melanoma cells, to be highly modulated through SUMOylation and subject to agonist-specific responses, factors that require consideration in optimizing the effectiveness of a potential treatment strategy.

BRAF inhibition and Cytokine Therapy for Melanoma: a novel rational combined approach

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Although the advent of BRAF inhibitors plus MEK inhibitors has revolutionized BRAF-mutant melanoma therapy, most patients relapse within 16 months due to the persistence of minimal residual disease (MRD), a key obstacle in cancer therapy. In our inducible genetically engineered mouse model (iBIP) doxycycline withdrawal results in BRAFV600E extinction causing tumors to shrink, but not to make

them completely disappear, achieving a status of MRD. Time-course microarray analysis revealed a set of immune hallmarks with acute and strong initial activation after BRAFV600E extinction, then a decrease through to MRD, suggesting that the MRD might be immune suppressive/evasive.

Bioinformatics analysis pointed CXCL9 as a central player in the orchestration of the process: CXCL9 is strongly induced within 8 h of BRAFV600E extinction, but dissipates just prior to MRD, paralleling the more general immune signature. Preliminary evidence also indicate that CXLC9 induction/shut down is likely coordinated by specific cell populations in the myeloid compartment. Data obtained from human samples and from other mouse models mirror what observed in iBIP mice, indicating that sustaining CXLC9 expression might support tumor immune infiltration and activation. Consistently, we have obtained promising preliminary results combining BRAF extinction with recombinant CXCL9 (rCXCL9) therapy, suggesting that CXCL9 can convert immunologically "cold" MRD back to "hot", making them susceptible to immune rejection. In order to overcome some known issues associated with cytokine therapy, we use a nanoparticle based approach that extends stability and half-life of rCXLC9. As a result, in this work we present an approach that utilizes a confluence of in silico and in vivo data to guide the rational identification of a molecularly-driven cancer treatment based on immune drivers with predicted molecular synergy.

FOXD3 regulates VISTA expression in melanoma

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Immune checkpoint proteins modulate anti-tumor immunity. Antibodies that inhibit the immune checkpoints PD-1/PD-L1 and CTLA-4 are FDA-approved to treat melanoma, but unfortunately many patients fail to respond. Additional immunomodulatory proteins are often upregulated in response to PD-1/PD-L1 or CTLA-4 blockade and their roles in cancer remain poorly characterized. Further investigation of these alternative checkpoint proteins can help us to determine their potential as therapeutic targets, as well as help us to more broadly understand anti-tumor immunity. VISTA is an immumodulatory protein that can act as a ligand or receptor to inhibit T cells. We observed that VISTA is expressed on melanoma cells in patient samples and cell lines. Further, tumor-specific VISTA expression promoted tumor initiation in an immune competent mouse model, but not in immune deficient NSG mice. This effect was associated with increased infiltration of T regulatory cells and an altered immune microenvironment. VISTA is linked to processes of stem cell differentiation, and we show that the stemness factor FOXD3 negatively regulated VISTA expression at both the protein and transcript level. Importantly, FOXD3 bound the VISTA gene locus, and a DNA-binding impaired mutant FOXD3 did not alter VISTA levels. FOXD3 was upregulated in response to BRAF inhibition, and BRAF inhibition also downregulated VISTA. These findings broaden our understanding of VISTA, and the effects of BRAF inhibitors on the immune profile of melanoma cells. Furthermore, we identify a surprising connection between a stemness factor and immune evasion pathways in melanoma.

Analysis of tumor mutation burden (TMB), PD-L1 status and clinical outcomes with checkpoint inhibitors (CPI) in acral melanoma (AM)

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The response rate (RR) to CPI correlates with TMB in many cancers, but this has not been assessed in AM. Thus, we compared clinical outcomes with CPI in AM patients (pts) with High versus Low TMB. A retrospective analysis was performed for 44 pts with metastatic AM who received anti-CTLA4 (ipilimumab) or anti-PD1 (pembrolizumab or nivolumab). PFS and OS were estimated using the Kaplan-Meier method. TMB was predicted from panel sequencing mutation data algorithm(*BMC Med. 2016;14:168*). Outcomes were compared between pts with Low (below the median, <15 mutations) vs High (>15 mutations) TMB. Baseline biopsies were analyzed by immunohistochemistry for PD-L1.

Median age was 63 years old (39–88); 60% were men. Median follow-up of 18 months (mo). For first-line anti-CTLA4 (n = 17), the RR was 17.8% by RECIST1.1 criteria; median PFS 6.7 mo (CI 95%, 2.8 to 17.2), and median OS 38.7 mo (CI 95%, 7.8 to 61.6). For first-line anti-PD1 (n = 15), the RR was 40%, median PFS 9.2 mo (CI 95%, 2.7 to 19.7), and median OS 60.1 mo (CI 95%, 12.4 to 67.4). Comparison of AM pts with High (n = 24) versus Low TMB (n = 20) showed no significant association with CPI agent, stage, serum LDH, or presence of brain metastasis. AM pts with Low TMB had improved OS (median 61.6 mo) vs pts with High TMB (20.0 mo; p = 0.033), and trends for improved PFS (median 11.8 vs 6.0 mo, p = 0.056) and RR (40% vs 25%, p = 0.291). There was no association between PD-L1 status and anti-PD1 RR (p = 0.982). Results of Nanostring, immune gene expression and TCR sequencing analyses will be analyzed and reported.

In contrast to many cancers there was no positive association observed between TMB and CPI outcomes in AM pts. Our data

suggests that other immune features may be critical to response to CPI in AM, and support the rationale to further evaluate the tumor microenvironment in these tumors.

Prognostic and predictive values of ALDH1A1 and ALDH1A3 in human melanoma

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Aldehyde dehydrogenase (ALDH) activity is a valuable marker for cancer cells with stem-like qualities and plays a critical role in drug resistance and disease progression in many tumors including melanoma. However, the role of ALDH enzymes in patient prognosis is unclear. To define the expression patterns, and prognostic and predictive values of the ALDH enzymes, we used patient RNA-sequencing expression data from The Cancer Genome Atlas (TCGA). We analyzed gene expression of all 19 ALDH isoenzymes and found that *ALDH1A1* and *ALDH1A3* were most highly expressed in melanoma. Through Kaplan-Meier Analysis, we found that *ALDH1A3* expression correlated with better overall survival in metastatic melanoma. Further, stratification of the TCGA cohort by distinct melanoma mutational subtypes revealed that the expression of *ALDH1A3* correlated with better prognosis in metastatic *BRAF*-mutant melanoma while that of *ALDH1A1* correlated with better prognosis in metastatic *BRAF* wild-type melanoma. Gene set enrichment analysis (GSEA) of these cohorts identified upregulation in oxidative phosphorylation, adipogenesis, and fatty acid metabolism signaling in *ALDH1A*^{lo} patients, suggesting BRAF/MEK inhibitor resistance in that subset of patients. Conversely, GSEA of *ALDH1A*^{hi} cohorts revealed upregulation in glycolysis, hypoxia, and angiogenesis, suggesting BRAF/MEK inhibitor sensitivity in that subset of patients. In order to determine the predictive value of *ALDH1A* expression, we performed gene expression analysis using pre-treatment tumor samples and found that high *ALDH1A3* expression prior to BRAF/MEK inhibitor treatment was predictive of better treatment response in *BRAF*-mutant melanoma patients. Our study provides evidence that *ALDH1A3* mRNA expression is both a prognostic marker and a predictive marker for BRAF/MEK inhibitor treatment response in *BRAF*-mutant metastatic melanoma patients.

Metabolic reprogramming drives H3K4me3 remodeling in adaptive melanoma drug resistance through *O*-GlcNAc transferase

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Accumulating evidence indicates adaptive cellular reprogramming is a major contributor to disease recurrence and acquired drug resistance in cancer. We recently have described a state-wise transition of parental cells to permanent resistant cells consisting of an early, slow-cycling state followed by a re-proliferative colony state. Metabolomics of targeted therapy induced cellular reprogramming resulted in separated clusters for the early slow-cycling and re-proliferative colony state following principle component analyses, while the untreated and permanent resistant cells clustered together. Detailed analyses of metabolic pathways showed that the glycolytic flux in the re-proliferating colony state is re-directed into the hexosamine biosynthesis pathway to produce UDP-N-acetylglucosamine, the substrate for *O*-GlcNAc transferase (OGT), an enzyme important for epigenetic remodelling in embryonic stem cells. OGT expression was highly increased at the colony state compared to the slow cycling state, concomitant with an increase in H3K4me3. H3K4me3 ChIP-seq of colonies compared to parental cells revealed differential marking at promotor regions and transcription factor binding motifs that have recently been described as O-GlcNAc chromatin consensus motifs, supporting the involvement of OGT in H3K4me3 remodeling. The adaptive process can be blocked by genetic or pharmacological inhibition of OGT or concurrent activation of AMPK to prevent metabolic adaption, subsequent epigenetic remodeling and permanent acquired drug resistance. This novel metabolic and epigenetic remodeling process is also observed following oncogene (NRAS^{Q61K}) induced stress and the OGT dependence of this adaptive process uncovered promising targets for combination therapy with current standard of care drugs that could significantly increase response duration and patient outcomes.

Guidelines for adjuvant treatment of cutaneous melanoma: a global perspective

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The management of cutaneous melanoma is continually evolving, with adjuvant treatment of earlier stage disease. Current clinical guidelines may not yet reflect newer treatment strategies. The objective of this review is to understand current global guidelines for disease management of stage II and III cutaneous melanoma in adults.

Systematic searches of Medline and Embase captured guidelines published between 01/01/2014-28/01/2019. Eligibility was determined by two reviewers and discrepancies were resolved by consensus. Non-English language texts were excluded.

Twenty publications were included for review. Treatment guidelines were identified for nine countries (England, Scotland, France, Spain, Switzerland, Poland, Australia, China and the US) plus one European guideline. All recommended adjuvant treatment for stage III disease including BRAF- and/or MEK inhibitors and anti-PD-1 therapies. Many focussed on adjuvant treatments for high-risk stages and few reported interventions for stage II. BRAF-V600 status was used to differentiate treatment pathways for stage III disease. For example, first-line combination treatment with a BRAF/MEK inhibitor was recommended for BRAF-V600 mutant stage III in Australia, Scotland and Spain. Australia and Spain also recommended second-line anti-PD-1 monotherapy with pembrolizumab (or nivolumab in Spain). Chinese, Swiss and EU country guidelines encouraged adjuvant immunotherapy via clinical trial participation. Australia, France and Poland recommended clinical observation but did not favour 'watch and wait' over adjuvant intervention.

In summary, lack of consensus in current guidance for adjuvant treatment of stage III disease and an absence of guidance for stage II highlights the challenge to improve management in eligible patient populations. Further results from clinical trials in the adjuvant setting will shape global perspectives and inform guidelines.

Health and cost impact of adjuvant therapy with pembrolizumab (PEMBRO) in completely resected stage III melanoma patients in a US health plan

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Patients with stage III melanoma remain at a high risk of disease recurrence or death after surgical resection. Adjuvant therapy with PD-1 inhibitor PEMBRO improves recurrence-free (RF) survival for these patients. We aimed to estimate the health and cost impact of adjuvant treatment with PEMBRO compared to watchful waiting (WW) from a US health plan perspective.

A cohort Markov model was used to project the clinical and cost outcomes for patients receiving adjuvant therapy with PEMBRO compared to WW over a 5-year period. The number of eligible patients was calculated based on a 1-million-lives health plan, disease incidence and prevalence, and treatment eligibility. Patients could transition through the following states: RF, locoregional recurrence, distant metastasis (DM) and death. Transition probabilities were estimated from EORTC1325/KN054 and Flatiron Health database. Data from EORTC1325/KN054, KN006 and published literature was used to estimate number of grade 3+ adverse events (AEs).

An estimated 16 plan members were eligible for PEMBRO following surgical resection. Compared to WW, treatment with PEMBRO resulted in 5.07 recurrences avoided (8.86 vs. 13.92) and 4 fewer patients requiring treatment in the DM state (6.78 vs. 10.86). A total of 2.3 deaths were avoided post-recurrence (3.97 vs. 6.27). The number of grade 3+ AEs with adjuvant PEMBRO was higher in the RF state (1.65 vs. 0.73) but these were offset by fewer AEs in the DM state (4.18 vs. 5.47). Most of the additional costs in the RF state ($2,142,994) were offset by cost saved from avoided recurrences ($1,481,169), resulting in an incremental cost of $661,825 over 5 years.

Adjuvant treatment with PEMBRO, although related to higher cost, leads to decreased recurrences and improved survival when compared to WW among completely resected stage III melanoma patients.

Sleeping Beauty mutagenesis drives therapeutic resistance in an *in vivo* model of $BRAF^{V600}$ mutant melanoma

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Cutaneous melanoma patients that harbor $BRAF^{V600}$ mutations (~50%) have routinely been treated using targeted therapies such as the selective kinase inhibitor vemurafenib. Although targeted therapy has been effective in extending overall survival, therapeutic resistance often occurs within a short timeframe. Multiple pathways have been implicated in the evolution of resistance, yet there is a critical need to identify alternative and targetable genetic alterations that can arise in melanoma. We performed a forward genetic screen using the Sleeping Beauty mutagenesis system in an effort to determine the genetic alterations causative for driving resistance *in vivo*. Using the A375 $BRAF^{V600E}$ melanoma cell line, we initiated Sleeping Beauty mutagenesis and engrafted

mutagenized cells in athymic nude mice. Once tumors arose, we enrolled the cohort on vemurafenib chow and monitored tumors for changes in growth. In comparison to non-mutagenized cells, Sleeping Beauty accelerated the rate of resistance to vemurafenib. Upon harvesting tumors, bulk sequencing and common insertion site analysis was completed to determine potential genetic drivers of resistance. We have previously conducted *in vitro* resistance screens, and hereby identify genetic drivers of resistance that are unique to the *in vivo* environment. We are currently pursuing the novel drivers of resistance *in vivo* for further validation and mechanistic comprehension. We also identified alterations in the insertional pattern of transposons into the *BRAF* locus, which we can attribute to additional growth factors present *in vivo*. Overall this study works to classify previously unidentified mechanisms of resistance to vemurafenib, while also giving key insight into how the microenvironment alters the genetic landscape of therapeutic resistance.

Siah2 control of T-regulatory cells limits anti-tumor immunity

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Understanding mechanisms underlying anti-tumor immunity is pivotal for improving immune-based cancer therapies. Here we demonstrate that growth of inoculated BRAF mutant melanoma cells was inhibited in *Siah2*$^{-/-}$mice, up to a complete loss. Melanoma grown in *Siah2*$^{-/-}$mice exhibited increased proinflammatory immune components, signified by enhanced intra-tumoral activated T cells, along with decreased expression of *Ccl17*and *Ccl22*, and decreased *Foxp3* expression. A marked reduction in Treg proliferation was associated with inhibition of Treg cell cycle progression. Correspondingly, G1 cell cycle arrest in *Siah2*$^{-/-}$Tregs coincided with elevated expression of the cyclin dependent kinase inhibitor p27, a Siah2 substrate. Growth of PD1-unresponsive melanoma was effectively inhibited up to complete tumor rejection in *Siah2*$^{-/-}$mice subjected to PD1 blockade, highlighting synergy between PD1 inhibition and *Siah2*loss. Low levels of Siah2 expression coincides with effective anti-tumor immunity seen in melanoma specimens. Siah2 regulation of Treg recruitment and cell cycle progression effectively controls melanoma development and confers synthetic lethality when combined with anti-PD1 therapy.

Inhibition of glutamine uptake by small molecular SLC1A5 inhibitor IMD-0354 inhibits melanoma

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Altered metabolic signaling is commonly seen in tumors, often serves their development and drug resistance mechanism. Glutamine metabolism is among pathways that confer tumor progression and responsiveness to therapy in a number of tumors, including melanoma and breast cancers. Among mechanisms underlying the increase in glutamine metabolism is improved glutamine uptake, mediated by the glutamine transporters, among which SLC1A5 (also known as ASCT2) has been shown to play a key role. Correspondingly, increased SLC1A5 expression coincides with decreased survival of breast cancer and melanoma patients. We thus set an imaging-based screen to identify small molecule inhibitors for SLC1A5. Small molecules that were able to prevent the localization of SLC1A5 at the plasma membrane, without impacting cell shape, were selected as positive hits that were further characterized. Out of 7,000 small molecules, 20 were selected as initial hits, of which one -IMD-0354 was selected for further assessment based on secondary and tertiary assays. Previously implicated in IKK signaling, our studies in melanoma cells identified IMD-0354 as potent inhibitor of glutamine uptake in multiple cancer cell lines resulting in sustained low intracellular glutamine and glutamate levels up to 72 h. Notably, IKK signaling was not involved in SLC1A5 inhibition, pointing to a novel, undisclosed role for IMD-0354. Concomitant with its inhibition of glutamine uptake, IMD-0354 attenuated mTOR signaling, decreased cancer cell growth, colony formation resulting in increases cell death. In addition to glutamine uptake inhibition, IMD-0354 attenuated fatty acid synthesis, via its inhibition of its rate-limiting enzyme, Acetyl-CoA carboxylase. The combined inhibition of glutamine uptake and fatty acid synthesis by IMD-0354 support its further development for preclinical assessment.

Improvement in survival of uveal melanoma patients with liver metastasis: A single institution's longitudinal experience

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Despite successful treatments of the primary tumor, up to 50% of affected patients will develop systemic metastasis, with the liver

involved in up to 90% of those patients. There is no FDA-approved treatment for metastatic uveal melanoma (UM) and overall outcomes are generally poor for those who develop liver metastasis.

We performed a retrospective single-institution chart review on consecutive series of UM patients with liver metastasis who were treated at Thomas Jefferson University Hospital between 1971–1993 (Cohort 1, *n* = 80), 1998–2007 (Cohort 2, *n* = 198), and 2008–2017 (Cohort 3, *n* = 452). Cohort 1 represents a systemic therapy dominant period, while Cohorts 2 and 3 represent earlier and more recent decades of liver-directed therapy dominant periods.

The median overall survival (OS) after diagnosis of liver metastasis (Mets-to-Death) was shortest in Cohort 1 (5.3 months, 95% CI: 4.2–7.0), longer in Cohort 2 (13.6 months, 95% CI: 12.2–16.6) and longest in Cohort 3 (17.8 months, 95% CI: 16.6–19.4). The one-year and two-year overall survival rates were different between Cohort 1 (one-year OS: 23%, 95% CI: 15–34%; two-year OS: 8%, 95% CI: 3–16%) and Cohort 2 (one-year OS: 59%, 95% CI: 52–66%; two-year OS: 28%, 95% CI: 22–35%), and between Cohort 1 and Cohort 3 (one-year OS: 67%, 95% CI: 63–72%; two-year OS: 35%, 95% CI: 31–40%).

Our retrospective study is one of the largest "real-world" data showing improvement in the outcomes of metastatic UM patients with liver metastasis over the last 47 years. We speculate that the shift of treatment strategy to liver-directed treatments is one of the major contributing factors responsible for the improved survival of uveal melanoma patients with liver metastasis.

Melanoma plasticity and its relationship to tumor progression and drug resistance

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The relationship between melanoma cellular plasticity and tumor progression and development of drug resistance is not well understood. We employed reprogramming to induced pluripotent cell-like state to study cellular plasticity in primary and metastatic melanoma cells and in development of mitogen-activated protein kinase (MAPK) inhibitor resistance. We found that expression of oncogenic BRAF(V600E) inhibits melanocyte plasticity. Similarly, tumor progression also diminished plasticity of melanoma cells. Interestingly, plasticity was restored by inhibition of the oncogenic pathway in BRAF(V600E)-inhibitor sensitive melanoma cells, but not BRAFi-resistant cells. Differentiation of melanoma-derived induced pluripotent stem cells produced dysplastic cells that showed mixed lineage of melanocytes and neural cells. These re-derived cells also acquired MAPK inhibitor resistance. Our data suggest that melanoma cell plasticity-dependent mechanisms may regulate melanoma tumor progression and recurrence of aggressive drug-resistant melanoma.

Anti-malaria drug derivatives enhance the efficacy of anti-PD-1 Ab in melanoma

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The <50% of response rate with single agent anti-programmed cell death protein (PD-1) antibody (Ab) in advanced stage melanoma, highlights the need for new strategies that can render the tumor microenvironment more sensitive towards immunotherapy. Therapy-induced autophagy is a major resistance mechanism to targeted therapy, and chloroquine (CQ) derivatives, which inhibit lysosomal palmitoyl-protein thioesterase 1 (PPT1), augment the efficacy of BRAF and MEK inhibitors in preclinical models. Here, we report that the CQ derivative, hydroxychloroquine (HCQ) enhanced the efficacy of anti-PD-1 Ab and the survival of immunocompetent mice bearing B16 mouse melanoma tumors. Unlike HCQ, genetic inhibition of the autophagy gene *Atg7* in tumor cells did not augment anti-PD-1 Ab response. HCQ had no effects on the percentage of effector T cells in tumors and the ability of melanoma- primed T cells to kill B16 cells. The enhanced efficacy of anti-PD-1 Ab + HCQ was associated with a change in the polarization of tumor associated macrophages (TAMs) from an M2 phenotype to an M1 phenotype and a significant reduction in the infiltration of polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) in tumors treated with combination therapy compared with anti-PD-1 Ab alone. The more potent CQ derivative DC661 induced macrophage activation that enhanced T cell mediated tumor cell death. Genetic PPT1 inhibition also changed macrophage polarization from an M2 to M1 phenotype. These findings were reproduced in a genetically engineered mouse model of *Braf* mutant melanoma. Together, this data indicates that in addition to direct antitumor cell activity, CQ derivatives elicit immunomodulatory properties that enhance tumor immunity when combined with anti-PD-1 Ab treatment. This therapeutic approach will be tested in the clinic which will start accruing patients by 2020.

Targeting the cyclin-dependent kinase 5 in metastatic melanoma

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Melanomas are notorious due to their propensity to form lethal metastases. Currently, no treatments are available to stop the metastatic spread. We observed that in malignant melanomas, a protein called cyclin-dependent kinase 5 (CDK5) is absolutely essential for melanoma metastasis. Using mouse and human melanoma cells, we demonstrated that in this tumor type CDK5 drives the metastatic spread by directly phosphorylating a mesenchymal-type intermediate filament, vimentin. We found that a genetic shutdown of CDK5 in a mouse model of melanoma completely abrogated metastasis, while chemical inhibition of CDK5 kinase in mice carrying patient-derived tumors strongly impeded the metastatic spread of human cells. Our results indicate that inhibition of CDK5 might represent an attractive therapeutic strategy to block the metastatic dissemination of melanoma cells.

Pharmacodynamic effect of tebentafusp (TCR–CD3 bispecific) on peripheral cytokines and association with OS in patients with advanced melanoma

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Tebentafusp (TEBE; IMCgp100) is a unique TCR–anti-CD3 bispecific ImmTAC molecule that is capable of redirecting polyclonal T cells against melanocyte-associated gp100. Activity has been seen in advanced melanoma, with rash and cytokine-related AEs observed. In this Phase I/II dose-finding study we also aimed to explore evidence of MoA of TEBE in the clinic. 84 HLA-A2+ pts with advanced melanoma (n = 61 cutaneous [CM], n = 19 uveal [UM], n = 4 other) received TEBE (NCT01211262). Serum (n = 40) and PBMC (n = 22) samples taken pre- and post-infusion were analyzed for changes in cytokines and circulating T cells. Baseline and on-treatment tumor biopsies were analyzed by IHC for gp100 (n = 16), CD3 (n = 11), CD4, CD8 and PD-L1 (n = 10 each) expression; tumor RNA (n = 2 partial response [PR] and n = 7 progressive disease [PD] pts) was analyzed for gene expression. TEBE induced a transient increase in IFNγ-inducible cytokines, notably CXCL10, which appeared associated with longer OS (p = 0.0002), tumor shrinkage (p = 0.003), and greater transient reduction in peripheral CXCR3+ CD8+ T cells (p = 0.001). Reduction in CXCR3+ CD8+ T cells also trended with longer OS (p = 0.02) and tumor shrinkage (p = 0.03). In tumor biopsies, increase in CD3+, CD4+ and CD8+ T cells was evident on-treatment; gp100 expression was unchanged. Enrichment analysis showed significant on-treatment increase in genes associated with T cell markers, immune-mediated cytotoxicity, and IFNγ-pathway in PR compared with PD pts. The association of clinical benefit with increased serum CXCL10 and decreased peripheral CXCR3+ T cells supports the hypothesized MoA of TEBE-induced T cell redirection and activation, and tumor biopsy results support TEBE redirection of T cells to antigen-positive tumors. Trials in CM (NCT02535078) and UM (NCT02570308 and NCT03070392) are ongoing.

Second primary melanomas found to occur despite anti—PD-1 immunotherapy, implicating a novel escape mechanism

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Immunologic therapies anchored in blockade of programmed death-1 (PD-1) receptor have reshaped treatment of advanced melanoma and now provide previously unprecedented rates of durable clinical response. However, we have observed a minority of patients currently receiving anti—PD-1 therapy present with new primary melanoma. We describe five such individuals: a 39 y/o female with stage IIIB superficial spreading melanoma of the back, placed on nivolumab, who developed melanoma in situ of the arm; a 59 y/o male with stage IIIB nodular melanoma of the leg, placed on nivolumab, who developed stage IA superficial spreading melanoma of the arm; a 54 y/o male with stage IIIC nodular melanoma of the thigh, placed on pembrolizumab, who developed stage 1A superficial spreading melanoma of the back; an 83 y/o male with melanoma in situ of the neck of the neck, placed on pembrolizumab for metastatic squamous cell carcinoma (SCC) of the neck, who developed stage IIB superficial spreading melanoma of the back; and a 73 y/o male with stage IV melanoma,

placed on pembrolizumab, who developed stage IIIA superficial spreading melanoma of the temple. The second primaries were discovered at a mean of 201 days after starting anti—PD-1 therapy (range 37 to 395 days). Cases of in situ or stage 1A melanoma were treated with wide local excision (WLE). The case of stage IIB melanoma underwent WLE and was continued on anti—PD-1 therapy for metastatic SCC. The case of stage IIIA melanoma was treated with WLE but discontinued anti—PD-1 therapy due to immune-refractory primary melanoma. These five patient cases, obtained from a single center within the past two years, demonstrate that the occurrence of second primary melanoma on anti—PD-1 therapy is not rare and necessitates diligent screening.

MITF and BRN2 negative melanoma cells represent an AXL positive cell population.

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Understanding the unique cell populations contributing to tumour heterogeneity is essential for improving treatment outcomes. The proteins MITF and AXL have been the focus of many recent studies looking at sub-populations of melanoma cells. MITF is a master transcriptional regulator of the melanocytic lineage and AXL a receptor tyrosine kinase. A sub-population of melanoma tumour cells with low MITF and high AXL expression ($MITF^{low}/AXL^{high}$) have been described as resistant to MAPK pathway inhibitors. The POU family transcription factor BRN2 is often linked to MITF expression with feedback between the two proteins resulting in mutually exclusive expression in many patient tumours. This inverse correlation between expression of BRN2 and MITF was reported in the AXL^{low} sub-population but not AXL^{high}.

Utilising cell lines with doxycycline inducible shRNA expression we have uncovered a complex relationship between MITF, AXL and BRN2. Depletion of MITF from melanoma cells resulted in a concomitant increase in AXL expression that was further increased by the additional depletion of BRN2.

These results indicate that depletion of MITF and BRN2 simultaneously from melanoma cells results in up-regulation of AXL expression to a level much higher than MITF depletion alone. The ongoing study of these three proteins has significance in determining the populations responsible for intrinsic resistance to MAPKi.

Expression of BAP-1 is associated with Activation of Canonical NFκB signaling pathway in metastatic uveal melanoma

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Purpose: Uveal melanoma is lethal cancer with a strong propensity to metastasize. The BAP1 gene localized on chromosome 3p21.1 is involved in epigenetic modulation of chromatin. Inflammation in uveal melanoma (UM) is linked to a bad prognosis and have a higher incidence of metastases. Canonical NFκB pathway is known to play a crucial role in tumor inflammation. Therefore, we aim to detect the association of BAP-1 with activation of C-NFκB proteins and how they are linked to metastasis in uveal melanoma.

Methods: Seventy-five patients are recruited in our study. Expression of BAP-1 and C-NFκB proteins (p65/RELA, p50/NFκB1 & c-REL/REL) was evaluated using immunohistochemistry and real-time PCR. Co-immunoprecipitation was performed on five each case of the metastatic and non-metastatic group to detect the presence of p65/p50 and c-Rel/p50 heterodimers. BAP-1 sequencing was performed on 10 cases.

Result: Activation of C-NFκB proteins found on 54%,60% and 41% of cases while the loss of BAP-1 was observed in 69% of cases. Loss of BAP-1 protein (93%) along with activation of C-NFκB proteins (81%) was seen in the metastatic group. Loss of BAP-1 along with activation of C-NFκB proteins was statically significant with inflammatory factors such as TILs-CD3+ ($p = 0.036$), TAM-CD68+ ($p = 0.012$) and epithelioid cell type ($p = 0.027$). In metastatic group fold-change value of REL-A (5.45), NFκB1 (4.95) & REL (2.85) genes was reduced to 3.85 (RELA), 2.74 (NFκB1) & 1.84 (REL) gene in non-metastatic group. Loss of BAP-1, along with the activation of C-NFκB proteins, was associated with reduced metastasis-free survival and overall survival ($p < 0.05$).

Conclusion: Our finding reveals that activation of C-NFκB and loss of BAP-1 are significantly associated with each other at expression levels.

Leveraging transcriptional dynamics to improve BRAF inhibitor responses in melanoma

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Melanoma is known to be a heterogeneous tumor, but the impact of this heterogeneity upon therapeutic response is not well

understood. In the current study, we used single cell mRNA analysis and our Single Cell Heterogeneity (SinCHet) platform to define the transcriptional diversity of melanoma and its dynamic response to both BRAF inhibitor therapy and treatment holidays. Our analyses showed melanoma cell lines and patient specimens to be composed of >3 co-existent, transcriptionally distinct states. The cell state composition was dynamically regulated in response to BRAF inhibitor therapy, with State #1 declining and States #2 and #3 increasing in response to drug. The percentage of cells in State #1 recovered following a drug holiday, allowing for a successful therapy re-challenge. Other melanomas that lacked State #1 had varying degrees of intrinsic drug resistance and were not amenable to re-challenge. We next leveraged the differences in fitness between the different transcriptional states in the absence and presence of drug to develop a mathematical model that optimized therapy schedules to retain the drug sensitive population. In vivo validation of the mathematical model demonstrated that the personalized adaptive dosing schedules were better at suppressing tumor growth than either continuous or fixed intermittent BRAF inhibitor schedules. Together our studies provide the first preclinical evidence that transcriptional heterogeneity at the single cell level predicts for the initial sensitivity to BRAF inhibitor therapy and the potential for response to therapy rechallenge. We further demonstrate that manipulating transcriptional heterogeneity through personalized adaptive therapy schedules can delay the time to resistance.

Serial proteomic analysis of CSF from patients with leptomeningeal melanoma metastases identifies signatures associated with disease progression and mediators of therapy resistance

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Purpose: The development of leptomeningeal melanoma metastases (LMM) is a devastating complication of the late-stage disease, for which no effective treatments exist. Here, we performed a multi-omics analysis of the CSF from LMM patients to determine how the leptomeningeal microenvironment shapes the biology and therapeutic responses of melanoma cells.

Patients and Methods: A total of 45 serial CSF samples were collected from 16 patients, 8 of these with confirmed LMM. Of those with LMM, 7 had poor survival (<4 months) and one was an extraordinary responder (survival >24 months). CSF samples were analyzed by mass spectrometry and incubated with melanoma cells, that were subjected to RNA-Seq analysis. Functional assays were performed to validate the pathways identified.

Results: Mass spectrometry analyses showed the CSF of most LMM patients to be enriched for pathways involved in innate immunity, protease-mediated damage, and IGF-related signaling. All of these were anti-correlated in the extraordinary responder. RNA-Seq analysis showed CSF to induce PI3K/AKT, integrin, B-cell activation, S-phase entry, TNFR2, TGF-b and oxidative stress responses in the melanoma cells. ELISA assays confirmed that TGF-b expression increased in the CSF of patients progressing with LMM. CSF from poorly resonding patients conferred tolerance to BRAF inhibitor therapy in apoptosis assays.

Conclusions: These analyses demonstrated identified proteomic/transcriptional signatures in the CSF of patients who succumbed to LMM. We further showed that the CSF from LMM patients has the potential to modulate BRAF inhibitor responses and may contribute to drug resistance.

Profiling the adaptive translational response to oncogenic BRAF inhibition in melanoma

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Despite the success of targeted therapies in melanoma clinical outcomes are limited by a residual disease that results in relapse. This residual disease is characterized by drug-induced adaptation, however the underlying mechanisms that control this adaptive cellular plasticity are largely unknown. We have recently uncovered a role for mRNA processing pathways in cellular adaptation to targeted therapy in melanoma. This is particularly intriguing given mRNA processing pathways are emerging as key determinants of gene expression programs activated in response to stress. Here, we investigated both transcriptional and post-transcriptional mechanisms underlying cellular adaptation to BRAF inhibition by analyzing both mRNA abundance and mRNA bound to ribosomes, as a read out of mRNA translation. Consistent with previous studies, our analysis revealed a strong transcriptional component during the early response to BRAF inhibition, including downregulation of gene sets associated with the cell cycle and MYC transcription. Notably, however, global analysis of the relationship between mRNA abundance and translation at later time points also indicated additional post-transcriptional modes of gene expression regulation during drug-induced adaptation. At a pathway level, our data provides evidence of an underappreciated mode of gene expression regulation known as “translational buffering”, whereby changes in mRNA abundance are not reflected in rates of mRNA translation. Intriguingly, this includes multiple pathways implicated in BRAF targeted therapy response and resistance in melanoma patients, including oxidative phosphorylation. We propose that post-transcriptional mRNA processing pathways represent an attractive therapeutic target to improve efficacy of MAPK pathway inhibitors by targeting the process of adaptation itself, rather than the outcome, as a next generation combination therapy.

Innate immune cells play a role in induction of therapy resistance to anti-PD1 in Hu-mice melanoma model.

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Immune checkpoint inhibitor therapy is rapidly emerging as a front-line treatment option for many solid tumors. However, only a third of melanoma patients respond to immune checkpoint blockade. Currently available mouse models have many short comings and are unable to address the basis of therapy resistance and immune non-responsiveness that are observed in patients.

Our laboratory has developed a novel humanized mouse melanoma model. Immuno-deficient NSG mice were reconstituted with human CD34+ cells and after 8–12 weeks, mice are fully reconstituted with human innate and adaptive immune cells. Humanized mice were then challenged with HLA-matched melanoma cells and the functional ability of human immune cells to restrict tumor growth was monitored. Restricted tumor growth was observed in humanized mice indicating *in vivo* sensitization of human immune cells to melanoma.

In therapy studies, tumor-bearing humanized mice treated with anti-PD-1 showed restricted tumor growth. Anti-PD-1 therapy resulted in enhanced infiltration of T-cells that correlated with tumor response. MassCyTOF studies was performed using a panel of immune markers to understand the mechanism of therapy non-responsiveness in some tumors. Results indicated downmodulation of HLA-class I molecules and increased presence of mast cells cells in the tumor region. In tumor-bearing mice, combination of therapy drugs targeting c-kit+ mast cells and anti-PD1 caused complete regression of tumor lesions. Tumor free mice were able to reject freshly challenged melanoma cells indicating presence of memory T-cell responses.

Our results suggest that humanized mouse melanoma model can be explored further to understand the causes of therapy resistance and immune non-responsiveness. Model will be useful for developing new therapeutic approaches.

BOP1 expression contributes to the proliferative/invasive phenotype in melanoma

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We recently performed whole exome sequencing of 30 melanocytic naevi which surprisingly revealed numerous regions of copy number loss. It was found that common reticular naevi (mostly with a junctional component) have a high incidence of copy number aberrations (CNA). Importantly, concurrent with the loss of tumour suppressor genes (e.g. *CDKN2A*, *TP53*, *and NF1*) was the loss of potent oncogenes (e.g. *NRAS*, *MITF*, *and MDM2*). We postulated that the balanced nature of these CNAs confers protection from transformation, thus keeping the lesion in the benign state. Along with copy-number loss of these well-known genes, further interrogation of these data revealed a loss on chr8q24.3 which was frequently observed in 7/30 (23%) naevi. In the smallest region of overlap, the block of proliferation 1 (*BOP1*) gene was identified as a candidate for functional validation. BOP1 is part of the trimeric "PeBoW" complex, essential for ribosome biogenesis and cell cycle progression. In melanoma cell lines (MM96L, HT144, and MM253), we used siRNA-mediated gene targeting to achieve differing BOP1 expression levels (high, low, absent), which resulted in altered cell states. For example, high BOP1 expression caused an increase in proliferation and colony formation, whereas low BOP1 expression conferred reduced proliferation, colony formation, and a senescent-like phenotype. Interestingly, low BOP1 expression also resulted in increased migration and invasive capacity. Complete ablation of *BOP1* eventually lead to cell death. BOP1 immunofluorescence assessment of benign naevi revealed limited expression whereas in early invasive melanomas, BOP1 expression was clearly evident in proliferative melanoma nests. These data support the role of BOP1 playing a role in melanoma progression by contributing to phenotype switching.

Increased frequency of albinism alleles in individuals with amelanotic/hypomelanotic melanoma

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Amelanotic/hypomelanotic melanoma (AHM) is a subtype with absent or minimal melanin. This study assessed previously reported coding variants in albinism genes (*TYR*, *OCA2*, *TYRP1*, *SLC45A2*, *SLC24A5*, *LRMDA)*and common intronic, regulatory variants of *OCA2* in individuals with AHM, pigmented melanoma cases (PM) and controls. Exome sequencing captured rare coding variants for 28 patients with AHM and 303 patients with PM, which were compared to whole exome data from 1144 control individuals from the Medical Genome Reference Bank (MGRB). Microarray genotyping was also available for an expanded set of 17 AHM and 86 PM patients, 147 unknown pigmentation cases and 652 unaffected controls. Rare deleterious variants in *TYR/OCA1* were more common in AHM cases than PM cases (SMMAT $p = 0.0088$). The *OCA2* hypomorphic allele V443I was more common in melanoma cases (1.8%) than controls (1.0%, X^2 $p = 0.02$), and more so in AHM (4.4%, X^2 $p = 0.007$). Furthermore, none of the AHM cases carried an extended intronic haplotype of *OCA2* present in 7.1% of PM cases ($p = 0.0005$) and 9.43% controls. The frequency of rare variants

in other *OCA* genes did not differ significantly. Variants in *TYR* and *OCA2* may play a role in AHM susceptibility.

Uncovering microtubule-driven mechanisms of melanoma invasion

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Metastatic melanoma is currently incurable and available therapies, although effective, result in resistance and recurrence. The majority of deaths are due to metastatic disease, highlighting the need for *'migrastatics'*, therapeutics which act to inhibit invasion. A recent paradigm shift positions the extracellular matrix as a key player in the metastatic cascade. Cell navigation of 3D matrix requires adaptive changes in cell and nuclear shape to fit matrix physical attributes in a process termed mechanosensing. This process incorporates dynamic remodelling of cell matrix adhesions and the cytoskeleton, to facilitate movement through confined spaces, via proteolytic matrix degradation or cell squeezing. Microtubules play a pivotal role in both of these processes. Our data show that the microtubule-binding proteins, CLASPs, are highly over-expressed in metastatic melanoma lines where they regulate the resistance of microtubule mechanical compression during melanoma invasion in 3D collagen matrices. Using high-resolution live-cell microscopy coupled to genetic alteration and substrate microfabrication, we have identified that patient-derived Melanoma cells utilise CLASP1 and CLASP2, for differing functions to drive 3D invasion. We report paralog specific depletion of CLASPs results in strikingly different 3D invasion phenotypes. Crucially, paralog specific depletion of CLASPs ablates the ability to inter-convert between adaptive invasion strategies by interfering with microtubule-dependent functions during 3D-invasion. Furthermore, pan-depletion of CLASPs within 1205Lu melanoma cells results in 3D migration stasis and reduced cell viability following conditions of 3D confinement, which we do not observe in 2D. These findings suggest that CLASPs function in melanoma cells to facilitate biomechanically regulated cellular processes of both invasion and survival in confined environments.

Development of a new molecular predictor for risk of melanoma brain metastases

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Despite therapeutic advances in the treatment of melanoma, development of brain metastases continues to be a major cause of treatment failure. Prognosis for patients with brain metastases is exceedingly poor, therefore the development of sensitive and specific biomarkers to predict which melanoma patients are at highest risk for disease progression are needed. To accomplish this goal, we embarked on an effort to generate a combined molecular/clinical predictor of brain metastasis risk. We analyzed multiple gene expression datasets including TCGA (n = 437) and a dataset from Australia (n = 183) and identified a list of 60 consensus genes that is robustly predictive of development of melanoma brain metastases ($p < 0.05$; FDR 5%). Next, we performed a similar analysis of association of miRNAs and brain metastasis risk which identified a set of miRNAs with significant predictive power. An optimized combined set of mRNA and miRNA markers was a better predictor of brain metastasis risk than either mRNA or miRNA alone when applied to the TCGA dataset. The combined predictor was most sensitive in separating patients with no metastases from those with either brain metastases or systemic metastases. Current efforts are focused on optimizing miRNA and mRNA separation of patients specifically with brain metastases from those with other mets, and with integrating the expression classifier with other clinical and pathologic predictive factors including: age, stage, thickness, location, histology, ulceration, and gender. The sensitivity and specificity of the resulting clinical/molecular predictor will be validated in an independent retrospective cohort, and subsequently implemented in a prospective screening trial to determine real-world utility of this approach in preparation for prospective brain metastasis adjuvant/chemoprevention trials utilizing both immunotherapy and targeted therapy approaches.

Surprising diversity of spontaneous MAPK pathway inhibitor resistance within a single melanoma cell line

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Despite the success of BRAF/MEK-targeted therapies for treatment of BRAF-mutant melanoma, resistance to MAPK pathway inhibitors (MAPKi) remains a major clinical challenge. A common strategy for investigating mechanisms of MAPKi resistance has been to derive MAPKi-resistant sublines via prolonged MAPKi treatment. However, the resistant populations obtained in such studies have often not been characterized systematically, and divergent resistance mechanisms have been reported, even for the same cell line. To systematically investigate the diversity of resistance mechanisms in a single cell line, we generated vemurafenib-resistant sublines of the widely utilized BRAF-mutant melanoma cell line, A375. Based on growth characteristics alone, two major classes of resistant cells were obtained. Rare, rapidly emerging clones, which appeared within 2–3 weeks of drug selection, were

isolated by cloning rings. More slowly growing cells, which were too numerous to clone and which resumed growth in vemurafenib by 5–6 weeks, were maintained as polyclonal populations. Drug-resistant cells were then analyzed biochemically and by Illumina RNA-seq. Among the rapidly growing clones, we identified multiple, mutually exclusive mechanisms, including BRAF N-terminal truncation, apparent BRAF fusions, and loss of NF1. The more slowly growing polyclonal drug-resistant populations shared a distinct gene expression profile that distinguished them from parental A375 or any of the rapidly growing clones. The promoters of strongly upregulated genes in the polyclonal populations were enriched for TEAD transcription factor binding sites. Thus, the methods used to derive drug-resistant cells and the size of the founder population can have a major impact on the nature of the resistant cells that are ultimately obtained from the same parental cell population.

Normal dermal fibroblasts and melanoma-associated fibroblasts differ in their sensitivity to melanoma cell produced exosomes

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Exosomes are small endosome derived lipid nanoparticles (30–150 nm) actively secreted by exocytosis in most living cells. These small vesicles containing nucleic acid, proteins, lipids and metabolites are released to extracellular fluids including blood, urine, saliva or breast milk. Multiple cell types have been also described to release exosomes in culture cell medium *in vitro*, including melanoma cells. Here we exploit isolation of melanoma cell exosomes from the conditioned medium by ultracentrifugation. The main aim of this study was the comparative analysis of the influence of exosomes produced by melanoma cells on the normal and melanoma-associated fibroblasts. Using antibody-array, we discovered that the secretory profile of melanoma-associated fibroblasts after the treatment with exosomes was significantly different from the profile of activated normal fibroblasts. The main difference was the up-regulation of both IL-6 and IL-8 expression in melanoma-associated fibroblasts and suppression of the thrombospondin-1 production. This result demonstrated the difference between normal and cancer-associated fibroblasts as concerning their sensitivity to melanoma produced exosomes. It indicates that melanoma-derived exosomes shape the landscape of tumour microenvironment which is consequently favourable for tumour progression, possibly for metastasis.

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Orthotopic xenograft mouse model established by splenic injection of metastatic uveal melanoma cell can be monitored with non-invasive live imaging

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Patients with liver metastatic uveal melanoma (MUM) usually die within one year. The lack of diverse MUM cell lines and appropriate animal models makes it difficult to develop new treatments for MUM. We previously demonstrated that orthotopic xenograft mouse model established by direct injection of MUM cell into the liver was useful for the analysis associated with tumor microenvironment. Here, we aim to establish new orthotopic xenograft models via hematogenous dissemination, and compare their characteristics with hepatic injection model. Next, we investigated whether hepatic tumor can be monitored with non-invasive live imaging. We injected MUM cells into the spleen and tail vein as hematogenous dissemination model. We demonstrated that the splenic injection model could establish hepatic tumors, but the tail vein injection model could not. While hepatic injection model established single localized tumor in the liver, splenic injection model established multiple hepatic tumors diffusely throughout the liver. IVIS imaging showed that splenic injection model had stronger fluorescent intensity compared to hepatic injection model. There were no significant differences of tumor growth between splenic injection with splenectomy and without splenectomy. Long term monitoring of splenic injection model demonstrated that tumor growth, tumor distribution in the liver and overall survival depended on the number of injected cells, and tumor could be monitored with fluorescence intensity. Our findings suggest that our new orthotopic liver metastatic mouse model via hematogenous dissemination may be applicable for the preclinical experiments of new treatment and the analysis of liver metastasis mechanisms.

Trends of intralesional T-VEC Use in the anti-PD1 Era: a multi-institutional retrospective chart review

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Talimogene laherparepvec (T-VEC) received FDA approval in 2015 to treat stage IIIB, C and IVa metastatic melanoma, regardless of treatment line. Use of PD1-based therapy for advanced melanoma has risen steeply. T-VEC in the era of widespread anti-PD1-based therapy requires further investigation.

A retrospective review of T-VEC for melanoma patients was conducted at 7 US institutions. Patients who received T-VEC on a clinical trial were excluded. Demographic and clinical information was collected for patients receiving an initial T-VEC injection between 1/1/17 and 3/31/18. All analyses were descriptive.

83 patients were identified. Median age at first T-VEC injection was 66 years (range 29–94); 51% were female and most patients were ECOG 1 or 0 (72%). Seventeen patients (21%) had BRAF V600E and 3 (4%) had V600K mutations. At first T-VEC injection, most patients were stage IIIB (17%), IIIC (35%) and IVc (13%).

Use of T-VEC with respect to anti-PD1-based therapy was distributed across 3 categories: 1) T-VEC used without PD1 (n = 29, 35%), 2) T-VEC given after at least one course of PD1-based therapy (n = 29, 35%) and 3) concurrent T-VEC and PD1-based therapy (n = 25, 30%). There were no significant differences in patient- nor disease-related characteristics across these 3 groups (Table 1). At the time of data abstraction, 89% of patients had discontinued T-VEC therapy, with the most common reasons being no remaining injectable sites (n = 21, 28%) and progressive disease (n = 30, 42%), with an additional 8 patients (11%) discontinuing due to adverse events. Among patients treated with T-VEC alone, 4% discontinued due to adverse events. Discontinuation of T-VEC was not affected by the use of anti-PD1-based therapy.

There has been a shift in T-VEC usage since commercial availability. It is now primarily used concurrently with or after anti-PD1-based therapy.

Clinical outcomes in melanoma patients with family or personal history of cancer

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About 5–12% of melanomas occur in individuals with a familial predisposition. The spectrum of hereditary melanoma-predominant syndromes, involving mutations in *CDKN2A*, *CDK4*, *TERT*, *BAP1*, *MITF* and *POT1*, also increase risk for pancreatic, kidney, ovarian, and CNS tumors. Recent data suggests that patients with a family history of cancer (FHC) may respond better to immunotherapy (IO) and have better overall survival (OS). 132 consecutive new melanoma consults at the Cleveland Clinic in 2016 were retrospectively reviewed. All patients had a three-generation FHC taken during a medical oncology visit and non-melanoma skin cancers were excluded. Overall survival (OS) was estimated by Kaplan-Meier and Cox proportional hazard models. Of 132 patients, 106 (80%) reported either FHC, personal history of multiple cancers (PHMC) or multiple melanomas (MM). 99 (74%) had FHC, 68 (52%) had ≥2 family members with cancer, and 79 (59%) had cancer in a first-degree relative. Patients with any FHC, PHMC, or MM received fewer lines of therapy as compared to those with no FHC or other cancers (p = 0.02). When analyzed individually, patients with a FHC received significantly fewer lines of therapy (p = 0.03). The presence of any FHC, PHMC, or MM did not have statistically significant different 5-year OS (74% vs. 64%; p = 0.31). Patients receiving IO with ≥2 family members with cancer demonstrated improved OS than those with ≤2 family members (83% 5-year survival vs. 63%; p = 0.04). Older age was associated with worse survival (p < 0.001); however, median age at diagnosis (59 years) was the same for all patients, irrespective of FHC, PHMC, or MM. A significant proportion of melanoma patients have FHC, PHMC or MM; within this subset of patients, improved therapy response and OS were observed. Family history may have value as a prognostic marker of clinical outcome; further studies are needed.

Incidence and outcome of venous thromboembolism in melanoma patients on immunotherapy stratified by presence of brain metastases

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Venous thromboembolism (VTE) in cancer significantly contributes to morbidity and a worse overall prognosis. Little is known about the incidence of VTE in melanoma patients (pts) receiving immunotherapy (IO). This study aims to assess the incidence of VTE in melanoma

pts on IO, interrogate potential association with brain metastases (BM), and ascertain its prognostic utility. We conducted a retrospective cohort study of melanoma pts who received any IO including ipilimumab (ipi), nivolumab (nivo) or pembrolizumab (pembro) from July 2015 to December 2017 at the Cleveland Clinic. VTE including deep venous thrombosis (DVT) and pulmonary embolism (PE) were identified by chart review. Overall survival (OS) was estimated by Kaplan-Meier and Cox proportional hazard models; association between VTE and BM was examined using the Pearson's chi-squared test. The study population comprised 230 pts with median age of 63 years (range 23–90) and 67.4% male. Pembro was most commonly used (37.4%), followed by ipi/nivo (26.4%), ipi (21.2%), and nivo (14.7%). Most pts had distant metastatic disease (80.4%), and 21.6% had BM. VTE occurred in 18.3% of pts, of which DVT comprised 52.4%, PE 21.4%, DVT+PE 16.7%, and visceral vein thrombosis 9.5%. VTE was observed in 40% of pts with BM and only 12% of non-BM pts; VTE was significantly associated with BM ($p < 0.001$). Pts with BM had similar OS regardless of VTE status ($p = 0.841$). Among pts without BM, VTE was associated with worse OS when adjusted for age, gender, and IO (median OS 5.6 years vs. NR; $p = 0.007$; HR 2.69 [95% CI, 1.31–5.53]). VTE risk may be elevated for melanoma patients receiving IO and consequently yield worse clinical prognosis. Nearly half of all patients with BM developed VTE. Thus, melanoma patients on IO, particularly with BM, experience elevated risk of VTE and may benefit from thromboprophylaxis.

Circulating tumor DNA (ctDNA) kinetics and survival outcomes in patients (pts) with metastatic melanoma (MM) and brain metastases (BM) treated with dabrafenib (D)+ trametinib (T) in the COMBI-MB trial

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Nearly 50% of pts with MM are diagnosed with BM. Although baseline ctDNA levels and changes during treatment predict clinical outcome after targeted and immunotherapy, no prospective trials have evaluated pre- and on-treatment ctDNA kinetics in pts with BM. We measured *BRAF* V600E ctDNA at baseline and in longitudinally collected plasma samples before progression for up to 40 weeks in 38 pts with intracranial (IC) and extracranial (EC) disease enrolled in cohort A (asymptomatic MM with BM; no previous local brain therapy; ECOG PS ≤1) of the phase 2 COMBI-MB trial (NCT02039947) evaluating D+T in pts with MM and BM. ctDNA was quantified using a validated mutation-specific droplet digital PCR assay (threshold, 0.25 copies/mL). Separately, 20 of 21 samples from 9 pts with isolated IC disease had no detectable ctDNA; those pts were excluded. Progression-free survival (PFS), overall survival (OS), and IC and EC RECIST responses were analyzed. Baseline ctDNA was detectable in 34 of 38 pts (89%); ctDNA copy numbers were correlated with EC (Pearson $r = 0.48$; $p = 0.0042$) but not with IC (Pearson $r = 0.16$; $p = 0.3257$) disease volume. Presence/absence of baseline ctDNA was not correlated with IC or EC best overall response (BOR); baseline ctDNA levels were significantly associated with PFS (HR, 1.17 [95%CI, 1.05–1.30]; $p = 0.0024$) and OS (HR, 1.21 [95%CI, 1.07–1.38]; $p = 0.0020$). ctDNA zeroconversion over time (including all longitudinal on-treatment samples) was significantly correlated with EC BOR (OR, 5.8; $p = 0.04$) but less so with IC BOR. Although these analyses confirm recent findings that ctDNA is not a good biomarker for monitoring CNS responses and associated clinical outcome, we found some associations between baseline ctDNA levels and PFS/OS as well as ctDNA zeroconversion and EC response.

Long-term survival benefit of nivolumab plus ipilimumab (NIVO+IPI) vs BRAF+MEK inhibitors (BRAF+MEKi) for patients (pts) with *BRAF*-mutant advanced melanoma (MEL)

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A population-adjusted indirect comparison was used to assess the relative long-term OS and PFS of NIVO+IPI vs BRAF+MEKi dabrafenib+trametinib (DAB+TRAM), vemurafenib+cobimetinib (VEM+COBI), and encorafenib+binimetinib (ENC+BIN) in treatment-naive pts with *BRAF*-mutant MEL. Pt-level data from the NIVO+IPI CheckMate 069 and 067 trials and published aggregated data from the BRAF+MEKi pivotal trials COMBI-d, COMBI-v, COLUMBUS, and coBRIM were used. Baseline characteristics were matched by reweighting pts in the CheckMate trials by their odds of enrollment in the BRAF+MEKi trials. Cox proportional-hazards models were fit to the weighted OS and PFS for NIVO+IPI and virtual event and censor times derived from published survival curves for BRAF+MEKi. To capture the nonproportionality in the survival curves, Cox models with HR for 0–12 and >12 months were fit. No adjustment was made for use of subsequent therapy. After matching, effective sample sizes of NIVO+IPI vs comparators were

106 for DAB+TRAM, 59 for VEM+COBI, and 60 for ENC+BIN. At 4 years, NIVO+IPI showed superior OS vs DAB+TRAM (HR, 0.62; 95% CI, 0.45–0.85) and VEM+COBI (0.52; 0.33–0.82), and a trend for superior OS vs ENC+BIN (0.71; 0.45–1.10). At 0–12 months, HRs for OS and PFS were similar for NIVO+IPI and BRAF+MEKi. At >12 months, NIVO+IPI showed superior OS vs DAB+TRAM (HR, 0.35; 95% CI, 0.21–0.60), VEM+COBI (0.25; 0.12–0.55), and ENC+BIN (0.35; 0.16–0.73). Similar long-term PFS benefits were observed with NIVO+IPI vs BRAF+MEKi. After adjusting for population differences, NIVO+IPI demonstrated a significant long-term OS benefit over BRAF+MEKi therapies, which emerged after 12 months of follow-up. These hypothesis-generating results will require validation in ongoing randomized trials that directly compare sequential combination approaches. Updated data will be presented.

Metabolic heterogeneity among melanoma cells confers differences in metastatic potential

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Metastasis requires cancer cells to undergo poorly-understood metabolic changes. We found that metabolic differences among melanoma cells confer differences in metastatic potential as a result of differences in Monocarboxylate Transporter 1 (MCT1) function. In vivo isotope tracing in patient-derived xenografts revealed differences in nutrient handling between efficiently and inefficiently metastasizing melanomas, with circulating lactate being a more prominent source of tumor lactate in efficient metastasizers. Efficient metastasizers had higher MCT1 levels and MCT1 inhibition reduced lactate uptake. MCT1 inhibition had little effect on primary subcutaneous tumor growth but depleted circulating melanoma cells and reduced metastatic disease burden in patient-derived xenografts and in mouse melanomas. MCT1 inhibition suppressed the oxidative pentose phosphate pathway and increased ROS levels. Anti-oxidants blocked the effect of MCT1 inhibition on metastasis. $MCT1^{high}$ and $MCT1^{-/low}$ cells from the same melanomas had similar capacities to form subcutaneous tumors, but MCT1high cells formed more metastases after intravenous injection. Metabolic differences among cancer cells thus confer differences in metastatic potential as metastasizing cells depend upon MCT1 to manage oxidative stress.

Treatment (trx) patterns and clinical outcomes for cutaneous melanoma patients (pts) with CNS metastases (mets): a real-world study

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Melanoma CNS mets significantly affect pt morbidity/mortality. Characteristics, trx patterns, and outcomes of real-world US melanoma pts with CNS mets are described. Pts with cutaneous melanoma and histologically confirmed unresectable stage III/IV diagnosis (Dx) on/after January 2011 were identified from Flatiron Health (New York, NY). Of 1666 pts identified, 383 with a subsequent CNS mets Dx and a clinical encounter ≤90 days after Dx were analyzed. Mean pt age was 61 years. Pts were mostly white (82%), male (67%), and treated at community practice (92%); 43% and 57% had a CNS mets Dx in 2011–2014 and 2015–2018, respectively. CNS tumor burden was equally distributed across low (≤3 CNS mets, 49%) and high (>3 CNS mets, 49%). 311 pts had initial trx ≤90 days after their CNS mets Dx. Most pts had a normal lactate dehydrogenase level (<250 units); this was balanced across high and low CNS burden. *BRAF* mutations were more frequent in pts with high (52%) vs low CNS burden (33%). Extracranial disease burden was greater in pts with high vs low CNS burden (median of 3 vs 2 sites). Most pts (83%) received local trx (stereotactic radiosurgery [SRS], craniotomy, whole brain radiotherapy [WBRT]) ± systemic trx as initial trx. Most common trx was SRS for pts with low CNS burden and WBRT for pts with high CNS burden. Pts who received any SRS ± systemic trx (no other local trx) had better survival compared to other trx categories. This was consistent across low ($p \leq 0.01$) and high ($p \leq 0.01$) CNS burden and across Dx year (2011–2014, $p \leq 0.001$; 2015–2018, $p \leq 0.001$). Survival for any SRS pts at year 1 was 48% for pts Dx in 2011–2014 vs 67% for pts Dx in 2015–2018. Use of any immunotherapy (IT) was higher in pts Dx in or after 2015 compared to 2011–2014 (28% vs 15%). Increasing use of IT, along with local trx, may improve survival outcomes.

dsRNA signalling via STAUFEN 1 as a new driver of melanoma progression

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Melanomas are notorious for their altered mRNA expression profiles. Yet, the specific contribution of RNA binding proteins (RBPs) to melanoma development is unclear. Moreover, specific roles of RBPs

in the resistance to targeted or immune-based therapies remain also largely unknown. The main complication in assigning individual RBPs to specific roles in malignant transformation and treatment failure is the very complex nature of these proteins (over 1500 RBPs have been described to date, most of which have yet to be functionally characterized). Mining large clinical datasets, and combining histological and functional studies, we have identified the dsRNA binding protein (dsRBP) STAU1 as a potential oncogene in melanoma. Here we will present mechanistic data uncovering new targets and functions of STAU1 beyond previously reported effects on mRNA decay reported in other systems. Specifically, we will discuss unexpected functions of STAU1 in the tumor microenvironment that fuel the inherent metastatic potential of malignant melanomas.

Somatic mutations and their impact on survival in metastatic uveal melanoma

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Extensive mutation analysis of primary uveal melanoma has identified several key mutations including GNAQ/GNA11, SF3B1, EIF1AX, and BAP1 mutations. However, the role of these mutations in metastatic uveal melanoma remains elusive.

In this study, we investigated somatic mutations of metastatic uveal melanoma and their impact on the survival of the patients. A total of 88 paraffin-embedded specimens were obtained from 87 metastatic uveal melanoma patients. Eleven specimens were analyzed for GNA11 and GNAQ mutations by Sanger sequencing, and 77 specimens were analyzed for 592 cancer-related genes by next generation sequencing. Two patterns of somatic mutations were identified in metastatic uveal melanoma: (1) mutations in GNAQ/GNA11, and (2) mutations related to epigenetic pathways. GNA11 (47%) and GNAQ (45%) mutations were the most dominantly identified in metastatic uveal melanoma. In GNA11 mutations, the majority of mutations were Q209L (88%). In contrast, GNAQ mutations consisted of both Q209L (36%) and Q209P (54%). In terms of mutations related to epigenetic pathways, BAP1 mutations were found in 44/77 specimens (57%) followed by SF3B1 (13%), SETD2 (2.5%), FBXW7 (2.5%) and PBRM1 (1.3%). No specimen had EIF1AX mutation.

There was no difference between metastasis carrying GNA11 or GNAQ mutations regarding time from initial diagnosis of primary uveal melanoma to development of metastasis. In contrast, survival after development of metastasis (Met-to-Death) was much longer in patients with GNAQ 209P mutation compared to those with GNAQ/GNA11 209L mutations (Log-rank test, $p = 0.006$). BAP1 mutation was also a major factor for shorter Met-to-Death compared to those with BAP1 wild type ($p = 0.006$).

These data indicate that strategies to block the GNAQ/GNA11 pathways and to target the epigenetic pathways are two key approaches to improve the outcome of metastatic uveal melanoma patients.

Multi-omic profiling demonstrates importance of B cells in immune checkpoint blockade response

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Tumor mutational load and PD-L1 have been shown to predict response to Immune Checkpoint Blockade (ICB), but robust biomarkers for guiding patient stratification are not yet available. In this study, we performed multi-omic analysis of data from a neoadjuvant melanoma ICB trial (NCT02519322) and demonstrate B cells as predictors of response to ICB. Patient tumor samples were collected at baseline (B) and on-treatment (OnT) and transcriptome profiling was performed. Immune cell signatures were generated using MCP-counter, a tool for immune cell deconvolution. Unsupervised clustering of single-cells was performed using *K*-means algorithm. Spatial organization of B cells was assessed using multiplex immunohistochemistry and B cell phenotype was queried through mass cytometry.

Differential expression analysis of bulk tissue transcriptome data identified genes related to B cell function as overexpressed in responders (Rs) to ICB in comparison to non-responders (NRs). The immune cell signatures demonstrated that B cell signature strongly predicted response (B-$p = 0.001$, OnT-$p = 0.01$) and this was also validated on the Renal Cell Carcinoma (RCC) cohort ($p = 0.001$). Single-cell transcriptome analyses showed increased B cell infiltration in Rs than NRs ($p = 0.004$) and clustering analyses identified four clusters related to B cell activation, metabolism, inflammation, and proliferation. Using mass cytometry, class-switched memory B cell

and plasma cell-like phenotypes were frequently observed in Rs and naïve B cell phenotype in NRs. Further examination of tissue sections revealed B cell organization into Tertiary Lymphoid Structures (TLS) and the TLS density was higher in Rs than NRs (B-p = 0.03, OnT-p = 0.003).

In conclusion, the multi-omics analyses demonstrate importance of B cells in ICB. Additionally, the data suggests a mechanistic role for B cells in response to ICB.

NR2F1 underlies persistence of residual disease in melanoma

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Despite the clinical success of targeted therapy and checkpoint inhibitors in melanoma, therapeutic responses are transient, followed by relapse that may be driven by a small subpopulation of residual or drug-tolerant cells. Understanding residual disease and metastasis mechanisms can provide clues both for developing improved versions of a drug and for guiding the selection of appropriate drug combinations for melanoma therapy. Here, we found that the well-known marker of tumor dormancy, Nuclear Receptor Subfamily 2 Group F Member 1 (NR2F1), was overexpressed in minimal disease residual cells following CDK4/6 and MEK inhibitors (CDK4/6i+MEKi) treatment *in vivo*. Furthermore, melanoma cells overexpressing NR2F1 were less sensitive to (CDK4/6i+MEKi) or BRAF and MEK inhibitors (BRAFi+MEKi) treatment *in vitro* and *in vivo* models, inhibiting apoptosis. Surprisingly, we did not find any evidence of decreased cell growth in our model. Using a three-dimensional tumor spheroid assay *in vitro*, we found the NR2F1 expression enhanced melanoma invasion following CDK4/6i+MEKi or BRAFi+MEKi treatments. Use of published RNA Seq data sets that were gathered from the GEO database and Single Cell Seq data sets from PDX melanoma samples showed that high expression of NR2F1 is enriched in the undifferentiated cell state and invasive cells, respectively. Furthermore, BRAF mutant patient sample with an acquired mutation in NRAS Q61R following BRAFi+MEKi+CDKi presented high expression of NR2F1. Altogether, these findings suggest that NR2F1 may play a role in residual disease persistence besides known features of tumor dormancy, especially important in determining responses to dramatic changes in the environment, such as changes induced by anti-cancer therapy.

Splicing Factor RBM10 as a new driver in melanoma

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RBM10 is an RNA binding protein implicated in the regulation of alternative splicing. In number of tumor types, other than melanoma, RBM10 promotes apoptosis and inhibits cell proliferation, attributes associated with loss-of-function mutations found in these tumors. Notably however, in melanoma, RBM10 is upregulated and its high expression level inversely coincides with survival of melanoma patients. RBM10 oncogenic-like role in melanoma implies that splicing events regulated by RBM10 may constitute driver functions in melanoma. We thus set to determine the significance of RBM10 in melanoma development. Inhibition of RBM10 expression using specific shRNAs decreased cell viability, colony forming ability and sphere formation of the human melanoma cell lines A375 and WM793, pointing to its importance in melanoma growth. RBM10 was identified to interact with SHARPIN, adaptor protein of the Linear Ubiquitin Complex, in both LC/MS and immunoprecipitation studies. In earlier studies we have found that melanoma expresses higher levels of SHARPIN, not coinciding with the expression of other LUBAC components, pointing to LUBAC-independent roles of SHARPIN in melanoma. Inhibition of SHARPIN expression by shRNAs resulted in reduced RBM10 protein expression. Inhibition of SHARPIN expression also resulted in reduced melanoma cell viability, which could be rescued upon re-expression of RBM10. These observation points to an undisclosed role of SHARPIN-RBM10 regulatory axis in melanoma. Supporting the importance of the SHARPIN-RBM10 pathway is the positive correlation between SHARPIN and RBM10 expression in a subset of melanoma patients, identified in TCGA database. The nature of transcripts that are differentially expressed upon altered RBM10 expression will be discussed. Overall, this study identifies a new player in melanoma biology, which mediates oncogenic activities, opposite to its tumor suppressor roles in other tumor types.

Treatment trends and variance among experts and community practitioners in advanced melanoma

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Best practices in the use of immune checkpoint inhibitors (ICIs) and targeted therapy in advanced melanoma continue to evolve. In 2019, we developed an online treatment (Tx) decision support tool designed to provide community practitioners (CPs) with case-specific Tx recommendations from 5 melanoma experts. To use the tool, CPs entered disease and patient (pt) characteristics (eg, disease stage and resectability, *BRAF* mutation status, LDH level) for their specific case, along with their intended Tx. Customized recommendations from each of the 5 experts were then presented, and users could indicate whether this online consultation would change their Tx plan.

An analysis of 384 cases entered into the tool found variation between the planned Tx of CPs and expert recommendations. This was noted in several areas of consensus among experts in the adjuvant setting; for example, for pts with stage III wild-type *BRAF* disease who would merit adjuvant therapy, all of the experts recommended a PD-1 inhibitor, while only 60% of CPs planned this Tx. Likewise, variance was observed for many cases in the metastatic/unresectable disease setting; for example, in select pt scenarios where all of the experts recommended more aggressive first-line combination ICIs (eg, symptomatic disease, high LDH), only 21% of CPs planned to use this Tx approach. Of note, there was variation among experts and CPs in the selection of BRAFi + MEKi vs ICI therapy for pts with *BRAF* mutations in both the adjuvant and frontline metastatic settings. In a post-tool use survey, 42% of CPs whose planned treatment differed from that of the experts indicated that they intended to change their Tx based on expert recommendations. A more detailed analysis of expert and CP Tx trends, as well as a comparison with trends observed with a 2016 version of the tool, will be presented.

Distinguishing melanophages from tumor in melanoma patients treated with talimogene laherparepvec (T-VEC)

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Talimogene laherparepvec (T-VEC) is the first FDA-approved oncolytic virus for the treatment of advanced melanoma. T-VEC is injected into melanoma lesions, but response may be difficult to assess because pigmented macrophages that have ingested melanoma cells (termed "melanophages") persist after immunotherapy at injected sites mimicking melanoma. Thus, novel methods are critically needed to clearly identify the nature of the cells found in tissue specimens from patients treated with T-VEC. We evaluated skin biopsy specimens which were obtained pre- and post- T-VEC therapy of the melanoma patients (stages III-IV) at Columbia University. We used quantitative immunofluorescence (qIF) to: 1) Distinguish melanophages from melanoma in biopsies from patients treated with T-VEC; 2) Evaluate tumor immune micro-environment pre- and post-T-VEC.

Tissue specimens were stained with the antibody panel: DAPI, CD3, CD8, CD68, HLA-DR, and Sox10. Multispectral images were acquired and analyzed using machine learning. Using multiplex staining, post T-VEC samples showed only a few residual melanoma cells within the skin biopsies confirmed by nuclear Sox10+ expression, and many melanophages with cytoplasmic co-staining of CD68, Sox10, and HLA-DR, with no nuclear expression of Sox10. This is a novel finding and highlights the phagocytosis of melanoma cells by macrophages following immunotherapy. QIF also revealed a dense immune infiltrate of CD3+ CD8+ and CD68+ cells in post T-VEC samples. QIF methods may assist pathologists in determining whether lesions from patients treated with immunotherapy contain residual viable melanoma. It also maybe helpful to assess immune response to the therapy.

Chloroquine sensitizes GNAQ/11 mutant uveal melanoma to MEK1/2 targeted therapy

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GNAQ and GNA11 (GNAQ/11) mutations are found in approximately 2% of melanoma, including more than 80% of uveal melanoma. Mutations in these G-alpha proteins lead to constitutive activation of multiple oncogenic pathways, including MAPK (RAF–>MEK1/2–>ERK1/2) signaling. Unfortunately, unlike cutaneous melanoma, metastatic uveal melanoma is refractory to all forms of pharmacologic treatment, such as FDA-approved targeted therapies inhibiting MEK1/2 (trametinib and binimetinib). We showed that combining MEK1/2 inhibitors with 4-aminoquinoline antimalarials, chloroquine or hydroxychloroquine, resulted in synergistic cytotoxicity in multiple GNAQ/11 mutant uveal melanoma cell lines. Interestingly, in contrast to our previous work in pancreatic cancer, this combination effect worked independently of the lysosomotropic role of chloroquine, as neither lysosomotropic agent, Bafilomycin A1, nor autophagy-specific and macropinocytosis-specific pharmacologic and genetic inhibition yielded a cytotoxic effect in combination with MEK1/2 inhibition. Nevertheless, MEK1/2 inhibitor and chloroquine treatment resulted in increased apoptosis as measured by Caspase 3/7 fluorescent imaging. Furthermore, we utilized a hepatic colonization model, in which luciferized human metastatic uveal melanoma cell lines, OMM2.5 and OMM1, were injected into the livers of immunocompromised mice. Daily treatment of trametinib with hydroxychloroquine in combination resulted in decreased tumor burden and increased overall survival *in vivo* compared to either treatment as monotherapy. Our findings suggest a potentially effective strategy combining two FDA-approved drugs for the treatment of metastatic uveal melanoma.

A retrospective cohord study of 1144 melanoma patients from 2007–2017

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Greek population is at intermediate risk for melanoma due to UV exposure and skin type.

The study analyzes epidemiological, histological and surgical data in the largest melanoma center in Greece.

Is a study of 1144 melanoma patients who diagnosed, surgically treated and had their follow up to our hospital from 2007 to 2017. The statistical analysis was carried out using the SPSS 24.

Mean age was 58.24. Females 51.6% and males 48.5%. Higher incidence to skin type III 51.8%, type II 45.9%, type I 1.8% and type IV 0.5%. Patients report indoor job 71.9% and outdoor 28.1%. UV exposure as low 13.6%, moderate 52.1% and high 31.6%. Referred pre-existence nevus 73%, lesions greater than 5 mm in diameter 81.4%, increasing size 61.3%, color change 47.8%, asymmetry 43%, urticarial 29%, redness 14.4%, bleeding 24.9%, regression 13%.

Primary sites: head 15.7%, neck 2.5%, shoulder-arm 11.2%, forearm 3.4%, hand 1.1%, palm 0.1%, subungual hand 0.6%, chest wall 4.3%, back 20.9%, west 6.6%, abdomen 4.2%, gennitalia 0.7%, thigh 6.7%, calph 9.9%, mucosa 0.2%, foot 6.8%, sole 2.6%, subungual foot 1.1% and unknown 1.4%.

The pathology shown: vertical growth in 90.3%, regression in 0.5% and in situ growth 6.0%, Type: ALM 4.5%, LM 4.0%, LMM 5.2%, MLM 0.1%, NM 17.6%, SSM 61.4% and other types 7.2%. Clark level: I 6.0%, II 11.4%, II/III 2.0%, III 24.6%, III/IV 5.8%, IV 40.4%, IV/V 1.7%, V 6.0% and 2% regressed and unknown primary site. Mean value for Breslow was 2.7 mm. Breslow <1 32.6%, ≥1 and <2 24.1%, ≥2 and ≤4 26.1% and >4% 14.5%. Ulceration 25.8%.

The patients treated surgically with wide excision and SLNB in 44.9% of which the 23.98% were positive and 76.02% negative. CLND performed in 145 cases positive SLNB and suspicious characteristics confirmed with FNAB. The sites of CLND was axilla 44.63% groin 38.02%, neck 15.70% and both axilla and neck 1.65%. The mean dissected lymph nodes was 17.07 and positive was 2.03.

Diagnostic accuracy of high resolution ultrasound and fine needle aspiration for recurrence and locoregional metastases in patients treated initially for stage I melanoma: a systematic review

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Cutaneous melanoma is the fifth most common cancer in the UK, and the incidence is increasing. The highest rates of local or distant recurrence (metastases) occurs in the first 5 years post diagnosis. Testing for suspicion of new or recurrent melanoma may involve a range of tests. We conducted a systematic review of the diagnostic accuracy of tests used to detect recurrences in patients who were treated initially for stage I melanoma. The tests considered were high resolution ultrasound and fine needle aspiration cytology.

Literature searches were conducted between 1998 and July 2019. We retrieved 2,250 unique citations. All citations were screened in duplicate by 2 of 3 researchers first by title and abstract and then for potentially eligible studies by screening full text papers. For included studies, data on diagnostic accuracy were extracted. The risk of bias

was assessed using the QUADAS-2 tool. The sensitivity and specificity of the tests were collated as Forest plots.

Two studies met the inclusion criteria. One study, at high risk of bias, assessed fine needle biopsy in 400 stage I patients. The second study, at low risk of bias, conducted 669 investigations (average 3/patient) using high resolution ultrasound and clinical investigation. The sensitivity of fine needle biopsy was 0.93, 95% confidence interval (0.88–0.97), and specificity 0.98 (0.95–0.99). Corresponding findings for ultrasound were sensitivity 1.00 (0.025–1.00), specificity 0.99 (0.97–0.99).

There is limited data on the diagnostic accuracy of fine needle biopsy and ultrasound for patients with stage I melanoma in recently published literature. Larger cohorts of these patients would be required to provide truer estimates of diagnostic performance.

The effectiveness of surveillance and follow-up strategies for AJCC stage 1 melanoma: a systematic review

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There is little consensus about the most effective way to follow-up people treated for melanoma. We aimed to identify various surveillance and follow-up strategies after surgical excision of AJCC stage I primary cutaneous melanomas in adults, and assess their relative effectiveness. We conducted a systematic review of comparative studies of surveillance strategies. We searched ten bibliographic databases, grey literature and guidelines from 2011 to May 2018. Outcomes included overall survival, progression or recurrence free survival, detection of recurrence, new primary tumours /metastases. Two randomised controlled trials; one from the USA and other from Netherlands met our inclusion criteria. The USA trial evaluated the effect of a structured skin self-examination (SSE) in 494 dyads of patients and their partners. Data reported for stages 0-2B showed the intervention was successful in increasing SSE by patient-partner dyads compared to standard physician care (mean difference of SSE frequency 0.94 [95% CI, 0.58 to 1.30], $p < 0.001$) at 24 months. A secondary outcome was detection of a new or recurrent melanoma by the dyad or physician. For stage 1 disease new primaries or recurrences were detected in 49/258 (19%) patients with stage IA or IB; 36 (18%) of 203 in the intervention group compared with 13 (24%) out of 55 in the control arm. Data from the Netherlands trial is unavailable as the trial is ongoing.

Evidence for the effectiveness of surveillance and follow-up strategies for stage 1 melanoma is limited. There are few comparative data and no evidence from well-designed comparative studies on most outcomes of importance. Current surveillance practice may be sub-optimal and well-designed comparative studies are needed to establish the most effective surveillance strategies.

PRIME002: Early phase II study of Azacitidine and Carboplatin priming for Avelumab in patients with advanced melanoma who are resistant to immunotherapy

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Melanoma patients with checkpoint inhibitor immunotherapy resistance have limited treatment options. Azacitidine (aza) results in DNA-demethylation and increase in neoantigen expression. Carboplatin increases DNA-damage and cellular stress. This trial aims to test if sequential aza and carboplatin "primes" for checkpoint inhibitor therapy rechallenge.

2 cycles of aza 40 mg/m^2 IVI/day × 5 days followed by carboplatin AUC 4.5 IVI D8/28 day cycle, followed by Avelumab 10 mg/kg IVI/2 weeks. RECIST 1.1 after 8 weeks and 22 weeks. Avelumab continued until iRECIST disease progression. Primary outcomes-complete response (CR), partial response (PR), stable disease (SD), objective response rate (ORR), disease control rate (DCR). A favourable response in 20% patients and minimal grade 4/persistent (>4 weeks) grade 3 treatment-related adverse events are required for continuation.

7 primary immunotherapy resistant metastatic melanoma patients were enrolled [median age, 64 (range, 63–76); 5M/2F; ECOG PS 0/1 (83%/17%)]. Pts received at least 6 cycles of prior pembrolizumab (median, 17.5; range, 6–44) or combination ipilimumab and nivolumab (4 cycles combo, 6–8 months nivolumab). All 7 pts received 2 cycles of aza/carbo and 6 doses of Avelumab. The ORR was 14.3% (1/7) and DCR was 100% (7/7) after aza/carbo. ORR was 28.6% (2/7) and DCR 71.4% (5/7) after 6 doses of Avelumab. Average follow-up 40 weeks (range = 62–26). 4 additional patients have enrolled. Pt 8 had SD in multiple intracranial metastases after 2 cycles of aza/carbo.

Sequential azacitidine and carboplatin limits the burden of disease for immunotherapy resistant advanced melanoma which may allow for effective re-treatment with checkpoint inhibitor therapy. The results meet the criteria for continuation and expansion. The authors acknowledge the support provided by Merck KGaA.

Development and classification of rare melanoma patient derived xenograft models and cell lines

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Patient-derived xenograft (PDX) and cell line models of melanoma are important tools in preclinical and translational cancer research. These models help to weigh drug efficacy and generate predictive data to drive cancer research, allowing for the study of melanoma in a rapid and cost-effective manner. PDX models are invaluable for preclinical studies of drug response, biomarkers, and tumor biology. While there are many PDX models and several cell lines available, most of them lack relevant clinical information such as age, stage, treatment history, sites of metastasis, survival and therapy response. We have developed over 100 PDX models and 27 cells lines with extensive clinical and genotype information. In addition to several pan negative models, our cohort includes *BRAFV600E*, non-V600E *BRAF*, *NRAS* and *NF1* mutations. Our PDX bank collection includes cutaneous models as well as several rare subtypes of melanoma including 12 acral, 12 mucosal and 13 unknown primary melanomas that have previously been unavailable elsewhere. Clinical, molecular, and whole exome sequencing data are available for the majority of samples. The cell lines that we have developed comprise of 16 cutaneous, five acral, four mucosal and two unknown primary melanomas. All of the cell lines are STR profiled against matched patient blood and known mutations are validated using Sanger sequencing. In summary, we have established an extensive collection of melanoma PDX models and cell lines with comprehensive clinical and genetic correlative data. Our collection includes several rare subtypes and genotypes which are seldom characterized and are useful in developing and evaluating novel treatment strategies. These are available to all qualified investigators at other institutions.

Single cell and neighborhood analysis for predicting outcome in metastatic malignant melanoma patients treated with anti-PD1

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Despite that anti-PD1 therapy has changed treatment paradigm for patients with malignant melanoma, still a substantial number of patients do not derive benefit from this type of treatment. The mechanisms how immunotherapy works takes place on a multifunctional and multicellular level and the exact composition of immune cells in responding lesions and their highly dynamic interplay remains largely elusive. As a result, our treatment predictive tools are suboptimal and the improvement of precision in terms of diagnostics and prediction is a demanding issue. We therefore performed multiplex immunofluorescence staining on FFPE material (Bolognesi et al, 2017) to visualize 85 immunological markers at single-cell level in the context of preserved tissue architecture in order to search for predictive markers in patients undergoing immune checkpoint therapy.
We characterized immune cell subpopulations in 13 pretreatment biopsies from 12 melanoma patients treated with anti-PD-1 therapy. Based on the expression of the 85 selected markers, we looked at the differences between responding and non-responding patients, correlating the abundance of immune cells, their functional status and spatial relationships using neighborhood analysis. A Nanostring transcriptomic analysis (>800 genes) was done in addition in order to confirm our multiplex results. We found that cytotoxic T cells have higher levels of exhaustion in responding patients with TIM3 expression being the most discriminative marker for response. Neighborhood analysis revealed more frequent B-T cell interactions in responders. Gene set enrichment analysis demonstrates upregulation of pathways involved in B-cell and T-cell receptor signaling in the responding patients. More in depth neighborhood analyzes will be presented.

ctDNA predicts early recurrence in advanced melanoma patients

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Melanoma is the deadliest form of skin cancer and the identification of metastatic progression is crucial for the management of patients. *BRAF*, *NRAS* and *TERT* promoter hotspot mutations affect more than two-thirds of melanomas and their detection in circulating tumor DNA (ctDNA) represents a possibility for detecting and monitoring the metastatic disease.
In this study, we aimed to standardize a platform to identify hotspot mutations (BRAFV600E/ K/R, NRAS Q61K/L/R/H183A>T/183A>C and TERTC250T/C228T) for liquid biopsy purposes. Then we investigated the ctDNA detection in a cohort of 19 advanced melanoma patients and whether it was associated with the patient's characteristics.
Here we performed droplet digital PCR using tumor cell lines for validating and determine the limit of detection (LOD) of assays. We determined somatic mutation status on paraffin-embedded tissue from melanoma patients followed by plasma analysis of ctDNA by ddPCR.
With this study, we established a specific and sensitive methodology with a LOD up to 0.01%. 17 (89%) patients had at least one somatic mutation, of whom 7 had detectable ctDNA on plasma. ctDNA detection was associated with the deeper primary lesion (>7.5 mm, $p = 0.048$), age <61 years ($p = 0.034$) and shorter progression-free

survival (median PFS, 50 days for detectable ctDNA and 146 days for undetectable ctDNA, $p = 0.014$)

Despite preliminary, our data demonstrate the use of ctDNA as prognosis biomarker, in which patients with detectable levels tend to have an unfavorable outcome. A larger cohort should be analyzed to validate this data so as longitudinal sampling to better understand changes in ctDNA levels over time and their associations with variables such as treatment and also survival.

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Wild type p53 is targeted by CEACAM1 to facilitate melanoma cell proliferation

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CEACAM1 is a multifunctional protein, which is associated with melanoma progression and poor prognosis. We have previously demonstrated that CEACAM1 serves as an immune evasion mechanism and facilitates cell cycle and proliferation of melanoma cells. Here we describe the downstream signaling leading to enhanced proliferation.

Co-immunoprecipitation showed strong interactions between CEACAM1, SHP-1 and RAP1 in melanoma cells. Point mutations in CEACAM1 phosphorylation sites not only abrogated the enhanced proliferation but also abolished the interactions with SHP-1 and RAP1. CEACAM1 interaction with RAP1 was confirmed with mass spectrometry and knockdown of SHP-1 or RAP1A inhibited CEACAM1-mediated proliferation. Conversely, CEACAM1 overexpression increased the active fraction of RAP1 as demonstrated by pull-down assays, and RAP1A inhibitor hindered proliferation in CEACAM1-positive cells. Thus, CEACAM1 recruits RAP1 to its cytoplasmic tail, leading to its activation and increased proliferation.

Comparative microarray analysis showed significant alteration in p53 and RAP1 signaling pathways following CEACAM1 overexpression. Expression of p53 effectors such as p21 was suppressed by CEACAM1 in a SHP1 dependent manner, confirmed by concomitant knockdown of SHP-1. Binding of p53 to pulled-down active RAP1A was confirmed by immunoblotting. Importantly, inhibition of RAP1A enhanced p53 phosphorylation and expression of p21. Studies in various melanoma lines showed that the effects of RAP1A and CEACAM1 depend on the presence of wild type p53. TCGA analysis identified strong association between RAP1 signaling and p53 wild type melanomas that exert p53 mutant-like phenotype.

In conclusion, as opposed to other cancers, p53 is infrequently mutated in melanoma. Our results show that wild type p53 is targeted by the commonly expressed CEACAM1, which sheds new light on the role of p53 in melanoma biology.

Initial results from a phase 1b study of PV-10 and anti-PD-1 in melanoma refractory to checkpoint inhibition

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PV-10 (rose bengal disodium) is a small molecule oncolytic immunotherapy in development for solid tumors; intralesional injection can yield immunogenic cell death and induce tumor-specific reactivity in circulating T cells.

PV-10-MM-1201 is a phase 1b/2 study of PV-10 in combination with anti-PD-1 for patients (pts) with advanced cutaneous melanoma; pts must have at least 1 injectable lesion and be candidates for pembrolizumab (pembro). The combination is administered q3w for 5 cycles followed by pembro alone for up to 24 months; the primary endpoint is safety and tolerability with objective response rate and progression free survival key secondary endpoints (assessed by RECIST 1.1 after 5 cycles then q12w). Correlative assessments are being performed on a subgroup of pts.

Accrual into an expansion cohort of pts relapsed or failing to achieve an objective response on checkpoint inhibition (CI) began in Dec 2018; this extends an exploratory group of CI-refractory pts enrolled into the main cohort of the study. All 8 pts (1 Stage IIID, 3 M1a, 1 M1b, 3 M1c; median age 77, range 64–90) had prior CI (2 refractory to CTLA-4, 2 to PD-1 and 4 to CTLA-4 and PD-1). Adverse events have been consistent with established patterns for each drug. Among this initial group, 3 pts withdrew due to progression during combination treatment; 2 pts (IIID and M1a, respectively) achieved a best overall response of PR and 2 pts (M1a and M1c) achieved SD. Three pts have completed correlative assessment: post-PV-10 serum exhibited elevation of High Mobility Group Box 1 (HMGB1), a Damage Associated Molecular Pattern (DAMP) associated with activation of dendritic cells.

Acceptable safety and tolerability have been observed, and enrollment is ongoing. Initial correlative results for this highly refractory population are consistent with prior evidence of immune activation by single-agent PV-10.

B cells sustain inflammation and predict response to immune checkpoint blockade in human melanoma

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Tumor associated inflammation predicts response to immune checkpoint blockade in human melanoma. Here we show that tumor-associated B cells (TAB) are vital to tumor associated inflammation.

In human melanoma, TAB are located at the invasive tumor-stroma margin arguing for a preferentially cell contact-independent communication with tumor cells. We therefore exposed *in vitro* peripheral blood- and melanoma-derived B cells to the secretome from autologous melanoma cells. In proteomics and RNA-seq data, we observed induction of several pro- and anti-inflammatory factors and differentiation towards a *plasmablast-like* phenotype. In human melanoma samples we could identify this B cell phenotype by 7 color multiplex immunostaining and as a distinct B cell cluster in public scRNA-seq data.

RNA-seq and multiplex immunostaining data also revealed that depletion of TAB by anti-CD20 immunotherapy of metastatic melanoma patients led to a pronounced decrease in tumor inflammation signatures and $CD8^+$ T cell numbers, in line with scRNA-seq data on expression of T cell chemoattractants CCL5, CCL4, CCL28 in plasmablast-like TAB.

The potential clinical implications of our observations are demonstrated in two independent large-scale whole-tissue (sc)RNA-seq datasets. Here, the frequency of plasmablast-like TAB in pretherapy melanoma samples predicted response and survival to immune checkpoint blockade. Consistently, in a surrogate assay of T cell activation, MCM-induced B cells significantly increased the activation of PD-1-expressing Jurkat T cells by PD-1 blockade.

Together, our data argue that tumor-associated B cells orchestrate and sustain tumor inflammation, recruit CD8+ T effector cells and may represent a predictor for response and survival to immune checkpoint blockade in human melanoma.

Adjuvant therapy and prognostic factors for conjunctival melanoma

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Background: Conjunctival melanoma (CM) is a rare tumor in Asian with only a few case reports. Adjuvant therapy have not been established for CM. This study is to determine the adjuvant therapy and prognostic factors of primary CM in China.

Methods: A retrospective analysis of CM patients(pts) were conducted from the database of Peking University Cancer Hospital between Jan 1998 and Dec 2019.

Results: Seventy-three pts were included for analysis with a median age of 57.0 years; Twenty-eight (38.4%) pts were male. When initial diagnosed, most pts presented with localized disease (76.7%). Predominant metastatic sites were distant nodes (28.3%), lung (26.4%), bone (17.0%) and liver (11.3%). There were 9.6% pts harbored C-KIT mutation and 17.8% BRAF. Surgical treatment was performed in 98.6% of all pts, with which 88.9% were wide excision and 11.1% orbital exenteration. 57.1% underwent adjuvant therapy after surgery. Thirty-seven of them were adjuvant high-dose-interferon (HD-INF) and only 5 pts administrated with adjuvant chemotherapy. By the time of July 2019, a recurrence of the disease was recorded in 52/72 (72.2%), with 50.7% local recurrence. Local recurrence was related with surgery time but not with operation modalities (Surgery before 2010 showed a dromaticaly high local recurrence 93.3% vs. 40.4% $p < 0.001$). In comparison, the control cohort of 28 patients was found to have a shorter RFS time of 14.3 months compared with adjuvant HD-INF (36.1 m, $p = 0.006$). But the local recurrence rate was not reduced by adjuvant HD-INF. Predictors of RFS were stage and HD-INF adjuvant therapy in multivariate analysis. By the time of the last data collection, the median OS was 84.2 months. Age, initial stage, adjuvant HD-INF and liver metastatic are survival predictors in univariate analysis.

Conclusion: Conjunctival melanoma have a high rate of local recurrence, adjuvant HD-INF may still a choice for this subtype.

Liver metastasis and treatment outcome with anti-PD-1 antibody monotherapy and TACE combination in patients with melanoma – a pooled analysis

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Background: This study is a pooled analysis of monotherapy clinical trials of anti PD-1 Ab aiming to analyze the efficacy of anti PD-1 Ab in melanoma pts with liver metastasis. And it was assumed that local therapy of TACE combination might improve the outcome of liver metastasis.

Methods: MM pts from clinical trials of anti PD-1 Ab monotherapy (second-line or more) were included in this pooled analysis. And a retrospective analysis of 12 patients with anti PD-1 and TACE (cisplatin) combination was done for efficacy compare.

Results: A total of 187 pts with MM received anti PD-1 Ab monotherapy in clinical trials from 2015 to 2018 were included. There were 46 pts got liver metastasis and 141 pts in non-liver metastasis group. LDH level was significantly higher in liver metastasis group

(56.5% vs. 27.0%, $p < 0.0001$). The response rate of anti PD-1 Ab was lower in liver metastasis group than non-liver metastasis group without significance,10.0% vs. 15.7%. The PFS and OS were significantly shorter in liver metastasis group, 2.8 m vs. 5.6 m, and 5.7 m vs. 14.9 m, $p < 0.0001$ respectively. In multivariate analysis, liver metastasis, ECOG > 0 and elevated LDH level were independent risk factors for PFS & OS, and the standard regression coefficient of liver metastasis (0.44) had the most significant effect. In liver metastasis group, LDH and ECOG were independent risk factors of OS for pts with liver metastasis.

The response rate of anti PD-1 and TACE combination group was 4.7%, which was similar when compared with monotherapy. But the PFS: 4.1 m was longer. The response of hepatic and extrahepatic lesions to treatment is consistent.

Conclusion: Liver metastasis is an independent prognostic factor for melanoma pts receiving anti PD-1 Ab. Further treatment strategy need to be explored for MM with liver metastasis.

Nab-paclitaxel or temozolomide combined with anti-angiogenetic drug in advanced melanoma patients progressing on PD1 immunotherapy

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Background: Immunotherapy have proved effective for advanced melanoma. However, resistance to PD-1 inhibitors is a new obstacle. This study aimed to examine characteristics and efficacy of nab-paclitaxel(nab-p) or temozolomide(TMZ) combined with anti-angiogenetic drugs in PD-1 inhibitors failed metastatic melanoma pts.

Methods: This study included metastatic melanoma pts who developed resistance to PD-1 inhibitors from 2015 to 2018. All pts received nab-p or TMZ combined with anti-angiogenetic drugs subsequently followed PD-1 inhibitors resistance. The response rate and PFS of chemotherapy(c-PFS) were stratified by the PFS of PD-1 (p-PFS),age, BRAF mutation status, lactate dehydrogenase (LDH) levels, ECOG, gender, time of receiving PD-1 and chemotherapy regimens.

Results: 70 pts were included with a median age of 52.2 years. 40% were males, 46.8% had LDH>ULN, 34.3% had ECOG 0, 91.4% were BRAF WT. 31.4% received PD-1 inhibitors as 1st line therapy. Primary drug resistance (p-PFS ≤ 3 m) was found in only 27.1% pts. All pts were divided into 2 cohorts: nab-p combined with anti-angiogenetic drugs (cohort A: 26 pts) and TMZ combined with anti-angiogenetic drugs (cohort B: 44 pts). The overall ORR was 5.7% and DCR was 37.1%. Compared with nab-p, TMZ based chemotherapy showed better ORR (3.8% vs. 6.8%), but was not significant. The median c-PFS was 2.75 months with no significant difference in two cohorts (2.8 m vs. 3.0 m, $p = 0.808$). In multivariable analyses, p-PFS ≥ 3 months, earlier line of anti-PD-1 therapy, acral pathology subtype, and normal LDH predicted better c-PFS. For primary anti-PD-1 resistance pts, nap-p had better PFS compared with TMZ (3.0 m vs. 1.0 m, $p = 0.009$).

Conclusion: Chemotherapy combined with anti-angiogenetic drugs has a promising treatment effect in pts with PD-1 inhibitors resistance. Further studies will be needed.

Paradoxical role for wild type p53 in driving therapy resistance in melanoma

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Metastatic melanoma is an aggressive disease. Despite recent improvements in therapy, drug resistant populations emerge even in drug-sensitive tumors. A subset of melanoma cells can transition to a slow-cycling state, rendering them resistant to most targeted therapy. Several markers of resistance have emerged however, it is still unclear what pathways define these subpopulations and promote this resistant phenotype. In the current study, we show that Wnt5A, a non-canonical Wnt ligand that drives a metastatic phenotype, stabilizes the half-life of p53 and uses p53 to initiate a slow-cycling state. Both Wnt5A and p53 are increased in these metastatic cells following stress (DNA damage, targeted therapy, and aging). Inhibiting p53 blocks the slow cycling phenotype and promotes sensitivity to targeted therapy. A single dose of a p53 inhibitor at the commencement of BRAF/MEKi therapy was sufficient to prolong sensitivity to BRAF/MEKi therapy in Yumm1.7 derived tumors grown in aged mice (> 52 weeks). Upon analysis of PDX tumors with WTp53, treated with BRAF/MEKi combination or BRAFi therapy, we observed an increase in p53 positive cells as well as an increase in Wnt5A expression, further linking Wnt5A and p53 with resistance to targeted therapy. These data suggest that melanoma cells require p53 to survive multiple types of stress and that taking the paradoxical approach of inhibiting rather than activating WTp53 may sensitize previously resistant metastatic melanoma cells to therapy by temporarily driving them into a rapidly cycling state.

Real world time to next treatment in advanced melanoma patients treated with pembrolizumab in the German ADOReg melanoma registry

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Melanoma is among those tumors very prone to the effects of immunotherapy. In a follow-up data cut of the ADOReg treatment registry, we analyzed real world time-to-next-treatment (rwTtnT) for characterizing pembrolizumab treatment benefit in advanced melanoma. Patients initiating pembrolizumab from Aug 2015-Dec 2017 for unresectable stage III or stage IV melanoma were identified from the ADOReg. rwTtnT was calculated as the interval between start of pembrolizumab treatment and the start of a subsequent treatment or death and evaluated by survival time analysis. We report on 664 patients among which 63/601 were unresectable stage III/stage IV melanoma; 386/278 were treatment-naïve/pretreated; 535/30/17/81 were cutaneous/ocular/mucosal/unknown primary origin. In first-line, the median rwTtnT was markedly longer than in second-line (12.9 vs. 8.0 months). However, in third-line or higher the median rwTtnT was 11.5 months. This was also reflected by 1-year next-treatment rates of 51.3% 41.4%%, and 48.9% in first-, second-, and third-line plus, respectively. Median rwTtnT was longest for unknown primary and cutaneous melanoma, but markedly shorter for ocular, and mucosal melanoma. In contrast, the presence vs. absence of brain metastasis did not significantly affect median rwTtnT. Capturing PFS as a clinical endpoint in the real world is impaired by imprecise and non-specific tumor measurements, pseudoprogression, and subjective judgements, in particular delimiting slightly progressive from stable disease. Moreover, in patients receiving immunotherapy, treatment beyond progression may be of value in certain patients to preserve some tumor control. In comparison to PFS, rwTtnT appears to be of higher relevance and discriminatory power and may be a valuable endpoint in real world populations receiving immunotherapy.

BRAF/MEK inhibitors induce potent ER stress-enforced apoptosis in BRAFwt/NRASmut melanoma cells – insights into mode of action and resistance mechanisms

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About 15–20% of all melanomas harbor activating NRAS mutations, which are associated with aggressive disease requiring rapid anti-tumor intervention that is not available so far. In a phase III trial, the MEK inhibitor (MEKi) binimetinib displayed a moderate anti-tumor activity in NRASmut melanoma, providing an ideal backbone for combination treatments. In our previous study, we showed that the anti-tumor effect of the MEKi binimetinib in NRASmut melanoma cells is potentiated by combination with the BRAF inhibitor (BRAFi) encorafenib through ER stress induction. In this study, we aimed to further analyse the mechanism of action of BRAF/MEK inhibitor (BRAF/MEKi) combinations in BRAFwt/NRASmut melanoma cells. We established three NRASmut melanoma cell lines that are resistant to different BRAF/MEKi combinations. BRAF/MEKi did not induce growth inhibition and apoptosis in resistant cell lines in contrast to the parental, sensitive cell line. Accordingly, the resistant cells did not upregulate or cleave apoptotic proteins such as Bim, caspase 9, caspase 3 and PARP under treatment with BRAF/MEKi. Levels of the ER stress proteins ATF4 and CHOP were similar in the resistant and sensitive cell lines. However, phospho-ERK levels were higher in the resistant cell lines, suggesting that BRAFi-mediated ER stress but not MEKi-mediated apoptosis is induced in the resistant cells after treatment. In conclusion, BRAFwt/NRASmut melanoma cells appear to acquire resistance to BRAF/MEKi by up-regulating downstream MAPK pathway molecules and demobilizing the mitochondrial pathway of apoptosis. Overall, BRAF/MEKi induce potent apoptosis in BRAFwt/NRASmut melanoma representing a promising new treatment strategy for this patient group.

Characteristics and outcomes of patients with advanced melanoma when retreated with anti-PD1 monotherapy after response to first course in clinical practice

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Clinical trial data suggest that patients with advanced melanoma who have stable disease (SD) or better after a first course of anti-PD1 monotherapy may benefit from retreatment with a second course. For clinicians in routine practice, the question arises whether to retreat with anti-PD1 monotherapy if disease progresses. We explored outcomes of retreatment with anti-PD1 monotherapy for patients with advanced melanoma treated at US community oncology practices.

The nationwide Flatiron Health EHR-derived database was used to identify adult patients prescribed pembrolizumab or nivolumab monotherapy for unresectable melanoma on or after 4Sep2014 and who were retreated with anti-PD1 monotherapy starting on or before 30Apr2018 after a ≥90-day treatment gap. Enhanced manual chart review was used to determine real-world tumor response (rwTR). Patients were eligible if they achieved best rwTR with first anti-PD1 course of complete response (CR), partial response (PR), SD, or unknown rwTR. Overall survival (OS) was determined using Kaplan-Meier.

Of 29 eligible patients, 18 (62%) were male; median age was 74 years (range 34–85). Of those with available data, 5/23 (22%) had elevated LDH, 12/28 (43%) had *BRAF*-mutant melanoma, and 14/20 (70%) and 6/20 (30%) had ECOG performance status 0–1 and ≥2, respectively. At data cutoff (31Oct2018), 18/22 patients (82%) with known best rwTR achieved CR, PR, or SD with second-course anti-PD1 monotherapy. From start of the second course, median OS was 30.0 months (95% CI 25.6–NR); 1-year survival was 90% (71–97%); 2-year survival was 77% (51–90%).

This small group of patients with advanced melanoma who had SD or better after the first treatment course benefited from retreatment with anti-PD1 monotherapy, showing high response rate and 90% survival at 1 year, with median OS of 30 months.

DGAT1: a novel and potent driver of melanoma growth and progression.

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Metabolic reprogramming is one of eight hallmarks of cancer, with alterations in lipid metabolism impacting on cell structure, signaling and energy metabolism. Identification of key alterations in melanoma cell lipid metabolism may lead to novel therapeutic targets. We have identified DGAT1, an ER localized enzyme critical for catalyzing the conversion of diacylglycerides into triacyglycerides, as a novel driver of melanoma cell growth and survival. We found DGAT1 to be among the most up-regulated genes in a Zebrafish model of RAS driven melanoma, and to be over-expressed in human melanomas and correlated with poor survival. Using Zebrafish transgenesis, we show that the specific over expression of DGAT1 in melanocytes leads to acceleration of NRAS driven melanoma formation and progression. Strikingly we have also demonstrated that in a p53 mutant background DGAT1 over-expression alone was sufficient to induce melanoma formation and progression. Using *in-vitro* melanoma models we find that this pro-oncogenic activity is mediated through both the ability of DGAT1 to catalyze the formation of lipid droplets, organelles that store neutral lipids, and through activation of S6K signaling. Inhibition of DGAT1 leads to reduced lipid droplet formation and increased beta oxidation that produces toxic metabolites, ultimately resulting in the loss of mitochondrial membrane potential, increased ROS production, and cell death. Further, the inhibition of DGAT1 also leads to decreased melanoma cell proliferation, which is caused by reduced activity of the TOR target S6K. Conversely, over-expression of DGAT1 increases formation of lipid droplets, and protects against cell death induced by both hypoxia and ROS inducing agents, while also increasing cellular proliferation through S6K. Overall these data demonstrate that DGAT1 plays a key and previously unappreciated role in melanoma development and progression.

Targeting the CoREST complex as a novel therapeutic strategy for melanoma and overcoming acquired BRAF inhibitor resistance

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The majority of cancers harbor both genetic and epigenetic alterations, and tumor cells routinely use epigenetic processes to ensure escape from targeted therapies and host immune surveillance. This

knowledge has led to the development of epigenetic agents as anticancer therapies, while the major limitation is the lack of target selectivity and specificity, resulting in a wide range of effects and a narrow therapeutic window. To combat this, we developed Corin, a small molecule inhibitor of CoREST chromatin-modifying complex, derived from a class I HDAC inhibitor (entinostat) and an LSD1 inhibitor (tranylcypromine analog). Cell-based assays revealed that Corin potently inhibited cell growth across a number of melanoma cell lines and outperformed the anti-proliferative effect of its parent HDAC and LSD1 inhibitors. Combination treatment with the parent HDAC and LSD1 inhibitors could not match the anti-proliferative action of Corin, suggesting Corin's unique pharmacologic action depends upon integration of the two inhibitory compounds. Gene expression analysis revealed that Corin was a potent inducer of tumor suppressor genes, many of which have been observed to be epigenetically silenced in cancer. Corin was also effective in slowing tumor growth in a melanoma mouse xenograft model. Finally, combination treatment with Corin and a BRAF inhibitor restored sensitivity to treatment in BRAF inhibitor-resistant melanoma cells and delayed tumor growth in BRAF inhibitor-resistant tumor xenografts. The dual action inhibitor demonstrates a novel, potent, and specific therapeutic approach to targeting epigenetic pathways in human melanoma and may be an important mechanism in overcoming acquired BRAF inhibitor resistance.

A common variant at chr7p21.1 confers tanning response and melanoma risk via regulation of *AHR*

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Genome-wide association studies have identified a melanoma-associated locus on chr7p21.1 near the *AGR3* and *AHR* genes with rs117132860 as the leading SNP ($p = 3.83 \times 10^{-21}$, OR = 0.71 for G allele). Notably rs117132860 is also associated with tanning ability ($p = 7.63 \times 10^{-23}$; OR = 1.30, G-> ease of tanning) and cutaneous squamous cell carcinoma ($p = 3.6 \times 10^{-8}$; OR = 0.68, G allele). As sun-exposure and pigmentation traits are major risk factors for melanoma, we set to determine the mechanisms by which this locus modifies melanoma risk. Bayesian fine-mapping identifies rs117132860 as the strongest candidate causal variant, with risk-associated A allele weakening a consensus AHR binding motif. Luciferase assays for rs117132860 in melanocytes and melanoma cells showed allele-specific transcriptional regulation with lower activity associated with A allele. Five genes are localized in the topologically-associated domain containing rs117132860, each > 200 kb from the SNP. Promoter- Capture-C data from melanocytes suggested an interaction between rs117132860 and a region upstream of *AHR*, and 3C confirmed a specific interaction between the SNP and *AHR* itself. As AHR plays important roles in response to dioxin and UV, we explored potential links between this SNP and *AHR* expression after TCDD or UV exposure. AHR CHIP showed allele-specific AHR binding to rs117132860-G following both UV and TCDD treatment, coinciding with increased *AHR* expression. We are presently knocking out this SNP to assess effects on *AHR* expression with UV/TCDD treatment, as well as its effect on BRAF inhibitor response in melanoma cells, given that AHR has been linked to BRAF inhibitor resistance. In summary, the association of chr7p21.1 with both melanoma risk and tanning response implies the mechanistic link between two processes through AHR and provides a system through which we can develop potential therapeutic targets.

Disappointing real-word experiences of target therapy combining or sequencing with immunotherapy for Chinese BRAF V600E mutated melanoma

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Backgrounds: Target therapy(TT) has been proved to improve outcomes of BRAF mutated melanoma. Immunotherapy(IT) combined with TT can theoretically extend benefits of the latter. However, whether, when or how to transfer either one to another is still unclear in clinical practice. In study, we collected our preliminary real-world experiences of Chinese melanoma.

Methods: Totally 11 patients with BRAF V600E mutation were recruited, 6 patients with M1a and 5 had M1c disease. All of them received Vemurafenib initially. According to different combination patterns, they were divided into three groups. Group A contained 3 patients who started Pembrolizumab after progression. Group B contained 3 patients who started IT once reached best response and stopped TT. Group C contained the other 6 who received both TT and IT at first or once reached best response.

Results: In Group A, the duration of TT was 4–14 months respectively. No response observed in this group after patients changed to IT. All of 3 died in 3 months after progression. In Group B, the duration of initial TT was 4–8 months and all patients received partial response. However, after stopping TT and starting IT, all patients had disease relapse within 1–2 months. Two reached another disease control after re-starting TT. One died after brain metastasis. In Group

C, 2 patients received both two types of therapy for 4–5 months and having ongoing PR. Another 3 added IT with TT once reached PR after 3–5 months. All of 3 then had disease progression only 2 months after combination. In all 11 cases, Grade 3 adverse events occurred with IT involved, mostly with severe pyrexia, rash and one case with hypophysitis.

Conclusion: It seems with quite disappointment outcomes when combining TT and IT simultaneously or sequentially. More efforts should be made to explore the optimal patterns.

Efficacy of anti-PD1 immunotherapy plus Anlotinib on metastatic melanoma: real-world data from Chinese population

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Backgrounds: Metastatic melanoma (MM) in China, especially for acral(AM) and mucosal(MCM) subtype, has relatively lower efficacy of anti-PD1 monotherapy compared to cutaneous subtype (CM), with nearly 15% ORR. Antiangiogenic therapy combined with immunotherapy has been proved to be effective in MCM and renal cancer. This study aimed to summarize the real-world data of MM treated by immunotherapy plus Anlotinib in our center.

Methods: Anlotinib is an orally administered tyrosine kinase inhibitor (TKI), targeting VEGFR, FGFR, PDGFR and c-kit. Patients received immunotherapy of Pembrolizumab 200 mg or Toripalimab 240 mg every 3 weeks, with Anlotinib 12 mg once daily also in 3-week cycles (14 days on, 7 days off) until disease progression or unacceptable toxicity.

Results: 15 patients, 8 males (53.3%) and 7 females, with median age of 55 years old, were recruited in this study. 7(46.7%) were AM, while 2(13.3%) were cutaneous (CM) and 6(40%) were MCM. On baseline, most patients had metastasis more than 3 organs (7, 46.7%) and elevated LDH level (8, 53.3%). Best overall response (BOR) were documented as 1(6.6%) CR, 6(40%) PR, 4(26.7%) SD and 4(26.7%) PD. Therefore, ORR and DCR was 46.6% and 73.3% respectively. Mean PFS was 4.5 months and longest PFS observed reached 8.5 months. 3 patients died, 5 changed to other therapy after PD while 6 patients with ongoing response. Any AE occurred in 66.7% patients including 3 fatigue, 2 myocardial damage, 1 pneumothorax, 1 hypertension, 1 pyrexia, 1 diarrhea and 1 hemorrhage. Only 1 patient with pneumothorax then developed multi-organ failure and died, which might relate to SAE.

Conclusion: Anti-PD1 immunotherapy plus antiangiogenic therapy might be an effective combination to treat metastatic melanoma, especially for AM and MCM. However, further benefit should be investigated through perspective clinical trials.™

Identification of UAP1L1 gene expression as a potential factor for development of melanoma

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Backgrounds: Malignant melanoma (MM) remains the leading cause of skin cancer related death with very poor prognosis. So far, the driven genetic alteration and effective genetic target for treatment of melanoma is still unclear, except for BRAF mutation. From previous bioinformatic study, gene UDP-N-acetylglucosamine pyrophosphorylase-1-like-1 (UAP1L1) was reported to be a critical factor for development of human hepatoma cells. In this study, we aim to analyze the role of UAP1L1 in MM development.

Methods: The expression of UAP1L1 was detected by IHC analyses. UAP1L1 knockdown cell lines (A375, OM431) were constructed and evaluated by qRT-PCR and western blotting. Cell proliferation was investigated by MTT. Cell apoptosis and cycle were detected by flow cytometry. Transwell assay was performed for evaluating cell migration.

Results: Through comparison between 166 melanoma samples and 30 normal control samples, the expression of UAP1L1 was significantly up-regulated in melanoma tissues. With 105 melanoma cases with detail tumor characteristics, it was demonstrated that high UAP1L1 expression was correlated with high risk of lymphatic metastasis and advanced tumor stage. In melanoma cell lines, namely A375, OM431, it was revealed that knockdown of UAP1L1 could significantly inhibit cell proliferation of melanoma cells. The results of flow cytometry showed that UAP1L1 knockdown promoted cell apoptosis, which may be contributed to G2 phase arrest. Finally, it was demonstrated that knockdown of UAP1L1 could inhibit the expression of EMT-related proteins and cell migration ability of melanoma cells.

Conclusion: Collectively, UAP1L1 may act as a tumor promotor for melanoma and knockdown of UAP1L1 could be used as a potential treatment strategy for treating melanoma. Further study will aim to explore the functional pathway for UAP1L1 gene in melanoma.

Modified iliac lymph node basin dissection techniques with inguinal ligament preserved for metastatic acral melanoma

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Backgrounds: Acral melanoma is the major subtype of Chinese melanoma. Groin lymphatic basin is often involved in patients with thick and ulcerated primary tumors at lower extremities. Combined dissection of both inguinal and iliac lymph nodes is frequently required

while high-burden superficial groin metastasis or positive Cloquet's node detected. Conventional ilioinguinal excision has high risk of post-operative hernia complication. In this study, we introduced a modified ilioinguinal dissection with inguinal ligament preserved in clinical practice.

Methods: Our procedure of iliac basin dissection also follows inguinal lymphatic dissection. Fixed Cloquet's nodal metastasis or severe invasion of sub-ligamental structure is contradiction of preserving the ligament. Excision is made along the transition line of external oblique abdominal muscle downwards till the inguinal ligament. Push the peritoneum towards midline and expose the iliac fossa upwards to the bifurcation of common iliac artery. Dissect the external iliac lymph node from lateral towards medial, and the obturator nodes from superior towards inferior. And finally lift up the inguinal ligament to dissect the nodes behind the ligament and surround the inferior epigastric vessels.

Results: We have succeeded to perform this surgical procedure in five cases of metastatic Chinese acral melanoma. Median number of inguinal and iliac nodes dissected were 12 and 6 respectively. Mean operative time was 45 min for whole ilioinguinal dissection. No difference of nodal number, blood loss and operative time between this technique and conventional technique.

Conclusion: Ilioinguinal lymph node dissection with inguinal ligament preserved can be safely performed in clinical practices. Further benefits of prevent post-operative hernia should be demonstrated by large-sample trial and long-term follow-ups.

Cancer stem cells in head and neck metastatic malignant melanoma

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Malignant melanoma (MM) accounts for 60–80% of deaths from skin cancers worldwide. There were 287,723 new cases worldwide in 2018 with Australia (33.6/100,000) and New Zealand (33.3/100,000) having the highest incidence. Tumor recurrence, metastasis and treatment resistance have been attributed to cancer stem cells (CSCs) which have been identified in many cancer types including metastatic MM to the brain. This study identified and characterized CSCs in metastatic head and neck MM (HNmMM) to the regional lymph nodes using induced-pluripotent stem cell (iPSC) markers. Immunohistochemical (IHC) staining performed on 20 HNmMM patient tissue samples demonstrated expression of iPSC markers OCT4, SOX2, KLF4 and c-MYC in all samples while NANOG was expressed at low levels in two samples. Immunofluorescence (IF) staining demonstrated an OCT4+/SOX2+/KLF4+/c-MYC+ CSC subpopulation within the tumor nests (TNs) and another within the peritumoral stroma (PTS) of HNmMM tissues. IF also showed expression of NANOG by some OCT4+/SOX2+/KLF4+/c-MYC+ cells within the TNs in an HNmMM tissue sample that expressed NANOG on IHC staining. *In-situ* hybridization and reverse-transcription quantitative polymerase chain reaction (RT-qPCR) on six HNmMM samples confirmed expression of all five iPSC markers. Western blotting of two primary cell lines derived from two of the 20 HNmMM tissue samples showed expression of SOX2, KLF4 and c-MYC but not OCT4 and NANOG, and these cell lines demonstrated the capacity for *in vitro* tumorsphere formation. This study demonstrates the presence of two putative CSC subpopulations within HNmMM which may be a novel therapeutic target in the treatment of this aggressive cancer.

An ARF6-modulated Calcineurin-NFAT axis is a potential therapeutic target for uveal melanoma metastasis

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Uveal melanoma (UM) is the most common primary ocular malignancy. Currently, there is no effective treatment for metastatic UM largely due to an insufficient understanding of the molecular mechanisms governing metastasis. Activating mutations in Gαq (GNAQ and GNA11) are found in over 90% of uveal melanomas and these mutations drive several oncogenic signaling pathways. We recently discovered that oncogenic GNAQ activates the small GTPase ARF6, which promotes the trafficking of GNAQ from the plasma membrane to cytoplasmic vesicles where oncogenic signaling is enhanced. Although the vast majority of uveal melanomas harbor Gaq mutations, only about 50% of patients develop metastatic disease, which usually occurs in the liver. Therefore, additional molecular pathways must be driving metastasis. Here we show that ARF6 is hyperactivated by either hepatocyte growth factor (HGF) or insulin-like growth factor 1 (IGF1), both of which are thought to play a role in UM invasion and metastasis. We show that ARF6 activation is both necessary and sufficient to promote invasion of UM cells and that the calcineurin-NFAT1 signaling pathway is upregulated in highly invasive UM cells following ectopic expression of constitutively active ARF6 ($ARF6^{Q67L}$). Moreover, the ARF6 effector ASAP1 promotes invasion of UM cells by activating the calcineurin-NFAT1 signaling pathway. Pharmacological inhibitors of calcineurin or the peptide inhibitor of NFAT significantly reduce UM cellular invasion induced by $ARF6^{Q67L}$, HGF, or IGF1. Interestingly, calcineurin inhibitors also reduce cancer cell proliferation in an NFAT-independent manner. This work demonstrates a key role for the calcineurin-NFAT signaling pathway in controlling UM invasion, suggesting that targeting this molecular pathway could potentially be used as a therapeutic strategy to reduce UM metastasis.

Analysis of prognostic factors for metastasis in acral melanomas

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Background: The purpose of current study was to investigate the prognostic factors for metastasis of acral melanomas.

Methods: We retrospectively reviewed all patients with melanoma located on acral sites from 1996–2016. Only patients who had followed-up for at least 3 years were included. We divided total 189 acral melanoma patients into groups of no metastasis, first lymph node metastasis, and first distant metastasis. Demographic and clinicopathological characteristics were obtained. This study followed AJCC 8th edition staging system. The degree of pigmentation of melanoma was also evaluated from amelanotic to heavy pigmentation with the 5-point scales. Analyses were carried out using SPSS Statistics.

Results: The median age was 63 years and 51.3% of patients were male. The number of patients who were diagnosed as melanoma in situ was 46 (24.3%), and that of invasive melanoma without metastasis was 29 (15.3%). Among 114 acral invasive melanomas with metastasis, the first metastasis to regional lymph nodes was predominant (99, 86.8%), whereas the first metastasis to distant organs was detected in only 15 patients (13.2%). Depth of invasion (Breslow thickness), ulceration, and mitotic rate were associated with metastasis with statistical significance. Acral melanomas deeper than T3 stages, ulceration, and higher mitotic rate more than 7 per square mm and melanomas ranging from amelanotic to mild pigmentation were more likely to metastasize to lymph node and other organs than invasive melanomas without metastasis with statistical significance. However, there was no statistically significant difference in age, sex, detail site (nail and volar sites), height, and duration of time to first metastasis among patient groups.

Conclusion: Breslow thickness, ulceration, mitotic rate, and the degree of pigmentation are significant prognostic factors for prediction of metastasis in acral melanomas.

β-catenin-YAP1 axis is critical for the maintenance of CAF phenotype and melanoma growth

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Tumor cells reside in a highly complex and heterogeneous tumor microenvironment (TME), in which cancer-associated fibroblasts (CAFs) function as the signaling center and remodeling machine. β-catenin is a dual-function protein playing critical roles in both cadherin-based cell-cell adhesion and Wnt-signaling-mediated gene expression. Previously, we showed that in vivo targeted ablation of β-catenin in melanoma-associated fibroblasts after melanoma occurrence significantly suppressed tumor growth. However, the mechanisms by which β-catenin regulates CAF phenotypes and melanoma progression remain to be elucidated. To understand the role of β-catenin in human stromal fibroblasts, we used a lentiviral doxycycline-inducible expression system to inhibit β-catenin expression by short hairpin RNA (shRNA). We discovered that β-catenin functions to activate stromal fibroblasts and promote melanoma growth by interacting with Yes-associated protein 1 (YAP1). Co-immunoprecipitation and proximity ligation assay results demonstrated that YAP1 as an important β-catenin-interacting partner in stromal fibroblasts. Mechanistic investigation revealed that YAP1 nuclear translocation is significantly modulated by Wnt/β-catenin activity in fibroblasts. In the absence of YAP1, the extracellular matrix (ECM) remodeling ability of stromal fibroblasts was greatly inhibited, which is consistent with the phenotypes caused by β-catenin deficiency. To assess the tumor-promoting role of β-catenin in fibroblasts, 3D spheroids co-culture was performed. After three-day co-culture, less melanoma cells were observed in melanoma spheroids co-cultured with β-catenin-deficient fibroblasts. Collectively, our data clearly demonstrated a new role of a β-catenin-YAP1 signaling axis in regulating the stimulation and tumor-promoting function of CAFs, ECM remodeling, and cancer cell phenotypes.

Melanotan II induces an aggressive tumor-initiating cell state in human melanoma cells.

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While genetic changes are responsible for the initial events of tumorigenesis, transcriptional reprogramming that occurs in the absence of additional alterations to the genome can drive further progression. Our research aims to monitor the transcriptional state of *BRN2* in melanoma and to determine how the modulators on *BRN2* states may change cell behaviors. We have found a recurrent transcriptional cell state spontaneously oscillating between a *BRN2*-high state and a *BRN2*-low state in multiple melanoma cell lines. We found that the *BRN2*-low state is more proliferative and expresses higher levels of the stem cell markers, SOX10 and PAX3. All *BRN2*-low cell lines consistently express reduced levels of MITF and p16, consistent with a neurocrest stem cell state. We found that a currently widely used cosmetic compound Melanotan II drives the kinetics of the switch to a more aggressive state both *in vitro* and *in vivo*, which is consistent with case reports suggesting that mole size can increase as a side-effect of the use of this compound. Application of this small molecule upregulates SOX10 expression, consistent with its upregulation in the *BRN2*-low state. The result indicates that shifting to a stem cell state may be a prerequisite to drive switching plasticity. Our research explores key facets of cell state plasticity to understand outcomes in melanoma progression following changes in cell state plasticity.

Regulation of 3D genome organization by the STAG2 tumor suppressor in melanoma

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Stromal antigen 2 (STAG2) is a core subunit of the cohesin complex that participates in the DNA looping interactions and facilitates 3D chromatin organization. Recentcancer genomics studies have revealed that inactivating mutations in *STAG2*frequently occur in various cancers. Our laboratory previously discovered loss of STAG2 as a novel genetic mechanism of BRAF inhibitor drug resistance (*Nature Medicine 2016*). More recently, we have combined RNA-Seq, ChIP-Seq and HiChIP analyses to systematically identify transcriptional targets of STAG2 and to characterize its role in the regulation of 3D chromatin organization in melanoma cells. Intriguingly, our analyses reveal Interferon Regulatory Factor 9 (IRF9) as one of the top targets of STAG2 in melanoma. We show that knockdown of STAG2 in melanoma cells led to increased IRF9 mRNA and protein levels, leading to activation of both interferon (IFN) alpha and gamma response pathways. We demonstrate that STAG2 binds to the boundary sites of an insulated neighborhood containing *IRF9*, whereas loss of STAG2 permits the interaction of a distant enhancer to with the *IRF9* promoter. Importantly, Interruption of the distant enhancer by the dCas9-KRAB mediated suppression approach reverses the effect of STAG2 loss on IRF9 expression and IFN signaling. Our study supports a critical role of STAG2 in maintaining the chromatin topology at the *IRF9*locus, which may contribute to the tumor suppressor function of STAG2 through IFN signaling-mediated immune evasion. Our findings also suggest that STAG2 could serve as a predictive biomarker for immune checkpoint blockade therapies.

Phenotype-specific drivers of adaptive resistance to MAPKi in melanomas with BRAF$^{V600E/K}$

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Adaptive resistance to MAPKi signifies the acute signaling alternations that enable melanomas to survive and eventually proliferate during MAPKi. Different melanomas utilize different adaptive resistance pathways, of which notable examples include Akt and cell-adhesion signaling. The diversity of adaptive resistance mechanisms is reflected in the heterogeneous response to BRAF-inhibitor treatment combinations across different melanomas. For instance, melanomas like A375 and COLO858 are exquisitely sensitive to the co-inhibition of BRAF and Src while others such as SKMEL28 and WM2664 are indifferent. To establish a framework that can account for different adaptive resistance mechanisms, we are performing forward genetic screens in a panel of melanoma cell lines against the BRAF/MEK inhibitors encorafenib/binimetinib and the pan-Raf inhibitor LY3009120. The melanomas we are studying differentially express classical transcription factors related to melanocyte lineage or cells of mesenchymal origin. These cell lines also show differences in their drug-induced differentiation trajectory with some becoming more neural-crest like and some becoming more pigmented upon MAPKi. Methodologically, we perform the genetic screens using the *Sleeping Beauty* transposon system. Our preliminary results in four melanoma cell lines suggest that adhesion signaling promoted specifically by VAV1 or MKLN1 represents the dominate mode of escape to MAPKi in melanomas that adopt a neural crest phenotypic state, typified by the upregulation of NGFR. Furthermore, this class of melanomas is vulnerable to the co-inhibition of BRAF and Src.

Minimally invasive adrenalectomy for solitary or non responding melanoma metastases: adrenal as a sanctuary site?

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Surgery for stage IV melanoma remains controversial. However, heterogenous responses to current systemic therapy may leave certain metastatic sites as solitary or non-responsive lesions. Minimally invasive adrenalectomy has facilitated resection of resistant to therapy adrenal metastases, with minimal morbidity and short hospital stay. The adrenal gland may function as a sanctuary site for metastatic growth in spite of appropriate systemic therapy, thus highlighting the importance of surgical metastasectomy in disease control and possible survival advantage.

Between 2014–2019, 15 patients underwent 16 minimally invasive adrenal metastasectomies. Mean time from primary melanoma diagnosis to surgery was 32 months. All patients received either immunotherapy or BRAF inhibition or both prior to surgery. 10 patients had solitary lesions, and 5 had multiple metastasis in which the adrenal was non responsive. Mean operative time was 130 min, and median length of stay was 2 days. There was no operative mortality. At a median follow up of 24 months, 7 patients have no evidence of disease, 6 patients had progressive disease with eventual mortality, while another 2 patients have stable disease with maintenance therapy.

The pattern of response to systemic therapy for melanoma remains unknown. Pulmonary lesions have been associated with a high objective response to treatment, while other lesions show more resistance to treatment, underscoring the contribution of tumor microenvironment to response. While the effect of current therapy on the adrenal microenvironment remains unknown, our data suggests that the adrenal gland may serve as a sanctuary site shielding the adrenal from the effect of contemporary melanoma therapies. Modern minimally invasive techniques have made adrenalectomy a safe option for selected patients, with a satisfactory oncologic outcome.