Society for Melanoma Research 2016 Congress

Efficacy of small MC1R-selective α-MSH analogs as sunless tanning agents that reduce UV-induced DNA damage

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The melanocortin 1 receptor (*MC1R*) is a major determinant of constitutive pigmentation and the tanning response to UV, and is a melanoma susceptibility gene. Additionally, activation of the MC1R on the cell surface of melanocytes, modulates the DNA damage response to UV, resulting in enhanced repair UV-induced DNA damage. Half of all Whites in the U.S. are carriers of a *MC1R* variant, which increases their risk for melanoma. Given the significance of *MC1R* in activating multiple photoprotective mechanisms, we developed tetra- and tripeptide analogs of its agonist α-melanocyte stimulating hormone (α-MSH) by modifications of the His-D-Phe-Arg-Trp, or His-D-Phe-Arg sequence, combined with terminal modifications of the of α-MSH peptide. We tested the efficacy of these analogs in activating the MC1R, as measured by stimulation of cAMP formation and the activity of tyrosinase in primary cultures of human melanocytes (hMC). Based on selectivity for MC1R, lipophilicity and potency, we selected analogs for further testing on hMC and cultured human skin substitutes (hSS). Two tetrapeptides were 100 fold more potent, and three tripeptides were only 10 fold less potent than α-MSH in activating the MC1R on hMC. These peptides also reduced DNA photoproducts and oxidative DNA damage independently of increasing pigmentation. Testing of the two tetrapeptides and one of the tripeptides showed that they enhanced pigmentation of hSS within 4 days of treatment, and treatment of hSS for 10–15 days enhanced repair of DNA photoproducts in melanocytes and the entire epidermis, without affecting the histology or melanocyte number. The unique properties of our small α-MSH analogs can potentially allow for their topical application to induce sunless tanning and enhance DNA repair in melanocytes, thus reduce the risk for melanoma in high risk individuals.

Tissue-specific CRISPR in zebrafish identifies *PVRL1* as a novel metastasis gene in melanoma

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Despite tremendous progress in the treatment of melanoma, the prognosis of patients with metastatic disease remains poor. Understanding the genetic determinants of the metastatic process is key. Next generation sequencing methods have uncovered recurrent genetic alterations in cancer but their functional relevance needs assessment. In this study, we focused on a frequent deletion on chr11q23.3 which contains a gene, *PVRL1*, homozygously lost in 5% of human melanomas. In order to identify new genetic modifiers of melanoma, we developed a vector system based on the CRISPR/Cas9 technology of genome editing that allows us to inactivate genes specifically in the melanocytes of a zebrafish melanoma model. As a proof of principle, we injected a vector targeting *pten* in Tg(*mitfa:BRAFV600E*), *p53*$^{-/-}$ zebrafish embryos and showed that it significantly accelerated tumor onset compared to a control vector. Using this system, *pvrl1* inactivation also accelerated tumor onset compared to control (median: 18 versus 20 weeks). Deep sequencing of the CRISPR sites in tumors showed frameshift insertions or deletions. Since PVRL1 is a membrane protein involved in adherens junctions, we reasoned that its loss could affect the metastatic potential of melanoma cells. Indeed overexpression of *PVRL1* in human melanoma cell lines increased cell aggregation. We designed a transplantation assay in which 300 000 primary zebrafish melanoma cells are injected subcutaneously along the dorsal line of irradiated Casper recipients. In this assay, *pvrl1* CRISPR melanoma cells robustly migrated away from the injection site while control cells did not. These results establish *PVRL1* as a new metastasis gene in melanoma. Our method can be easily extended to the functional evaluation of entire regions of the genome frequently deleted in human cancers, and provides a powerful means to uncover new tumor-suppressor genes *in vivo.*

Targeting CDK4 in melanoma: mechanisms of resistance and novel combinational therapies

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Activation of the Cyclin-Dependent Kinase 4 (CDK4) pathway is a frequent occurrence in cancer and is a major risk factor for the development of melanoma. Activation of this pathway occurs mostly as a result of mutations in the CDKN2A locus that encodes for p16, which is a potent inhibitor of CDK4. The frequency of CDK4 pathway-activating events in melanoma and other cancers has led to the development of specific CDK4 small molecule inhibitors. These inhibitors have shown promising antitumor activity especially in combination with other targeted therapeutics, such as inhibitors of the MAPK/ERK pathway. Despite the substantial advancements in targeted therapeutics, the efficacy of these agents is limited by the emergence of intrinsic or acquired resistance, which remains a major challenge in oncology.

In this project we are focusing on understanding the mechanism underlying acquired resistance to CDK4 inhibitors in melanoma. We have developed several CDK4 inhibitor-resistant cell lines and have assessed changes in both gene and protein expression levels that accompany the emergence of resistance. Furthermore, we have explored the efficacy of different combinational-targeted therapy in overcoming or preventing the development of CDK4 inhibitor resistance. We have identified that increased activation of Cyclin-Dependent Kinase 2 (CDK2) pathway leads to the development of resistance. Furthermore, we have identified several targets whose inhibition helps in overcoming resistance to CDK4 inhibition; these include protein arginine methyltransferase-5 (PRMT5) and RNA polymerase I (POL1).

In summary, the outcome of this research will allow the development of novel combination strategies to overcome or delay the resistance to CDK4 inhibitors and identify predictive biomarkers of response, which will improve the personalization of treatment regimens for melanoma patients.

Identification of unique molecular signatures of differentially cycling tumor cell subpopulations in a 3D melanoma model

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Microenvironment-driven intra-tumoral dynamic heterogeneity is a leading cause of drug resistance acquisition in melanoma. 3D spheroids generated from fluorescent ubiquitination-based cell cycle indicator (FUCCI)-transduced melanoma cell lines revealed two differentially cycling subpopulations within the spheroid: a central G1-arrested and a peripheral proliferating subpopulation. Confocal microscopy of sectioned spheroids showed that expression of the Microphthalmia-associated transcription factor (MITF) exclusively co-localized with the peripheral cycling population. To elucidate the molecular mechanism behind this phenomenon, we isolated cells from each subpopulation by Hoechst dye diffusion and FACS and then confirmed their accurate separation by their respective MITF expression pattern. RNA seq analysis of cells isolated from these two different subpopulations revealed that the melanocyte- and melanoma-specific isoforms of MITF (MITF-M, MITF-Mdel), several upstream and downstream effectors of MITF, DNA repair and cell cycle promoting genes were significantly downregulated in the central G1-arrested compared to the peripheral cycling subpopulation. Pathway enrichment analysis of the RNAseq data suggested that the PI3K-AKT pathway is downregulated and the non-canonical Wnt/β-catenin pathway is upregulated in the central G1-arrested compared to the peripheral cycling subpopulation. Our ongoing studies aim to decipher the PI3K-AKT and non-canonical Wnt/β-catenin pathway driven regulatory mechanism behind the differential expression pattern of MITF in these differentially cycling tumor subpopulations. In addition, we will also investigate the downstream effectors of MITF to understand how differential expression of MITF and its activity in these two subpopulations regulate their segregation within spheroids.

Metabolic consequences of the aged microenvironment in melanoma

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The peak age of diagnosis for melanoma is from 55 to 74 of age, making it a disease of the elderly. Furthermore, older patients (>55 years old) have a poorer prognosis and worse response rates to therapy compared to young (<40 years old). In this study, we investigate the effects of the aged and young microenvironment on the metabolism of melanoma cells. Nutrient availability changes in melanoma cells grown in an aged microenvironment versus a young one. Metabolic fuels, glucose, lipids and amino acids, were measured in melanoma cells in culture of different microenvironments. Melanoma cells in the presence of the aged microenvironment, showed rewiring of their metabolic pathway via increased glucose consumption, hyper activation of the PI3K/AKT/mTORC1 pathway, increased AMPKα and elevated GLUT1R protein levels. Accumulation of lipid droplets was observed in the melanoma cells after being exposed to the aged microenvironment. Extracellular amino acid digestion was increased in melanoma cells in the aged microenvironment compared to the young microenvironment and control, through macropinocytosis. Our data shows how different microenvironments in melanoma cells change their metabolic activity. This study focuses on finding how changes in our body as we aged affect how patients respond to therapy, with the goal of providing a therapeutic window for elder melanoma patients.

High response rates with combined BRAF and autophagy inhibition: results of 2 phase I trials

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Autophagy is a resistance mechanism to BRAF inhibitors that can be targeted with hydroxychloroquine (HCQ). We launched a Phase I trial of vemurafenib and HCQ in *BRAF^{V600}* melanoma patients. 7 patients in the 1st dose level (vemurafenib 960 po bid + HCQ 400 po bid) had 2 dose limiting toxicities (DLT; grade 3 rash and grade 3 transaminitis in 2 different patients) preventing further dose escalation. 6/6 patients evaluable for response had PR or CR (median max target shrinkage -62% [-38- -100%]). Prolonged PFS was seen in 1 CR (30+ months) and 1 PR (20 months). Combined BRAF/MEK inhibition was adopted widely so this trial was closed, and a multi-institution phase I/II trial of dabrafenib (D), trametinib (T) and HCQ was opened. Phase I was completed with no DLT (n = 7). Recommended phase II dose was HCQ 600 bid with D+T. Phase II enrollment continues. D+T+HCQ was well tolerated, with no evidence of visually significant ocular toxicity. Striking responses were observed: 6/7 patients responded and 5/6 patients had a CR. The only non-responder was found to have *BRAF^{V600E}* amplification, and had pyrexia that required frequent dose interruptions. Only 1 of 6 responders has progressed (PFS for responders 7–19 months, ongoing, censored as of July 2016), with brain metastases harboring a *BRAFV600E* and *PIK3CA* mutation after 15 months. A cell line created from this resected metastases showed continued sensitivity to D+T+HCQ *in vitro*. The patient developed progressive CNS disease despite brain radiation and restarted D+T+HCQ, benefiting from second response that continues to date. Patient derived xenografts were created from 4/4 pretreatment tumor biopsies from the 7 patients. A randomized PDX trial with all combinations of treatments is underway with PDX from responding and resistant patients to determine if the addition of HCQ to D+T is significantly contributing to the activity of this regimen.

Treatment with neoadjuvant + adjuvant dabrafenib and trametinib (D+T) is associated with improved relapse-free survival (RFS) versus standard of care (SOC) therapy in patients with high-risk resectable BRAF-mutant melanoma

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The treatment of stage IV melanoma has been revolutionized by targeted therapy and immune checkpoint blockade, and there is a strong rationale to evaluate these agents in earlier stages of disease. The current SOC in patients (pts) with high-risk resectable melanoma (stage IIIB/IIIC) is upfront surgery +/- adjuvant therapy, but relapse rates are high. We hypothesized that treatment with neoadjuvant + adjuvant D+T in this population would result in lower relapse rates compared to SOC.

We conducted a prospective randomized clinical trial (NCT02231775) in pts with resectable Stage IIIB/C or oligometastatic stage IV BRAF-mutant melanoma. Pts were randomized in a 1:2 fashion to SOC (Arm A) or neo + adjuvant D+T (Arm B, 8 weeks neoadjuvant + 44 weeks adjuvant). Planned enrollment was 84 pts. Primary endpoint was RFS.

Randomization was halted after 21 pts were enrolled (arm A = 7, arm B = 14). Arms were well matched for gender and stage of disease, though pts in arm A were younger (median age 46 versus 59, $P = 0.02$). Perioperative complication rates were similar and toxicity in arm B was manageable (27% ≥ grade 3). At week 8 the RECIST response rate with D+T was 77% and the pathologic complete response (pCR) rate was 58%. Early analysis revealed a significantly higher RFS in the D+T arm over SOC (HR 12.96, 95% CI = 11.27–391, $P < 0.0001$), with 6-month survival estimated at 100% in Arm B and 28.6% in Arm A, leading to trial closure.

Treatment with neoadjuvant + adjuvant D+T is well tolerated, results in high clinical response and pCR rates, and markedly improves RFS in pts with high-risk resectable metastatic melanoma. Correlative analyses are underway to characterize mechanisms of response and resistance to neo + adjuvant D+T.

Interim analysis (IA) of a phase 2 multicenter single arm clinical trial to evaluate biodistribution and shedding of talimogene laherparepvec (T-VEC) in patients (pts) with unresected Stages IIIB-IV melanoma

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T-VEC is a modified herpes simplex type-1 virus (HSV-1), first-in-class oncolytic immunotherapy for treatment (tx) of melanoma. This trial evaluates biodistribution, shedding, and risk of T-VEC transmission to close contacts and caregivers (CCC) in 60 pts with unresectable stage IIIB-IV melanoma (NCT02014441). Here we present results of the IA.

T-VEC was injected intralesionally into cutaneous, subcutaneous, and nodal lesions up to 4 $mlx10^6$ pfu/ml (3-w cycle), 3 w later up to 4 $mLx10^8$ pfu/ml every 2 w (2-w cycles) for at least 6 months unless other tx was required or until there were no injectable lesions. T-VEC DNA was evaluated by qPCR in blood, urine, and swabs (injection site, dressing exterior, oral swabs, and suspected herpetic lesions) during tx and 30 days after end of tx (EOT). Presence of infectious virus in swabs was confirmed by cell culture infection assay.

Among 30 pts: 57% men; median age 65 y (range 24–93); 73% HSV seropositive; 13% Stage IIIB, 67% IIIC, 20% IVM1a. Including all time points assessed at data cutoff 23-Feb-2015, T-VEC DNA was detected in blood and urine in 90% and 20% of pts, respectively, with the highest levels on the day of tx during the 2nd cycle, and none after EOT. Infectious T-VEC was detected at the injection site in 4 (13%) pts, all within the 1st w after the day of injection, but never on outside of the occlusive dressing, indicating an effective barrier was established. Of 20 reported suspected herpetic lesions from 11 pts and one caregiver, only one that was adjacent to and under the same dressing as an injected lesion was positive for T-VEC DNA but not infectious virus. Low level of T-VEC DNA but no infectious virus was detected once in 1 pt in an oral swab.

These data suggest low risk of T-VEC transmission from pt to CCC.

Ex vivo and *in vitro* analyses of tumorigenic capabilities of primary melanoma tumour cells and primary melanoma metastatic cells of MeLiM model in comparison with human melanoma cell lines

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Hereditary spontaneous animal tumours appear to be preferred and highly suitable models to study human oncology and cancer therapy. MeLiM pig strain of hereditary melanocytic lesions is characterised by a high tumour incidence and malignant behaviour on the one hand together with the occurrence of spontaneous regression. Most of the highly invasive melanomas regressed spontaneously in the first year of the piglet's life and the regression was followed by hair and skin depigmentation.

We isolated primary cells from progressive and regressive melanoma tumours and from metastasis in lung or lymphatic nodes. These primary cells were tested for melanoma markers and melanoma stem cell markers by immunocytochemistry, Western blot, FACS, and q-PCR. The migration and invasion capabilities were tested by invasion and migration assays. All above mentioned analyses were done also with human melanoma cells lines BLM, G-361, A2058, and heathy keratinocyte cell line HaCaT, as controls.

The final test was done by implantation of selected primary cells into Crl:CD1-Foxn1nu immunodefficient mice. Our experiments suggest that both cell types obtained from progressive and regressive melanoma were not able to form new tumours in Crl: CD1-Foxn1nu mice.

Supported by the Czech Science Foundation project no. GA16-05534S, by the projects no. LO1609 and LQ1605 from the National Program of Sustainability I and II (MEYS CR), and by the project FNUSA-ICRC no. CZ.1.05/1.1.00/02.0123 (OP VaVpI).

A Phase 2 multicenter trial to evaluate efficacy and safety of HF10, oncolytic virus immunotherapy and ipilimumab in patients with unresectable or metastatic melanoma

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HF10 (intratumoral injection) shows activity in injected lesions and non-injected metastatic lesions presumably via an antitumor immune response elicited by viral destruction of injected lesions. An ongoing Phase 2 study of HF10 combined with ipilimumab (ipi) in melanoma pts is assessing whether the antitumor effect of HF10 is enhanced by concurrent ipi treatment. Efficacy and safety of HF10 + ipi treatment are reported herein. An immunologic correlative data analysis is currently ongoing. Ipi naïve adults with stage IIIB, IIIC or IV unresectable melanoma with measurable non-visceral lesion(s) received HF10 injections into single or multiple tumors ($1x10^7$ $TCID_{50}$/ml, up to 5 ml/dose); 4 injections qwk; then up to 15 injections q3 wk. Ipi (4X at 3 mg/kg, IV) was administered per SOC. Tumor responses were assessed at 12, 18, 24 weeks, and 36, 48 weeks for pts continuing HF10 monotherapy. Best Overall Response Rate (BORR) was determined at 24 weeks. Of 46 pts treated, 20% were stage IIIB, 43% stage IIIC, and 37% stage IV. Most HF10-related AEs were ≤G2, similar to HF10 monotherapy. No DLTs were reported; 3 G4 AEs reported, all not treatment related. 30.4% had G3 AEs. HF10-related G3 AEs (n = 3) were left groin pain, thromboembolic event, lymphedema, hypoglycemia, and diarrhea. Of 43 efficacy evaluable pts, preliminary BORR at 24 weeks per irRC was 41.8% (11.6% CR, 30.2% PR), disease stability rate 67.4% (25.6% SD). 8 responders (53%) were stage IV. Overall study BORR, including those after 24 weeks, by irRC was 48.8% (18.6% CR, 30.2% PR), disease stability was 67.4% (18.6% SD). In summary, HF10 + ipi treatment does not appear to exacerbate ipi toxicity, is safe and well tolerated, has both local and systemic antitumor activity, with promising response rates when combined with ipi.

A phase 2 multicenter trial to evaluate efficacy and safety of HF10 oncolytic virus immunotherapy and ipilimumab in patients with unresectable stage IIIB-IV melanoma

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HF10 is a bioselected herpes simplex virus type-1. Intratumoral injection of HF10 shows activity in injected and uninjected metastatic lesions. An ongoing phase 2 trial of HF10 and ipilimumab (ipi) in unresectable metastatic melanoma patients is assessing whether the antitumor effect of HF10 is enhanced by concurrent ipi treatment. Efficacy and safety of HF10 + ipi treatment are reported herein. An immunologic correlative data analysis is currently ongoing.

Ipi naïve adults with Stage IIIB, IIIC or IV unresectable melanoma with measurable non-visceral lesion(s) received HF10 injections into single or multiple tumors ($1x10^7$ $TCID_{50}$/ml, up to 5 ml/dose); 4 injections qwk; then up to 15 injections q3 wk. Ipi infusions (3 mg/kg) were given q3 weeks for 4 doses. Tumor responses were assessed at 12, 18, 24, 36, and 48 weeks. Best Overall Response Rate (BORR) was determined at 24 weeks. Of 46 pts treated, 20% were stage IIIB, 43% stage IIIC, and 37% stage IV. Most HF10-related AEs were ≤G2, similar to HF10 monotherapy. No DLTs were reported; 3 G4 AEs reported, all not treatment related. 30.4% had G3 AEs. HF10-related G3 AEs (n = 3) were left groin pain, thromboembolic event and lymphedema, hypoglycemia, and diarrhea. 21 (46%) pts had received at least 1 prior therapy for metastatic melanoma. Of 43 efficacy evaluable pts, preliminary BORR at 24 weeks per irRC was 41.8% (11.6% CR, 30.2% PR), disease stability rate 67.4% (25.6% SD). 8 responders (53%) were Stage IV. Overall study BORR, including those after 24 weeks, by irRC was 47.7% (15.9% CR, 31.8% PR), disease stability was 65.9% (18.2% SD).

In summary, HF10 + ipi treatment does not appear to exacerbate ipi toxicity, is safe, well tolerated and has both local and systemic antitumor activity, with promising response rates when combined with ipi in patients with prior therapy.

Blood PAX3d, MITFm, TGFβ2 mRNA as useful biomarkers in malignant melanoma

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Malignant melanoma (MM) is the most malignant tumour of skin and mucous membranes mainly due to its tendency to generate early metastases. The earliest the advanced disease can be diagnosed the best results are achieved in terms of treatment median overall survival. Blood-based biomarkers capable of

detecting melanoma patients at risk for developing distant metastases can improve outcomes for melanoma patients. In this study we serially measured circulating transcript copies of three potential biomarkers previously identified by our group, namely PAX3d, MITFm and TGFβ2. Three ml of peripheral blood from 74 MM patients at each time point (baseline, 6 and 12 months) were collected and the respective RNA were tested by multi-marker qRT-PCR assay on LightCycler 480 instrument. MM patients were homogenous for stage categories (I-IV) and gender (53% male and 47% female). 37% of patients with stage IV MM showed relevant high expression level of PAX3d at baseline (P = 0.05). The 19.3% underwent to a relapse during the period of observation, and at baseline the expression of PAX3d in relapsing patients was significantly higher than those not relapsing (P = 0.002). In details, one patient showed the highest level of PAX3d at baseline (14.44 copies/μl) while other 2 patients after an initial decrease, detected at the time of the second draw, showed again a significant raise at the last time point and predicted the following relapses occurrence in these patients. A strong correlation between MTIF transcript and the positivity to the sentinel lymph node was observed (P = 0.009). This results confirmed what we previously hypothesized about PAX3d and its capability to be an early useful prognostic and diagnostic marker to monitor treatment outcome. Furthermore, mean transcript value of PAX3d correlated with stage categories (I-II versus III-IV, P = 0.03) in the 85% of the patients.

Deciphering distinct roles of RASA2 in melanomagenesis

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Melanoma is the deadliest form of human skin cancer. The incidence of melanoma continues to rise. Recent advances in knowledge of melanoma genetics, genomics and biology has led to an optimistic view of the therapeutic outlook for melanoma patients. We analyzed sequence data from >500 melanoma genomes/exomes to identify novel tumor suppressor genes in melanoma. *RASA2* was identified as the most highly somatically mutated novel tumor suppressor gene. *RASA2* was mutated in 5% of melanomas and deleted in an additional 16.4% of cases. RASA2 is a GTPase Activating Protein (GAP) that regulates RAS; which is one of the most highly mutated oncogenes in melanoma but drugs targeting RAS have as yet shown poor efficacy. The role of RASA2 has not been investigated in melanoma. *NF1*, which encodes another RAS- specific GAP, was found to be frequently mutated in melanoma. Interestingly, mutations in *RASA2* and *NF1* co-occur in the same patients with high frequency. We plan to elucidate the roles of RASA2 in melanomagenesis and to understand why RASA2 and NF1 mutations co-occur despite the fact that both proteins are RasGAPs. Ras includes three isoforms: NRas, KRas and HRas. Our preliminary data show that RASA2 is more specific to NRAS and that NF1 is more specific to KRAS and HRAS. This finding highlights the existence of a paradigm of cooperativity in which combined loss of multiple negative regulators (RASA2 and NF1) of the RAS pathway is required for melanoma development. Therefore, this type of enhancement of RAS signaling is possibly selected for in some melanomas. We will apply a proteomic screen using BioID to identify RASA2 and NF1 binding partners to provide insights into the functional effects and consequences of alterations in RASA2 and NF1. We expect that these studies will not only identify the cellular components that contribute to the Ras signaling pathway but will also identify potential novel therapeutic targets.

Efficacy of binimetinib in patients with *NRAS*-mutant melanoma: subgroup analysis of the phase 3 NEMO study

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NEMO is a randomized phase 3 trial of binimetinib (BINI; n = 269) versus dacarbazine (DTIC; n = 133) in patients (pts) with advanced *NRAS*-mutant melanoma. The primary endpoint, progression-free survival (PFS), was (median; 95% CI) 2.8 (2.8–3.6) months with BINI versus 1.5 (1.5–1.7) months with DTIC (hazard ratio [HR; 95% CI], 0.6 [0.5–0.8]; P < 0.001). Elevated lactate dehydrogenase (LDH), advanced stage, multiple organ involvement, visceral disease and poor performance status (PS) are associated with worse outcomes in melanoma. Cox multiple regression modeling and subgroup analyses assessed predictive and prognostic relevance of these variables for PFS. Pts randomized to BINI (45 mg oral BID) or DTIC (1000 mg/m^2 IV Q3W) were stratified by stage (IIIC/M1a/M1b [n = 152] versus M1c [n = 250]), Eastern Cooperative Oncology Group (ECOG) PS (0 [n = 293] versus 1 [n = 109]) and prior immunotherapy (yes [n = 85] versus no [n = 317]). Stratified Cox model results for PFS were consistent with those in the broader melanoma population, although only BINI (HR, 0.6 [0.4–0.8]; P < 0.001) and LDH (HR, 1.1 [1.0–1.1]; P = 0.020 for each 125-IU/L increase) were statistically significant. Point estimates for PFS favored BINI in most subgroup analyses; HRs were 0.5 (0.3–0.7) for elevated LDH (n = 113), 0.5 (0.4–0.7) for stage M1c disease, 0.4 (0.3–0.6) for ≥3 organs involved (n = 199), 0.5 (0.4–0.7) for visceral disease (n = 334) and 1.0 (0.6–1.6) for ECOG PS 1. In subgroups with more favorable prognostic characteristics, HRs

were 0.7 (0.5–0.9) for normal LDH (n = 269), 0.8 (0.5–1.3) for stage IIIC/M1a/M1b disease, 0.9 (0.6–1.3) for ≤2 organs involved (n = 203), 1.3 (0.6–2.6) for no visceral disease (n = 66) and 0.6 (0.4–0.8) for ECOG PS 0. NEMO analyses suggest that BINI treatment is a stronger prognostic factor for improved PFS than other variables evaluated, and provides clinical benefit in pts with unfavorable prognostic characteristics.

Role of interferon g- secreting macrophages in UV-induced melanomagenesis

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Cutaneous malignant melanoma is a complex and highly aggressive form of skin cancer which is known to become rapidly chemo-resistant to currently available therapies. Ultraviolet radiation is a major etiological factor involved in the development of malignant melanoma. Apart from direct DNA damage, UV radiation also causes inflammation and immunosuppression. However, the contribution of these non-mutational and microenvironmental molecular mechanisms to UV induced melanomagenesis remains poorly understood. Interferon-g is a cytokine that is conventionally associated with anti-tumor immunosurveillance mechanisms. However, in our previous studies, we found a microenvironmental 'pro-tumorigenic' role of this cytokine in the context of UV-induced melanomagenesis. In our preliminary observations, dual immunohistochemistry (IHC) with anti-CD68 and anti-IFNg antibodies on single case FFPE sections of human malignant melanoma tissue samples showed presence of tumor-associated macrophages expressing IFNg. We also performed dual IHC on 2 human melanoma tissue microarrays (TMAs). On first TMA, 39 out of 60 cores (65%) and on the second TMA, 32 out of 57 cores (56%) were found to be positive for tumor-associated macrophages expressing IFNg. Flow cytometry analysis of single-cell suspensions from back skins of mice pups UVB-irradiated at post-natal day 1/ PD1 and harvested at PD5 also showed the presence of these IFNg secreting macrophages. Interestingly, flow cytometry analysis of single-cell suspensions from fresh, surgically resected tumors from melanoma patients also showed presence of these IFNg secreting macrophages. Thus, these observations strongly suggest that IFN g – expressing macrophages were present in significant percentages in human melanoma patient samples and thus, may be utilized as microenvironmental biomarkers for prognosis of melanoma.

Clinical predictors of survival with cobimetinib (C) combined with vemurafenib (V): pooled analysis from BRIM7 and coBRIM

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The phase 1b BRIM7 study showed the preliminary safety and efficacy of C+V in patients (pts) with *BRAF*^{V600}-mutated metastatic melanoma. The phase 3 coBRIM study demonstrated that C+V significantly improved progression-free (PFS) and overall survival (OS) versus V. Data from pts receiving first-line C+V (C 60 mg QD, days 1–21 and V 960 mg BID, days 1–28) in both studies (n = 274) were pooled to identify baseline characteristics prognostic for survival. Kaplan-Meier estimates and Cox proportional hazard models were used to evaluate PFS and OS in subgroups defined at baseline by age (<65 versus ≥65 years), ECOG status (0 versus 1), LDH level (normal versus elevated), and disease stage (IIIC/ M1A/ M1B versus M1C). Median PFS (95%CI) and OS (95%CI) in the pooled C+V population were 12.7 months (10.5–14.7) and 23.8 months (21.1–31.8), respectively, versus 7.2 months (5.6–7.6) and 17.4 months (15.0–19.8) with V, as reported previously. Elevated LDH (hazard ratio $[HR]_{PFS}$ = 1.73; 95%CI, 1.28–2.32; HR_{OS} = 2.66; 95%CI, 1.86–3.81) and advanced disease stage (HR_{PFS} = 1.65; 95%CI, 1.21–2.27; HR_{OS} = 2.03; 95% CI, 1.38–2.99) were associated with reduced PFS and OS, while the PFS and OS benefits were similar in pts with ECOG status of 0 versus 1 (HR_{PFS} = 1.23;95%CI, 0.88–1.72; HR_{OS} = 1.09;95%CI, 0.73–1.63). In the pooled C+V population, 51.7% and 28.8% were progression-free at 1y and 2y, while 75.6% and 49.6% were alive at 1y and 2y. Among patients with normal/elevated LDH levels, 61.3/38.9% and 35.4/18.6% were progression-free at 1y and 2y, while 88.8/59.7% and 63.1/32.2% were alive at 1y and 2y. Among pts with non-M1c/M1c disease, 60.8/46.0% and 39.0/22.4% were progression-free at 1y and 2y, while 87.5/68.4% and 63.3/41.4% were alive at 1y and 2y. The combination of C+V showed efficacy in all pt subgroups. In particular, a high proportion of pts with better prognostic factors were alive and disease-free at 1 and 2y.

Management of MEK inhibitor (MEKi) toxicities of binimetinib (BINI) in the NEMO trial

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MEKi toxicities are not well understood in the oncology community. Currently approved MEKi's are combined with BRAF inhibitors, which ameliorates many MEKi toxicities. Management of BINI-related adverse events (AEs) are described for NEMO (NCT01763164), a randomized phase 3 trial of BINI versus dacarbazine that met its primary endpoint, increased progression-free survival in patients (pts) with advanced *NRAS*-mutant melanoma. Per-protocol guidelines for dose modification (interruption or reduction) were provided for ocular disorders, elevated creatine kinase (CK), decreased left ventricular ejection fraction (LVEF), cutaneous reactions, diarrhea, and liver function abnormalities. Guidelines for prophylaxis/management of cutaneous AEs and diarrhea also were provided. One dose reduction for BINI was permitted; pts

requiring additional reduction were discontinued. Doses were not modified for grade 1 AEs; pts received symptomatic treatment. Dose re-escalation was allowed except after dose reduction for decreased LVEF. Treatment interruptions >21 days mandated permanent discontinuation unless the investigator (with sponsor) concluded BINI could be beneficial. Grade 3/4 AEs occurred in 68% of pts; the most common were elevated CK (19.3%), hypertension (7.4%), and rash (4.1%). AEs resulted in permanent discontinuation in 24.5%. Dose modification occurred most frequently in pts with elevated CK (18.2%), rash (9.3%), and acneiform dermatitis (7.1%). Dose modification allowed most pts to remain on BINI, with more dose modifications versus discontinuations for elevated CK (18% versus 2%), rash (9% versus 0.7%), and acneiform dermatitis (7% versus 1.1%). Pts with vascular eye events (2.2%) were permanently discontinued per protocol; no AEs of permanent blindness occurred. The BINI safety profile was consistent with known class effects and manageable with dose modification and supportive care.

Inhibition of CXCR4-CXCL12 chemotaxis by trametinib to prevent melanoma dissemination within the cutaneous microenvironment

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Progression of radial growth phase melanoma to vertical growth phase is associated with unfavourable prognosis for which, the bidirectional interplay of the CXCR4-CXCL12 chemokine axis between the primary tumour and stromal cells within the cutaneous microenvironment may play a key role. Deciphering mechanisms mediating melanoma CXCR4-CXCL12-mediated chemotaxis within the cutaneous microenvironment, the potential for autocrine signalling and cross talk with MAPK activation may reveal novel therapeutic strategies to limit tumour dissemination. To this aim CXCL12 secretion was quantified in supernatants from melanoma cells, primary melanocytes, keratinocytes and fibroblasts, with CXCR4-CXCL12 autocrine cell signalling investigated by co-culture of melanoma cells with a CXCL12 neutralising antibody and evaluation of p-CXCR4 and p-ERK expression. Results revealed significant CXCL12 secretion by melanoma cells and primary dermal fibroblasts and reduced p-CXCR4 and p-ERK expression in melanoma cells cultured with anti CXCL12, collectively suggesting dermal fibroblasts mediate melanoma CXCR4-CXCL12 chemotaxis in the cutaneous microenvironment and that melanoma CXCL12 secretion drives autocrine CXCR4-CXCL12 signalling and activation of MAPK signalling. Transwell chemotaxis assays of $CXCR4^+$ melanoma cells towards CXCL12 rich primary dermal fibroblast supernatant further revealed significantly increased migration of BRAF mutant and wildtype melanoma, the effect of which was prevented by anti-CXCL12, or clinically achievable concentrations of the MEK specific inhibitor, trametinib. Taken together, these data suggest MEK inhibition in wildtype or BRAF/RAS mutant melanoma presents a viable strategy to inhibit autocrine CXCR4-CXCL12-mediated MAPK activation, chemotaxis in the localised cutaneous microenvironment and tumour dissemination from the primary site.

RNF4 ubiquitin ligase regulates melanoma progression and chemo-resistance

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Despite major advances in melanoma therapies, durable response for advanced melanoma remains a challenge, in part due to therapy-resistance. Thus, deciphering new molecular pathways that are crucial for melanoma progression and therapy-resistance are of utmost importance. Ubiquitylation and SUMOylation are post-transcriptional modifications involved in tight regulation of protein turnover and function. Both pathways have been implicated in melanoma tumorigenesis, but their connection in melanoma is unknown. These processes can be linked by RNF4, a SUMO targeted E3 ubiquitin ligase (STUbL), that recognizes SUMOylated proteins and targets them for ubiquitylation. Importantly, analyses of melanoma tumor samples from patients and cultured melanoma models suggest that RNF4 plays a central role in melanoma development as in its chemo-resistance. Analysis of human melanoma biopsies identified that high level of RNF4 (mRNA and protein) correlates with reduced over-all survival ($n = 330$, $P < 0.05$). Using cultured melanoma cells and mouse xenografts confirmed that RNF4 is required for tumor survival and promotes melanoma development. Knockdown of RNF4 in human melanoma cells attenuated cell proliferation, migration and clonogenicity. Likewise, conditionally-induced-targeting of RNF4 blocked vemurafenib-resistant tumor growth *in vivo*. Conversely, conditional expression of RNF4 in melanoma cells promoted their growth, resulting in larger tumors. Taken together, our results establish the importance of RNF4 in melanoma development. The mechanisms underlying RNF4 contribution to melanomagenesis will be discussed.

Unique photodocumentary of evolving childhood Spitzoid melanoma

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A 3-year-old girl presented with a left shin lesion that grew over 6 months, and her mother took serial photographs of its evolution. It was initially thought to be a mollusca (during her concurrent infection), and then a keloid scar. At presentation to dermatology, examination revealed a 1.6 cm red, slightly friable nodule with peripheral scale. Excisional biopsy showed a severely atypical compound Spitzoid melanocytic proliferation, consistent with Spitzoid melanoma. The tumor had 3.5 mm maximum thickness, level IV, no ulceration, mitotic rate of 14/mm2, and no lymphovascular invasion or regression. Sentinel lymph node biopsy including inguinal and external iliac nodal basins showed 4/4 positive nodes. PET-CT imaging was negative, and the patient underwent completion

lymphadenectomy with no evidence of melanoma. She is currently undergoing interferon treatment.
Amelanotic melanomas are commonly mistaken for benign nevi, pyogenic granulomas, warts, and dermatofibromas, which can lead to delayed diagnosis and worse prognosis. While an uncommon variant in adults, a significant number of pediatric melanomas are amelanotic. Pediatric melanomas are commonly symmetrical, <6 mm, with regular borders and uniform color, and modified ABCD criteria for childhood melanoma has been proposed.
The photodocumentary that this patient's mother provided, showing changes over several months, helped in evaluation of a rare challenging tumor. Home photography provides far more information than can be obtained in office visits alone, and has previously been utilized to determine the time course of superficial infantile hemangioma growth. We endorse home photodocumentary as a useful tool for both research and clinical practice – it can inform diagnosis and management of pediatric dermatologic conditions, and images may also be useful to understand disease and counsel future patients.

Inhibition of age-related melanoma progression and therapy resistance by rosiglitazone-mediated induction of Klotho

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Malignant melanoma is highly aggressive and has a much greater incidence and far poorer outcome in older patients. Older melanoma patients are also more susceptible to therapy resistance. Therefore, it is crucial to understand the molecular mechanisms underlying age-related melanoma aggressiveness and therapy resistance for the identification of novel targets and treatment options suitable for older patients. Klotho is a secreted molecule, with levels decreasing dramatically with the progression of age. Klotho is also reported to inhibit tumor progression. We hypothesize that this decrease in Klotho levels in the ageing tumor microenvironment impacts melanoma tumor growth and therapy resistance. We have previously shown that exogenous klotho can inhibit the internalization and signaling of Wnt5A, a driver of metastasis in melanoma which has also been shown to promote resistance to targeted therapy. Increasing Klotho levels in the aged microenvironment may therefore provide an effective strategy to overcome both metastasis and therapy resistance. Peroxisome proliferator-activated receptor gamma (PPARg), also known as the glitazone receptor, regulates Klotho expression. In this study, we show that glitazones increase klotho in aged fibroblasts. Our results also demonstrate that Rosiglitazone, a FDA approved anti-diabetic drug and a PPARg agonist, increases klotho in mouse serum and reduces tumor burden of BRAF-resistant tumors in aged mice, but not young mice. Thus, use of glitazones as an adjuvant therapy for melanoma may provide a new treatment strategy for patients over the age of 50 who have developed resistance to Vemurafenib.

Text message reminders to increase skin self-examinations

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Skin self-examination (SSE) is important for the early detection of melanoma. Our study investigated whether cell phone text message reminders, sent weekly and monthly, increased the rate of SSE in patients at risk for skin cancer. The secondary aim was to identify additional variables that may mediate adherence to SSE.
The study group contained 105 subjects (mean age = 46 ± 14 years) recruited from Emory melanoma clinics. Most subjects were Caucasian (98%), male (69%), married (69%), employed (64%), and had a history of melanoma (74%). Forty-three subjects reported performing complete SSEs at baseline (42%) and 59 subjects performed partial SSEs at baseline (57%). Overall, subjects examined 4.7(±2.6) out of seven body parts at baseline. There was no significant difference in demographics or SSE compliance between the intervention and control groups (P = 0.24–0.91).
Thirty subjects in the intervention group and 25 subjects in the control group completed the study; 50 total were lost to follow-up. Subjects in the texting group reported examining significantly more body parts at follow-up compared to baseline (4.4 ± 2.5 versus 5.2 ± 2.5, P = 0.049), which trended toward significance when compared to the improvement seen in the non-texting group (P = 0.05). Subjects who received text messages also reported greater increase in awareness (P = 0.01) and cancer worry (P = 0.0494) than controls. Logistic regression analyses demonstrated an increased self-efficacy (confidence in one's ability to perform SSE), response efficacy (confidence in utility of SSE), skin cancer awareness, knowledge, and worry.
We concluded that text messaging reminders are an effective method to increase the number of body parts examined in SSE and to optimize mediators of adherence to SSE. Although text messaging as an intervention is no longer avant-guarde within the changing landscape of social media, newer technology (e.g. apps) can utilize lessons from our study.

GMPR is a novel MITF target that mediates suppression of melanoma invasion

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Metastatic melanoma is a highly invasive type of cancer. It has been established that depletion of microphthalmia-associated transcription factor (MITF) induces melanoma cells invasion albeit *via* not fully elucidated mechanisms. Through gain- and loss-of-function experiments in melanoma cells, we demonstrate that MITF suppresses intracellular GTP levels leading to the decreased amounts of active (GTP-bound) RHO-GTPase proteins, suppresses invadopodia formation, and ultimately, reduces invasion. These phenotypes stem from the direct MITF-dependent transcriptional activation of the gene encoding for guanosine monophosphate reductase (*GMPR*). Accordingly, *GMPR* transactivation is necessary for inhibition of melanoma cell tumorigenicity and lung colonization by MITF. Additionally, we establish that the increased invasion of $BRAF^{V600E}$ melanoma cells resistant to vemurafenib treatment is due to a downregulation of GMPR levels, achieved in the course of vemurafenib selection. Our data identify a novel mechanism connecting MITF-mediated regulation of invasion, increased

invasion in therapy-refractory melanoma cells, and regulation of GTP biosynthesis.

Leptomeningeal disease (LMD) from metastatic melanoma: a single institution experience and predictors of survival

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Several studies have demonstrated that the presence of LMD correlates with short overall survival (OS) in metastatic melanoma (MM) patients (pts) with central nervous system (CNS) metastasis. However, identifying predictors of outcomes in pts with LMD has been limited by the relatively small number of such pts in most CNS cohort studies. Thus, we reviewed the clinical features, treatments, and OS of MM pts diagnosed with LMD by CSF cytology or radiographic findings from 2000 to 2015. Cox proportional hazard regression analysis was performed in order to identify factors significantly associated with OS. We identified 178 pts with LMD. Median age at diagnosis was 51 and 74% of pts were male. 39% had elevated LDH, and most patients had concomitant brain (76%) and extracranial (74%) metastases. 101 pts underwent mutation testing, and 38% had a BRAF mutation. Neurological deficits were reported in 47%, and 48% had positive CSF cytology. Treatments received after LMD diagnosis included intrathecal (IT) (38%) therapy, chemotherapy (51%), immunotherapy (IMT) (7%), and targeted therapy (34%). Median OS from LMD diagnosis was 2.7 months, and 1-, 3-, and 5-year OS rates were 22%, 9%, and 9%, respectively. Positive CSF cytology (HR 2.26, CI 1.27–4.00, P = 0.006), presence of neurological deficits (HR 2.21, CI 1.60–3.05, P < 0.0001), uncontrolled systemic disease (HR = 1.68; CI1.16–2.44, P-0.006) and elevated LDH (HR 1.44, CI 1.04–2.00 P = 0.03) were associated with shorter OS. Median OS was 8.2 months for pts treated with targeted therapy, 7.1 months for IT, 4.7 months for chemotherapy, and 3.7 months for IMT. These results provide candidate prognostic markers for analysis in other MM cohorts, and for the appropriate design and interpretation of future trials for pts with LMD.

Copy number alteration (CNA) analysis of melanoma primaries based on next generation sequencing

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Detailed copy number analysis of small, formalin-fixed paraffin embedded (FFPE) melanoma has not been feasible to date because of the limited quantity and poor quality of extracted DNA. We have attempted to remedy this through Next Generation Sequencing. We have examined CNA of primary melanoma FFPE samples from the Leeds Melanoma Cohort, investigating a range of preparation techniques for obtaining reliable DNA.

We identified all participants (with primary melanoma Breslow thickness ≥ 0.75 mm) who had died from melanoma (at the time of the study) and, as a comparison, participants who had survived for at least 5 years from diagnosis and whose Breslow thickness distribution was similar to that of the deceased group (total of 868 participants). Primary tumour was available for 46% of participants from the pathology laboratory. A 0.6 mm diameter tissue microarray needle was used to sample each tumour consistently and DNA extracted. With respect to DNA input, 93% of samples with <1000 ng of DNA produced libraries as compared to 65% if the input was only 20 ng or less.

A total of 333 NGS libraries were sequenced on an Illumina GAII or HiSeq sequencer to produce >100 bp paired-end reads (either 5 or 1 per lane). DNA reads were aligned and mapped achieving approximately 1x coverage; the number of reads falling within each 10 kb window was adjusted for GC content and mappability. Replicate samples showed high reproducibility. Focused analysis of the *CDKN2A* region yielded high quality, biological plausible data revealing multiple copy number events. 67% of samples showed no CNA across the 4 Mb region covering *CDKN2A* while a small proportion of CNAs did not involve *CDKN2A*. In total 4 common distinct patterns were observed. Copy number loss ranged from 20 kb to > 4 Mb. A germline CNA could reliably be identified.

Funded by Worldwide Cancer Research and Cancer Research UK.

Evaluation of pembrolizumab in the Dutch expanded access program

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Before pembrolizumab was approved for advanced melanoma in Europe, an expanded access program (EAP) was conducted. We present the data from the Dutch EAP in terms of clinical efficacy and safety. Patients could participate in the EAP (July 2014 till Aug 2015) if they progressed on standard treatment with ipilimumab, and when indicated a BRAF/MEK inhibitor. To be eligible patients should have an ECOG performance score of 0 or 1 and no active brain metastases. Pembrolizumab 2 mg/kg was administered intravenously every 3 weeks in the EAP. Data was collected for 152 patients of the 190 patients that were enrolled in the EAP in 6 centers. Median age was 58 years, 30.9% of patients was ≥65 years. Most patients had stage M1c melanoma (77.3%). Forty patients were known with brain metastasis at baseline (26.7%). Median amount of courses was 6 (1–34) and a median FU duration of 15 months. Objective response occurred in 37/146 patients (25.3%), with nine complete responses. Twelve-month overall survival (OS) was 54% (CI 95% 45.6; 62.4%), median OS was not reached. Baseline LDH level, C-reactive protein and ECOG performance status were significant prognostic factors for OS; (LDH normal (n = 81) versus elevated (n = 55): median OS 16.3 versus 10.5 months, (C-reactive protein normal(n = 40) versus elevated (n = 70): median OS not reached versus 10.0 months and ECOG score 0 (n = 72) versus 1 (n = 56) versus 2 (n = 15): median OS 18.3 versus 11.7 versus 3.6 months). Baseline fifty-five treatment-related adverse events were reported in 152 patients, grade 3 or 4 adverse events occurred in 9 patients (5.9%). In this 'real world' cohort of advanced melanoma patients objective responses were seen in 25.3% of the patients. This is comparable with the efficacy of pembrolizumab seen in the Keynote trials and other cohort evaluations of the EAP. The treatment was well tolerated, which is illustrated by the low number of (serious) adverse events.

(Neo-)adjuvant ipilimumab + nivolumab (IPI + NIVO) in palpable stage 3 melanoma – the OpACIN trial

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IPI+NIVO induces high response rates and improved overall survival in late stage melanoma. T cell checkpoint inhibition is of greatest value at the moment of TCR triggering and therefore depends on the amount of antigen present, arguing for that adjuvant immunotherapy will work most efficiently, when initiated prior to surgery.

Two-arm Phase 1b feasibility trial consisting of 20 high risk AJCC stage 3B/C melanoma patients with palpable nodal disease receiving the combination of IPI 3 mg/kg and NIVO 1 mg/kg, either adjuvant four courses after surgery, or split neo-adjuvant and adjuvant.

To date, 17 patients are evaluable (9 neoadjuvant; updated data and first analyses of melanoma specific T cell responses will be presented.). Neo-adjuvant application of IPI+NIVO was feasible and no surgery-associated adverse events were attributed to (neo-)adjuvant therapy.

15/17 patients (88%) had to stop earlier due to grade 3/4 toxicities.

ORR in the neo-adjuvant IPI+NIVO arm was 78% (3 pCR, 3 near pCRs [minimal remaining micrometastasis], 1 pPR [remaining metastasis of 0.5 mm], 1 SD and 1 PD).

So far, post-surgery, none of the responders in the neoadjuvant arm has relapsed. Relapse was observed for 1 neoadjuvant SD patient and for 3 patients within the adjuvant arm.

The combination of IPI+NIVO in the (neo-)adjuvant treatment setting for high risk stage 3 melanoma patients is feasible. However, severe grade 3/4 toxicity was more frequent than expected from stage 4 melanoma patient study data. In parallel, response rate and depth of response also may be higher than in stage 4 melanoma patients.

These results indicate that IPI+NIVO is a promising combination for neo-adjuvant treatment in stage 3 melanoma, which will be tested in adjusted schemes in the upcoming phase 2 OpACIN-neo trial, with the aim of preserving efficacy, but reducing toxicity.

Impact of baseline serum lactate dehydrogenase (LDH) concentration on efficacy in the KEYNOTE-006 study of pembrolizumab versus ipilimumab

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An elevated baseline serum LDH concentration is a known poor prognostic factor for advanced melanoma and is associated with poor treatment outcomes. We assessed outcomes by baseline LDH level in the KEYNOTE-006 study of pembrolizumab versus ipilimumab for advanced melanoma (NCT01866319). 834 patients (pts) were randomized 1:1:1 to pembrolizumab 10 mg/kg Q2W or Q3W for 2 y or ipilimumab 3 mg/kg Q3W for 4 doses. Response was assessed per RECIST v1.1 by independent central review. Elevated LDH was defined as >1 × ULN. The pembro arms were pooled for this analysis. Baseline LDH was elevated in 179 of 556 (32%) pts in the pembrolizumab arms and 91 of 278 (33%) pts in the ipilimumab arm. ORR was higher with pembrolizumab compared with ipilimumab for both normal (40% versus 17%) and elevated (28% versus 7%) LDH. Duration of response was the same regardless of baseline LDH with pembrolizumab (median [range] NR [8–99+ weeks] for normal, NR [11–99+ weeks] for elevated) and ipilimumab (median [range] NR [5+ to 97+ weeks] for normal, NR [27+ to 103+ for elevated]). PFS was improved with pembrolizumab over ipilimumab for both normal (HR 0.64 [95% CI 0.52–0.80], median 7.0 versus 2.9 months) and elevated (HR 0.55 [95% CI 0.40–0.74], median 2.8 versus 2.5 months) LDH. Similar results were seen for OS (normal LDH: HR 0.77 [95% CI 0.57–1.02], median NR in either group; elevated LDH: HR 0.55 [95% CI 0.40–0.75], median 14.7 versus 6.2 months). Although PFS and OS are shorter and ORR lower in pts with elevated versus normal LDH, pts with elevated LDH can achieve durable responses. Pembrolizumab was associated with improved efficacy over ipilimumab in advanced melanoma regardless of baseline serum LDH concentration.

Correlation between baseline parameters and overall survival in patients with advanced melanoma treated with ipilimumab

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Ipilimumab (ipi) has revolutionized treatment options for patients (pts) with advanced melanoma. Recently, we proposed a framework consisting of 7 parameters (PMs) describing different interactions between cancer and the immune system (the 'cancer immunogram'). Using Kaplan-Meier- and Cox-regression-analysis correlations between 6 of these PMs at baseline and overall survival (OS) were investigated in a single

center cohort of pts treated with 3 mg/kg ipi. PMs assessed were: LDH (≤2xULN versus >2xULN), erythrocyte sedimentation rate (ESR,≤ULN versus >ULN), absolute lymphocyte count (ALC, <1000/mm^3 versus ≥1000/mm^3) and IHC on tumor material for CD8+ T-cell infiltration (<median versus ≥median), PDL1 expression (<1% versus ≥1% positive cells) and MHC-class I expression (loss versus weak/positive). We analyzed a series of 179 pts treated with ipi at the NKI between May 2010 and June 2013. Mean FU was 13.9 months, range 0.3–71.1. Median age was 55 years (19–88) and 61% of pts were male. Data were available as follows: in 177 cases LDH, 159 ALC/ESR, 118 PDL1, 99 CD8 staining and 68 MHC-class I. Median OS of the cohort was 7.1 months. In univariate analysis LDH, ALC, ESR and CD8 were significant for OS ($P < 0.01$). PDL1 and MHC expressions were not significant. Pts with all 4 favorable PMs had a median OS of 25.1 (95%CI 0–59.1), while pts with 3 PMs 11.4 months (95%CI 3.3–19.5), 2 PMs 7.9 months (95%CI 3.8–12.1), 1 PMs 4.8 months (95%CI 0–9.9), and no favorable PM a median OS of 1.7 months (95%CI 0–4.5). In multivariate analysis LDH (HR3.8 95%CI 1.6–8.9) and ESR (HR2.4 95%CI 1.1–5.0) were the only independent PMs. Pts with a normal LDH and ESR had a median OS of 24.4 (95%CI 21.5–27.4) versus 2.5 months (95%CI 0.8–4.3) for pts that did not ($P < 0.01$). In conclusion, low values of baseline LDH and ESR are the strongest PMs associated with a favorable outcome in pts with advanced melanoma, treated with ipi.

Peptide vaccine with resiquimod for patients with resected melanoma: a pilot study

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A vaccine that could expand melanoma-specific T cells might reduce the risk of recurrence of high risk melanoma following curative-intent surgery. We tested the safety and immunogenicity of a vaccine coupling a melanoma-associated peptide with a xenogenic peptide (to promote epitope spreading) and/or topical resiquimod (to activate antigen-presenting cells).
HLA-A2-positive patients with stage II, III, IV melanoma with no evidence of disease were assigned sequentially to treatment on one of three schedules. All patients received 3 doses of the melanoma-associated antigen peptide MART-1a mixed with Montanide in a subcutaneous vaccine. Patients on Schedule 1 also received the xenoantigen peptide Gag267-274, patients on Schedule 2 received topical resiquimod, and patients on Schedule 3 received both Gag267-274 and resiquimod. Pre- and post-treatment blood samples were tested for frequency of antigen-specific T cells via tetramer assay, as well as immune cell subtypes and plasma cytokine levels.
Patients enrolled from October, 2012 to December, 2014, with 10 patients enrolling to each schedule. No grade 3 or higher adverse events were noted. The most common grade 2 adverse effects were injection site reaction (7 patients) and hypersensitivity reaction (2 patients). Tetramer analysis revealed antigen-specific responses (defined as doubling of MART-1a-specific T cells from pre-treatment to post-treatment) in 2 of 10, 6 of 10, and 4 of 10 patients treated on Schedules 1, 2, and 3, respectively.
Vaccine treatment consisting of MART-1a peptide, Gag267-274, Montanide, and topical resiquimod was generally well-tolerated. The addition of the Gag267-274 xenoantigen was not associated with an increase in the response to MARt-1a, whereas the addition of topical resiquimod was associated with a numerical increase in MART-1a-specific T cell responses.

Specific elimination of invasive and multidrug-resistant melanoma by a novel anti-AXL antibody drug conjugate

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AXL is overexpressed in many types of cancer, including melanoma, and is associated with EMT and increased invasiveness of tumors. AXL is also upregulated upon resistance to a variety of therapies including the oft-used BRAF and MEK inhibitors.
Drug resistance and upregulation of AXL occur concurrently with the downregulation of MITF. Moreover, due to the high intra-tumoral heterogeneity in melanoma, AXL-expressing, drug-resistant cells can already be detected prior to therapy resistance. Thus, we hypothesize that targeting AXL-expressing melanoma cells may specifically eliminate invasive and drug-resistant cancer cells, whereas the MITF-high melanoma cells can be targeted with MAPK inhibitors.
To this end, we developed a novel antibody drug conjugate: HuMax-AXL-ADC, consisting of an AXL antibody coupled to the microtubule-disrupting agent MMAE. We show that HuMax-AXL-ADC has promising tumor-eliminating effects in AXL-expressing melanoma cells both *in vitro* and *in vivo*, which include a variety of patient-derived melanoma xenografts. Also, we show that combining HuMax-AXL-ADC with BRAF inhibitors yields superior anti-tumor effects compared to either single agent alone. Importantly, through immunohistochemical analysis of 42 human melanoma biopsies we can detect AXL-expressing cells prior to targeted therapy and find that they are enriched in the majority of BRAF inhibitor resistant melanomas.
These findings suggest that drug-resistant melanoma fractions, whether present prior to therapy or upon acquisition of resistance, can be effectively targeted with HuMax-AXL-ADC. Furthermore, combining HuMax-AXL-ADC with MAPK inhibitors may prevent or delay onset of resistance to targeted therapies by specifically eliminating drug-resistant clones in heterogeneous melanomas.

In situ evaluation of microdissected tumor infiltrating lymphocytes (TILs): remarks to the current morphologic classification of the TILs patterns

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Although the morphological classification of tumor-infiltrating lymphocytes (TILs) in melanoma into brisk, non-brisk and absent patterns is considered an independent prognostic factor, controversy exists on the real impact of TILs as *in-situ* immunohistochemistry (IHC) has shown a mixture of phenotypes among TILs, and as very few studies have examined their *in-situ* functional status. Such an evaluation might however be valuable to stratify patients for immunotherapy.
qPCR, proteomics and IHC were performed after microdissection of intratumoral and extratumoral lymphocyte clusters from frozen cutaneous metastatic melanomas with

different TILs-patterns (10 brisk, 10 non-brisk, 3 absent) collected from the Dept. of Pathology, University Hospitals Leuven. Markers of T lymphocyte activation (INF-gamma, OX-40, CD40L) and inhibition (PD-1, PD-L1, TIM3, LAG3) were studied at transcriptomic and proteomic level.

According to their functional status, two types of TILs clusters could be identified, i.e. one with high expression of activation markers and low expression of inhibition markers, and the other with low expression of activation markers and high expression of inhibition markers. Surprisingly, clusters of predominantly exhausted T cells were found both in melanomas with a 'brisk' and with a 'non brisk' TILs-pattern, indicating that the 'brisk' pattern of TILs may harbor exhausted T-cells, and that different clusters of TILs in 'non-brisk' melanomas may vary in activation status, thereby suggesting functional heterogeneity in the local immune response in 'non-brisk' melanomas.

Our data indicate that, in addition to morphological classification and immunophenotypic characterization of TILs, their functional evaluation may identify new subgroups of morphological TILs-patterns and thereby carry added value in prognostication.

Prevention and early detection of melanoma

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Skin awareness campaigns aimed at reducing sun exposure and promoting early diagnosis of skin cancer have been carried out in many western countries. However, the incidence of skin cancer continues to rise. Euromelanoma is a dermatologist–led campaign carried out annually in over 30 European countries. During a Euromelanoma Screening Day anyone can have a skin examination by a dermatologist and participants are counseled about sun smart behavior, skin protection and warning signs of skin cancers. Finland participated for the first time in 2015. Campaign materials were delivered via various media, cancer and skin societies and there were free check-ups in five major cities. All skin examination appointments were booked within hours. 985 individuals were examined during 1 day. Clinical diagnoses of 19 melanomas, 14 basal cell cancers, one squamous cell cancer and 32 actinic keratoses were made. 2016 campaign results will be presented at the Society for Melanoma Research Meeting.

The impact of organized screening by total-body skin examination for asymptomatic individuals on melanoma morbidity and mortality is not well studied. Large-scale public campaigns, screening programs and retrospective studies have provided valuable data but randomized screening trials are scarce. Due to relatively low melanoma incidence and mortality rates in most western countries, randomized controlled screening trials would take a large population, long follow-up time and large funding. There is some evidence that screen-detected melanomas are thinner but whether it translates into reduction in mortality remains to be seen. The current evidence is insufficient to support organized screening for general public. Screening focused on high-risk populations would probably be more effective. The effect of increased public awareness on skin protection and early detection of skin cancers remains almost impossible to measure and motivates future campaigns.

A common intronic variant of *PARP1* confers melanoma risk and mediates melanocyte growth and senescence escape via regulation of *MITF*

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Prior genome-wide association studies have identified a melanoma-associated locus on chr1q42.1 that encompasses a ~100 kb region spanning the *PARP1* gene. Unique amongst melanoma GWAS loci, the protective alle at this locus is also associated with poor melanoma outcome. Expression quantitative trait (eQTL) and allele-specific expression analyses in multiple tissue types, including melanoma cell lines, melanoma tumors, as well as sun-exposed and sun-protected skin, consistently demonstrated that the 1q42.1 melanoma risk allele is correlated with higher *PARP1* levels. *In silico* fine-mapping and functional validation identified a common intronic insertion/deleltion variant, rs144361550 (-/GGGCCC, minor allele frequency = 0.33 in Europeans), that drives allele-specific transcriptional activity. A proteomic screen identified RECQ1 as an allele-specific binding protein that modulates *PARP1* expression. In human primary melanocytes, expression of PARP1 promotes cell proliferation and rescues $BRAF^{V600E}$-induced senescence phenotypes. PARP1 expression also transforms *TERT*-immortalized melanocytes expressing $BRAF^{V600E}$. PARP1-mediated senescence rescue occurs through activation of *MITF* via histone modulation of the MITF-M promoter in a PARylation-independent manner. Our data provide a molecular mechanism for the biological underpinnings of a common melanoma susceptibility locus and elucidates a novel role for PARP1 in regulation of a key melanoma oncogene.

Predictors of toxicity associated with combination BRAF/MEK inhibition

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Activating BRAF mutations are present in ~50% of advanced cutaneous melanomas. For pts who possess this mutation, combined BRAF/MEK inhibition is an effective treatment option and though responses can be durable in a subset of pts, toxicity can be considerable.

We retrospectively reviewed 2 cohorts of BRAF-mut pts treated with BRAF/ MEK inhibition. The 1st cohort was comprised of pts treated at Massachusetts General Hospital (MGH) with commercially available drugs and the 2nd of pts enrolled at MGH and Dana Farber Cancer Institute (DFCI) on protocol 10-056, a ph I/II study assessing the safety and efficacy of combination therapy. In total, 76 pts treated between 2010 and 2015 were evaluated to determine the rate of toxicity and the factors predictive of treatment limiting toxicity (TLT) with TLT defined as an event that forced dose interruption or reduction. A multivariable logistic regression model was fit to identify predictors such as age, gender, ECOG status, LDH level and line of therapy.

Of the 76 pts, 31 were treated in the commercial drug cohort (CC) and 45 in the trial cohort (TC). Baseline functional limitation

and extent of disease was more pronounced in the CC as 23% had an ECOG of 2 and 58% had CNS disease versus 0% and 20% in the TC, respectively. However, the rates of TLTs were similar: 64.5% (95% CI 45–81) in the CC and 69% (95% CI 53–82) in the TC. Age at which therapy was started was predictive as pts ≥55 years were 4x more likely to experience a TLT (OR: 3.8, 95% CI 1.2–12.2, P = 0.02). The presence of CNS disease, advanced ECOG status and LDH level did not correlate with TLT occurrence.

This study demonstrates the toxicity associated with BRAF-targeted therapy and the significance of age on development of TLT. As melanoma treatment continues to evolve with use of combinations and now the emergence of triple therapies, the identification of pts vulnerable to toxicity will be paramount.

Stromal neuregulin-1 modulates the response to MEK inhibitors in WT BRAF/WT NRAS (WT/WT) melanomas

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MEK-ERK1/2 signaling is elevated in the majority of WT/WT melanomas. However, the activity of MEK inhibitors in WT/WT metastatic melanoma (MM) patients (pts) is quite low. In order to determine the potential contribution of the tumor microenvironment (TME) to resistance, we tested the effects of five growth factors on WT/WT melanoma growth in the presence of the MEK inhibitor (MEKi) trametinib. Minimal effect on cell growth by the growth factors was observed in the absence of MEKi. However, neuregulin-1 (NRG1) protected multiple WT/WT melanoma cells from growth inhibition by MEKi treatment. Reverse Phase Protein Array analysis implicated adaptive activation of ErbB2 in protection from MEKi, and ErbB3 or ErbB2 blocking antibodies blocked the protective effects of NRG1. Conditioned medium from fibroblasts and cancer-associated fibroblasts (CAF) contained NRG1 and activated ErbB3/ErbB2 signaling. ErbB3 and ErbB2 antibodies prevented the protective effects of fibroblast- and CAF-derived NRG1 *in vitro*, and they co-operated with MEK inhibitors to block xenograft growth *in vivo*. Finally, analysis of the TCGA RNA seq data from WT/WT melanomas and a transcription factor database (MotifMap) predicted regulation of ErbB3 expression by SOX10. Knockdown of SOX10 in WT/WT melanoma cell lines reduced ErbB3 expression and NRG1 effects on cell growth. Together our results provide a rationale for the treatment of WT/WT melanomas expressing SOX10 and NRG1 with the combination of MEK inhibitors and ErbB3/ErbB2 antibodies.

GDF6-induced BMP signaling reawakens a neural crest identity in melanoma to prevent cell death and differentiation

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It has been known for some time that melanoma cells express neural crest markers, but the importance of this expression and the means of activating these markers have been poorly understood. Recently, studies in zebrafish have shown that neural crest gene expression is found in the cell of origin of melanomas, indicating that a fundamental change in cell identity occurs at the earliest stages of tumor onset. Whether this change facilitates tumor progression has not yet been established. In comparative oncogenomic studies to uncover new melanoma genes, we identified the recurrently amplified BMP ligand gene *GDF6*. GDF6 protein is not expressed in normal melanocytes but is present in nearly 80% of melanomas, where its level of expression correlates with patient survival. In knockdown and gain-of-function studies, we found that *GDF6* promotes melanoma initiation and outgrowth. Expression analyses found that *GDF6*-induced BMP signaling maintains a trunk neural crest identity in melanomas. In maintaining this identity, *GDF6* induces SMAD1/5/8 phosphorylation, and phospho-SMAD1/5/8 directly bind to and repress transcription of *MITF* to prevent differentiation of melanoma cells. *GDF6* also represses *SOX9* expression. Melanoma cells undergo apoptosis upon *GDF6* knockdown, and this cell death can be abrogated by concomitant knockdown of *SOX9*. During embryogenesis *GDF6* is expressed in the neural crest, where it regulates normal melanocyte development. Our study uncovers an important role for *GDF6* and BMP signaling in reawakening an embryonic cell identity to promote melanoma progression and provides new opportunities for targeted therapy of GDF6-positive cancers.

Targeting melanoma metastasis by modulating tumor-associated macrophages at lymphovascular niches

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Current therapies against melanoma focus primarily on two main strategies: (i) targeting tumor cells based on their genetic fingerprint, or (ii) deactivation of intrinsic mechanisms of protection against immune recognition. Unfortunately, responses to these treatments are either transient or limited to a restricted subset of melanoma patients. Therefore, there is still the need to find alternative strategies to attack tumor cells and their microenvironment. Tumor-associated macrophages (TAMs) are an attractive target for their long-reported, but still poorly understood impact in melanoma development. First, TAMs support tumor progression by secreting anti-inflammatory and pro-tumorigenic cytokines. Moreover, TAMs have also been described to contribute to pathologic lymphangiogenesis, a key event in tumor cell dissemination and metastasis. Nevertheless, macrophages are highly dynamic cells, and can shift phenotypic profiles towards anti-tumoral cell populations. Mechanisms that modulate this macrophage polarization, particularly at lymphovascular sites are unclear. Here, we present a thorough study of melanoma-associated macrophages and their role on melanoma metastasis and lymphangiogenesis. Using mouse models engineered to monitor neo-lymphangiogenesis and metastatic dissemination, and analyzing the proteome of melanoma cells we have identified a novel class of melanoma-secreted factors that control the homing and polarization of macrophages. Furthermore, we have identified a nanoparticle-based compound that can re-educate TAM and block both their pro-tumorigenic and pro-lymphangiogenic activity *in vivo*. Synergistic effects with immne checkpoint blockers will also be presented.

Altered mitochondrial activity in uveal melanoma cells with monosomy3

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Uveal melanoma (UM) is the most frequent primary intraocular cancer in adults and its metastasis occur preferentially in liver with very poor prognosis. The most important genetic alterations associated with poor prognosis in UM are *BAP1* gene alterations and loss of an entire copy of Chromosome 3 (monosomy3), which are most often concurrent, *BAP1* is encoded on chromosome 3. It is unknown whether monosomy 3 and *BAP1* alteration are independent or interdependent mechanisms.

As BAP1 has been speculated to play a role in mitochondrial metabolism, we hypothesized that BAP1 alteration or monosomy3 would lead to aberrant mitochondrial metabolism in human UM cells. A mitochondrial Oxidative Phosphorylation inhibitor, IACS-1131, developed at MD Anderson Cancer Center, was used to treat cells with or without *BAP1* alteration/monosomy3, results show that the three cell lines with monosomy3 were more resistant to this inhibitor when compared with three lines with normal chromosome 3 copy number. This resistance vanishes under hypoxic conditions or in galactose media, when oxidative phosphorylation is not the predominant form of sugar metabolism. Further studies with mitochondria show more active mitochondria and a larger mitochondrial reserve capacity in monosomy3 cells. Re-expression of wild type and nuclear localized BAP1 failed to rescue these altered mitochondrial properties.

In conclusion our results show that UM cells with monosomy3 are more resistant to mitochondrial oxidative phosphorylation inhibitor due to larger mitochondrial reserve capacity. We are now in the process of elucidating the underlying molecular basis that can account for this difference in monosomy3 containing UM cells. Genes/proteins responsible for this resistance can be developed as a target for combination therapy with the oxidative phosphorylation inhibitor, IACS-1131.

Transcriptome analysis of primary melanoma cell cultures reveals heterogeneity between patients and treatments

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Melanoma is a highly heterogeneous cancer that often becomes resistant to therapy. Although the majority of driver mutations reside in the MAPK pathway, patients with the same mutation do not respond similarly. Patients with a BRAFV600E generally received a specific BRAF inhibitor but response can be very variable, thus there might be other factors that influence response to therapy. Patients with an NRAS mutation can receive a MEK inhibitor under a clinical trial setting but also have variable response. To assess this heterogeneity, we have sequenced 92 primary melanoma cell cultures from 56 patients undergoing various kinase inhibitor treatments and immunotherapy. 67 of the samples had a BRAF V600E mutation and 28 had an NRAS Q61 mutation and 3 had a cKIT mutation. Preliminary analysis of this dataset revealed no specific mutation clustering for BRAF V600 and NRAS Q61 mutated patients. Multiple samples from a single patient generally clustered together but in some cases the samples from a single patient were vastly different. This analysis suggests that patient specific gene expression has a greater impact that treatment specific gene expression changes.

Safety results from an expanded access protocol (EAP) of talimogene laherparepvec (T-VEC) for patients (pts) with unresected, stage IIIB–IVM1c melanoma in the United States

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T-VEC is a genetically modified herpes simplex virus-1-based oncolytic immunotherapy for local treatment (tx) of unresectable sub-/cutaneous and nodal tumors in pts with melanoma recurrent after initial surgery. The EAP was proposed to allow T-VEC access until US FDA approval. Here, we report results of the EAP.

Intralesional T-VEC dosing: ≤4 ml 10^6 PFU/ml at day 1, then ≤4 ml 10^8 PFU/ml 21 days later, then every 14 days. Tx continued until complete response, no injectable tumors, progressive disease (PD), intolerance, or US FDA approval. Adverse events (AEs) were graded during and 30 days after the end of tx by CTCAE v3. Suspected herpetic lesions were tested for T-VEC DNA by qPCR.

Between Sep 2014 and Oct 2015, 41 pts were enrolled at stages: IIIB (22%), IIIC (37%), IVM1a (34%), IVM1b (5%) and IVM1c (2%). Pts were: 68% ECOG performance status (PS) 0, 32% PS 1, 54% male, and median age 72 (range 32–96) years. Median tx duration was 13 (range 3–41) weeks. T-VEC was used as 1st (51%), 2nd (17%), or ≥3rd (32%) line of tx. AEs occurring in ≥25% of pts were fever, chills, fatigue, influenza-like illness, and myalgia. Three (7.3%) pts had grade 3 T-VEC-related AEs; no AEs of grade >3 occurred. One death occurred 44 days after last dose and was attributed to PD. Most common concomitant medications were paracetamol (acetaminophen), diphenhydramine, and ondansetron, used in 49%, 27%, and 22% of pts, respectively; most use occurred in the first 3 tx cycles. Suspected herpetic lesions were reported and swabbed in 5 (12%) pts. Suspected herpetic lesions not injected with T-VEC all tested negative for T-VEC.

In the clinical practice setting, T-VEC has a safety profile comparable to previously reported clinical trials. T-VEC (IMLYGIC®) is now approved in the US and EU.

Interim analysis of a randomized, open-label phase 2 study of talimogene laherparepvec (T) and ipilimumab (I) versus I alone in unresected, stage IIIB-IV melanoma

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T is a herpes simplex virus 1-based oncolytic immunotherapy designed to selectively replicate in tumors, produce GM-CSF and stimulate antitumor immune responses. I (anti-CTLA-4 Ab) blocks inhibition of antitumor T-cells. Both T and I monotherapy are approved in the US and EU for the treatment of advanced melanoma.

The primary endpoint for the phase 2 part was ORR by immune-related response criteria. Key secondary endpoints are safety, progression-free survival, time to response, duration of response, and survival. Key entry criteria are unresectable stage IIIB-IV melanoma, with ≤2 prior tx, measurable/injectable tumor(s), and no symptomatic autoimmunity or clinically significant immunosuppression. T was given ≤4 ml x 10^6 plaque forming units (PFU)/ml on d1 w1; ≤4 ml x 10^8 PFU/ml d1 w4, then q2w in arm 1 until no injectable tumors, disease progression, or intolerance. I started with the 3rd dose of T in arm 1 or alone in arm 2 at 3 mg/kg IV q3w x 4. An interim analysis (IA) for efficacy was performed when 82 patients (pts) had ≥48 w of follow up.

173 pts were randomized: 88 T+I; 85 I. Characteristics for all pts were similar: 54% stage IIIB-IVM1a, 45% IVM1b/c. Median follow up time for 82 pts in the efficacy set was 61.2 w (range: 0.14–113.9). Confirmed ORR was 35.7% (T+I) and 17.5% (I); unconfirmed ORR was 50% (T+I) and 27.5% (I). Of 165 pts in the safety set (85 T+I, 80 I), most common adverse events (AEs) for T+I, I (%) were fatigue (52, 39), chills (51, 3), diarrhea (39, 34), pyrexia (39, 8), rash (39, 31) and pruritus (38, 35). 20% T+I and 18% I pts had grade 3/4 tx-related AE. A grade 5 autoimmune hepatitis occurred in the T+I arm (investigator attributed to I).

ORR was higher for T+I versus I alone at this IA. AEs were comparable between arms except for increased fatigue, chills, and pyrexia in the T+I arm.

A mutation in the *Cdon* gene potentiates congenital nevus development mediated by $NRAS^{Q61K}$

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Congenital nevi develop before birth, and sometimes cover large areas of the body. They are presumed to arise from the acquisition of a gene mutation in an embryonic melanocyte that becomes trapped in the dermis during development. To better understand the development and biology of congenital nevi we have used a mouse model carrying an $NRAS^{Q61K}$ mutation in all melanocytes. These transgenics develop melanocytic lesions ostensibly identical histologically to many human congenital nevi. The murine lesions start to form at post-natal day 10, and by day 40 are fully formed. The lesions emanate from melanocytes escaping hair follicles. We combined the nevus-prone transgenics with the Collaborative Cross (CC), a resource of ~100's of genetically diverse mouse strains that enable rapid mapping of quantitative trait loci and is specifically designed to permit the discovery of genes for complex diseases. We examined variation in nevus cell density in 70 CC strains and mapped a large effect quantitative trait locus controlling nevus cell density to murine chromosome 9. We stratified candidate genes within the interval by cataloguing DNA variants that vary between the susceptible and resistant strains. This revealed a very strong candidate gene (*Cdon*) carrying a missense mutation. *Cdon* is a positive regulator of sonic hedgehog (Shh). No genome wide association studies have been performed to help us understand the susceptibility to the development of congenital nevi. For the first time, we utilized a systems genetics approach to define innate variation that dramatically influences the density of congenital nevi. Understanding genes that control the development of these lesions will be a first step in the development of new treatments and control measures for these lesions.

Simultaneous identification of candidate melanoma risk variants using massively parallel reporter assay

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Recent melanoma Genome-Wide Association Study (GWAS) has identified 20 common susceptibility loci via meta-analysis of >15 000 cutaneous malignant melanoma cases. While GWAS is extremely powerful in identifying genomic regions contributing to melanoma risk, teasing out functional variants and unraveling molecular mechanisms by which the risk is conferred presents a tremendous challenge due to limited resolution of genetic structure and lack of high-throughput assay system. To simultaneously identify functional risk variants from multiple GWAS loci, we employed recently developed Massively-Parallel Reporter Assays. Based on the hypothesis that many GWAS functional variants regulate gene expression through transcriptional mechanism, we systematically examined allele-specific transcriptional activities of genetically indistinguishable candidate variants. Out of >2800 melanoma-associated variants (R^2 > 0.4, EUR) from 16 melanoma loci for which transcriptional mechanism could be applied 835 variants were prioritized based on their relevance as melanocyte-/melanoma-specific cis-element based on ENCODE annotation as open chromatin or putative promoter/enhancer histone marks in melanocytes and melanoma cell lines. Pooled oligoes of 145 bp encompassing each variant with either melanoma risk or protective allele coupled with 10 different unique sequence tags were cloned into luciferase vectors with or without minimal TATA promoter and transfected into melanoma cell lines. Resulting expressed tag counts were subsequently measured using massively parallel sequencing to compare allelic transcriptional activity. The results from this analysis will greatly accelerate the identification of functional melanoma risk variants and further shed light on molecular mechanisms of genetic susceptibility of melanoma in population.

Co-targeting HGF-cMET signaling with MEK inhibitors in metastatic uveal melanoma

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Metastatic uveal melanoma (UM) patients usually die within 1 year and the liver is the most predominant site of metastasis. MEK inhibitors show only modest efficacy in UM patients, emphasizing an urgent need to develop new treatment strategies. Previously, we demonstrated that HGF-cMET signaling provides resistance to MEK inhibitors in metastatic UM. In this study, we investigated the mechanisms of HGF-driven resistance to MEK inhibitors and including dependency on phosphoinositide 3-kinase (PI3K) isoforms. We also tested new clinical grade cMET inhibitors for their capacity to revert the resistance mediated by HGF. RNA and phospho-proteomic profiling were carried out in metastatic UM cells to implicate a role of Bcl2 family members in trametinib-mediated apoptosis and protection by HGF. We determined that two BH3-only family proteins and pro-apoptotic factors, Bim-EL and BMF, are transiently upregulated in trametinib-treated cells, but then returned to basal levels upon HGF treatment. The PI3Kβ-sparing inhibitor GDC0032 effectively blocks HGF-induced AKT phosphorylation and resistance to trametinib in growth assays, indicating that activation of PI3K$\alpha/\gamma/\delta$ isoforms mediates resistance to trametinib. Targeting HGF-cMET signaling with LY2875358, a neutralizing and internalizing anti-cMET bivalent antibody, and LY2801653, a dual cMET/RON inhibitor reversed resistance to trametinib by exogenous HGF and by conditioned medium from primary hepatic stellate cells. Co-treatment of metastatic UM explants with LY2801653 and trametinib decreased AKT phosphorylation and promoted pro-apoptotic PARP cleavage in ex vivo explants from metastatic UM. Together, our data support the strategy for selective blocking of cMET signaling or PI3K$\alpha/\gamma/\delta$ isoform activities to antagonize HGF-mediated resistance to MEK inhibitors and intrinsic resistance to MEK inhibitors provided by stromal cells in the liver.

Skin lesion analysis toward melanoma detection

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We describe the results of a public challenge for automated analysis of dermoscopic images hosted at the 2016 International Symposium on Biomedical Imaging (ISBI). 1279 annotated images were provided, with 900 for training, and 379 as a test set. A subset of 100 from the test set were evaluated for diagnostic classification by 8 dermatologists as a baseline comparison. Participants were invited to submit automated predictions for lesion segmentation, attribute classification, and diagnostic classification.

On the 379 test images, the top performing individual submission yielded 85.5% accuracy, 50.7% sensitivity, 94.1% specificity, 0.637 average precision, 0.804 area-under-curve, and 22.7% specificity when evaluated at the 95% sensitivity binary operating threshold. An average fusion of all participants submissions produced 85%, 45.3%, 94.7%, 0.703, 0.858, and 28%, respectively. A voting fusion produced 86%, 53.3%, 94.1%, 0.71, 0.858, and 31.9%, respectively.

On the subset of 100 test images also evaluated by dermatologists, the top participant result demonstrated 72%, 52%, 92%, 0.839, 0.793, and 18%, respectively. The average fusion of all participants produced 69%, 46%, 92%, 0.869, 0.856, and 58%, respectively. The voting fusion produced 73%, 56%, 90%, 0.863, 0.859, and 24%, respectively.

The 8 expert dermatologists produced average accuracy of 70.5%, sensitivity of 82%, and specificity of 59%. Two dermatologists with the highest accuracy of 76% produced an average sensitivity of 95% (92% and 98%), with corresponding average specificity 57% (60% and 54%).

This is the largest public comparative study of automated melanoma diagnosis in dermoscopic images to date. Results show that automated disease prediction can yield results comparable to the performance of specialists. While preliminary, this study supports the value of automated analysis to augment care and the need for standardized and publicly available benchmarks.

Correlation between toxicity and outcome in melanoma patients (pts) treated with ipilimumab plus nivolumab (ipi/nivo)

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Immune checkpoint inhibitors have become the standard of care for treatment of metastatic melanoma. However immune-related adverse events (irAEs) remain a serious concern. We report our experience investigating the potential correlation between degree of toxicity and progression-free survival (PFS). 74 pts were treated with the combination of ipi/nivo as part of the phase I trial, [NCT01024231], an expanded access protocol [NCT02186249] or with commercially available drugs from Dec. 2009 to Oct. 2015. irAEs were graded according to the CTCAE v4.0 and steroid use was studied as a surrogate for overall toxicity. 69 (93%) pts experienced an irAE of any grade. 39 pts (53%) had a grade 3 irAE and 4 (5%) had a grade 4 irAE. Pts often experienced >1 irAE. Females tended to get more toxicities than males. The median PFS in the patient population was 9 months (range 0–65). The median OS was 16 months (range 3–65). The objective response rate was 55%. 70 pts survived >6 months. There was a statistically significant difference in the PFS in pts who experienced no irAEs when compared with those who had any irAEs (log-rank $P < 0.0001$). Similar findings were seen in the analysis of OS. PFS and OS were also stratified by length of time on steroids. Any steroid requirement at all was associated with a reduced risk of disease progression (log-rank $P = 0.0185$) but the number of days on steroids above the median (23 days) corresponded with an increased risk of progression. Pts treated with the combination of ipi/nivo who received steroids to treat autoimmune toxicity had improved outcomes when compared with those pts who received no steroids, suggesting that pts who have some irAEs from immunotherapy may have improved outcomes. However, a fine balance between autoimmunity and anti-tumor response may be necessary for optimal long-term outcomes.

Clinical impact of a 31-gene expression profile test on guidance of sentinel lymph node biopsy, imaging and oncology referral

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A 31-gene expression profile (GEP) test to predict metastasis risk has been validated with over 900 cutaneous melanoma (CM) tumor samples. The test generates Class 1 (low-risk) or 2 (high-risk) results, which are associated with significantly different 5-year distant metastasis free survival rates (91% versus 57%, resp). 157 dermatologists who attended a national educational dermatology conference were presented with the clinical validity evidence for the 31-GEP test, followed by a description of a 30-year-old male with a thigh lesion that was biopsy confirmed melanoma, without ulceration or atypical features and no family or personal history of skin cancer. They were asked to provide the Breslow thickness (BT; ranging from 0.7–1.5 mm) at which they would recommend sentinel lymph node biopsy (SLNB), oncology referral, or imaging. The majority of respondents (62%, 57%, and 55%, resp) indicated a BT of 1.0 mm as the thickness to manage a patient with each modality, reflecting an adherence to current management guidelines. Respondents were then asked the same question in the context of a Class 1 or Class 2 result. After inclusion of a Class 1 result, responses reflecting the BT inflection points for guiding SLNB, oncology referral and imaging were changed in 23%, 18% and 19% of cases, resp, with risk-appropriate changes (increased BT) of 87%, 83% and 59%. Following addition of a Class 2 outcome to patient characteristics, the initial BT used to guide SLNB, medical oncology referral, and imaging was changed in 47%, 50% and 47% of the responses, resp, with 95%, 84% and 97% of the cases changed in a risk-appropriate direction (decreased BT). The results indicate that the 31-GEP test may have a significant and appropriate impact upon CM patient management while remaining within the context of the established practice guidelines that exist today.

ALK expression and treatment response in distinct subtypes of melanoma

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Anaplastic Lymphoma Kinase (ALK) is an oncogenic kinase which is not typically expressed in melanomas and other cancers, but can be expressed and activated by mechanisms such as gene fusions. *ALK* fusions have not been identified in common cutaneous melanomas, but recently a novel alternate ALK transcript (ALK^{ATI}) was found in 10% of cutaneous melanomas. Exogenous ALK^{ATI} expression in cell lines and one patient harboring ALK^{ATI} responded to ALK inhibitors, but treatment response in patient samples with ALK^{ATI} has not been well studied. Additionally, the prevalence and treatment response of wild-type ALK, ALK fusions, and ALK^{ATI} in patient samples with different melanoma subtypes has not been determined. In this study, we used qPCR and western blotting to characterize ALK expression in 45 melanoma patient-derived xenograft (PDX) tumor models. ALK^{ATI} was expressed in 2/3 acral lentiginous, 2/5 mucosal, 3/13 nodular, 1/14 superficial spreading, and 0/10 unknown primary melanomas, for a total of 8/45 (18%). Interestingly, we saw different patterns of co-expression between ALK^{ATI}, ALK fusions, and wild-type ALK. Two out of the eight tumors co-expressed ALK^{ATI} and wild-type ALK, and one mucosal melanoma co-expressed ALK^{ATI} and an EML4-ALK fusion. Three tumors expressed only wild-type ALK. We treated four ALK^{ATI} cell lines and one wild-type ALK cell line derived from PDX tumors with ALK inhibitors ceritinib and crizotinib, and only the mucosal melanoma cell line co-expressing the EML4-ALK fusion responded. We are currently expanding ALK-expressing PDX models to examine other ALK inhibitors *in vivo*. Our study identified novel patterns of ALK expression across different melanoma subtypes. The finding that not all patient samples harboring ALK^{ATI} responded to currently available inhibitors has strong clinical implications and warrants further investigation into the role of ALK expression in melanomas.

Real world utilization and outcomes of pembrolizumab for the treatment of patients with advanced melanoma in US community oncology practice

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PD-1 inhibitors are promising immunotherapies for the treatment of patients (pts) with unresectable or metastatic melanoma. As the first US FDA approved PD-1 inhibitor, pembrolizumab (pembro) has demonstrated efficacy and safety in clinical trial settings. However, the real world utilization and pt outcomes associated with pembro are unknown. Adult pts with advanced melanoma who received pembro between 9/1/ 2014 and 12/31/ 2015 were identified retrospectively from electronic health records (EHR) of McKesson Specialty Health and chart abstraction, with follow-up through March 31, 2016. Pts in clinical trials were excluded. Overall survival (OS) and progression (physician reported) free survival (PFS) were analyzed from pembro initiation using Kaplan Meier analysis. 168 pts were included in the analysis. Median age at pembro initiation was 66.4 years (range 26.2–93.9); 31.0% had an elevated lactate dehydrogenase (LDH); 24.4% had brain metastases and 78.6% had an ECOG performance status of 0 or 1. The most common pembro discontinuation reason was progression (45.4%) followed by treatment-related toxicity (18.2%). Death occurred in 16.7% of the population. With a median follow-up time of 5.3 months (range 0.03–16.8), median PFS from pembro initiation was 4.3 months (95% CI = 3.0–5.8) and median OS was not reached however, 12-month survival was 64.5% (95% CI = 54.8–72.6) in the overall population. Characteristics predictive of worse survival in multivariable analyses included later line of therapy ($P = 0.0233$ for 3L+), presence of brain metastases ($P = 0.0293$), and elevated LDH ($P = 0.0275$).

The study illustrates results utilizing real world data examining treatment patterns and outcomes in pts treated with pembro. Overall, the efficacy and safety of real world use of pembro for advanced melanoma is consistent with findings from clinical trials.

OrienX010 oncolytic viral therapy in phase Ib trial of intralesional injection in unresected stage IIIC–IV acral melanoma patients

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Acral melanoma was the main subtype in Asia, which was prone to intralymphatic diseases available for intralesional injection. The safety and efficacy of OrienX010, a herpes simplex virus type 1-derived oncolytic immunotherapy with expression of gene encoding human GM-CSF, was tested in phase Ib trial of unresected stage IIIC to IV (M1a/M1b) acral melanoma pts. Phase 1b08 (n = 10) studied safety of OrienX010 (8×10^7 pfu/ml) at $\leq 4 \times 10^8$ pfu q2w according to size of injectable lesions (1–10 cm). ORR was evaluated q12w. Without DLT, the maximum dose would be escalated to $\leq 8 \times 10^8$ pfu q2w in phase 1b09 (n = 10). Treatment continued until DLT, intolerance, or disease progression per Immune Related Response Criteria. DLT was any grade (gr) ≥ 4 adverse event (AE) or gr ≥ 3 immune-related AE within first 6 weeks. From Feb 2014, 12 pts received OrienX010 up to 4×10^8 pfu: 50% stageIIIC, 42% M1a, 8% M1b. Mean injection times were 10 (3–27). Mean size of all injectable lesions was 65.9 mm(18.0- 99.0). AEs were all gr 1/2, pyrexia 66.7%, injection site pain 50%, leucopenia 25%, rash 16.7%, nausea16.7%. No DLTs were reported. ORR was 16.7% (2 PR), SD 41.7%. Time to response was 6–12 weeks, median PFS 12.0 weeks (95%CI 9.3–14.7), and duration of response 24.0 weeks (95%CI 19.3–28.7). OS was not reached. From May 2015, another 11 pts with stage IIIC/M1a were enrolled in dose escalation up to 8×10^8 pfu, with mean injectable size of 77.0 mm (14.0–108.0). A shorter time to regression (4–8 weeks) could be seen. ORR was 27.3% (3 PR); 36.4% had SD. Most AEs were similar. By Jan 2016, the duration of response had reached 24.0 weeks (95%CI 19.3–28.7). OrienX010 has shown its activity in acral melanoma pts without DLTs. Higher dose might result in shorter time to response, and longer duration of response. Phase 2 and combination trial are pending.

Parkinsons disease and melanoma: confirming and reexamining an association

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This study examined an association between melanoma and Parkinson's disease (PD). In phase I, Rochester Epidemiology Project (REP) medical records were used to identify all PD patients in Olmsted County, MN from 1/1/1976-12/31/2013, with 3 age- and sex-matched controls each. Records were reviewed for cases of cutaneous and uveal melanoma. JMP statistical software was used to assess relative risk (RR) of melanoma in PD patients compared to controls. In phase II, all REP cases of melanoma were identified from 1/1/1976-12/31/2014, with one control each. Records were reviewed for PD cases. RR of PD in melanoma patients was calculated, and Kaplan-Meier analysis was performed to determine 35-year cumulative risk of developing PD. RR of death from metastatic melanoma was calculated in melanoma patients without PD compared to those with PD. In phase I, there were 32 melanoma cases in 974 patients with PD compared to 63 melanomas in 2922 controls. PD subjects had a 1.5-fold increased RR of melanoma (CI 1.0–2.3, P = 0.03). Subset analysis suggested a 9-fold RR of uveal melanoma (CI 0.9–86.4, P = 0.05). PD was associated with a significantly younger age of melanoma diagnosis, and in most cases melanoma diagnosis preceded diagnosis of PD (3.7-fold RR of melanoma pre-index date, CI 2.1–6.6, P < 0.0001). In phase II, 43 of 1544 melanoma patients had PD compared to 14 of 1544 controls (3.1-fold RR of PD in melanoma patients, CI 1.7–5.6, P < 0.0001). Kaplan-Meier analysis revealed an increased 35-year cumulative risk of PD in melanoma patients (11%) compared to controls (2%), P < 0.0001. Of all melanoma patients, those without PD had a 10.5-fold increased RR of dying from metastatic melanoma compared to PD patients (CI 1.5–72.2, P = 0.02). This study provides compelling evidence for both counseling melanoma patients regarding PD risk and implementing careful cutaneous and ocular melanoma surveillance in PD patients.

Melanoma cells are sensitized to targeted therapies by inhibition of autophagy

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Early detection of cutaneous melanoma gives good prognosis, but disseminated disease is associated with poor prognosis due to drug resistance. Treatment with BRAF inhibitor vemurafenib has shown promising clinical results but relapses are common. Overexpression of receptor tyrosine kinases have been identified before as resistance mechanisms to vemurafenib. The multi kinase inhibitor crizotinib has been shown to abrogate invasion in uveal melanoma cells and also to induce autophagy in lung cancer. Autophagy has emerged as an important drug resistance mechanism for cancer cells. Therefore, understanding the underlying molecular mechanisms of these drugs can be used to design optimal drug therapy regimes to combat drug resistance. We have demonstrated that the combination of vemurafenib and crizotinib have an additive effect compared to any of the drugs alone in both BRAF V600E mutant melanoma cell line A375 and it's vemurafenib resistant subline A375VR4 using cell proliferation assay. Also, AnnexinV/PI analysis confirmed this combination effect showing an increased apoptosis. Our experiments also revealed that when autophagy was blocked with chloroquine, the cells were further sensitized towards the combination supporting the cyto-protective role of autophagy. We are currently investigating the mechanisms underlying the efficiency of the combination and also studying morphological changes in the cells after crizotinib treatment. Combination therapy regimes seem to be clinically more effective than single treatments. It is therefore important to explore better combination therapies to improve treatment outcome. By addressing these issues, the project aims at contributing to the development of personalized medicine in melanoma management.

Characterization of responders to cobimetinib (C) and vemurafenib (V) in patients (Pts) with *BRAF*-mutated metastatic melanoma (MM) in the coBRIM study

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In the coBRIM study, C+V significantly improved overall response rate, progression-free survival, and overall survival versus placebo + V (P+V) in pts with previously untreated *BRAF*^{V600}-mutated MM. We did an exploratory analysis (data cutoff: August 2015) to characterize pts with complete or partial response (CR/PR) versus stable or progressive disease (SD/PD). Pts were randomly assigned to C+V (n = 247) or P+V (n = 248). In the C+V arm, 171 pts had CR/PR and 64 had SD/PD; in the P+V arm, 124 had CR/PR and 116 had SD/PD. CR/PR pts were less likely than SD/PD pts to have stage M1c disease (58% versus 64% and 51% versus 74% in the C+V and P+V arms, respectively), or have elevated lactate dehydrogenase levels (40% versus 59% and 29% versus 54%). Incidence of common, any grade, adverse events (AEs) was higher in CR/PR pts than SD/PD pts, including rash (47% versus 28%; 43% versus 34%), photosensitivity (43% versus 17%; 23% versus 16%), alopecia (22% versus 6%; 41% versus 21%), diarrhea (66% versus 50%; 37% versus 30%), nausea (47% versus 28%; 23% versus 26%), and fatigue (40% versus 28%; 38% versus 26%). However, the incidence of most common grade ≥3 AEs was similar between CR/PR and SD/PD pts. In both arms, CR/PR pts were more likely to have dose modification for AEs than SD/PD pts but less likely to discontinue because of AEs. Median exposure to C was 602, 370, 128, and 47 d in CR, PR, SD, and PD pts, respectively, in the C+V arm, and median exposure to P was 668, 273, 120, and 49 d in the P+V arm; median exposure to V was 609, 383, 140, and 47 d in the C+V arm, and 675, 273, 122, and 56 d in the P+V arm. Quality of life was similar between CR/PR and SD/PD pts in both arms. Pts with CR/PR had fewer poor prognostic factors, remained on treatment longer, and had a lower treatment discontinuation rate than pts with SD/PD.

Distinct roles of c-Jun N- terminal (JNK) isoforms in melanoma

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Overexpression of c-Jun-N-terminal kinases (JNK) isoform has been found to promote keratinocyte transformation, suggesting an important role of JNK signaling in promoting tumor development. However, it is unclear how different JNK isoforms, especially the ubiquitously expressed JNK1 and JNK2, function in melanoma. Our previous study has found that C116S mutations in both JNK1 and JNK2 rendered them insensitive to the pan-JNK inhibitor JNK-IN-8, without affecting their activity. To delineate the specific roles of JNK1 and JNK2 on melanoma cell proliferation and invasiveness, we expressed WT and C116S mutants in melanoma cell lines and used JNK-IN-8 to enable chemical-genetic dissection of JNK1 and JNK2 activity. We found that JNK-IN-8 dramatically increased colony formation and invasiveness in cells overexpressing the C116S JNK2 mutant compared to cells overexpressing WT JNK1, WT JNK2 and C116S JNK1. When cells stably expressing WT or C116S JNK were subcutaneously implanted into each side flank of immunodeficient mice, we found expression C116S JNK2 upon administration of JNK-IN-8 significantly increased tumorigenicity *in vivo*. In addition, using phospho-cJUN as a reporter of JNK pathway activity, we observed high JNK activation in some human melanoma resistant cells to BRAF inhibitor (BRAFi) compared to paired sensitive cells, mainly due to activation of JNK2. JNK-IN-8 significantly enhanced the response to dabrafenib in resistant cells overexpressing WT JNK1, WT JNK2 and C116S JNK1, but had no effect on C116S JNK2, suggesting JNK2 signaling is also crucial for BRAFi resistance in a subset of melanoma cells. Collectively, our study shows that JNK2 activity is specifically required for melanoma cell proliferation, invasiveness and BRAFi resistance and can be a potential therapeutic target for the treatment of melanoma.

Adjusting overall survival when accounting for treatment switch in the KEYNOTE-002 study of pembrolizumab versus chemotherapy in patients with ipilimumab-refractory advanced melanoma

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In the randomized phase 2 KEYNOTE-002 (NCT01704287) study, pembrolizumab 2 mg/kg and 10 mg/kg Q3W significantly improved PFS versus investigator-choice chemotherapy at the second interim analysis in ipilimumab-refractory advanced melanoma ($P < 0.0001$), with no difference between pembrolizumab doses ($P = 0.44$). Intent-to-treat (ITT) analyses showed a numeric, but not statistically significant, trend toward improved OS with pembrolizumab 2 mg/kg Q3W versus chemotherapy (hazard ratio [HR] = 0.86 (95% confidence interval [CI], 0.67–1.10). Final analysis of the KEYNOTE-002 study will be presented at this year's European Society for Medical Oncology congress. Here, we aim to adjust for the bias introduced by crossover from the chemotherapy to pembrolizumab arms. We applied both the 2-stage method for crossover adjustment to OS, and the rank-preserving structural failure time (RPSFT) correction as prespecified in the statistical analysis plan. The resulting adjusted survival curves for chemotherapy were evaluated against external literature-based evidence. With crossover correction for the pembrolizumab 2 mg/kg arm, the HR was 0.79 (95% CI, 0.50–1.03) for the RPSFT and 0.58 (95% CI, 0.44–0.78) for the 2-stage method. When comparing against external literature-based evidence, the adjusted OS for chemotherapy from the 2-stage method was found to fit best with external historical data. Results of the pembrolizumab 10 mg/kg arm will be included in the presentation. In conclusion, the OS benefit of pembrolizumab

versus chemotherapy using the best-fitting crossover adjustment was statistically significant.

Pro-tumorigenic role of HACE1 in melanoma

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RAC1 is the third most common somatic mutation in melanoma and is considered as an oncogene in this neoplasm. RAC1 functions in several key cellular processes, including cell migration, cell proliferation and survival. The activity of RAC1, small GTPase, is mainly controlled by GAPS, GEFs and E3 ligases. HACE1 – a HECT-domain containing E3-Ubiquitin ligase that interacts preferentially with GTP-bound RAC1 – catalyzes its polyubiquitylation and promotes its degradation. Therefore, by favoring the degradation of RAC1, HACE1 might act as a tumor suppressor, as this has been described in several human cancers. However, the role of HACE1 in melanoma has not been considered so far. In order to characterize how HACE1 functions in melanoma, we studied its role on cellular function. In multiple melanoma cell lines and in primary cells, RNAi-mediated depletion of HACE1 inhibits cell migration and adhesion, whereas HACE1 overexpression enhances colony formation and migration. Analysis of public clinical data, shows that HACE1 expression correlates negatively with the survival of patients with melanoma. In addition, microarray analysis demonstrates that HACE1 affects integrins expression that could explain the effect observed on the migration. This data suggests that HACE1 behaves as an oncogene rather than as a tumor suppressor, in melanoma cells. To confirm this hypothesis, we showed that HACE1 invalidation significantly decreases the number of infiltrating cells in lung colonization compared to control mice. Co-immunoprecipitation assays allowed us to identify fibronectin as a new HACE1 interactor. Our results show that HACE1 favors fibronectin secretion thereby providing a direct molecular link between HACE1 and melanoma cell migration. In summary, our study provides a critical tumorigenic role for HACE1 in melanoma progression and establishes a new role of HACE1 in this process.

The H3 lysine 9 tri-methylation mark denotes early stress-induced drug tolerance in melanoma

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Besides somatic mutations or drug efflux, epigenomic reprogramming leads to acquired drug resistance. We recently have identified early stress-induced multi-drug resistant melanoma cells termed induced drug-tolerant cells (IDTCs). IDTCs were generated over 12 to 15 days using cancer cell lines corresponding to four different cancer types including melanoma, lung, breast, and colon cancer. A common loss of H3K4me3 and H3K27me3 but a gain of the H3K9me3 mark was observed in all cell lines as a distinct response to drug exposure or nutrient starvation in IDTCs. The global increment of the H3K9me3 mark under treatment was confirmed in a xenograft IDTC melanoma model. Histone modifiers, which are specific for the H3K4me3 (KDM5B) and H3K27me3 (EZH2) decrease but increase in H3K9me3 (SETDB1, SETDB2) were altered accordingly in matched paired samples from melanoma patients treated with targeted therapies. The epigenetic changes were reversible upon drug holidays. Microarray and qPCR data confirmed up-regulation of histone methyltransferases and down-regulation of histone demethylase contributing to the accumulation of H3K9me3 in different cancer types including melanoma. Conversely, genome-wide CpG site-specific DNA methylation arrays showed no common changes at the IDTC state due to intrinsic differences of the methylome. This suggests that distinct histone methylation pattern rather than DNA methylation is driving the transition from parent to IDTCs. These epigenetic modifications were perceived not only in melanoma but also in other cancer types. Alterations of histone marks in early IDTCs with a characteristic increment in H3K9me3 is neither exclusive for any particular stress nor cancer type specific but rather a generic response.

Exosomal microRNAs as putative predictive biomarkers for targeted therapy in Stage IV cutaneous malignant melanoma (CMM)-updated results

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MicroRNAs (miRNAs) are short, non-coding RNAs regulating gene expression at the post-transcriptional level. Exosomes are nanometer sized extracellular vesicles secreted by all types of cells. MiRNAs are carried within exosomes as intercellular communication vesicles. MAP-kinase inhibitors (MAPKis) are used to treat patients with metastasized *BRAFV600* mutated CMMs, inducing rapid tumor responses but with limited durable benefit due to resistance. Our aim was to identify candidate predictive biomarkers of treatment response to MAPKi in Stage IV CMM.

Plasma samples were obtained *before* (*BT*) and *during* (*DT*) treatment from 25 patients. Detailed clinical data was collected. Exosomes were isolated from plasma, RNA was extracted and used for miRNA expression profiling using Exiqon PCR Array. Differential expression of the identified miRNAs in repeated sampling was correlated with therapy response and PFS.

Exosomal miR-125b-5p, miR-497-5p, miR-26a-5p and miR-20a-5p *during* MAPKi therapy were correlated with PFS. The median PFS was 288 versus 97 days in the patient groups with high and low miR-125b-levels, respectively (HR = 4.6; P = 0.008). Low expression of miR-497-5p was correlated with median PFS of 13 days versus high expression of miR-497-5p with median PFS of 288 days (HR = 13; P < 0.0001). Analyzing the *fold change*, i.e. *DT/BT-ratio*, of exosomal miRNAs, a high ratio of let-7 g-5p

was associated with longer PFS (227.5 days) while lower *DT/BT ratio* with shorter PFS of 95 days (HR = 2.6; P = 0.02). A lower *DT/BT ratio* of miR-106-5p was related to longer PFS (288 days) while higher *DT/BT ratio* with shorter PFS of 133 days (HR = 0.32; P = 0.02).
MiRNAs were identified in the *during* therapy and in *fold change* analyses that could be predictive for treatment outcome in Stage IV CMM patients receiving MAPKis.

Vemurafenib resistance increases melanoma invasiveness and modulates the tumor microenvironment by MMP-2 upregulation

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The *BRAF*^{V600E} mutation confers constitutive kinase activity and accounts for > 90% of *BRAF* mutations in melanoma. This genetic alteration is a current therapeutic target; however, the antitumorigenic effects of vemurafenib, the BRAF^{V600E} inhibitor, are short-lived and the majority of patients presents tumor relapse in a short period after treatment. Characterization of vemurafenib resistance has been essential to the efficacy of the next generation therapeutic strategies. Herein, we found that acute BRAF inhibition induced a decrease in active MMP-2, MT1-MMP and MMP-9, but did not modulate the metalloproteinase inhibitors TIMP-2 or RECK in naïve melanoma cells. In vemurafenib-resistant melanoma cells, we observed a lower growth rate and an increase in EGFR phosphorylation followed by the recovery of active MMP-2 expression. Furthermore, we found a different profile of MMP inhibitor expression, characterized by TIMP-2 downregulation and RECK upregulation. In the 3D spheroid model, the invasion index of vemurafenib-resistant melanoma cells was more evident than in its non-resistant counterpart. We confirmed this pattern in a matrigel invasion assay and demonstrated that use of a matrix metalloproteinase inhibitor reduced the invasion of vemurafenib resistant melanoma cells but not drug naïve cells. Moreover, we did not observe a delimited group of cells invading the dermis in vemurafenib-resistant melanoma cells present in a reconstructed skin model. Acute vemurafenib treatment induces the disorganization of collagen fibers and consequently, extracellular matrix remodeling, with this pattern observed even after the acquisition of resistance. Altogether, our data suggest that resistance to vemurafenib induces significant changes in the tumor microenvironment mainly by MMP-2 upregulation, with a corresponding increase in cell invasiveness.

Presence of immune cells, low tumor proliferation and wild type *BRAF* mutation status is associated with a favourable clinical outcome in stage III cutaneous melanoma

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The variable prognosis in stage III cutaneous melanoma is partially due to unknown prognostic factors and tumor heterogeneity. Improved prognostic tools are required to define patients with an increased risk of developing metastatic disease who might benefit from adjuvant therapies. The aim was to examine if cellular immune markers in association with tumor proliferation rate and *BRAF* mutation status have an impact on prognosis in stage III melanoma. We have used two cohorts of patients with stage III disease: 23 patients with short survival (≤ 13 months) and 19 patients with long survival (≥ 60 months). Nodal metastases were analyzed for Ki67, CD8 and FOXP3 expression using immunohistochemistry. *BRAF* mutation status was analyzed in a previous study on the same cohort. Low tumor proliferation rate was significantly associated with a better prognosis (P = 0.013). Surprisingly, presence of FOXP3+ T cells was not correlated to adverse clinical outcome. Patients with a combination of high expression of CD8+ and FOXP3+ T cells, low tumor proliferation and *BRAF* wildtype status showed a highly significant trend for a longer survival (P = 0.0005). Presence of at least three of these four markers was found to be an independent favorable prognostic factor (P = 0.012), when adjusting for ulceration and number of lymph node metastases. Proliferation alone remained significant in multivariate analyses (P = 0.013) but with a wider confidence interval. We have demonstrated that presence of immune cells in association with tumor proliferation and *BRAF* mutation status may further contribute to identify stage III melanoma patients with high risk of relapse.

NFIB mediates BRN2 driven melanoma cell migration through the regulation of EZH2 and MITF

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Metastatic melanoma is a complex and heterogeneous disease, which is highly resistant to conventional chemotherapies and radiation. Characterisation of the cellular programs that underpin melanoma growth, invasion and metastasis is crucial for identifying novel therapeutics to treat this disease. Metastatic melanoma is one of the most aggressive cancer subtypes, and the switch that confers the ability of tumour cells to metastasize is poorly understood. Accumulating evidence from both invivo and invitro models suggest that metastatic melanoma contain two distinct subpopulations characterised by an inverse expression profile between the MITF and BRN2 transcription factors. BRN2 has been shown to play a key role in driving increased invasiveness and tumourigenicity of tumour cell populations, however the downstream signalling targets through which it functions remain to be identified. The NF-I genes are a family of four transcription factors that have been shown to be oncogenic drivers in various cancer models, but their function has yet to be investigated within melanoma. Our data investigates the novel function of NFIB in driving a highly

invasive and metastatic phenotype downstream of BRN2 by directly modulating the expression of the epigenetic modifier EZH2, and MITF. Moreover, we show that NFIB colocalises with highly invasive BRN2 populations in both primary and metastatic human melanoma tumours, and that in silico gene expression analysis reveals a correlation between NFIB and an invasive genetic signature. Using cultured melanoma cells and a melanoma sphere model, we are also able to show that mimicking tumour-like conditions such as nutrient deprivation, hypoxia, and invasive melanoma cell conditioned media drives an increase in melanoma cell migration/invasion, with the ability of these cells to respond to these conditions being contingent on this BRN2/NFIB axis.

Testing the functional significance of pigment heterogeneity in melanotic melanomas

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Intratumoral heterogeneity (ITH), defined by the presence of phenotypically-, genetically- and/or epigenetically-distinct cell sub-populations within a tumor, is a feature of most human cancers, including melanoma. ITH has important clinical implications if distinct cancer cell sub-populations differ in their capacities to propagate disease and/or respond to treatments.

During investigation of ITH in melanoma, we found that these tumors are highly heterogeneous for production of melanin pigment. Histologically, 84% of patient melanomas contained melanin and, of these, 91% were comprised of mixtures of cells with low and high pigment content (LPC and HPC, respectively). Similar patterns of melanin pigment heterogeneity were found in mouse and human melanoma cell lines and in patient-derived xenograft (PDX) melanomas.

To test functional consequences of melanin pigment deposition in melanoma cells, we developed a novel method to prospectively separate LPC from HPC melanoma cells using fluorescence-activated cell sorting (FACS). Using this approach, we found that LPC cells are relatively abundant and have enhanced clonogenic potential *in vitro* and tumorigenic potential *in vivo*, compared to HPC cells. Moreover, LPC- but not HPC-derived cells recapitulated the pigment heterogeneity of the original tumor or cell line.

These findings are inconsistent with proposed plastic relationships between pigmented and non-pigmented melanomas cells, and suggest that tumorigenic LPC cell populations must be eradicated in the effective treatment of patients with melanotic melanomas. Future studies defining mechanisms associated with pigment deposition and loss of tumorigenicity in melanoma cells will identify novel opportunities for enhancing melanoma treatment.

Deciphering the interplay between signaling pathways and MITF in melanoma

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The importance of *Microphthalmia*-associated transcription factor (MITF) in melanoma biology is well established. MITF-M is the major isoform expressed in melanoma cells and, unlike its family members, shows a constitutively nuclear staining pattern. Nuclear localization of MITF-M is dependent on four arginines (R214–217) in its DNA binding domain. Consequently, genetic manipulation of this nuclear lcoalization signal (NLS) results in exclusive cytoplasmic localization of the mutant protein. The molecular mechanisms that link signaling and the subcellular localization of MITF-M remain, however, poorly understood. It is thus the scope of this study to identify kinases that target this multifaceted transcription factor and to determine how phosphorylation events govern its nuclear import and/or export. Computational kinase prediction analysis revealed an array of serine sites in MITF-M that may become phosphorylated by specific kinases. Indeed, our findings in human melanoma cell lines confirm that MITF-M is phosphorylated by mitogen-activated protein kinases, mTOR and glycogen synthase kinase 3, among others. Interestingly, the cytoplasmic NLS mutant MITF-M exhibits a phosphorylation pattern distinct from that of its wild type counterpart. Furthermore, the subcellular localization of MITF-M is notably altered upon inhibition of particular kinases. Similarily, alanine mutants mimicking the dephosphorylated state of MITF-M at specific serine residues, such as S73, show a significant effect on its subcellular localization when compared to the wild type protein. Unraveling the phosphorylation pattern of MITF-M in response to pathway inhibitors will ultimately help us to better understand the interplay of signaling networks and MITF in melanoma.

Investigating the impact of PREX1 pathway inhibition in melanoma

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Melanoma is the most lethal form of skin cancer, representing more than 75% of skin cancer-related deaths. Progress in the understanding of the disease has led to the development of targeted and immune therapy, but intrinsic and acquired resistance is problematic. Therefore, there is an urgent need for biomarkers that assess a patient's prognosis and that can predict the response to a given therapy. We have previously shown that mice lacking PREX1, a Rac-specific Rho GTPase guanine nucleotide exchange factor, are resistant to metastasis when crossed to a murine model of melanoma. Furthermore, we showed that inhibition of the GTP exchange protein Rac1 suppresses tumour growth, invasiveness and metastatic spread in NRas-induced melanoma. We are currently investigating the components and interactions within the RAC and mTORC2 pathways in melanoma progression and invasion, and seek to identify prognostic and predictive biomarkers in this pathway. We have validated several antibodies targeting proteins within these pathways and demonstrate that high PREX1 and p4eBP1 expression correlated with shorter overall survival and recurrence-free survival in melanoma patients, which makes these proteins potential new drug targets. In the absence of commercially available PREX1 inhibitors, we turned to indirect inhibition of pathways that lead to PREX1 signalling activation. This therapeutic approach resulted in a long-term survival advantage and reduced tumour burden in a genetically engineered mouse model of melanoma. Moreover, targeting this pathway in human melanoma derived cell lines had a dramatic impact on cellular survival. We will further investigate whether the targeting of pathways upstream of PREX1 activity

exerts this anti-tumour effect in a PREX1 or RAC1 dependent manner, and hypothesise that targeted inhibition of PREX1 may offer a therapeutic opportunity for a selected number of melanoma patients.

New strategies to overcome BRAF resistance in melanoma: extended BRAF or BRAF/MEK inhibition? A pooled analysis

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Despite BRAF/MEK inhibitors trigger early anti-tumor response, the majority of patients develop resistance to the therapy and the question of management beyond BRAF inhibitor (BRAFi) is still unanswered.

We conducted an eletronic search on studies evaluating the management of patients (pts) affected by BRAF-mutant metastatic melanoma (MM) after BRAFi disease progression to investigate the prognostic role of extended BRAF or BRAF/MEK inhibition beyond disease progression.

Four studies were included in the analysis for BRAFi, involving 229 MM patients with progressive diseas (PD). 45% of pts received BRAFi treatment beyond progression (TBP), 55% underwent other therapeutic approaches. Median overall survival was 7.9 months in BRAFi TBP group (4.9–11.6) versus 2.2 months (1.4–3.4). For the evaluation of extended BRAF/MEK inhibition data from CoBrim trial are not available. In Combi-d trial more pts in the dabrafenib–trametinib group than in the dabrafenib-only group continued TBP (19% and versus 16% respectively), while in Combi-v trial 23% of pts in each study group continued to receive study treatment and median duration of post progression study treatment was 1.5 months (0–11) in BRAF/MEK Combo versus 1 month (0–7) in vemurafenib group. Survival data are not mature for these subgroups in both trials.

The prognosis of MM patients after progression on BRAFi is poor. However, subsets of patients treated beyond BRAFi live longer, showing that treatment beyond BRAFi and BRAF/MEK Combo progression should be further explored. The definition of clinical determinants to best select patients to treat with extended BRAF or BRAF/MEK inhibition should include the evaluation of the nature of PD, the clinical response to first line therapy and the identification of prognostic factors, to personalize treatment and choose between different therapeutic options the optimal approach.

Accumulation of amyloid protein is identified in melanoma and may contribute to resistance to targeted therapy

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Protein dysregulation occurs in stressful cellular states (such as cancer) and may lead to protein misfolding and amyloid accumulation. There are a growing number of reports demonstrating that the tumor suppressor p53 can adopt an amyloid fold, and evidence that this may mediate therapeutic resistance in cancer – which may be targeted. There is also evidence that certain drugs (including MEK inhibitors) may contribute to protein dysregulation and amyloid accumulation. Based on this data and the central role of p53 in melanoma, we sought to determine if misfolding of p53 into amyloid aggregates occurs in melanoma, and further sought to test its impact on therapeutic resistance.

To study this, we assessed amyloid content by staining in melanoma tumors (n = 95) and by biophysical methods in cell lines (n = 6). Tumors and cell lines were co-stained for amyloid and p53 co-localization. Given the central role of the BRAF/MEK pathway and its connection to protein homeostasis, we investigated cellular amyloid burden in response to BRAF/MEK inhibition.

In these studies, we found that over two thirds of melanomas were positive for amyloid aggregates (66/95) and that this proportion was increased with disease stage and decreased with patient age. Importantly, these aggregates co-localized with p53 in the majority of melanoma tumors (n = 56) and cell lines tested. We found that upon MEKi in cell lines, p53 and amyloids co-localized, the amyloid burden increased and p53 mediated apoptosis decreased. Using paired pre- and on-treatment tumors, we found that p53 mediated apoptosis was likewise decreased in half of this cohort (8/16).

These studies suggest that amyloid aggregates are pervasive in melanoma, and may contribute to p53 dysfunction and therapeutic resistance. These provocative results need to be validated in larger cohorts.

The role of endothelin 3 during melanoma lung premetastatic niche formation

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A number of studies have shown that there is an intricate crosstalk between primary tumors and future sites of metastases in order to create a suitable microenvironment in the distant organs for the tumorigenic cells. This microenvironment is called the premetastatic niche, and is set to receive disseminating cancer cells, and support the growth of the metastatic colony. Melanoma is a highly metastatic cancer which preferentially establishes secondary lesions in the lungs. Human melanoma initiation is mainly driven by activation of oncogenic protein BRaf and deletion of PTEN gene and has recently been modeled in mouse cell lines. The cytokine Endothelin 3 (Edn3) and its receptor Endothelin receptor b (Ednrb) have been implicated in melanoma metastasis. Our laboratory has created a mouse model (*K5-Edn3*) that overexpresses Edn3. In order to establish whether Edn3 drives melanoma lung premetastatic niche formation we injected 3 different $BRaf^{V600E/+}$;$PTEN^{-/-}$ murine melanoma cell lines (D4M, YUMM1.1 and YUMM1.7) into *K5-Edn3* and control mice. The appearance of bone marrow derived cells (BMDCs) clusters and metastatic cells in the lungs was monitored by immunofluorescence and flow cytometry at different time points of tumor progression. Analysis of the earlier stages of tumor development has demonstrated there are no differences in the numbers of BMDCs clusters and metastatic cells between *K5-Edn3* and control mice. Although none of the three cell lines express Ednrb *in vitro*, upon injection into *K5-Edn3* mice YUMM1.1-derived tumors expressed Ednrb. Interestingly, YUMM1.1 tumors produced into *K5-Edn3* mice were statically larger than the control mice and these tumors were able to metastasize to the lungs. These data suggest that Edn3/Ednrb signaling may be an important player in melanoma progression and the metastatic potential of a $BRaf^{V600E/+}$;$PTEN^{-/-}$ murine melanoma cell line.

Defect in S phase cell cycle checkpoint renders melanoma cells vulnerable to CHK1 inhibitor single-agent treatment *in vitro* and *in vivo*

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CHK1 inhibitors are being investigated as chemosensitising agents with agents that increase replication stress. Here we have investigated the molecular basis of sensitivity to CHK1 inhibitors as single agents in melanoma. We have found that sensitivity *in vitro* and *in vivo* to single agent CHK1 inhibitor is loss of the S phase cell cycle checkpoint response. This is through a number of mechanisms including the uncoupling of CHK1 activation with the destabilisation of CDC25A. Loss of checkpoint by over-expressing components of the checkpoint or inhibition of Wee1, covert CHK1 inhibitor insensitive cells to sensitive, and similarly depletion of CDC25A reduces CHK1 inhibitor sensitivity in sensitive lines. This results in severely elevated replication stress. Loss of the S phase checkpoint provides cells with an adaptive advantage through introduction of moderate levels of genomic instability. The increased DNA damage found with CHK1 inhibitor treatment is not sufficient to induce cell death, but also involves a mechanism that is dependent in part on DNAPK activity. Loss of S phase checkpoint function is predicted for 25% of all melanomas. This is independent of other known risk factors, suggesting a significant proportion of melanoma patients could benefit from treatment with these drugs.

Sarcoidosis-like granulomatous inflammation induced by pembrolizumab

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A 36 year-old woman with advanced melanoma of unknown primary was started on pembrolizumab after progression despite surgical intervention, radiation, interferon alpha-2b, and ipilimumab. PET/CT imaging before therapy demonstrated normal lung parenchyma and no mediastinal or hilar lymphadenopathy. PET/CT imaging performed 5 months into treatment revealed multiple, predominantly upper lobe, bilateral lung nodules and mediastinal and hilar lymphadenopathy. The patient was asymptomatic. Bronchoscopy with hilar and mediastinal fine needle aspiration and broncho-alveolar lavage demonstrated non-necrotizing granulomas with negative cytology and cultures. Repeat PET/CT imaging showed progression of the lung parenchymal changes. Pembrolizumab was held; 7 months after treatment cessation, the patient remained clinically asymptomatic, and chest CT revealed stable mediastinal and hilar lymph nodes and interval improvement of the pulmonary nodules. Ipilimumab, a cytotoxic T-lymphocyte-associated protein 4 inhibitor, was the first immune checkpoint inhibitor associated with a sarcoidosis-like granulomatous reaction. Recently, similar reactions were attributed to nivolumab and pembrolizumab, both anti-programmed death (PD-1) inhibitors, where pulmonary non-caseating granulomas developed that improved after cessation of immunotherapy. Sarcoidosis is a multisystem granulomatous disease made up of a predominantly hyperactive Th1 immune response. PD-1 inhibition drives antigen-induced cellular reactivity towards a proinflammatory Th1 response, which may explain the development of sarcoidosis-like inflammation with the use of these checkpoint inhibitors. This adverse effect should be recognized and distinguished from disease progression as the use of checkpoint inhibitors for immunotherapy in advanced melanoma increases.

Melanoma in adolescent and young adult patients: a nineteen-year retrospective analysis

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Melanoma in the adolescent/young adult (AYA) population is rare, accounting for 1% to 4% of melanomas. However, it is the third most common cancer in AYAs, with increasing incidence. We sought to characterize the epidemiology of melanoma in patients 15 to 40 years old at University Hospitals Case Medical Center compared to pediatric (0–14 years of age) and adults (over age 40 years) from January 1, 1996 to December 31, 2014. We also examined cases by six age subgroups within the AYA cohort. We identified 7 patients between 0 and 14 years, 582 patients between 15 and 39 years, and 2323 patients over 40 years. In the entire cohort, most patients were Caucasians (93.2%). Within the AYA group, the frequency of melanoma was higher in women than men. Women 18–24 years old had the highest frequency of melanoma (76.6%). Interestingly, the frequency was higher in men than women ages 15–17 years, matching what we observed in the pediatric group. The most common type of melanoma in all AYA age groups was superficial spreading. The frequency of Spitzoid melanomas was highest in the 0–14 year age group (42.8%) and lowest in the 35–39 year age group (1.0%) ($P < 0.0001$ for difference between these groups). The majority of patients in all age groups (82.7%) presented with early stage disease; this did not statistically significantly differ between groups (all $P > 0.05$). In all patients, melanoma occurred most frequently on the trunk (45.4%); this also did not differ between groups (all $P > 0.05$). There were no ulcerated melanomas in patients under age 17 years, although the frequency was low in all groups combined (8.8%). Our AYA cohort had similar characteristics as those reported by other institutions, with some features that are unique to this age group. Differences between the AYA population and pediatric/adult age groups should be further explored, including through molecular characterization of tumors.

Prognostic significance of patterns of tumor infiltrating lymphocytes (TIL) in cutaneous melanoma

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Many studies have demonstrated the prognostic relevance of TIL in melanoma. Patients with tumors that have 'brisk' TIL have a better prognosis than those with 'nonbrisk' or 'absent' TIL, however, there remains unexplained variability in survival among patients in each group. In this study of patients from 5 institutions' cohorts and 1 clinical trial, we characterized 974

primary lesions with TIL infiltration for its location, intensity and distribution. Survival analysis was used to investigate the prognostic significance of these factors. Among patients with brisk TIL, 36% had peripheral brisk (P-brisk) TIL and 62% had both peripheral and central TIL (PC-brisk). Patients with P-brisk TIL had significantly better prognosis than those with PC-brisk TIL (P = 0.04) with 5-year melanoma-specific survival (MSS) rates of 90% and 78%, respectively. P-brisk remained a significant prognostic factor after controlling for thickness, mitotic rate and ulceration (adjusted hazard ratio = 0.47, P = 0.01). Among patients with nonbrisk TIL, 62% had peripheral nonbrisk (P-nonbrisk) TIL and 37% had peripheral and central (PC-nonbrisk) TIL; these two survival curves were not significantly different (P = 0.19) and the overall 5-year MSS rate was 74%. In addition, 12% has focal-nonbrisk TIL (F-nonbrisk), 70% had multi-focal nonbrisk TIL (MF-nonbrisk) and 18% had segmental nonbrisk TIL (S-nonbrisk). Patients with F-nonbrisk TIL had significantly better prognosis than those with MF- or S-nonbrisk TIL (P = 0.02) with 5-year melanoma-specific survival (MSS) rates of 87% and 74%, respectively. TIL intensity was not a significant prognostic factor alone or in combination with the different TIL patterns. A phenotypic and functional characterization of the immune microenvironment that includes location and distribution may yield immune signatures that can be used to enhance prognosis and prediction.

MAP Kinase inhibition triggers melanoma mechanosensitive properties and induces the production of a BRAF-inhibitor protective extracellular matrix

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Cutaneous melanoma remains one of the most challenging cancer to treat due to its heterogeneity, high plasticity, metastatic potential and resistance to treatment. Combined therapies targeting BRAFV600E and MEK kinases have shown remarkable clinical efficacy. However, patients eventually relapse due to the acquisition of drug resistances linked to tumor phenotypic plasticity and heterogeneity. Using an *in vitro* model of BRAF inhibitors (BRAFi) resistant melanoma cells, we found that acquired resistance to vemurafenib is associated with a phenotype switching that confers to a subset of resistant cells a fibroblast-like mesenchymal morphology associated with increased adherence properties and metastatic potential. In contrast to their sensitive counterparts, BRAFi-resistant cells exhibit increased beta1 integrin expression, elevated FAK activity as well as YAP nuclear translocation and display higher sensitivity to changes in microenvironment stiffness. In addition, they produce and remodel a pro-fibrotic ECM that protects drug-naive melanoma cells from vemurafenib. Using pharmacological and RNAi approaches, we demonstrated that targeting the beta1/FAK pathway affects the mechanophenotype of resistant cells and decreases both their adherence and invasive properties. Finally, we found that treating sensitive melanoma cells with BRAF, MEK or ERK inhibitors triggered the rapid expression of various extracellular matrix components, as well as the activation of both integrin and YAP/TAZ pathways. Our results may provide an explanation to the rapid onset of resistance observed in patients treated with BRAF inhibitors and suggest that the combined inhibition of the MAP Kinase pathway and the FAK/YAP axis may represent a valuable approach to delay the onset of resistance and restrain metastatic melanoma spreading.

Genetic polymorphisms on *PDCD1* gene, regulator of lymphocyte activity, in cutaneous melanoma susceptibility

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This study aimed to evaluate herein whether the PD1.1 (c.-606G>A), PD1 (c.627+252C>T), PD1.5 (c.804C>T) and PD1.9 (c.644C>T) single nucleotide polymorphisms (SNPs) of *PDCD1* gene influence the risk, clinicopathological manifestations, and survival of patients with cutaneous melanoma (CM). We evaluated 250 patients diagnosed with CM at the University of Campinas, between 2007 and 2015, and 250 blood donors taken as controls. DNA was analyzed by real-time polymerase chain reaction (PCR) for genotyping. The statistical significance of differences between groups was calculated using the Fisher's exact or chi-square test. The prognostic impact of genotypes of SNPs on recurrence-free survival (RFS) and overall survival (OS) of CM patients were examined using the Kaplan Meier probability estimates, and Cox regression analyses. Individuals with CC genotype PD1 isolated and associated with PD1.5 CC genotype were under 2.20 (95% CI: 1.00–4.82, P = 0.04) and 2.51 (95% CI: 1.04–6.03, P = 0.03) times greater risk of developing CM, respectively. C allele of SNP PD1 was more common in patients than in controls; C allele carriers were at 2.15 (95% CI: 1.04–4.44, P = 0.03) fold increased risk of CM than others. The median follow-up time of CM patients was 90 months (range: 1.0–179.0 months). At 60 months of follow-up, a shorter RFS was observed in patients with PD1.1 AA genotype compared to those with GA + GG genotypes (33.3% versus 71.8%; P = 0.02; Kaplan-Meier estimates) the result was confirmed by univariate Cox analysis. The data suggest, for the first time, that SNPs in *PDCD1* gene influence the risk and prognosis of CM patients. These findings, once validated in additional studies, will contribute to identify individuals at high risk of developing CM and tumor patients with poor prognosis, deserving special attention in prevention, early diagnosis and differentiated treatment.

Non-invasive monitoring of treatment response to immunotherapy in patients with metastatic melanoma

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Current methods for monitoring melanoma dynamics during treatment are limited to LDH levels and imaging to estimate tumour burden. In particular, responses to immune checkpoint inhibitors vary greatly in timing and extent, and may not be accurately reflected through radiological examination. The analysis of circulating tumour DNA (ctDNA) through sensitive methods can provide a rapid, accurate and quantitative method to determine therapeutic effect as early as possible. In this study

mutation-specific droplet digital PCR was used to measure plasma concentrations of oncogenic BRAF, NRAS and TERT variants in 47 patients with advanced metastatic melanoma before commencing treatment with the immune checkpoint inhibitors pembrolizumab (N = 30) or ipilimumab (N = 17). Tumour-associated ctDNA was detected in 32/47 (68%) plasma prior to treatment, in 76% (N = 17) of BRAF mutant and 100% (N = 2) of NRAS mutant melanomas. In addition, TERT promoter mutations were detected in the plasma of 61% (N = 28) of BRAF/NRAS wild-type cases. Monitoring of ctDNA levels in patients positive at baseline, showed a decrease in ctDNA in response to therapies prior to or concurrently with radiographic response. However, a delayed pattern of response was observed in comparison with our previous data in patients treated with MAPK inhibitors, and consistent with clinical measures of response in ipilimumab and pembrolizumab clinical trials. In conclusion, this study demonstrates the utility of ddPCR assays to monitor ctDNA in the plasma of melanoma patients undergoing immunotherapy.

PD-L1 expression on circulating melanoma cells is predictive of response to pembrolizumab

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Immune checkpoint inhibitors (ICIs) have revolutionized melanoma treatment. However, only one third of patients achieve durable disease control and long-term survival. Treatment is costly and associated with novel immune related adverse events. Therefore, there is an urgent need for predictive biomarkers to better select patients most suited to treatment with ICIs.

Tumour PD-L1 expression is a clinically validated biomarker of response to anti-PD-1 therapy. However, tissue biopsies are invasive and not suited for frequent longitudinal monitoring during therapy. Furthermore, intra-tumour heterogeneity may not accurately capture the PD-L1 status of the whole tumour burden through a single biopsy. Circulating tumour cells (CTCs) are a promising biomarker for predicting response and monitoring dynamic changes in response to therapies. We therefore used multiparametric flow cytometry to identify CTC subpopulations based on the co-expression, in individual CTC, of melanoma markers MCAM and MCSP, tumour initiating markers ABCB5, CD271 and RANK in 22 metastatic melanoma patients prior to commencing treatment with pembrolizumab. In particular, we evaluated the expression of PD-L1 on CTCs and its relation to treatment response.

We found that CTCs were heterogeneous within and between patients, with limited co-expression between the five markers analysed. Majority of the CTCs detected expressed ABCB5 and RANK. A reduction of CTCs was not apparent at first follow up (6–10 weeks) after treatment initiation. Expression of PD-L1 was detectable in 8/22 patients and was significantly associated with response to pembrolizumab (P = 0.02, Fisher's exact test). 7/8 (88%) cases with PD-L1 positive CTCs responded to treatment, compared to only 5/14 (33%) cases with PD-L1 negative CTCs. Our results provide first evidence that detection of PD-L1 on CTCs predicts response to anti-PD1 therapy in melanoma.

An open-label phase 2a study of combination dabrafenib (D) and trametinib (T) in Asian patients (pts) with advanced *BRAF* V600–mutant acral lentiginous melanoma (ALM) or cutaneous melanoma (CM)

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ALM, 1 of 4 major histological subtypes of CM, occurs most frequently in individuals with darker skin with CM (40–50%), including in Asian populations. Randomized phase 2 (BRF113220) and phase 3 (COMBI-d, COMBI-v) studies demonstrating clinical benefit of D+T over BRAF inhibitor monotherapy in pts with *BRAF* V600–mutant unresectable or metastatic CM led to the approval of the combination in the United States, Australia, Canada, and Europe; however, these studies were conducted primarily in Caucasian populations. Thus, data for D+T in non-Caucasian populations and in pts with ALM are limited. As efficacy and safety of D+T still needs to be evaluated in Asian pts with melanoma, an urgent unmet medical need remains in countries where this combination is not yet approved. Here, an ongoing single-arm, open-label, multicenter phase 2a study evaluating D+T in Asian pts with histologically confirmed stage IIIC (unresectable) or stage IV *BRAF* V600–mutant ALM or CM (NCT02083354) is described. Prior systemic anticancer treatment in the adjuvant or metastatic setting is permitted; however, pts with previous exposure to BRAF or MEK inhibitors are ineligible. Enrolled pts are orally administered D 150 mg twice daily and T 2 mg once daily until disease progression, death, unacceptable toxicity, withdrawal of consent, or study completion. The primary endpoint of this study is overall response rate per RECIST v1.1. Secondary endpoints include progression-free survival, duration of response, overall survival, pharmacokinetics, pharmacodynamics, and safety. This ongoing trial will be conducted at 14 centers in the following cities: Beijing, Changchun, Guangzhou, Hangzhou, Kunming (mainland China); Hong Kong; Kaohsiung, Taipei, Taoyuan (Taiwan); Seoul (Korea); Bangkok, Hat Yai (Thailand).

Efficacy and safety of nivolumab (NIVO) alone or combined with ipilimumab (IPI) in patients with melanoma (MEL) metastatic to the brain in a phase 1 study

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Up to 75% of patients (pts) with advanced MEL develop brain metastases (BMts). NIVO alone or combined with IPI has demonstrated clinical benefit across multiple tumor types; however, there are limited data on the impact of these therapies in pts with BMts. Here we report efficacy and safety data in a cohort of pts with untreated MEL BMts enrolled in one part of a phase 1 study (CA209-038). A total of 20 pts were enrolled; at the time of database lock (March 24, 2016), 19 pts had received either NIVO 1 mg/kg + IPI 3 mg/kg Q3W × 4 then NIVO 3 mg/kg Q2W (n = 10) or NIVO 3 mg/kg Q2W (n = 9). Tumor assessment based on RECIST v1.1 criteria was performed at weeks 7, 13, and 23, then Q8W. Baseline characteristics were generally balanced across groups. In the NIVO+IPI group, 5 of 10 pts (50%) completed the induction phase and continued on to the maintenance phase with NIVO monotherapy. In the NIVO group, 7 of 9 pts (78%) received >4 doses. The confirmed objective response rate (ORR) in target lesions was 40% (95% confidence interval [CI]: 12, 74) for NIVO+IPI and 33% (95% CI: 7, 70) for NIVO; the overall ORR was 50% (95% CI: 19, 81) and 44% (95% CI: 14, 79), respectively. Median time to response was 8.8 weeks for NIVO+IPI and 12 weeks for NIVO. Grade 3/4 treatment-related AEs were reported in 8 pts (80%) with NIVO+IPI versus 1 patient (11%) with NIVO. The incidence of treatment-related AEs leading to discontinuation (NIVO+IPI: n = 4, 40% versus NIVO: n = 2, 22%) was similar to that reported in phase 3 studies. Data from this small cohort of pts with MEL BMts demonstrate efficacy and an acceptable safety profile with NIVO+IPI and NIVO. Further updates, including progression-free survival, will be presented for all 20 pts enrolled in this cohort of CA209-038.

Efficacy of pembrolizumab (pembro) in patients (pts) with advanced mucosal melanoma (mucMEL): data from KEYNOTE-001, 002, and 006

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Pembro has demonstrated efficacy and a manageable safety profile in advanced MEL. We assessed outcomes of pts with advanced mucMEL enrolled in KEYNOTE-001 (NCT01295827), KEYNOTE-002 (NCT01704287), and KEYNOTE-006 (NCT01866319). Pts received pembro 2 mg/kg Q3W, 10 mg/kg Q3W, or 10 mg/kg Q2W. Response was assessed per RECIST v1.1 by independent central review. Of the 1567 pts in the pembro arms who received ≥1 pembro dose, 84 (5%) had mucMEL. 57% of pembro-treated pts with mucMEL were women, 49% were aged ≥65 y, 32% had ECOG PS 1, 48% had elevated LDH, 8% had $BRAF^{V600}$ mutant tumors, 81% had M1c disease, 58% had baseline tumor size ≥77.7 mm (ie, median in total population), and 70% with known PD-L1 status had PD-L1–positive tumors. 90% of pts received ≥1 prior therapy: 37% received 1, 45% received 2, and 8% received ≥3; 39% received prior ipilimumab (ipi). In pts with mucMEL, ORR was 19% (95% CI 12–29%), DCR was 31% (95% CI 22–42%), median PFS was 2.8 months (95% CI 2.7–2.8), and median OS was 11.3 months (95% CI 7.7–16.6). In the 16 responders, median time to response was 12.4 weeks (range, 11.1–84.1), 12 (75%) were alive without subsequent progression, and median response duration was 27.6 months (range 1.1+ to 27.6). In ipi pretreated pts with mucMEL, ORR was 15% (95% CI 7–31%), DCR was 27% (95% CI 15–44%), 4 of 5 (80%) responders were alive and without subsequent progression, and median response duration was 27.6 months. In the 1483 pembro-treated pts with non-mucMEL, ORR was 33% (95% CI 30–35%), DCR was 47% (95% CI 44–49%), median time to response was 12.4 weeks (range 3.7–144.0), 72% of responders were alive and progression free, median response duration was NR (range 1.3+ to 38.8+), median PFS was 4.2 months (95% CI 3.6–5.5), and median OS was 23.5 months (95% CI 21.1–26.8). Pembro is active in advanced mucMEL and provides durable activity regardless of prior ipi.

Acetylsalicylic acid governs the effect of Sorafenib in mutant NRAS melanoma

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To date no therapies directly targeting mutant NRAS melanoma have been approved, leaving chemotherapy with very low response rates or immunotherapy for the treatment of mutant NRAS melanoma patients. Here we report a novel strategy to target mutant NRAS melanoma by combining the multi kinase inhibitor Sorafenib and the non-steroidal anti-inflammatory drug acetylsalicylic acid (Aspirin), both of which are clinically tested and approved. The addition of Aspirin, but not isobutylphenylpropanoic acid (Ibruprofen) or Celecoxib significantly increased the *in-vitro* cytotoxicity of Sorafenib resulting in a fivefold reduced effective Sorafenib dose in WM1366, WM832, and WM1361 mutant NRAS melanoma cells. Mechanistically, combined exposure resulted in the simultaneous hyperactivation of AMPK and ERK pathways. Combining Sorafenib with other AMPK activators like

Metformin or A769662 was not sufficient to induce cell death due to sole activation of the AMPK pathway. Accordingly, cytotoxicity of Sorafenib and Aspirin was blocked by concurrent inhibition of AMPK or ERK pathways using pharmacological inhibitors of RAF (LY3009120), MEK (Trametinib) and AMPK (Compound C) or shRNA targeting BRAF or AMPKα1/2. The combination was found to be specific for mutant N*RAS* and had no significant effect in wild type *RAS* keratinocytes or melanoma cells. *In-vivo* the treatment of SCID mouse xenografts with Sorafenib and Aspirin significantly reduced tumour volume compared to single treatment alone. Combined Sorafenib and Aspirin selectively target mutant N*RAS* melanoma cells by simultaneously affecting two independent pathways. The combination represents a novel treatment strategy for mutant N*RAS* melanoma by repurposing clinically approved drugs with the potential to reduce Sorafenib induced adverse effects while maintaining clinical efficiency.

Mutational burden and neoantigen load predict response to adoptive T cell therapy in melanoma

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Adoptive cell therapy (ACT) using autologous tumor-infiltrating lymphocytes (TILs) is a highly personalized treatment modality that has yielded objective response in 40–50% and complete response in 10–20% of melanoma patients in clinical trials. However, this therapy requires time and resources, and biomarkers aiding in selection of patients most likely to benefit from ACT are in demand. This study investigated potential association of tumor molecular characteristics to response to ACT in a phase I/II clinical trial (1) comprising 24 stage IV melanoma patients, who had received previous therapy, most commonly other immunotherapy including IL-2 and ipilimumab. Somatic mutations were identified in the pre-treatment tumors using whole-exome sequencing of tumor and matched normal tissue. Expressed neoantigens formed by the non-synonymous mutations in combination with patient-matched HLA type were bioinformatically predicted using additional RNA-seq. Analysis revealed correlation of both higher mutation load and higher neoantigen load to long-term benefit from ACT. Furthermore, tumors in non-responders demonstrated downregulated antigen processing and presentation pathway, while responders' tumors were enriched for the immune cell component. In conclusion, tumor genetic and microenvironmental profiles are predictive of response to ACT in melanoma patients.

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CADM1 is negatively regulated by TWIST1 and promotes melanoma anoikis

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Melanoma is the deadliest form of skin cancer; however, with early detection prior to metastatic dissemination, patients generally have a good prognosis. Recently, our group has implicated the transcription factor TWIST1 in dermal invasion, a step within the metastatic cascade. In mutant BRAF melanoma cells we found that RAS-RAF-MEK-ERK (ERK1/2 pathway) signaling increases TWIST1 expression, promoting invasion in the dermal microenvironment, at least in part, by enhancing levels of the matrix metalloproteinase, MMP1. Other TWIST1 regulated targets are poorly described. In this study, we compared expression profiling data to identify the TWIST1 regulated transcriptome. KEGG and GO analysis revealed that TWIST1 expression associated with cellular adhesion and, in particular, an inverse correlation was found with cell adhesion molecule 1 (CADM1) (aka NECL-2, IGSF4, TSLC1, SynCAM). Chromatin immunoprecipitation (ChIP) studies and promoter assays demonstrate that TWIST1 physically interacts with the CADM1 promoter, and this interaction is linked with reduced CADM1. We also demonstrate that RAF inhibitor treatment reduced TWIST1 levels and concurrently increased CADM1. CADM1 expression reduces serum directed migration and invasion through Matrigel coated boyden chambers. Interestingly, CADM1 expression was found to induce anoikis, a process which may be regulated in part by the presence of C-terminal CADM1 species in the mitochondria. Furthermore, examination of a panel of melanoma cell lines suggests CADM1 levels are inversely associated with tumor staging, and analysis of the TCGA SKCM dataset reveals that patients with high levels of CADM1 have better overall survival. Taken together, these data support the proposed role of CADM1 as a tumor suppressor in melanoma.

Interest and uptake of *MC1R* testing in primary care

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Germline variants in the *MC1R* gene are very common and confer moderate melanoma risk in those with varied skin types. Given low melanoma awareness in the general population, we are conducting a randomized controlled trial examining uptake and outcomes (screening, sun protection) of *MC1R* testing in Albuquerque primary care clinics. Our study website, surveys and risk feedback materials underwent usability and cognitive testing to confirm comprehensibility in the target population. Of 1276 patients (English or Spanish speakers) approached to date, 20% have agreed to participate. Study participants (N = 254; 46% Hispanic, 48% non-Hispanic White, 69% female, mean age = 57) are randomized 1:6 to either a usual care condition (NCI skin cancer information for diverse skin types) or an *MC1R* test offer (invitation to log onto our study website to learn about the rationale, benefits and drawbacks of *MC1R* testing). They can then elect testing via buccal cell test kit. Of the 225 patients

randomized to the *MC1R* offer to date, 40% (n = 91) have elected to learn about *MC1R* testing by logging onto the website; most that log onto the website go on to request testing (93%). Logging onto the website is also associated with higher education, non-Hispanic ethnicity and home internet access (all $P < 0.05$), but not with age, gender, or skin type (all $P > 0.05$). Logging onto the website is also associated with psychosocial factors (e.g., lower cancer fatalism, skin cancer misconceptions, and superstitious thinking about cancer risk, all $P < 0.05$). Outcomes including level of comprehension of *MC1R* risk feedback (average *versus* higher risk) and 3-month sun protection and screening outcomes (target N = 885) will be examined. This study represents an example of the behavioral and epidemiological research collaboration necessary to maximize the public health benefit of skin cancer genomic information as research evolves.

MC1R variants in the New Mexico population

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In 2016, 76 380 new melanoma cases will be diagnosed in the U.S. and 10 130 people die from their disease. Germline variants or single nucleotide polymorphisms (SNPs) in the melanocortin 1 receptor (*MC1R*) increase the risk of developing skin cancer. Previous studies have summarized *MC1R* risk variants within Caucasian European populations; however, few studies of *MC1R* variant prevalence have been conducted among diverse ethnic groups. We present preliminary data from a randomized controlled trial investigating the personal utility and reach of genomic testing for melanoma among Hispanics and non-Hispanics in New Mexico. We present preliminary comparisons of *MC1R* sequencing and risk factors for skin cancer in this population. Patients recruited in Albuquerque, New Mexico primary care were offered *MC1R* genetic testing. DNA extracted from buccal cell samples was genotyped for *MC1R* by Sanger sequencing. There was no significant difference in the frequency of *MC1R* risk variants between Hispanics and Non-Hispanics in this population ($P = 0.60$). Results from 48 participants, 26% Hispanic and 76% non-Hispanic show no difference in the prevalence of 'risk' genotypes between ethnic groups. Additionally, there is no statistically significant difference in Fitzpatrick skin type. These findings suggest that Hispanics and Non-Hispanics share similar frequencies of *MC1R* variants and potentially share similar melanoma risk. Thus, behavior should be further studied between the groups to understand different rates of melanoma in these populations. Possibly more studies of gene-environment interactions are warranted to evaluate these relationships further. Understanding melanoma risk in different ethnicities may offer insights for public health policy and clinical prevention.

Multiple primary melanoma patients: cancer risks and survival in association with familial melanoma and germline *CDKN2A* mutation status

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Approximately 5% of melanoma patients develop multiple primary melanomas (MPM). The aim of this study was to investigate how family history of melanoma and germline *CDKN2A* mutation status of MPM patients affects their prospective risks of developing subsequent melanomas and other cancers as well as their survival.

The MPM patients in the study were identified through a preventive program starting in 1987 in Sweden. Comprehensive data on cancer diagnoses and deaths of MPM patients, their first-degree relatives and matched controls were obtained through the Swedish Cancer Registry and Cause of Death Registry.

Of the familial MPM cases (n = 100), 43 (43.0%) had germline *CDKN2A* mutations. Of the sporadic MPM cases (n = 131), only one case (0.8%) had a *CDKN2A* mutation. *CDKN2A* mutated cases were youngest at diagnosis of a second melanoma (median age 42 years) and had the highest relative risks among the MPM cohorts of developing >2 melanomas (RR 238.4, 95% CI 74.78–759.92). Subsequent melanomas in the head and neck area were prominent in the non-mutated cohorts, but not in the *CDKN2A* mutated MPM cases. Familial non-mutated MPM cases had the highest relative risks of squamous cell skin cancers (RR 23.93, 95% CI 11.39–50.26). Only the *CDKN2A* mutated MPM cases and their first-degree relatives had increased relative risks of non-skin cancers (RR 3.6, CI 1.9–7.1 and RR 3.2, 95% CI 1.9–5.6, respectively). The first-degree relatives of sporadic non-mutated MPM cases had no increased prospective risk of melanoma. When adjusted for age, sex and T classification of melanoma tumors, *CDKN2A* mutated MPM cases had worse survival compared to both familial (HR 3.0, 95% CI 1.3–8.1) and sporadic non-mutated MPM cases (HR 2.63, CI 1.3–5.4).

This study demonstrates that *CDKN2A* mutation status and family history significantly affects outcomes in MPM patients.

Immune stimulation following the delivery of plasmids encoding cytokines as a potential treatment of melanoma

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There have been major advances in the area of immunotherapy of cancer in recent years. Positive results have been achieved with the use of checkpoint inhibitors and other immune modulators and the number of indications continues to grow. Predominately, agents are delivered in the form of recombinant proteins. However, there is the potential for gene-based approaches. Recently T-VEC, an oncolytic virus encoding GM-CSF, achieved FDA approval. Although approved, there were concerns related to viral shedding and possible side effects. Developing non-viral gene transfer approaches that can enhance immune stimulation without unwanted side effects would be a major improvement. To this end, we have developed effective protocols utilizing gene electrotransfer (GET) to deliver plasmid

DNA encoding cytokiness directly into tumors. Delivery of plasmids encoding cytokines has been shown by our lab and others to not only generate a potent anti-tumor local immune response, but a systemic one as well. GET is a reliable and effective physical method for *in vivo* delivery of plasmid DNA. It is particularly attractive for use in anti-cancer cytokine therapy as it does not incur the side effects associated with conventional protein cytokine therapy. The positive responses were directly related to the ability to achieve the appropriate expression profile following delivery of the plasmid. Interestingly, expression levels necessary to achieve a local and systemic response were not the highest. To enhance this immune response, current work is further analyzing the immune response to determine if there is a marker that would indicate appropriate delivery of the plasmid as well as combining with checkpoint inhibitors. A more complete understanding of the process and identifying other agents that could further augment this approach would be an important step.

Whole exome sequencing identifies recurrent SF3B1 R625 mutation and co-mutation of NF1 and KIT in mucosal melanoma

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Melanomas in mucosal tissue account for <1% of all melanoma cases. Currently, there is little known about the molecular background of these rare melanomas, except that they lack common driver mutations used to target and treat cutaneous melanoma. To better understand the molecular background and identify new potential therapeutic targets, we performed whole exome sequencing on tissue samples obtained from 18 mucosal melanoma patients.

We found that mucosal melanomas arising in different anatomic sites (nasopharyngeal, anorectal, and vulvovaginal) have distinct mutational signatures and mutational burdens. Among these subgroups, anorectal had a significantly higher mutational burden compared to the nasopharyngeal ($P = 0.04$). Based on their mutational signatures, we found three distinct subclasses of mucosal melanomas according to the primary tumor's anatomic location. These three subgroups of mucosal melanoma should be considered as individual diseases for future studies into possible causes and therapy.

The mutational landscape of mucosal melanoma included *KIT* and *NF1* mutations frequently co-mutated in the vulvovaginal (50%) and anorectal (60%) groups, significantly higher than in cutaneous melanoma (1%). This study found potential novel therapeutic targets for mucosal melanoma including *SF3B1*. *SF3B1* R625H/S/C mutations were present in 7/18 (39%) mucosal melanoma patients. This mutation has been reported in uveal melanoma but not in cutaneous or mucosal melanomas. Overall mutations in the spliceosome and antigen presentation and processing pathways were found to be enriched in mucosal melanomas when compared to cutaneous melanomas. This study has provided molecular insight and potential therapeutic targets for treating mucosal melanoma.

Identification of novel melanoma predisposing genes by next-generation sequencing

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Melanoma is the most dangerous form of skin cancer and its incidence is steadily increasing in many populations. In Sweden, the increase in incidence is accompanied also by a rise in mortality. It is therefore of great importance to improve strategies for prevention and early diagnosis.

Approximately, 10% of the melanoma cases are considered to have an inherited predisposition for this disease. In Sweden, <20% of the melanoma families carry a known genetic aberration. The most common germline mutation in the Swedish melanoma families are a specific founder mutation in the *CDKN2A* gene that also associates with high risk of other cancer types and poor prognosis. However, for a majority of melanoma families, the genetic background is still unknown. The overall aim of the project is to discover novel melanoma-predisposing genes that can be used as a tool to identify individuals at increased risk of developing melanoma.

To identify melanoma susceptibility genes, we have executed whole-exome and targeted gene sequencing of high-risk melanoma patients. Identified gene variants were filtered based on co-segregation with the melanoma phenotype, minor allele frequency (MAF) <1% and predicted functional effects. Several of the strongest candidate genes were involved in chromatin modification, cell cycle regulation and autophagy. For example, we observed several rare/novel gene variants with a predicted functional effect in the histone methyltransferase gene, *EHMT1* that is involved in gene silencing. Genotyping of a melanoma case control cohort showed that these variants were more frequent among melanoma patients compared to controls. We have also identified novel truncating mutations in other genes including the putative tumor suppressor gene *NPAT* that is involved in cell cycle regulation. Functional investigations of candidate genes are ongoing.

Natural progression of hepatotoxicity after immune checkpoint inhibitor therapy in metastatic melanoma: case series from a large referral center

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Immune checkpoint inhibitors have improved survival in patients with metastatic melanoma compared to prior therapies. Notably they have been associated with an increased incidence of immune related adverse events in up to 44% of patients. We aimed to report the clinical features, treatment and outcomes of patients with immune checkpoint inhibitor-induced hepatotoxicity. In this retrospective observational study, we identified patients with metastatic melanoma seen in consultation and/or treated between March 2011 and March 2016. Hepatotoxicity was assessed using the Common Terminology Criteria for Adverse Events, v4.0. Seventeen patients were identified as having hepatotoxicity by history (grade 1–4). Twelve of 17 were diagnosed after ipilimumab; 3 were diagnosed after pembrolizumab; and 2 after ipilimumab combined with nivolumab. Median time from first dose of immune therapy to hepatotoxicity was 52 days. Clinical

symptoms were variable. Eight patients had concurrent adverse events including colitis, hypophysitis, pneumonitis, and/or rash. Immune therapy was discontinued in 14 patients. The patients were most commonly treated with systemic corticosteroids. Immunosuppression was discontinued by taper over a median of 42 days; in three patients steroids had to be reinitiated based on clinical or laboratory worsening of liver tests. Normalization of liver tests was seen within a median of 31 days of immunosuppression initiation. One patient with grade 4 hepatotoxicity had normalization with the addition of cyclosporine. Melanoma patients treated with immune checkpoint inhibitors should be monitored regularly for hepatotoxicity. Treatment with discontinuation of therapy and initiation of corticosteroids is indicated with grade 3 or 4 hepatotoxicity. Cyclosporine may be beneficial in steroid-refractory hepatotoxicity.

Safety and clinical activity of atezolizumab combined with cobimetinib in metastatic melanoma

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Atezolizumab (A) is an anti-PDL1 monoclonal antibody that inhibits binding of PD-L1 to its receptors PD-1 and B7.1. A has demonstrated promising clinical activity (ORR, 33%; mPFS, 5.5mo) in patients (pts) with cutaneous metastatic melanoma (mM; Hodi, *SMR* 2014). Given that targeted inhibition of MEK with cobimetinib (C) promotes intratumoral T-cell infiltration, MHC class 1 upregulation and PD-L1 expression in preclinical models (Ebert, *Immunity* 2016) and clinical tumor biopsy specimens (Bendell, *ASCO* 2016), A+C together may enhance clinical benefit over A alone. In this Ph Ib dose-escalation and dose-expansion study of A+C in advanced solid tumors, C was escalated from 20 to 60 mg daily (21d on/7d off) and combined with A dosed at 800 mg IV q2w. Tumor-specific expansion cohorts were enrolled at MTD (C 60 mg/d 21/7; A 800 mg IV q2w). At the time of data cutoff (April 26, 2016), 22 pts with mM (2 ocular, 20 non-ocular with 10 each *BRAF*-mutant and wild-type) who received no prior anti-PD-1/PD-L1 therapy were evaluable for safety and efficacy. Median safety follow-up was 12.7mo (range, 2.4–17.6). All-grade (Gr) AEs occurred in 100% of pts; treatment-related Gr3–4 AEs occurred in 50% of pts, with diarrhea and dermatitis acneiform being the most common (2 pts each [9.1%]). All AEs were manageable. No treatment-related Gr 5 AEs occurred. 18% of pts had treatment-related serious AEs and 14% of pts discontinued treatment due to AEs. Among pts with non-ocular mM (n = 20), ORR was 45% (confirmed, RECIST v1.1), DCR (CR+PR+SD) was 75% and mPFS was 12mo (95% CI: 2.8, 16.7). Pts with *BRAF*-mutant and wild-type mM had similar ORRs. These results suggest that A+C may lead to higher ORR and DCR, and longer PFS than A or C monotherapy in pts with mM. With the biomarker data from other study cohorts, these data suggest that C may alter the immune contexture thereby enhancing A activity. NCT01988896

Investigating transcriptional cyclin dependent kinases as novel melanoma drug targets

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Advanced melanoma carries a poor prognosis despite recent advances in therapy. BRAF-driven melanoma initiation and growth relies heavily on several transcription factors which control gene expression, suggesting that BRAF-driven melanoma may be exquisitely sensitive to transcriptional inhibition. Transcriptional Cyclin Dependent Kinases (CDKs) are nuclear kinases that regulate transcription and therefore offer a way to 'drug' previously 'undruggable' transcription factors. Compounds inhibiting CDK7, CDK8, and CDK9 demonstrated promising results in preclinical models for several difficult-to-treat cancers that rely heavily on transcription. Transcriptional CDKs have yet to be studied in melanoma. We have created a zebrafish melanoma model in which human BRAFV600E is overexpressed under the melanocyte-specific *mitfa* promoter in the presence of a *p53* mutation. We recently developed a melanocyte-specific CRISPR/Cas9 transgenic system and are generating melanocyte-specific transcriptional *CDK* mutations in the *BRAF/p53* melanoma model. Our preliminary data demonstrate that *CDK* loss of function leads to later tumor onset and tumors that grow more slowly than control tumors. CRISPR/Cas9 directed mutations and CDK protein levels are being assessed to confirm target loss of function. Our work will inform whether existing CDK inhibitors may have efficacy in melanoma while also elucidating whether transcriptional CDKs without inhibitors are worthy of inhibitor development.

Age, sex and response to immune checkpoint inhibitors in patients with advanced melanoma

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Immune checkpoint inhibitors targeting cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and programmed cell death-1 (PD-1) have been shown to improve survival in patients with metastatic cutaneous melanoma. Previous studies have demonstrated the association between somatic mutation load and clinical benefit to these agents. Increase in age and male sex is associated with higher somatic mutation burden in cutaneous melanoma.

We reviewed clinical trials of FDA-approved immune check point inhibitors in patients with advanced melanoma to explore the efficacy of immune checkpoint inhibitors by age and sex. Eligible studies were limited to clinical trials of single agent immune check point inhibitors reporting subgroup analysis in response rate, progression-free survival or overall survival by age and sex. Trials comparing anti-CTLA-4 antibody to anti-PD-1 were excluded.

A total of 4 trials were selected for review. All trials included patients with histologically confirmed unresectable or metastatic cutaneous melanoma. Subgroup analyses by age and sex were reported in 2 studies for overall survival and 1 study for progression free survival and response rate, respectively. Male sex was associated with numerically higher response rate and lower Hazard Ratio (favoring immune checkpoint inhibitors)

compared to female across the studies. There was no significant difference in response rate, progression free survival or overall survival by age across the studies. (see attached table)
There could be gender disparity in response to immune checkpoint inhibitors among patients with advanced melanoma which warrants further study.

Phase 2 trial of the BRAF inhibitor encorafenib (LGX818) using a pulsatile dosing schedule in patients (pts) with BRAFV600-mutated melanoma

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BRAF inhibitors effectively inhibit ERK signaling in melanoma cells harboring a BRAF V600 mutation. However, this leads to loss of negative feedback and re-activation of ERK. Using the BRAF inhibitor encorafenib (Enc) which has a 3-h half-life, we explored a pulsatile dosing schedule (PDS) in a phase II trial. Pts with metastatic or unresectable melanoma who harbored a BRAF V600 mutation who had not received prior BRAF or MEK inhibitor therapy received Enc (300 mg once daily) for 6 weeks and then on a PDS of 2 weeks off/2 weeks on. The primary endpoint was progression-free survival at 10 months. Of the 7 pts enrolled, 4 were male. Median age was 58 years (range; 53–75). Five pts had V600E mutations, two had V600K mutations. Four pts received prior immunotherapy for metastatic disease. Mean no. of cycles was 6 (range 1–16). Best response was complete response (CR) in two V600E pts, partial response (PR) in two V600K pts, stable disease in two V600E pts, and progression in one V600E pt. All pts had at least one grade 1 adverse event (AE). Grade 2 AEs included hand foot syndrome; n = 5, arthralgia/myalgia n = 4, hyperkeratosis n = 3, SCC n = 2, pruritus n = 2, rash n = 1, xerosis n = 1 and keratoacanthoma n = 1. Grade 3 AEs were nausea (1) and Bell's palsy (1) with no grade 4/5 AEs. Reasons for ceasing therapy were toxicity (n = 2), progression (n = 4), change in therapy to continuous dosing for growth off drug in PDS (n = 1). Both pts with CR stopped therapy for toxicity but remained in CR for 3 and 23+ months after ceasing Enc for toxicity. Duration of PR for the 2 pts who had a PR was 5 months each. Overall, Enc was poorly tolerated with toxicity necessitating halting of accrual. As a result, we were not able to test the utility of PDS. However, 2/7 (29%) pts were progression-free at 10 months and achieved CR, one ongoing 23 months off therapy.

Thio-redox imbalance mediates TKI-resistance via ER-stress induced autophagy in melanoma

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Early stage melanoma can be cured by surgery, but metastatic disease is lethal. For BRAFV600E positive patients, targeted combination therapies using small molecule tyrosine kinase inhibitors (TKI) such as BRAFV600E inhibitors (Vemurafenib, Dabrafenib); MEK inhibitors (Cobimetinib, Tramatenib); and non-specific TKI (Sorafenib, Pazopanib) increase progression free survival by months, but resistance develops eventually. Emerging evidence suggests that an unfolded protein response (UPR)-induced autophagy response plays an important role in the development of resistance to Vemurafenib. In the present study, a reduction in glutathione levels that leads to the UPR-mediated autophagy response is identified as a general mechanism of acquisition of resistance to TKI. Results demonstrate that chronic exposure to TKI leads to a thiol-redox imbalance that leads to an oxidative ER (endoplasmic reticulum) lumen; accumulation of mis-folded proteins (ER stress); and consequent activation of an UPR-induced autophagy response that results in resistant melanoma. Experiments conducted using FDA approved drugs to simultaneously attenuate ER stress using 4-phenylbutyric acid (4-PBA) and inhibit autophagy using hydrochloroquine (HCQ) sensitized TKI-resistant melanoma cells (generated by chronic exposure to TKI); supporting the hypothesis that UPR-mediated autophagy plays a significant role in the development of resistance to TKI. It was further observed that attenuation of ER stress and inhibition autophagy prevented acquisition of resistance to TKI *in vitro*. These results support the hypothesis that TKI-inhibition induces a thiol imbalance in melanoma cells that leads to an ER stress-mediated autophagy response that plays a significant role in the development of drug resistance; and allow designing new therapies that can and prevent acquisition of resistance to tyrosine kinase targeted melanoma drugs.

Identification of c-Jun targets in melanoma by ChIP-Seq

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A fundamental event in melanoma development and progression is the deregulation of cancer-relevant transcription factors. Subsequently, DNA-protein interactions have been investigated using a variety of biochemical approaches. Chromatin immunoprecipitation combined with sequencing (ChIP-Seq) is the most promising technology to perform genome-wide analysis of DNA-protein interactions.
Recent studies have indicated that c-Jun, one of the most important members of the AP-1 transcription factors in melanoma, acts by regulating cancer-relevant genes promoting the malignant phenotype. To further investigate direct c-Jun targets we screened pre-existing ChIP-Seq data of non-melanoma cells and identified 44 direct c-Jun targets. By various experimental setups we showed that 6 identified genes were differentially regulated in melanoma in a c-Jun dependent manner and ChIP experiments confirmed the direct interaction between c-Jun and the promoter regions of the identified genes. Interestingly, we detected a direct target gene regulation via c-Jun independent of the classical AP-1 consensus sequence.
Further, we established ChIP-Seq with melanoma cell lines to investigate c-Jun activity and its role in melanoma. By now, no ChIP-Seq data with human melanoma cells are available and we successfully established ChIP-Seq with these for the first time and independently verified the pre-existing ChIP-Seq data in terms of c-Jun targets. Our ChIP-Seq data indicate a crucial role of c-Jun and its targets in melanoma and thus analysis of c-Jun targets and their functions in melanoma is necessary to determine if c-Jun could serve as a therapeutic target.
In summary, the results of this study indicate c-Jun to play a critical role in melanoma by regulating cancer-relevant genes and the results of the newly established ChIP-Seq with melanoma cells give deep insights into the regulatory role of c-Jun and thus its function in melanoma.

Fine tuning of pro-metastatic factors by RNA complexes controlled by p62/Sequestosome-1 in melanoma

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The melanoma field is witnessing unprecedented clinical responses with genetically targeted and immune-based therapies. Still, melanoma remains the deadliest form of skin cancer with poor survival in advanced stages. Increasing evidence support the progression to metastasis involves a series of phenotype-switch changes in melanoma cells, but the underlying mechanisms remain unclear. Autophagy is of interest for its ability to favor cellular plasticity and controlling cell dissemination. Sequestosome-1/p62 is an adaptor in autophagy and a scaffolding hub in signaling cascades. Cancer cells hijack the scaffolding function of p62 to stabilize and maintain oncogenic signaling pathways. The contribution of p62 to melanoma metastasis is unknown. Expression studies and analysis of patient prognosis revealed that p62 is progressively increased in advanced melanomas, representing a risk factor for metastasis. Inducible mouse models were then generated for *in vivo* analyses of the specific contribution of p62 to this disease. Allelic loss of p62 significantly abrogated melanoma development in the Tyr::CreERT2;BrafCA;Pten$^{fl/fl}$ mouse strain. This effect was found to differ to roles of the classical autophagy factor ATG5 we addressed also in animal models. These data therefore support autophagy independent functions of p62 controlling melanoma progression. Genome-wide high-throughput proteomic and transcriptomic analyses were then performed to define downstream pro-metastatic targets of p62 that favor melanoma dissemination. These unbiased analyses uncovered an unexpected set of RNA binding proteins as direct p62 interactors, which were found to fine-tune the transcription of key pro-metastatic factors. Together, these results provide new insight of p62 as a tumor specific modulator of gene expression in melanoma, and as such, a putative target for therapeutic intervention.

HAPLN1 mediates collagen remodeling during aging to influence melanoma and immune cell motility

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Melanoma is an aggressive disease and is known to become increasingly invasive with age, particularly in those over 50. While various studies have detailed the changes in architecture of the skin due to aging, little research has focused on how aging affects the migratory capabilities of the cells in the skin. In human skin, stromal/interstitial matrices are known to be primarily composed of collagen I, which is crosslinked to form a stable extracellular matrix (ECM). We have characterized the secretome of aging dermal fibroblasts to assess changes in proteins involved in the structure and function of ECM. We found that among the ECM proteins, various collagen crosslinking proteins, such as HAPLN1 and LOXL (lysyl oxidase) are differentially expressed between young and aged fibroblasts. This led us to propose that age-related physical changes in the skin ECM could promote the metastasis of melanoma cells, while inhibiting the migration of immune cells that use these components to infiltrate the tumor. Indeed, our data indicates that as the stiffness of the collagen increases, the 3D spheroids become less invasive and the invading tumor cells change their morphology. Further, since T cells infiltrating the mouse skin are known to be guided by local ECM fibers; we observed the effect of ECM matrix on the motility of the T cells. Our results indicate that collagen remodeling by HAPLN1 affects the interstitial motility and cytotoxic activity of the T cells. Overall, our data is anticipated to elucidate the role of extracellular matrix proteins in modulating melanoma invasion and immune resistance to improve treatment options for elderly melanoma patients.

Anti-PD1 treatment in patients with metastatic melanoma (MM) and coexisting cancer (CC)

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Melanoma patients (pts) have an increased risk of developing second primary cancers. Therapeutic clinical trials often exclude pts with more than one cancer diagnosis. Anti-PD1 (aPD1) therapy is now approved across several tumor types, but there are limited data regarding clinical outcome and prognosis to aPD1 in melanoma pts with CC. In this single institution retrospective study, we describe our experience with aPD1 therapy in pts with MM and CC. Eligible pts with MM and CC who received aPD1 for MM after the diagnosis of CC were identified from the Moffitt Cancer registry from 1/2005–1/2016. Fifteen pts, median age 72 years (61–86) met eligibility criteria. 13 pts had stage IV disease; 2 were stage IIIc. The CC diagnosis was thyroid (3), prostate (2), kidney (2), CLL (2), bladder, head/neck, breast, esophagus, endometrial and DLBCL (all 1 each). No solid CC was metastatic. Ten pts received definitive treatment for CC before aPD1 therapy for MM; 5 remained therapy naïve for the CC (2 CLL, 2 prostate and 1 kidney cancer). Six pts received prior systemic therapy (range, 1–2) for MM; all 6 had received ipilimumab. Overall response rate to aPD1 treatment was 60%, including 4 complete responses. No patient had relapse or disease progression of the CC during aPD1 therapy. At a median follow-up of 11.2 months, the median progression-free (PFS) and overall survival (OS) on aPD1 therapy had not been reached. The 1-year PFS and OS rates were 70% and 91%, respectively. The most common adverse events were fatigue (n = 7) and rash (n = 5). There were 5 G3 adverse events (fatigue, rash, pruritus, arthritis and hypophysitis). In this small cohort of pts with MM and CC, aPD1 therapy can be administered safely and is efficacious. If this is confirmed in a larger cohort, future trials of aPD1 therapy should reconsider whether exclusion of a second primary cancer is necessary for eligibility.

Sharpin regulation of PRMT5 affects SOX10 and PAX3 expression and melanoma growth

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Understanding the mechanistic link between genetic alterations and epigenetic pathways is expected to identify new targets for monitoring the progression of and treating cancer. We have identified Sharpin, the adaptor protein for the linear ubiquitin chain assembly complex (LUBAC), as a PRMT5-associated protein. Sharpin association with PRMT5 occurs independently of other LUBAC components and results in increased PRMT5 activity by enhanced formation of multimeric complex of PRMT5. Notably, certain melanomas are vulnerable to cell death upon Sharpin knockdown. Mechanistically, Sharpin interaction with PRMT5 enables upregulation of the transcriptions factors SOX10 and PAX3, which play key roles in melanoma through regulation of microphthalmia-associated transcription factor (MITF) and related genes. The effect of PRMT5 on SOX10 and PAX3 is mediated by methylation-dependent repression of Ski, a negative regulator of PAX3 and SOX10. Thus, inhibition of Sharpin expression attenuates PRMT5 activity and limits the growth of melanoma cells via repression of SOX10 and PAX3 expression. Altogether, we have identified a mechanism by which PRMT5, SOX10, and PAX3 activity and/or expression could be restored in melanomas and a plausible target for inducing synthetic lethality.

Survival and clinical characteristics of patients with melanoma brain metastasis (BM) in the era of checkpoint inhibitors and targeted therapies

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Patients with melanoma with BM have a poor prognosis with historical series showing a median overall survival (OS) of ~5 months. Novel BRAF/MEK inhibitors and checkpoint inhibitors have dramatically improved OS in patients with advanced melanoma, but demonstrated mostly in those without BM. We evaluated OS and clinical outcomes of patients with BM in the era of these novel therapies. We performed a retrospective review of the medical records of melanoma patients with BM diagnosed between 01/2011 and 06/2015. We identified 79 patients with BM. The median time from primary melanoma diagnosis to BM was 3.2 years (range, 0–29.8 years), and the median time from stage IV diagnosis to BM was 2 months (range, 0–103 months). The stage prior to the BM diagnosis was II in 6 (7.6%) IIIA in 1 (1.3%), IIIB in 9 (11.4%), IIIC in 3 (3.8%), IV(M1a) in 6 (7.6%), IV(M1b) in 8 (10.1%) and IV (M1c) in 25 (31.6%). Six (7.6%) patients had brain metastasis as the only site of distant metastasis, and 36 (45.6%) patients had neurological symptoms at BM diagnosis. Twenty-nine (36.7%) had *V600 BRAF*-mutant melanoma. Thirty-four (43.0%) underwent surgical resection, and 52 (65.8%) were treated with stereotactic radiosurgery. 39 (49.4%), 28 (35.4%) and 24 (30.4%) patients received CTLA-4Ab, PD-1Ab and BRAF(+/-MEK) inhibitors, respectively. Of 55 (69.6%) patients who have died of melanoma progression, 30 (40.0%) died with progressing BM. The median OS from the diagnosis of BM was 12.8 months (range, 1.1–64.8 months), and the median OS from the time of a stage IV diagnosis was 18.2 months (range, 1.4–158.4 months). The median OS from the time of the first stereotactic radiosurgical treatment was 14 months (range, 1–63 months). Our finding suggests that OS of patients with BM has been significantly improved in the new era of the novel melanoma therapies.

Role of R-Ras activation via RasGAP inactivation in melanoma tumorigenesis

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Ras is a small GTP binding protein that is frequently activated by mutations in melanoma as shown for *NRAS* (20%), *KRAS* (2%) and *HRAS* (1%). Ras can also be activated by inactivation of its negative regulators, Ras GTPase activating proteins (RasGAPs), such as *NF1, RASA1, and RASA2*. In our recent study, we observed that inactivation of RASA1 (RAS p21 protein activator 1, also called p120RasGAP) suppressed melanoma via its RasGAP activity toward the R-Ras (related RAS viral (r-ras) oncogene homolog) isoform and that R-Ras was required to promote anchorage-independent growth driven by RASA1 inactivation. Moreover, a low level of *RASA1* mRNA expression is associated with decreased overall survival in melanoma patients with *BRAF* mutations. Based on these observations, we hypothesized that, although not mutated, R-Ras is activated in melanoma by inactivation of RasGAPs and that BRAF activation cooperates with this RasGAP/R-Ras pathway activation in melanoma tumorigenesis. In this study, we addressed the importance of R-Ras, a previously less appreciated member of the Ras small GTPases, in melanoma tumorigenesis. We have shown that R-Ras is frequently activated in BRAF mutant human melanoma cell lines and siRNA-mediated reduced expression of R-Ras suppresses anchorage-independent colony growth and tumor growth. Moreover, among the 3 major RAS effector pathways, reduced R-Ras expression suppressed Ral-A activation, which may explain the mechanisms of Ral-A activation in BRAF mutant melanoma. Interestingly, Ral inhibitor BQU57 treatment suppressed anchorage-independent growth driven by R-Ras activation downstream of RASA1 inactivation. This study supports the importance of R-Ras activation in BRAF mutant melanoma by activating the Ral-A pathway and the possible combinatorial treatment targeting BRAF/MAPK and Ral pathways.

Enrichment for melanocytic cells improves the performance of a gene expression test in the diagnosis of malignant melanoma

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Recently, a gene expression signature was developed to differentiate benign nevi and malignant melanoma based on the expression of 14 tumor marker genes. The resultant score provides adjunctive information for the diagnosis of melanocytic lesions that are difficult to diagnose by traditional methods. However, certain histopathologic subtypes have very low melanocytic content (e.g. lentigo maligna) or low tumor content within the lesion. This has the potential to dilute the malignant cells with non-malignant cells and subsequently produce a false negative score.

In this study, we first determined whether low melanocytic content may produce a false negative score. Clinical testing involves review of an H&E stained slide by an anatomic pathologist to identify representative areas of the lesion to be tested. The extracted RNA from all identified sections is pooled for gene expression measurement. Here, subsections of five lentigo maligna lesions with variable density and distribution of atypical melanocytes were tested separately. All samples had at least one subsection that produced a benign score, confirming that malignant lesions with low melanocytic volume can produce a false negative result.

We next determined the lowest concentration of melanoma cell RNA that would produce a true positive score. This was done by creating a series of composite samples containing known quantities of malignant and non-malignant RNA. Each composite sample contained malignant RNA from a different histopathologic subtype. These studies showed that the gene signature produced a positive score when at least 2–5% of the RNA was from malignant cells.

Overall, this shows that lesions tested with this gene signature that contain <2–5% melanocytic cells can produce a false negative test result. As such, a clinical melanocytic testing threshold is appropriate.

Differential metastatic potential of Akt mutants in a mouse model of melanoma

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Aberrations that lead to over-activation of the PI3K/AKT signaling pathway are common events in human cancer. In melanoma, oncogenic alterations of this pathway are observed in up to 70% of patient tumors and are associated with disease progression. AKT, a major signaling hub in the PI3K/AKT pathway, executes a myriad of biological responses employed through numerous effectors linked to transcription, glucose metabolism, cell migration, cell proliferation, apoptosis, and angiogenesis. The 3 AKT isoforms are highly conserved with substantial sequence homology but evidence suggests they have distinct cellular functions. Somatic, activating mutations occurring within the pleckstrin homology (PH) domain of AKT1 (E17K, E40K and Q79K) and AKT3 (E17K) have been found in human melanoma but their role in melanoma formation and metastasis has not been assessed. Prior work in our lab has demonstrated that constitutively active (myristoylated) forms of AKT1 and 3 promoted melanoma formation and metastasis to the brain and lung in an autochthonous mouse model. This occurred in the context of $BRAF^{V600E}$ and Cdkn2a loss and the metastatic phenotype was enhanced upon Pten loss. Myristoylated AKT2 promoted the formation of melanoma and lung metastases however no brain metastases were detected. Our current work has focused on assessing the tumor formation and metastatic potential of physiologically relevant mutations E17K, E40K, and Q79K in all three AKT isoforms, using our autochthonous mouse model. Thus far, all mutants tested are capable of inducing tumors in this context but differences in metastasis have been observed.

OPTIMIzE: a US multi-site observational study in patients with unresectable and metastatic melanoma (MEL)

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Although the treatment landscape has changed markedly for MEL over the past 5 years, there is limited information regarding treatment patterns and real-world outcomes for these patients (pts). The OPTIMIzE study was designed to address this unmet need. The primary objectives of this US-based, observational study of pts with newly diagnosed MEL are to (1) describe demographic and clinical characteristics, (2) assess patterns of care, and (3) track outcomes, including overall survival, in pts receiving treatment for MEL. Secondary objectives are to describe the use of healthcare resources, measure pt-reported outcomes (EQ-5D, functional assessment of cancer therapy-MEL, and work productivity), and characterize caregiver burden. Exploratory objectives are to assess treatment-related adverse events and the influence of pt characteristics on the incidence and severity of these events.

The study will enroll 2200 pts diagnosed with stage III/IV MEL (treatment-naive or previously treated) from community and academic settings. Prospective group pts (n = 1600) scheduled to receive immune checkpoint inhibitors or targeted antitumor agents will be recruited over 2 years and will be followed for ≥3 years from their study start date until death, withdrawal of consent, data unavailable, or end of study. Data will be collected for retrospective pts (n = 600) who received therapies other than immune checkpoint inhibitors or targeted agents during the 4-year period before ipilimumab approval (3/2011). These pts will be followed from 1 to 3 years as a benchmark for treatment patterns and outcomes before immune checkpoint inhibitors became available. The results of this study will provide a valuable reference resource for newer approved agents, and will document changes in treatment patterns for pts with MEL. Enrollment began in October 2015.

Clinical trial registration: NCT02780089.

Characteristics of cutaneous adverse drug reactions in vemurafenib early post-marketing phase vigilance study in Japan

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Recent advances in immune checkpoint inhibitors and targeted therapies have drastically changed treatments for advanced melanoma patients (pts). Vemurafenib (VEM) was approved in Japan in Dec 2014, after the approval of nivolumab. Therefore, many Japanese pts receiving VEM had prior nivolumab because

the order of approval of the two agents are reversed in the United States and European countries. Here we report the first adverse drug reaction (ADR) data from Japan. Data were collected in the VEM early post-marketing phase vigilance (EPPV) study. EPPV is a Japanese system of post-approval surveillance performed in the first 6 months after product launch to promote careful use and collect ADR data.
We collected ADR data for all patients treated with VEM (960 mg bid) from 26 February to 25 August 2015.
Data were collected for 95 pts; 118 ADRs were reported in 46 pts including 24 serious ADRs in 13 pts. Cutaneous squamous cell carcinoma (cuSCC), a characteristic ADR of BRAF inhibitors, occurred in 1 pt, the first case in a Japanese pt from 95 pts in the real world post-marketing setting plus Japanese Phase I/II clinical study. In contrast, the incidence of cuSCC was 19.3% in the Phase 3 BRIM3 study. The most common serious ADRs were skin reactions in 7 pts, 6 of whom had prior treatment with anti-PD-1 antibodies (discontinued 5–35 days before starting VEM), most occurring in pts with prior anti-PD-1 agents. Further research is needed to clarify whether prior treatment with anti-PD-1 agents or racial differences affect cutaneous ADRs of BRAF inhibitors.

Safety profile of nivolumab in Japanese patients with unresectable malignant melanoma: interim results from prospective post-marketing surveillance in Japan

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Nivolumab is an indispensable agent at present for unresectable malignant melanoma (MM) treatment, but the real world evidence after launch is insufficient. Since July 2014, when nivolumab was approved in Japan, prospective post-marketing surveillance (PMS) that is a system specific to Japan is being conducted for all MM patients treated with nivolumab in Japan. PMS data were collected from all MM patients treated with nivolumab in Japan in the period from July 2014 to May 2016 (data cut-off). The surveillance data on follow-up for 6 months or longer (maximum 458 days) were available on 680 patients. Interim data analysis was conducted. Of 680 MM patients, 389 cutaneous melanomas (57.2%) and 208 mucosal melanomas (30.6%) were recorded. Patients with ECOG performance status (PS) of 2–4 and patients with preexisting autoimmune disorders, who are not able to participate in most clinical trials, accounted for 16.9% (n = 115) and 11.8% (n = 80), respectively. The incidences of drug-related adverse events (AEs) in any grade was 53.5% (n = 364) and grade 3–5 was 12.4% (n = 84). Notable drug-related AEs included colitis/diarrhea (n = 34), interstitial lung disease (ILD) (n = 22), type 1 diabetes mellitus (T1DM) (n = 5), myasthenia gravis (MG) and myositis (n = 4). In terms of the patient characteristics, there was no difference in incidence of drug-related AEs when stratified according to age, gender, PS, pre-LDH and preexisting autoimmune disorders. Based on the surveillance data collected at this time, the tolerability of nivolumab in Japanese patients with MM was largely favorable despite the fact that patients with preexisting autoimmune disorders and patients with PS 2 or higher were included. However, serious immune-related AEs such as colitis, ILD, T1DM and MG should be carefully monitored.

Whole genome sequencing identifies recurrent somatic *DGKZ* mutations in primary mucosal melanoma

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Mucosal melanoma (MM) is a common subtype of melanoma in Asians with extremely poor prognosis, and therapeutic strategy has not been clearly established for MM. Whole genome sequencing (WGS) has provided insight into the genetic underpinnings of cutaneous melanoma but little is known about MM. The aim of this study is to perform genomic profiling of MM to obtain the comprehensive genomic view of this subtype of melanoma.23 fresh MM tumors and matched germline DNA samples underwent either whole genome sequencing (WGS; n = 15) or whole exome sequencing (WES; n = 8). Recurrently mutated genes were subsequently Sanger sequenced in an independent extension cohort of 145 MM. For potential gain-of-function mutation, *in vitro* sensitivity of MM cells harboring the mutation to specific inhibitors was analyzed. A wide range of somatic mutation rates was observed. Notably, a recurrent somatic non-synonymous hotspot mutation in the diacylglycerol kinase, zeta (*DGKZ*) gene was discovered in 4/15 WGS cases and 1/8 WES (5/23, 22%). Sequencing of the independent extension cohort identified this recurrent *DGKZ* mutation in 20.7% (30/145) of samples. Strikingly, the proliferation of HEK293T cells with stable expression of *DGKZ* mutants was inhibited by a PI3K-AKT-mTOR inhibitor. In summary, we discovered a new recurrently mutated gene in mucosal melanoma by whole-genome sequencing, suggesting that PI3K-AKT-mTOR inhibition may be a potential avenue of targeted treatment for patients harboring this gene mutation.

Emerging targets in resistance to BRAF-inhibition: tackling the RSK in malignant melanoma

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The clinical availability of small molecule inhibitors specifically targeting BRAF mutated at V600 marked a significant breakthrough in the therapy of melanoma carrying such mutations. Despite a dramatic anti-tumour activity and improved patient survival, rapidly emerging resistance to these inhibitors, however, greatly limits their clinical benefit. A large number of different resistance mechanisms have already been described, yet common to many of them is the reactivation of the MAPK signalling pathway. The p90 ribosomal S6 kinase (RSK) is a downstream effector of the MAPK signalling cascade and has been reported to enhance cell survival of melanoma cells to chemotherapy by inducing anti-apoptotic mechanisms. Based on that, the aim of this study was to asses a potential role of the RSK in resistance to the $BRAF^{V600E}$ inhibitor vemurafenib (PLX4032).
Comparing melanoma cell lines with acquired resistance to vemurafenib to their sensitive counterparts reveals that the phosphorylation as well as the activity of the RSK is significantly enhanced in the resistant cells, which seems to be mainly based on elevated MAPK signalling. Intriguingly, RSK inhibition can

resensitize vemurafenib resistant melanoma cells and seems to be effective both in 2-dimensional and in 3-dimensional culture systems, especially when applied over a longer time period. The effect of RSK inhibition can be partly reproduced by downregulation of the Y-box binding protein 1 (YB-1), an important target of the RSK. Intriguingly, RSK inhibition also seems to retain its efficacy in melanoma cells with combined resistance to vemurafenib and trametinib.
These data suggest that active RSK signalling might be an attractive, novel therapeutic target in melanoma cells with acquired resistance to MAPK pathway inhibitors.

Immune checkpoint inhibitor responses in humanized mouse melanoma models using patient-derived xenografts

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Melanoma patients develop resistance to both chemo- and targeted-therapy drugs. Promising pre-clinical and clinical results with immune checkpoint inhibitors using antibodies directed against CTLA4 and PD1 have re-energized the field of immune-based therapies in melanoma. However, only subsets of melanoma patients respond to immune checkpoint blockade. Currently available mouse xenograft and transgenic mouse melanoma models have a number of short comings and are unable to address the basis of drug resistance and immune non-responsiveness frequently observed in melanoma. Thus there is an urgent need to establish a mouse model with an immune microenvironment that can address the above issues encountered in melanoma patients. For this, our laboratory has developed a humanized mouse melanoma model using patient-derived xenografts (PDX). Immunodeficient NSG mice were reconstituted with human CD34+ cells and after 7–9 weeks, mature human CD45+ cells are observed in mouse peripheral blood and in the lymphoid organs. Humanized mice with optimum number of mature human CD45+ cells in peripheral blood were challenged with HLA-matched melanoma PDX and the immune response to melanoma associated antigens were monitored. Lymphoid cells derived from humanized mice that are challenged with human leukocyte antigen (HLA) matched melanoma cells *in vivo* showed enhanced cytokine expression to *in vitro* stimulation with peptides derived from melanoma antigens. In addition, cytotoxic T-cells were able to functionally lyse tumor cells *in vitro,* infiltrate and restrict *in vivo* tumor growth. We are currently refining our model to establish an autologous mouse melanoma model. Our innovative humanized mouse melanoma model will enable one to understand the causes of therapy resistance and immune non-responsiveness in patients.

Differences in the young and aged immune microenvironment may affect the efficacy of immune based therapies in melanoma patients

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Skin cancer is the most commonly diagnosed cancer in the U.S. with over 3 million cases last year alone. However, the vast majority of these are non-melanoma skin cancers which are slow growing, easily treatable, and have very low risks of mortality. Melanoma skin cancer is the most lethal form of the disease, and while it represents the minority of skin cancer cases (<5%), it accounts for over 80% of skin cancer related deaths. Unlike most other types of cancers which almost exclusively occur later in life, melanoma is one of the most common cancers in young adults under the age of 30. As a result, a much younger patient population exists within melanoma that must be considered when designing and analyzing therapies. It is now known that younger patients respond better to the FDA approved kinase inhibitors designed to treat the mutant BRAF form of melanoma. This is in part due to factors secreted in the aged tumor microenvironment that counteract the effect of therapy. Recently, treatment of melanoma as well as other cancer types have shifted towards immune based therapies which have somewhat lower response rates but offer more frequent long term response. Thus, we investigated the differences in infiltrating immune cells between the aged and young tumor microenvironment that may affect the response and tolerability of immune based therapies. Not surprisingly, differences were observed in the immune populations of tumors in aged and young mice, as well as in available patient data. The results presented here stress the importance of considering age when investigating immune checkpoint inhibitors and other immune based therapies.

Predicting response to vismodegib (VISMO) based on baseline (BL) characteristics in patients (pts) with locally advanced basal cell carcinoma (laBCC) in the STEVIE study

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VISMO, a first-in-class Hedgehog pathway inhibitor, is approved for use in adults with laBCC and metastatic BCC. STEVIE, a multicenter, phase 2 study is evaluating safety of VISMO in a real-world setting in advanced BCC pts. BL characteristics of pts with laBCC in STEVIE were analyzed to determine their influence on response to VISMO treatment. Adults with laBCC received oral VISMO 150 mg/day until progressive disease, unacceptable

toxicity or withdrawal. Safety was the primary objective. Influence of selected BL characteristics (lesion size/number, location [head, neck, trunk, extremities, other], age, sex and Eastern Cooperative Oncology Group performance status [ECOG PS]) on response (any or complete [CR]) to VISMO was assessed by uni- and multivariate analyses. The analyses included 863 pts with laBCC (excluding pts with Gorlin syndrome). Univariate analysis showed pts with neck/trunk lesions versus no neck/trunk lesions or ≥3 versus 1–2 lesions at BL were more likely to respond to VISMO. Tumor burden, measured by sum of lesion diameters, was not predictive of response. Pts with ECOG PS ≥2 were less likely to respond. Univariate analysis of complete responders showed that pts with ECOG PS 0 were more likely to have CR, but the odds of achieving CR decreased with increased tumor burden. None of the explored factors demonstrated statistical significance in the multivariate analysis for overall response; while for complete responders, multivariate analysis confirmed the findings of the univariate analysis. To conclude, pts with smaller lesions and better ECOG PS were more likely to achieve CR. While some BL characteristics were predictive of response independently, multivariate analysis did not identify any strong predictive markers of treatment response from demographic data and tumor characteristics.

Braf inhibitor resistance selects for recurrent aneuploidy in a mouse model with telomere dysfunction

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Telomere dysfunction leads to genomic instability including copy number aberrations, events which serve as substrates for the selection of both cancer-initiating[1] and cancer-progressing genetic lesions[2,3]. To date however, the role of telomere dysfunction in the genomic evolution of drug resistance is undescribed. To address this knowledge gap, we describe here the effects of telomerase loss on a *BRAF*-mutant mouse model of melanoma[4,5] in the context of pharmacological Braf inhibition. In the genomically stable *BRAF*-mutant melanomas, resistance to Braf inhibitors is acquired without increased mutation rates or aneuploidy, but by increased expression of mutant BRAF. By contrast, when telomerase-deficient mice experiencing telomere dysfunction and genomic instability acquired *BRAF* inhibitor resistance, these unstable melanomas selected for highly recurrent gains of chromosomes 11 and 15. These two chromosomes are syntenic to regions frequently gained in human melanoma[6] and are coincident with reactivation of MAPK signaling in the mice. We conclude that the presence of telomere dysfunction alters the genomic profile of drug resistance towards recurrent aneuploidies.

Efficacy of nivolumab (NIVO) plus ipilimumab (IPI) combination in patients with advanced melanoma (MEL) and elevated serum lactate dehydrogenase (LDH): a pooled analysis

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SpainBaseline LDH is a well-established, independent prognostic factor for poor outcomes in patients (pts) with MEL, but limited efficacy data exist for immuno-oncology agents in pts with elevated LDH. We present efficacy outcomes in this subgroup from phase 2 (CheckMate 069) and 3 (CheckMate 066, CheckMate 067) trials. Pooled data from treatment-naïve pts (N = 1270) who received NIVO+IPI (n = 407), NIVO (n = 507), or IPI (n = 356) were stratified by baseline LDH levels; treatment arms were balanced for other prognostic factors. Endpoints included median progression-free survival (mPFS) and objective response rate (ORR). LDH impact on overall survival was not evaluated as data remain immature for CheckMate 067. Of 1270 pts, 455 (36%) had LDH>ULN and 327 (26%) had *BRAF* mutations. With ≥18 months follow-up, mPFS in all pts with LDH>ULN treated with NIVO+IPI, NIVO and IPI was 5.2, 2.7 and 2.6 months, respectively (HR for NIVO+IPI versus NIVO, 0.75, P = 0.12; HR for NIVO+IPI versus IPI, 0.48, P < 0.01); in pts with a *BRAF* mutation and LDH>ULN, mPFS was 4.1, 2.8 and 2.7 months, respectively (HR for NIVO+IPI versus NIVO, 0.69, P = 0.26; HR for NIVO+IPI versus IPI, 0.56, P = 0.07). PFS rates for all pts with LDH>ULN treated with NIVO+IPI, NIVO and IPI were 47%, 34% and 15% at 6 months; 38%, 29% and 7% at 12 months; 35%, 28% and 6% at 18 months, respectively. ORR in all pts with LDH>ULN versus those with LDH>ULN and a *BRAF* mutation was 45% versus 48% for NIVO+IPI, 31% versus 15% for NIVO and 10% versus 11% for IPI. While MEL pts with LDH≤ULN had better efficacy outcomes, those with LDH>ULN still benefited from NIVO+IPI with a similar safety profile. NIVO+IPI provided higher ORR and longer mPFS than NIVO or IPI, irrespective of LDH level or *BRAF*

status. Additional data will include duration of response, LDH >2x ULN, and relative LDH changes as an early outcomes marker.

Arginyl-tRNA-protein transferase 1 (ATE1) in melanoma growth and drug response

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Arginyl-tRNA-protein transferase 1 (ATE1) transfers arginine from arginyl-tRNA to proteins, resulting in protein arginylation. While initially thought to take place at the N-termini, implicated in protein stability, recent studies suggest the arginylation can also take place along the protein coding sequence, thereby pointing to its plausible impact on broader range of processes.

Loss of ATE1 resulted in decreased cell migration and invasion, which were associated with impaired actin arginylation. Conversely, mouse embryo fibroblasts (MEFs) obtained from ATE1 knock-out mice exhibit increased tumorigenic potential, compared to their wild-type counterparts.

To determine the possible role of ATE1 in melanoma, we assessed ATE1 expression in cohort of melanoma cell lines and tumors. ATE1 expression was found to inversely correlate with melanoma patient survival and deregulated ATE1 expression was noted in 20% of melanoma specimens. ATE1 expression was found to be higher among NRAS mutant melanomas. Correspondingly, inhibition of ATE1 expression attenuated growth of melanoma in culture. Further sensitization was observed in both MEFs and melanoma cells that were subjected to serum starvation. Surprisingly, ATE1 knock-down conferred drug resistance to NRAS but not BRAF melanoma cells, pointing to distinct nodes that are ATE1-regulated in each of the melanoma genotypes. These data suggest that ATE1 plays a role in melanoma growth and response to therapy, likely, by arginylating genotype specific regulatory proteins. The mechanisms underlying ATE1 regulation and function in melanoma will be discussed.

The effect of systemic treatments on immune accessibility in melanoma: a retrospective histopathological investigation

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Histology and immunohistochemistry of melanoma mets represents the immunological status of a tumor and has prognostic relevance. In addition it might have also predictive value for subsequent immunotherapy. Since targeting the PD-1/PD-L1 interaction has been FDA approved for various cancer types, staining for these proteins is of particular interest. In this study, we focus on varying TIL infiltration and the relationship to IDO expression. Tumor samples of 42 melanoma patients paired pre- and post reatments including targeted- and immunotherapies were stained for markers including PD-L1, IDO, and CD3 and were analyzed with focus on altering TIL infiltration under treatment.In total 42 paired tissue samples were available for complete histological analysis and clinical correlation. 18 patients underwent immunotherapy, 24 targeted therapy including sequential combinations of both[DR1]. TIL infiltration was increased in 18 out of 42 patients, (6 out of 18 after immuno-, 9 out of 24 after targeted therapy). However, IDO-positivity at any time in a patient (n = 13) was associated with shorter median survival as compared to patients without (n = 30) with 19 versus 27 months. When exploring IDO-positive tissue samples (n = 17, 13 patients), 23.5% displayed moderate to severe TIL infiltration, 40.7% with determined IDO status (n = 81, from 42 patients), showed moderate to severe TIL. In 4 of 5 cases with PD-L1 expression >1%, they were also IDO-positive and displayed a moderate to severe level of TIL infiltration (4/5). To conclude, we found a coincidence of increased TIL infiltration, PDL1 and IDO 1 expression that is associated with improved tumor control and has to be interpreted as a marker for immunosusceptibility. A switch from a previous grenz zone like pattern to infiltration can be observed repeatedly and is often found with targeted therapy.

Impact of depth of response to targeted therapy on survival outcomes in patients treated with vemurafenib (V) or cobimetinib + vemurafenib (C+V): a pooled analysis

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Monotherapy with V or combination therapy with C+V has shown improved survival outcomes in patients with *BRAF*^{V600}-mutated metastatic melanoma. In this exploratory analysis, we pooled data from BRIM-2, -3, -7 (BRAF inhibitor-naive patients), and coBRIM studies to evaluate the association of depth of tumor response on survival outcomes in cohorts of patients given V or C+V and in previously identified prognostic subgroups (Larkin JM, et al. ASCO 2016 abst 9536). Depth of tumor response was assessed by maximum percentage change in the sum of longest diameters (SLD) between baseline and post-baseline SLD before disease progression (Max%SLD), and by time to Max%SLD (TimeMax%SLD). Association with survival outcomes was estimated using Cox proportional hazard ratios adjusted for age, sex, race, geographic regions, ECOG PS, lactate dehydrogenase, liver metastases, disease stage, and baseline SLD. Depth of response was significantly associated with PFS when evaluated as a continuous variable ($P < 0.0001$ for both Max%SLD and TimeMax%SLD in both V and C+V cohorts). Each 1-month incremental increase in TimeMax%SLD was associated with a 14% (V) or 16% (C+V) reduction in relative risk of progression. Median depth of response was greater in the C+V cohort versus V (−73% Max%SLD; IQR −51% to −92% versus −50% Max%SLD; IQR −28% to −71%, respectively), as was median TimeMax%SLD (7.4 months, 95% CI 6.7–8.3, for C+V versus 5.1 months, 95% CI 4.9–5.6, for V). Improved depth of response associated with C+V versus V was observed across the prognostic groups. Increasing depth of response is associated with improved survival outcomes (PFS) across

prognostic groups. These data, together with results from extended follow-up of coBRIM, confirm the persistent clinical activity and improvement in outcomes with long-term C+V combination therapy.

Enhanced efficacy of image-guided targeted α-particle therapy for human metastatic melanoma by co-treatment of MAPK and HDAC inhibitors

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Melanocortin subtype 1 receptor (MC1R) has long been considered a promising target for delivery of peptide-receptor targeted radionuclide therapy for melanoma. Previous published studies showed promising results using radiolabeled α-MSH analogs as theranostic agents in B16 murine tumor xenograft models. However, the potential of MC1R-targeted ligands has been limited by heterogeneous expression of MC1R in human melanoma cells compared to B16 cells. In the present work, we demonstrate that FDA-approved BRAF inhibitor Vemurafenib (Vem) and histone-deacetylase inhibitor phenylbutyric acid (4-PBA) can be used to enhance MC1R expression and the efficacy of MC1R-targeted imaging-guided α-particle therapy for melanoma. In *in vitro* studies, enhanced MC1R expression was observed (flow cytometry) in 3 human melanoma cell lines treated with Vem and 4-PBA. MC1R upregulation was confirmed by saturation binding assays using [^{125}I]NDP-α-MSH peptide ligand. In human melanoma tumor-bearing mice (A2058 xenografts), theranostics efficacy studies were performed using [^{203}Pb/^{212}Pb] labeled α-MSH analogue DOTA-VMT-MCR1 that specifically binds to MC1R. 2 h post-injection SPECT/CT imaging showed significantly higher tumor uptake of [^{203}Pb]DOTA-VMT-MCR1 when pretreated with Vem (10 mg/kg, PO) and 4-PBA (120 mg/kg, IP). In [^{212}Pb]DOTA-VMT-MCR1 therapy studies, significantly extended survival and tumor progression control were achieved in a combination therapy group ([^{212}Pb]DOTA-VMT-MCR1 + Vem + 4-PBA) compared with other groups (control, Vem only, 4-PBA only, [^{212}Pb]DOTA-VMT-MCR1 etc.). In conclusion, pretreatment of mice with Vem and 4-PBA induced a robust upregulation of MC1R expression that led to significantly improved SPECT/CT imaging and significantly enhanced MC1R-targeted alpha particle therapy for human metastatic melanoma in mice.

Dermal fibroblasts with mTOR activation modulate melanoma growth in xenograft mouse models

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Melanoma is composed of malignant cells and supporting stroma, which includes fibroblasts, immune cells and the extracellular matrix. Increased understanding of interaction between melanoma cells and the microenvironment could substantially benefit development of effective treatment for melanoma. In preliminary studies, we found the presence of variable numbers of fibroblasts at the base of melanomas that stained strongly for phospho-ribosomal protein S6 (pS6), a marker of mTORC1 activation. The results suggested that dermal fibroblasts with mTOR activation may influence the development and growth of melanoma. To investigate the effect of mTOR activated fibroblasts on melanoma growth, we added melanoma cells (MM200) and neonatal foreskin keratinocytes on the top dermal equivalents containing NFF-shTSC2 (human neonatal foreskin fibroblasts with TSC2 knock down and mTOR activation) or NFF-shNT (control). We grafted the dermal-epidermal composites to immunodeficient mice and found that the area of grafts with NFF-shTSC2 (26.2 ± 14.3 mm^2) is significantly smaller than the grafts with NFF-shNT (44.8 ± 6.8 mm^2) 8 weeks after grafting. In another approach, we subcutaneously injected melanoma cells (DO8) mixed with dermal fibroblasts derived from *Tsc2* knockout mice (NMF-Tsc2 cKO) or control cells (NMF-control).The size of tumors with NMF-Tsc2cKO (264.9 ± 97.2 mm^3) is significantly smaller than the tumor with NMF-control (393.9 ± 37.1 mm^3) 6 week after injection. Our results indicated that dermal fibroblasts with mTOR activation may negatively modulate melanoma growth in our xenograft mouse models. The findings could benefit further investigation and understanding of interaction between melanoma cells and cancer-associate fibroblasts in the tumor microenvironment.

Identification and characterization of the metastatic cell populations in a mouse model of melanoma

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Melanoma is the deadliest form of skin cancer due to its high propensity to metastasize and resistance to current therapies. We have created an inducible mouse model of metastatic melanoma (*Dct-Grm1/K5-Edn3*) where metastasis to the lungs is 80% penetrant. The primary tumors of these mice present cellular heterogeneity with cells at varying levels of differentiation. The main goal of this study is to determine if the primary tumor resident Tyrosinase positive cells are the major contributor to lung metastases and evaluate the dynamic pattern of gene expression as those cells move from the primary tumors to the sites of metastasis. To accomplish this aim we crossed the *Dct-Grm1/K5-Edn3* mice to *CreERT2/ ROSAmT/mG* mice to indelibly label Tyrosinase cell populations within the primary tumor and perform lineage tracing in the metastatic lesions. We found that Tyrosinase positive cells enter the circulation at the very early stages of tumor progression and establish close interactions with blood vessels. Metastatic cells in close association with the inner wall of the blood vessels loose pigmentation and do not express melanocytic markers. In the lung tissue they are capable of establishing successful metastases and express melanocytic markers. The results of this study will increase our understanding of the etiology and pathogenesis of melanoma metastasis. Further characterization of those more aggressive cells in melanoma will allow for the development of new prognostic tests and novel therapeutic strategies to eliminate metastasis.

RNF5 inhibits melanoma development by altering the gut microbiota and immune checkpoint activity

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To date, the success of current immune checkpoint inhibitors in cancer therapy has been limited to a few targets and tumor types, underscoring the need for greater understanding of immune checkpoin regulation. Here, we establish a link between the ubiquitin ligase RNF5, composition of the gut microbiota, and immune checkpoint control. We found that growth of mouse melanoma cells *in vivo* is attenuated, while tumor infiltration of effector CD^4+/CD^8+ T cells and dendritic cells is increased, in R*nf5*$^{-/-}$ compared with R*nf5*^{wt} mice. This phenotype was directly linked to the increased abundance of select bacterial strains in the gut of R*nf5*$^{-/-}$ tumor-bearing mice. We will discuss the importance of RNF5 in controlling the gut microbiome composition and its role in the immune checkpoint-mediated restraint of melanoma growth.

The natural history and patterns of metastases from mucosal melanoma: an analysis of 706 prospectively-followed patients

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We examined whether mucosal melanomas are different in their clinical course and patterns of metastases when arising from different anatomic sites.

Clinical and pathological data from 706 patients were compared for their stage distribution, patterns of metastases, CKIT/BRAF mutation status, and overall survival.

The anatomic sites of the primary mucosal melanomas were from the lower GI tract (26.5%), nasal cavity and paranasal sinuses (23%), gynecological sites (22.5%), oral cavity (15%), urological sites (5%), upper GI tract (5%), and other sites (3.0%). At initial diagnosis, 14.5% were stage I disease, 41% Stage II, 21.5% Stage III, and 23% stage IV. Predominant metastatic sites were regional lymph nodes (21.5%), lung (21%), liver (18.5%), distant nodes (9%). Oral cavity mucosal melanoma had a higher incidence of regional nodal metastases (32% versus 20%, $P = 0.009$), and a higher incidence of lung metastases (33% versus 19%, $P = 0.007$) compared to other primary mucosal melanomas. There was a 10% incidence of CKIT mutation and 12% BRAF mutation. Mucosal melanomas from nasal pharyngeal and oral, gastrointestinal, gynecological and urological had a similar survival with a 1-year survival rate (88%, 83%, 86%), 2-year survival rate (66%, 57%, 61%), 5-year survival rate (27%, 16%, 20%), respectively.

ConclusionThe largest sample size allows, for the first time, a comparison of primary melanoma stage and patterns of metastases across anatomical sites. With few exceptions, the presenting stages, incidence of nodal and distant metastases, or the site of predilection of distant metastases were similar despite different primary anatomic sites. These findings suggest that clinical trials involving mucosal melanomas and the administration of systemic therapy can be applied equally to mucosal melanomas regardless of their primary anatomic site.

Luteolin inhibits melanoma growth *in vitro* and *in vivo* via mitochondrial ROS induction

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Luteolin is a dietary flavonoid which shows inhibitory effect towards some cancer types. Luteolin inhibits melanin synthesis in mouse B16 cells; its anti-melanoma effect was not well studied and results were controversial in the limited publications. We show luteolin significantly inhibits the short-term proliferation in all 5 melanoma cell lines in a dose-dependent manner. The IC_{50} values ranged from 6~15 mM. Luteolin also inhibited colony formation in A375 and Wm3211 cell lines at low micromolar range. Oral luteolin administration in mice significantly inhibited the growth of xenografted tumors derived from A375 and Wm3211 cells. Although luteolin inhibited protein accumulation of PDE4B, AKT2 and AKT3 in both cell lines, over-expression of neither AKT2 nor AKT3 altered luteolin sensitivities. On the other hand, luteolin induced cellular reactive oxygen species (ROS, superoxide in particular) in a dose- and time-dependent manner. Further analysis indicated that the ROS was derived from mitochondria, rather than from the plasma membrane-located NADPH Oxidase 1 (NOX1). Consistently, luteolin induced the mitochondrial SOD2 but not the cytosolic SOD1. Further combination treatment using mitochondrial ROS scavenger and luteolin are being carried out to determine if the mitochondria is the target for luteolin in melanoma cells. In conclusion, our *in vitro* and *in vivo* results suggested that luteolin was able to effectively inhibit growth of a subset of melanoma cells, accompanied by inhibition of PDE4B, AKT2 and AKT3 at the protein level, which resulted in increased cAMP signaling. However AKT2 or AKT3 was not the critical luteolin target. Rather the luteolin-induced mitochondrial ROS played a critical role. To the best of our knowledge this is the first report showing *in vivo* efficacy of luteolin for melanoma, with a possible mechanism through provoking mitochondrial ROS generation.

Healthcare resource utilization and costs in first-line treatments for patients with metastatic melanoma

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Given the recent approval of newer immuno-oncology therapies (IO) for unresectable or metastatic melanoma (MM), the clinical and economic burden is not well documented for MM patients (pts) receiving PD-1 inhibitors. The aim of this study was to describe healthcare resource utilization (HRU) and costs among MM pts treated with IO, BRAF/MEK inhibitors (BRAFi/MEKi), and traditional chemotherapies. Adults with ≥2 claims for MM (ICD-9/10 codes 172.x/C43.x) and ≥1 claim for metastasis (196.x-198.x/C77.x-C79.x) were identified between 1/1/2011 and 12/31/2015 from a US claims database. Per patient per month (PPPM) HRU and costs were calculated during first-line (1L; first claim to

30 days prior to 2L or end of follow-up) by treatment (tx) group. There were five 1L tx groups identified among 812 MM pts with ≥1 month of tx: A = PD-1 inhibitor (n = 13), B = ipilimumab (n = 296), C = BRAFi monotherapy (n = 161), D = BRAFi/MEKi combination (n = 54), and E = other chemotherapy (n = 288). The majority were male (63%), mean (SD) age at metastatic diagnosis was 62.1 years (13.1), and 16% had brain metastasis. One pt (8%) in group A had a hospitalization during 1L, while 41%, 50%, 48%, and 48% of pts in groups B-E had ≥1 hospitalization during 1L. Overall mean PPPM healthcare costs during 1L were $14 763, $32 259, $9593, $25 205, and $7655 among pts in groups A-E, respectively, which were driven by mean MM tx costs ($10 893, $28 920, $6038, $20 819, and $2507, respectively). Treatment costs of B largely occurred in first 12 weeks, while costs for other MM tx occurred over longer periods, influencing PPPM costs during 1L. This study of real world evidence suggests costs vary substantially across 1L therapies for MM. Further data are needed on a larger number of MM pts treated with IO who have longer follow-up to better understand the long term clinical and economic burden in this population.

Oncologist preferences for attributes of drug therapy in advanced melanoma

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Multiple treatments options are available for advanced melanoma, yet factors that oncologists believe are important in making decisions for patients remain unknown. Our aim was to elicit oncologists' preferences for drug treatment attributes in advanced melanoma.

A discrete choice experiment (DCE) was conducted in US-based oncologists who treated 4 or more patients diagnosed with advanced (unresectable or metastatic) melanoma each month. A series of scenarios asked respondents to choose between two hypothetical medications, each with 7 attributes: mode of administration, dosing schedule, duration of therapy (3, 8, and 12 months), objective response rate (ORR) (15%, 33% and 65% chance of response), progression free survival (PFS) (3, 5, and 11.5 months), overall survival (OS) (45, 55, and 75% survival to 12 months), and grade 3/4 toxicities/adverse events (AEs) (10%, 32%, and 55% likelihood). Each attribute had 3 levels except dosing schedule (8 levels). Bayesian logistic regression models were used to estimate preference weights for each attribute.

A total of 1113 oncologists were invited, 362 responded (33%), and 226 met screening criteria and completed the study. They had practiced for a mean (SD) of 15.4 (6.4) years, and reported prescribing medications as first line therapy in the past 3 months as follows: nivolumab (58%), pembrolizumab (56%), ipilimumab (73%), and ipilimumab plus nivolumab (49%). The relative importance of medication treatment attributes were: AEs (49%), OS (34%), ORR (12%), PFS (3%), dosing schedule (2%), duration of therapy (0%) and mode of administration (0%). An improvement from 55% to 75% in 1-year OS was valued similar in magnitude to a 23% decrease (from 55% to 32%) in likelihood of AEs.

In summary, oncologists valued OS and AE as most important drug therapy attributes for advanced melanoma, while ORR and PFS were considered relatively less important.

Patient preferences for treatment options in advanced melanoma

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Multiple treatment options exist for patients with metastatic melanoma however it remains unclear what factors of individual therapies are most important to patients. We conducted a discrete choice experiment (DCE) to better understand aspects of therapy most important to US-based patients.

Patients diagnosed with advanced melanoma who had an ECOG performance status of 0–3 completed an online DCE survey. Scenarios were presented where they had to choose between two hypothetical profiles of different treatment options, in patient-friendly language, that related to seven attributes: mode of administration, dosing schedule, duration of therapy (3, 8, and 12 months), objective response rate (ORR) (15%, 33% and 65% chance of response), progression free survival (PFS) (3, 5, and 11.5 months), overall survival (45, 55, and 75% survival to 12 months), and grade 3/4 toxicities/adverse events (AEs) (10%, 32%, and 55% likelihood). Relative preference weights for medication features were estimated using a Bayesian logistic regression model with effects coding parameterization.

Of 935 invitees, 273 (29%) responded, and 200 (21%) completed the study. The majority of respondents were female (61%), white (84%), diagnosed in past 5 years (80%), and currently undergoing treatment (60%). In comparing preferences for treatment, pembrolizumab was preferred by 45.5% while nivolumab plus ipilimumab was preferred by 55.5%. The factors that had the most impact on treatment decisions were overall survival (33%), AEs (29%), ORR (25%), and to a lesser extent, PFS (12%). The relative importance of an increase in likelihood of toxicity from 10% to 32% was -3.4 whereas the relative importance of an increase in OS from 45% to 55% was 3.3.

Patients valued overall survival, AEs and ORR important when making a treatment decision, while dosing schedule, duration of therapy and mode of administration were relatively unimportant.

Identification of hypoxia-induced HIF1A targets in melanocytes reveals a gene profile associated with poor prognosis for melanoma

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HIF1A is a critical regulator of cellular response to changes in oxygen concentration within the tissue microenvironment. HIF1A signaling regulates multiple cellular processes critical to tumor progression, including metabolism, cellular proliferation, chromatin remodeling, vascularization and invasion. However, gene-specific responses to both hypoxia and HIF1A signaling are highly tissue dependent. Given the complexity found with HIF1A gene regulation across tissues, we utilized an integrated genomics approach to identify a melanocyte-specific response to hypoxia, and investigate the role of HIF1A-responsive genes in melanoma progression. We identified a cohort of 81 HIF1A direct target genes regulated by HIF1A signaling under hypoxia, 19 of which are novel HIF1A targets. Analysis of individual gene expression levels for this cohort within the cutaneous melanoma

primary tumor dataset generated by TCGA Research Network (http://cancergenome.nih.gov/) found that individual expression levels for 12 HIF1A direct targets were significantly correlated with reduced time of Disease Free Status (DFS). The cumulative expression profile of the 12 targets also finds significant correlation with DFS by logistic regression (P-value = 0.0013) and ROC curve analysis (AUC = 0.849, P-value<0.0001). This panel of HIF1A-responsive genes identifies a microenvironment-driven expression profile correlated with primary melanoma tumor progression to metastasis, and defines a melanocyte-specific response to regulation of metabolism, cellular proliferation, chromatin remodeling, and vascularization. This 12 gene panel provides targets for evaluation and refinement of diagnostic markers associated with primary melanoma tumor metastatic potential, and also provides targets for therapeutic strategies targeting HIF1A and hypoxia-driven metastatic disease progression.

Outcomes in patients (pts) treated with ipilimumab (ipi) after pembrolizumab (pembro) in KEYNOTE-006

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Several studies, including the KEYNOTE-001 and 002 trials of pembro, have established the efficacy of PD-1 inhibition in advanced melanoma refractory to the CTLA-4 inhibitor ipi. The efficacy of ipi following anti–PD-1 therapy is not clearly established. We assessed outcomes in pts enrolled in KEYNOTE-006 (NCT01866319) who received ipi monotherapy as the next therapy after pembro. Pts in KEYNOTE-006 were randomized to pembro 10 mg/kg Q2W (n = 279) or Q3W (n = 277) or ipi 3 mg/kg Q3W (n = 278). After study treatment discontinuation, subsequent anticancer therapy and outcomes were reported. As of Dec 3, 2015, ipi was recorded as a subsequent therapy for 129 pembro-treated pts. Of the 97 pts who received ipi monotherapy as their first post study therapy, 64% had M1c disease, 33% had elevated LDH, and 16% had *BRAF*^{V600}-mutant tumors at baseline. Median duration of pembro before ipi was 18 weeks (range 0–90). Best response to pembro (RECIST v1.1, central review) was PR in 16%, SD in 12%, nonCR/nonPD in 5%, PD in 62%, and nonevaluable/not assessed in 4%. Median time between the last pembro and first ipi doses was 5 weeks (range 1–42). Median duration of ipi was 8 weeks (range 0–17). Reported ORR for ipi was 14%; best overall response was CR in 3%, PR in 11%, SD in 33%, PD in 33%, and unknown in 23%; 40% of the pts with PR and 48% with SD had subsequent progression. Best response to pembro (central RECIST v1.1) in the 13 pts who responded to ipi was SD in 1, nonCR/nonPD in 2, PD in 8, and not assessed in 2. Best response to ipi in the 16 responders to pembro who received ≥1 ipi dose was SD in 8, PD in 3, and unknown in 5. 57 of the 97 pts had died, and median OS from randomization was 19.6 months (95% CI 16.4–23.5). Ipi has antitumor activity following pembro in pts with advanced melanoma, including those whose best response to pembro was PD, with an ORR consistent with historical data.

Functional impact of targeting the SLAMF6 immune receptor on anti-melanoma T cells

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The SLAM family of immune receptors (SFRs) is a conserved and partially redundant set of six receptors expressed on hematopoietic cells. They include SLAMF1 (SLAM), SLAMF6 (NTB-A), 2B4 (SLAMF4, CD244), SLAMF3 (Ly-9), SLAMF5 (CD84) and SLAMF7 (CRACC). All SFRs except 2B4 are homotypic receptors which contain tyrosine-based switch motifs in their cytoplasmic tail. The role of SFRs in cancer immunity is hard to decipher due to the duality of the switch motif and the *cis-trans* interaction options. Single receptor knockout yields minimal immune derangement, but multiple SFRs silencing or mutations of their adaptor protein, SAP, result in profound T and NK cell defects. We show that engagement of the receptor by its soluble ectodomain (seSLAMF6) specifically enhances anti-melanoma CD8 T cell activity while inducing SLAMF6 de-phosphorylation, markedly reduces activation-induced cell death, serves as a substitution for IL-2 and gives preference to the growth of anti-tumor T cells from bulk tumor infiltrating lymphocytes. *In vivo* experiments have shown that SLAMF6 triggering of T cells before adoptive transfer delays melanoma growth, confirming *in vivo* that SLAMF6 receptor triggering generates CD8 T cells with anti-melanoma reactivity which is comparable to IL-2. Interestingly, aberrant SLAMF6 expression on melanoma cells reduced T cell anti-melanoma response, thus inhibiting *in trans* CD8 T cell activation. These exciting observations, obtained in human and mouse studies, provide the rationale for our overall hypothesis, that SLAMF6 is a highly relevant, albeit unexplored, target for the immunotherapy of melanoma.

Lymphatic vessels are required for cutaneous immunity: implications for melanoma

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Lymphatic vessel remodeling is correlated with melanoma progression and lymph node metastasis. While lymphatic vessels provide an important route for disseminating tumor cells, their role in responding to immunological challenge has yet be appreciated with respect to tumor progression. We recently demonstrated that in the absence of dermal lymphatic vessels, the tumor microenvironment of murine melanoma remains completely uninflamed and fails to induce a robust T cell response. Furthermore, in an analysis of cutaneous metastatic melanoma taken from the TCGA database, we identified positive correlations between expression of genes associated with lymphatic vessels and immune infiltrate. Importantly, those patients that respond to immunotherapy enrich for infiltrating lymphocytes and genes associated with potent local immune response. We therefore hypothesize that lymphatic vessels are a biomarker of *in situ* immune responsiveness and therefore response to therapy. To simultaneously evaluate immune and vascular components in human melanoma we use a multiplex-immunohistochemistry-based approach, which allows for

examination of up to twelve markers by sequential staining of single sections. Tissue regions that include tumor/stroma borders and show high $CD8^+$ T cell infiltrates are selected for analysis. This is followed by tissue segmentation and automated detection of cell populations within intra-tumoral and peritumoral regions. Using this approach, we have demonstrated a correlation between infiltrating lymphocytes and tumor-associated lymphangiognesis or lymphatic remodeling. Our work taken all together indicates that the lymphatic vasculature is an important, active component of the anti-tumor immune response and may represent a biomarker to stratify patient response and survival for effective clinical application of immunotherapy.

Dermoscopy of a hypomelanotic melanoma masquerading as a squamous cell carcinoma

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72 year-old post transplant Caucasian woman with history of multiple squamous cell carcinomas (SCC) presented with an asymmetric, two-toned pale brown and pink-white shiny patch with hyper-reflective scale on the right upper shoulder. Dermoscopy revealed non-specific asymmetric pale brown pigmentation that covered <25% of the lesion as well as irregular, linear, and focally distributed polymorphous vessels. Based on clinical history and dermoscopic features the differential included a collision lesion of squamous cell carcinoma and pigmented actinic keratosis versus a hypomelanotic melanoma. The lesion was biopsied and pathology revealed superficial spreading melanoma measuring 0.23 mm Breslow thickness, Clark Level III with positive surgical margins. Specifically, the presence of atypical melanocytes in irregular array were observed in the epidermis and superficial dermis.

Melanocytic lesions with minimum to no pigment represent a diagnostic challenge clinically and dermoscopically, as they can mimic both benign tumors, such as a dermal nevi, in addition to malignant tumors, including basal cell carcinomas, SCCs, Bowen's disease, and actinic keratoses. Given that our patient had a history of multiple SCCs and this lesion presented as a scaly, pink-white shiny patch was more consistent initially with superficial SCC. However, the presence of atypical polymorphic vessels and faint non-specific pigmentation on dermoscopy suggested an alternative diagnosis. Dermoscopy allows the visualization of vascular features, which can be key to lesion diagnosis, especially when typical pigmented patterns are not observed. Histology confirmed a diagnosis of hypomelanotic melanoma, which is commonly misdiagnosed on clinical examination alone.

Results of the Spanish EAP of pembrolizumab in melanoma. Spanish Melanoma Group (GEM)

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The programmed death (PD-1) inhibitor pembrolizumab has demonstrated a significant improvement in terms of median overall survival (OS) in melanoma patients (p). We evaluated the clinical activity of pembrolizumab in melanoma p treated under the expanded access program (EAP) in Spain.

Patients with unresectable stage III/IV melanoma who had failed to previous ipilimumab, were treated with pembrolizumab 2 mg/kg iv every 3 weeks. Patients with brain metastases were not excluded.

Data from 67 advanced melanoma p (46 cutaneous, 8 uveal, 7 mucosal and 6 from unknown primary) were analyzed. Forty nine p were stage M1c (73.1%), 37 p had high LDH levels (55.2%), 27 p had ECOG 0 (40.3%) and 51 p were treated in 3–4th line (76.1%). Median overall survival (OS) was 8.73 months (m) (CI 95% 2.3; 15.14), and $OS_{12\ m}$ 44.2%. For cutaneous melanoma p, median OS was 14 m (CI 95% 4.3; 23.6) and $OS_{12\ m}$50.5%. Objective responses were obtained only in the subgroup of cutaneous melanoma p with an overall objective response of 27%, including three (6.5%) complete responses (CR). Median response duration was 14.3 m, with 83.3% of responses ongoing (3.5 m+ to 20.4 m+). From ten p included with brain metastases, four (40%) p had an objective response (OR), two (20%) of them CR. For p who achieved objective response (OR), stable disease (SD) and progressive disease (PD), $OS_{18m}$was 80%, 64.6% and 7%, respectively (P = 0.0001). Other significant prognostic factors for OS were LDH levels (elevated versus normal LDH had a median OS NR versus 3.7 m, respectively; P = 0.00001) and ECOG PS (median OS in ECOG 0, 1 and 2 was NR versus 6.8 m versus 1.1 m, respectively; P = 0.0001). There were no serious adverse events.

Activity of pembrolizumab at the approved dose and schedule, was confirmed in the real clinical setting, with long-term responders, including also patients with brain metastases.

Peripheral blood cells do count for melanoma patient prognosis

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Inflammation is involved in tumor development and progression, and can be estimated through surrogate markers easily computable from routine blood tests. Here, we analyzed the associations of peripheral blood cell counts and their ratios with disease characteristics and outcome of 584 melanoma patients at

any stage of the disease. Survival was estimated with the Kaplan-Meier method, and univariate and multivariate Cox proportional hazard models were applied. We show that the presence of distant metastases is associated with higher leukocyte, neutrophil and monocyte counts, and lower lymphocyte counts, and we confirm at a single-patient level that on disease progression from regional to distant metastatic, significant changes in absolute and relative blood cell counts occur. In early-stage patients, peripheral blood cell counts were not associated with the survival. Instead, in stage IV patients, higher leukocytes ($P = 0.001$), neutrophils ($P = 0.0002$), monocytes ($P = 0.002$) and ratio of neutrophils to lymphocytes (NLR, $P < 0.0001$) were all significantly associated with increased risk of mortality, independently of other known prognostic factors. Our findings suggest that these parameters might be exploited to improve patient stratification in randomized trials.

High body mass index (BMI) is associated with improved clinical outcomes in metastatic melanoma patients treated with anti-PD1: differences by gender

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Obesity is increasingly recognized as a prognostic factor across several malignancies. We previously reported a link between obesity and improved outcomes in BRAF-mutant metastatic melanoma (MM) patients treated with dabrafenib and trametinib that was more pronounced among men. In a multi-national cohort of 331 MM patients treated with anti-PD1, we examined WHO BMI categories at immunotherapy treatment initiation in relation to survival outcomes. Underweight patients (<2%) were excluded due to possible cancer-related cachexia. Kaplan-Meier analysis was used to estimate time to progression (TTP) and overall survival (OS). Hazard ratios (HR) and 95% confidence intervals (CI) were estimated within multivariable Cox models.
73% of males and 63% of females were overweight (BMI 25–29.9) or obese (BMI ≥30). With the exception of age, known prognostic factors (M stage, LDH, ECOG PS, gender, mutation status) did not differ by BMI. Median TTP and 3 year OS were 4.1 months and 37% respectively for lean (BMI 18.5 to <25), 6.5 months and 46% for overweight and 6.6 months and 47% for obese. Compared to lean status, overweight/obese phenotype was modestly associated with improved OS [3-year OS 46% versus 37%, HR and 95% CI: 0.7 (0.5–1.0); $P = 0.05$)] and TTP [median 6.5 versus 4.1: 0.8(0.6–1.0); $P = 0.07$] among all patients, but significant associations were only observed among men [median TTP 8.1 versus 2.8 months: 0.6 (0.4–0.9); $P = 0.01$; 3-year OS 43% versus 32%: 0.6 (0.4–0.9); $P = 0.02$;). No significant associations were observed among women (TTP $P_{interaction} = 0.06$ and OS $P_{interaction} = 0.26$).
Higher BMI is associated with improved outcomes in male MM patients treated with anti-PD1. This finding is similar to results observed in MM pts treated with targeted therapy. Mechanisms underlying this association are under investigation.

Precision medicine by targeting cell adhesion in melanoma

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The ability to distinguish between lethal cancers that need treating and non-lethal cancers that don't is an important challenge that – if met – could revolutionize the way we diagnose and treat cancer. Here we aim to address this challenge in the context of cutaneous melanoma by applying our knowledge on a cancer-defining biologic system, i.e. cell adhesion, to identify high-risk melanoma at the time of diagnosis. We present data from a multi-institutional study to discover new molecular risk factors associated with SLN positivity and melanoma recurrence. Gene clusters with functional roles in melanoma metastasis were discovered by next-generation sequencing and validated by quantitative PCR. We then used PCR to quantify a targeted set of genes in >500 consecutive melanoma samples from unique patients. Outcome of interest was i) SLN biopsy metastasis within 90 days of melanoma diagnosis and ii) disease recurrence after the initial work-up phase. Logic and logistic regression analyses were used to develop a model for the likelihood of SLN metastasis and disease recurrence from molecular, clinical and histologic variables. A model to identify SLN positive melanoma that included $\beta 3$ integrin, laminin B1, tissue-type plasminogen activator, and tumor protein p53 expression in combination with clinicopathologic variables (patient age, Breslow depth and tumor ulceration) performed significantly better than a model that only considered clinicopathologic variables and also performed well in a validation cohort. The aforementioned molecular model was also useful to assess relapse free survival after an initial work-up period of 90 days. We conclude that the addition of cell adhesion-linked gene expression variables to clinicopathologic variables improves the identification of patients with SLN metastases within 90 days of melanoma diagnosis and may aid in the identification of patients at risk for disease recurrence.

Global identification of genes targeted by DNMT3b in melanoma

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The purpose of this study is to identify the targets of the de novo methyltransferase Dnmt3b in melanoma. Expression of Dnmt3b increases with melanoma progression, is associated with poor survival and regulates key proliferative pathways, such as PI3K/Akt signaling in melanoma. Despite the prominent role of Dnmt3b, the targets of Dnmt3b in melanoma and other cancers are largely unknown. In this study, we identified the DNA methylation and expression changes (using RNASeq and RRBS) that occur with loss of Dnmt3b in a mouse model of melanoma. We validated putative Dnmt3b targets *in vitro*, in a human ENCODE dataset from ICF patients, and other validated studies of DNA methylation in melanoma. We examined global changes in 5-mC and 5-hmC, enrichment with histone modifications, and performed comprehensive unbiased identification of differentially expressed and methylated genes. We uncovered that inactivation of Dnmt3b resulted in a global decrease of 5-methylcytosine ($P < 0.01$, Student's t-test), hypomethylation of H3K27me3 marked areas ($q = 2.98E^{-48}$), and PRC2 target genes ($q = 8.36E^{-23}$), and sparing of H3K36me and H3K4me genomic loci. Integrating our results with findings

from human melanoma, we discovered that Dnmt3b likely targets nearly half of the genes known to be specifically hypermethylated in most melanomas, such as COL1A2, COL1A1, LXN, CYP1B1, and genes that have biomarker value in melanoma such as WIF1 and SOCS1. Furthermore, we identified novel Dnmt3b targets, such as TET1 and IDH1, which are known to play important roles in melanoma. These findings identify the DNMT enzyme mediating many well recognized aberrant methylation changes in melanoma, address an unresolved question in the field, and provide a comprehensive resource of Dnmt3b targets, including immunomodulatory genes with potential prognostic and therapeutic value in melanoma.

Sonidegib for patients with advanced basal cell carcinoma: long-term efficacy and safety results in the BOLT 30-month analysis

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Sonidegib 200 mg daily is US Food and Drug Administration approved for the treatment of patients with locally advanced basal cell carcinoma (laBCC) that recurs following surgery or radiation therapy and for those ineligible for surgery or radiation therapy. Sonidegib was approved based on the observation of meaningful responses and a manageable safety profile in the phase 2 BOLT study (NCT01327053), in which patients (N = 230) with laBCC (aggressive or nonaggressive subtypes) or metastatic BCC (mBCC) were randomized to sonidegib 200 mg (n = 79; laBCC, n = 66; mBCC, n = 13) or 800 mg (n = 151; laBCC, n = 128; mBCC, n = 23). Here we report updated 30-month data from BOLT (data cutoff, July 10, 2015; median follow-up, 38.2 months). At the data cutoff, the median durations of exposure were 11.0 months (200 mg) and 6.6 months (800 mg). In patients with laBCC, objective response rates (complete + partial response, assessed by central review using modified RECIST) were 56% (200 mg) and 45% (800 mg) and were comparable in patients with aggressive or nonaggressive histology. In patients with laBCC, Kaplan-Meier–estimated median durations of response were 26.1 months (200 mg) and 23.7 months (800 mg); median progression-free survival was 22.1 months (200 mg) and 22.0 months (800 mg); 2-year overall survival rates were 93% (200 mg) and 91% (800 mg). Considering all patients (laBCC and mBCC), the most common adverse events (AEs) of any grade were muscle spasm (200 mg, 54%; 800 mg, 69%), alopecia (49%; 58%), and dysgeusia (44%; 60%). Grade 3/4 AEs (43%; 64%) and AEs leading to discontinuation (30%; 40%) were less common with 200 versus 800 mg. Together, the durable responses and long-term safety observed in the BOLT 30-month analysis continue to support the use of sonidegib in patients with advanced BCC per local health authority guidelines.

Biphasic sarcomatoid transdifferentiation in a case of metastastic melanoma investigated by next generation sequencing

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We describe a highly unusual case of a metastatic melanoma manifested as groin lymph node metastasis in a 61 years old female with two distinct concomitant subcutaneous sarcomatoid lesions on the same leg, whereas no evidence for a conventional primary tumor was found.

All three tumorous lesions were formalin-fixed paraffin embedded and examined histologically on Hematoxylin & Eosin staining. Further characterization encompassed extensive immunohistochemical staining, in particular for known melanocytic markers as well as investigation of *BRAF* (Exon 15) and *NRAS* (Exons 2–4) genes by Sanger sequencing. Additionally, next generation sequencing was performed using the Ion AmpliSeq™ Colon and Lung Cancer Panel (22 genes) and Comprehensive Cancer Panel (409 genes).

The lymph node metastasis showed obvious morphological and immunohistochemical differentiation of melanoma. The two subcutaneous lesions were conventionally consistent with high-grade myxofibrosarcoma and primarily soft tissue mixed tumor, respectively. All three lesions were *BRAF* wildtype and harbored a *NRAS* p.Q61R mutation. Next generation sequencing data could confirm these findings and reveal further concordant mutations of 22 genes in all three lesions.

Although there was no morphological or immunohistochemical conclusive evidence of melanoma in the mesenchymal lesions, the concordant genetic profile indicate biphasic sarcomatoid transdifferentiation of melanoma in this case.

Cutaneous melanoma with brain metastasis: report of 193 patients with new observations

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Brain metastasis is a common endpoint in patients suffering from malignant melanoma. However, little is known about factors that predispose to brain metastases.

We performed a retrospective clinical and pathological investigation of melanoma patients with brain metastases in order to better characterise this patient population.

193 melanoma patients with brain metastasis histologically diagnosed between 1990 and 2015 at the University Hospital Zurich were retrospectively identified and further specified for sex, age at diagnosis and detection of brain metastasis, and

localisation. In addition, data were extracted regarding the subtype of primary melanoma, Breslow tumour thickness, Clark Level, mutation status, extent of metastatic spread and history of a second melanoma.
We found a significant male predominance (n = 126/193; 65%; P < 0.001). Breslow tumour thickness showed a wide range from 0.2 to 12.0 mm (n = 99; median 2.3 mm). 14 of 101 melanomas (14%) were classified as T1, thereof 11 (79%) were found in men. In 32 of 193 patients (17%), the primary melanoma was unknown.
Of special interest in our series is the high incidence of male predominance (79%) in cases of thin metastasing melanoma (14%), implicating genetic or epigenetic (hormonal) gender differences underlying tumour progression. Additionally, the high percentage of unknown primary melanoma (17%), at least partly representing completely regressed melanomas, indicates the importance of immune surveillance in melanoma progression

Primary cutaneous melanoma in elderly patients: potential prognostic markers

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Cutaneous melanoma is an immunogenic cancer and its interaction with the aging immune system could have an impact on biologic behaviour of this disease. Compared with younger patients (pts), in elderly ones melanoma tend to present in a more advanced stage. TILs (Tumor infiltrating lymphocytes), PD-L1 (Programmed Death-Ligand) and COX-2 (Cyclooxygenase-2) are potential markers of host immune response to the tumor, inflammation and carcinogenesis.
Our study analysed 113 consecutive cases of early melanoma occurred in pts aged ≥65 years at the time of diagnosis, observed between Jan 2010 and Mar 2014 at the Department of Oncology of Udine. We tested the association of TILs and other pathological data (PD-L1 and COX-2 expression) with DFI and MSS.
Median thickness was 3.3 mm, the ulceration was present in 38% melanomas and number of mitosis was ≥1/mmq in 86%. Lower TILs grade was observed in 65% of cases, higher grade in 35%. PD-L1 expression on tumor cells, on immune infiltrate and COX-2 expression were positive in 28%, 47% and 41% of case, respectively. With a median follow-up of 52 months, 36% of pts developed recurrence. Presence of ulceration was associated with shorter DFI (HR 8.3, 95% CI 3.02–22.70, P < 0.0001) and MSS (HR 9.5, 95% CI 2.96–30.11, P < 0.0001). Lymph nodes involvement was associated with shorter DFI (HR 3.4, 95% CI 1.44–7.96, P < 0.0001) and MSS (HR 3.3, 95% 2.16–17.63, P < 0.0001). A higher TILs grade was associated with better DFI (HR 0.29, 95% 0.09–0.88, P = 0.011). Positive PD-L1 expression on immune infiltrate was associated with longer MSS (HR 0.32, 95% 0.11–0.93, P = 0.043), that was confirmed in multivariate analysis. No association was observed for COX-2 expression.
In our study, ulceration, lymph nodes involvement, TILs grade and PD-L1 expression on immune infiltrate were prognostic factors in elderly patients with a primary melanoma diagnosis.

Interferon gamma induces CTLA4 expression in melanocytes and melanoma cells

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Therapeutic antibodies targeting Cytotoxic T Lymphocyte Antigen 4 (CTLA4) have shown promising antitumor activity in patients with advanced melanoma. CTLA4 is one of the inhibitory receptors that negatively regulate T cells activation by supressing CD28-B7 co-stimulatory signals via competitively binding to B7 molecules. CTLA4 expression is not restricted to immune cells, but is also expressed by melanocytes and melanoma cells. We have previously identified CTLA4 as the highest upregulated gene in melanocytes from UVB-irradiated neonatal mouse skin, in the context of an Interferon-gamma (IFNg) responsive gene expression signature. Here, we show that human melanoma cell lines have much higher baseline CTLA4 expression levels than primary human epidermal melanocytes (HEMn) and a variety of other types of tumor cell lines. Human recombinant IFNg robustly induced CTLA4 expression in both HEMn cells and human melanoma cells by RT-qPCR and flow cytometry analysis. IFNg-induced CTLA4 expression is blocked by JAK1/JAK2 inhibitor Roxulitinib pretreatment. Knock-down of STAT1 in human melanoma cells by siRNA leads to inhibition of IFNg-induced CTLA4 expression. Analysis of human CTLA4 gene promoter revealed several gamma-activated sequence motifs (GAS) for STAT1. We have comfirmed that STAT1 binds to CTLA4 promoter in IFNg-dependent fashion by ChIP-qPCR on human primary melanocytes. Also, recruitment of coactivator/histone acetyl transferase CREB-binding protein (CBP) closely paralleled STAT1 binding. IFNg-stimulated increase of histone 3 and histone 4 acetylation and RNA polymerase II recruitment at CTLA4 promoter were observed as well. In conclusion, these results suggest that IFNg induces chromatin changes and recruits RNA Pol II to CTLA4 promoter to initiate trasncription in a JAK/STAT-dependent manner. It may have significant clinical implications in personalized anti-CTLA4 immunotherapy.

Adjuvant dabrafenib (dab) in patients (pts) with surgically resected stage IIIC BRAF$^{V600E/K}$ mutated melanoma (mel)

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The benefit of adjuvant RAF inhibition is unknown. Pts with surgically resected stage IIIC mel are at a high risk of recurrence. Data from our institution shows that the relapse free survival (RFS) for stage IIIC disease is 24% at 2 years. Our hypothesis was that a 4 month course of adjuvant dab would result in significant death of residual tumor cells and improve 2 year RFS from 24% to 51%. We enrolled pts with stage IIIC BRAF$^{V600E/K}$ mutated mel with surgical resections within 90 days prior to starting adjuvant dab and with no radiographic evidence of disease. The treatment plan was adjuvant dab 150 mg po bid x 4 months. Pts were evaluated monthly x 6 and then q3 months; imaging was obtained at baseline and every 3 months until disease relapse or 2 years, whichever came first. Serial blood samples were collected for evaluation of BRAF $^{V600E/K}$ cell free DNA (cfDNA) detected and quantified by droplet digital PCR. 21/

23 pts enrolled were evaluable. 15 male, 6 female. Median age 54 (range 18–76). 17 pts with $BRAF^{V600E}$ and 4 pts with $BRAF^{V600K}$ mutations. Median days from surgical resection to dab start was 42 (range 25–81). 17/21 pts completed 4 months of adjuvant dab. 7 pts have not yet reached 2 years of follow-up. The estimated 2 year RFS is 31% (95% CI 12–53%). The estimated overall survival at 2 years is 75%. CfDNA was detected in 7/13 pts who have relapsed within 2 years. CfDNA has not been detected among the 7 pts who remain relapse-free to date. The null hypothesis was that 4 months of adjuvant dab does not improve 2 year RFS. Despite incomplete pt follow-up, it appears we cannot reject the null hypothesis which suggests that RAF inhibition may have limited efficacy in the adjuvant setting. We await results from randomized trials. CfDNA was identified in 54% with disease recurrence; there was no false positive cfDNA detection. Support: NCCN

Recurrent activating mutations of G-protein-coupled receptor *CYSLTR2* in uveal melanoma

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Uveal melanoma arises from melanocytes of the uveal tract and is the most common intraocular tumor in adults. While cutaneous melanomas frequently have activating mutations in *BRAF*, *NRAS, KIT* or *NF1,* these mutations are absent in uveal melanoma. Instead, uveal melanoma is characterized by activating mutations in *GNAQ* and *GNA11*, two highly homologous a subunits of $G\alpha_{q/11}$ heterotrimeric G proteins, and in *PLCB4* (phospholipase C b4), the downstream effector of Gαq signaling. We analyzed genomics data from 136 uveal melanoma samples from The Cancer Genome Atlas, Cancer Research UK, QIMR Berghofer Medical Research Institute, and University of Duisburg-Essen cohorts and found a recurrent mutation in *CYSLTR2* (cysteinyl leukotriene receptor 2). The recurrent mutation encodes a p.Leu129Gln substitution in 4 of 9 samples that lacked mutations in *GNAQ*, *GNA11*, and *PLCB4* but in 0 of 127 samples that harbored mutations in these genes. We showed that the Leu129Gln CysLT2R mutant protein constitutively activates endogenous Gαq and is unresponsive to stimulation by leukotriene. Expression of Leu129Gln CysLT2R in melanocytes enforces expression of a melanocyte-lineage signature, drives phorbol ester–independent growth *in vitro*, and promotes tumorigenesis *in vivo*. Our findings implicate *CYSLTR2* as a uveal melanoma oncogene and highlight the critical role of Gαq signaling in uveal melanoma pathogenesis.

Lymphatic invasion and angiotropism in primary cutaneous melanoma

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Lymphatic invasion (LI) and angiotropism within primary cutaneous melanoma (PCM) may correlate with sentinel lymph node (SLN) metastasis and outcome. Immunohistochemistry (IHC) for S100/D2-40 and CD31, respectively, may be used to highlight such foci. We reviewed 125 PCMs from patients who underwent SLN biopsy and used dual S100/D240 and CD31 IHC to detect LI and angiotropism. The presence of LI or angiotropism was correlated with known melanoma staging parameters (i.e. primary tumor thickness, mitoses/mm^2, and ulceration) and disease status at last follow-up. LI was detected in 2% of cases on H&E stains and in 26% of cases using dual S100/D2-40 IHC. Cases with LI were thicker (2.5 versus 1.6 mm; P = 0.01), showed more mitoses (6.3 versus 2.9/mm^2; P = <0.01), and more often were ulcerated (36% versus 11%; P = <0.01). 33% of cases with LI and 10% of cases without LI were associated with a positive SLN (P = <0.01). More patients without primary tumor LI were disease-free at last follow-up as compared to those with LI (79% versus 55%; P = <0.01). Angiotropism was detected in 13% of cases on H&E stains and 37% of cases using CD31 IHC. Cases with angiotropism showed a significantly greater tumor thickness (2.0 versus 1.4 mm; P = 0.04). No significant difference in presence of LI, SLN positivity, or adverse events was detected between tumors with and without angiotropism. This study supports prior evidence that LI as detected on S100/D2-40 dual IHC predicts SLN metastasis and adverse outcome in PCM. Thus, the detection of primary tumor LI may impact therapeutic planning in melanoma, such as the decision to perform a SLN biopsy. While no significant association between angiotropism and SLN metastasis or adverse outcome was identified, larger studies are needed to further investigate its clinical significance.

Higher serum 25-hydroxyvitamin D levels at melanoma diagnosis are associated with better prognosis in patients with intermediate VDR-expressing tumours, with transcriptomic evidence for inhibition of β-catenin signalling

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1α,25-dihydroxyvitamin D_3 is a fat-soluble hormone that signals via its high-affinity receptor Vitamin D Receptor (VDR). Studies in the Leeds Melanoma Cohort (LMC) as well as others showed that lower serum levels of 25-hydroxyvitamin $D_{2/3}$ ('vitamin D' henceforth) were associated with thicker tumours, more frequent ulceration and poor prognosis. However, the molecular mechanisms associated with the protective effect of vitamin D signalling in melanoma have not been explored. The LMC database includes transcriptomic data from 703 melanoma primaries for which serum vitamin D level at diagnosis, Breslow thickness, mitotic rate, AJCC staging and follow-up Melanoma Specific Survival (MSS) were recorded. Transcriptomic VDR expression was strongly correlated with better MSS, both in the LMC and the Cancer Genome Atlas (TCGA) data sets. VDR expression-based stratification into high, intermediate and low VDR-expressing groups revealed that higher serum vitamin D level conferred a beneficial survival effect only in the intermediate-VDR group. The tumour expression of 2100 genes (including those previously reported to contain vitamin D response elements- VDRE) was significantly correlated with serum vitamin D levels, only in the intermediate VDR group. The expression of 510 genes was shown to have a significant statistical interaction (FDR<0.05) with vitamin D on MSS and were subjected to subsequent pathway analysis using Metacore™ and STRING, both of which showed evidence of vitamin D associated perturbations in cell cycle, immune-related and DNA-damage pathways. In addition, both serum vitamin D and VDR expression were inversely correlated with β-catenin pathway components, suggesting β-catenin-signalling inhibition by the vitamin D-VDR axis.

Linking melanoma autophagy to lymphatic endothelial cell metabolism, lymphangiogenesis and metastasis

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Lymphangiogenesis serves as a mediator of melanoma cell dissemination to distant lymph nodes. Linked to tumoural cytokine secretion leading to enhanced lymphatic endothelial cell (LEC) proliferation, LECs also require sustained Fatty acid oxidation and mitochondrial function to induce vessel sprouting, suggesting activated autophagy in melanoma cells within the nutrient-deprived environment of the lymph node/tumour microenvironment may fuel LEC metabolism, thereby facilitating tumour lymphangiogensis. Semi-quantitative immunohistochemical analysis of p62 expression (a marker of autophagic activity) in a cohort of formalin fixed paraffin embedded (FFPE) primary melanomas and patient-matched lymph nodes revealed a significant increase in mean % p62 expression in primary tumours compared to patient-matched metastatic lymph nodes ($P < 0.0001$). Additionally, immunohistochemical expression of the LEC marker D240 revealed the presence of few lymphatic vessels in primary melanomas compared to increased expression in patient-matched metastatic lymph nodes, indicating melanoma metastasis to the lymph node is associated with the increased density of lymphatic vessels. Collectively these pilot data highlight the potential for cross talk between increased autophagy in melanomas and the increased generation of lymphatic vessels in a draining lymph node. Ongoing studies are evaluating the metabolite spectra of melanoma cells undergoing starvation-induced autophagy and the ability of derived metabolites to promote LEC sprouting as well as studies to inhibit a rate limiting enzyme key to LEC cell metabolism with the aim of testing the possibility that dual inhibition of melanoma autophagy and LEC metabolism may represent a novel therapeutic strategy to prevent melanoma lymphangiogenesis and limit the development of metastatic disease.

TERT promoter mutations in melanoma survival

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Despite advances in targeted therapies, the treatment of advanced melanoma remains an exercise in disease management, hence a need for biomarkers for identification of at-risk primary melanoma patients. In this study, we assessed the prognostic value of *TERT* promoter mutations in primary melanomas. Tumors from 300 patients with stage I/II melanoma were sequenced for *TERT* promoter and *BRAF/NRAS* mutations. Cumulative curves were drawn for patients with and without mutations with progression-free and melanoma-specific survival as outcomes. Cox proportional hazard regression models were used to determine the effect of the mutations on survivals. Individually, presence of *TERT* promoter and *BRAF/NRAS* mutations associated with poor disease-free and melanoma-specific survival with modification of the effect by the rs2853669 polymorphism within the TERT promoter. Hazard ratio (HR) for simultaneous occurrence of *TERT* promoter and BRAF/NRAS mutations for disease-free survival was 2.3 (95% CI 1.2–4.4) and for melanoma-specific survival 5.8 (95% CI 1.9–18.3). The effect of the mutations on melanoma-specific survival in noncarriers of variant allele of the polymorphism was significant (HR 4.5, 95% CI 1.4–15.2) but could not be calculated for the carriers due to low number of events. The variant allele *per se* showed association with increased survival (HR 0.3, 95% CI 0.1–0.9). The data in this study provide preliminary evidence that *TERT* promoter mutations in combination with *BRAF/NRAS* mutations can be used to identify patients at risk of aggressive disease and the possibility of refinement of the classification with inclusion of the rs2853669 polymorphism within *TERT* promoter.

SPOP enhances resistance of BRAFV600E melanoma cells to targeted BRAF inhibition

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Acquired resistance against BRAF inhibitors in $BRAF^{V600}$-mutant melanoma has produced an urgent need for new treatment options. Targeting additional kinases within the MAPK pathway has not circumvented resistance as single mutations can overcome multiple inhibitors and MAPK is regulated via complex feedback networks. Defining regulators of key oncogenic signaling pathways, including the MAPK and PI3K/AKT cascades, may provide new opportunities to restore kinase inhibitor sensitivity. We recently conducted a human cDNA overexpression screen and identified the E3 ubiquitin ligase SPOP (Speckle-type POZ protein) as a potential regulator of BRAF/MEK inhibitor response in melanoma. SPOP functions as a critical hub regulating MAPK and PI3K/AKT signaling pathways by promoting the degradation of the MAPK and PI3K negative regulators DUSP6 and PTEN, respectively. In this study we investigated the contribution of SPOP to BRAF inhibitor sensitivity in $BRAF^{V600E}$ mutant melanoma. We generated two melanoma cell models, with inducible expression of wild type SPOP. As expected, SPOP induction led to the loss of DUSP6 and PTEN protein expression and this was associated with activation of MAPK and PI3K/AKT signaling, as indicated by the phosphorylation of downstream targets, including p-ERK and p-AKT. The induction of SPOP expression also enhanced the survival of melanoma cells in response to BRAF inhibition. SPOP-induced BRAF inhibitor resistance was associated with increased MAPK and PI3K signaling, as indicated by the persistence of p-ERK and p-AKT, in dabrafenib-treated cells. Our data indicate that overexpression of SPOP can alter the activity of MAPK and PI3K signaling to promote resistance to BRAF inhibitors, and highlight the significant role of protein ubiquitination in the regulation of oncogenic signaling activity.

Acetate dependency in melanoma

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Tumors rely on multiple nutrients to meet cellular bioenergetics and macromolecular synthesis demands of rapidly diving cells. Although the role of glucose and glutamine in cancer metabolism is well understood, the relative contribution of acetate metabolism remains to be clarified. We show that glutamine supplementation is not sufficient to prevent loss of cell viability in a subset of glucose-deprived melanoma cells, but synergizes with acetate to support cell survival. Glucose-deprived melanoma

cells depend on both oxidative phosphorylation and acetate metabolism for cell survival. Acetate supplementation significantly contributed to maintenance of ATP levels in glucose-starved cells. Unlike acetate, short chain fatty acids such as butyrate and propionate failed to prevent loss of cell viability from glucose deprivation. *In vivo* studies revealed that in addition to nucleo-cytoplasmic acetate assimilating enzyme ACSS2, mitochondrial ACSS1 was critical for melanoma tumor growth in mice. Our data indicate that acetate metabolism may be a potential therapeutic target for a subset of melanoma.

Patient characteristics associated with ≥36-months clinical benefit with combination dabrafenib and trametinib (D+T) in COMBI-d

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Analyses of factors that predict clinical outcomes with D+T in patients with *BRAF* V600E/K–mutant melanoma across phase 2 and 3 registration trials (median follow-up, 20 months) previously identified baseline lactate dehydrogenase (LDH) and number of disease sites as the most influential factors for progression-free survival (PFS) and overall survival (OS), and LDH as the strongest predictor of PFS or OS lasting ≥24 months (Long et al. SMR 2015; Davies et al. ESMO 2016). Longer-term follow-up analyses are needed to further characterize the durability of PFS and OS achievable with D+T. We explored factors that may influence clinical benefit lasting ≥36 months in the phase 3 COMBI-d study (NCT01584648; ≥36 months follow-up) in patients who received D+T (n = 211; cutoff, 15Feb2016). Long-term PFS (n = 183) and OS (n = 185) analyses excluded patients censored prior to 36 months. A total of 31/183 patients (17%) had PFS ≥36 months, and 76/185 (41%) had OS ≥36 months. At data cutoff, 23/31 (74%) and 36/76 (47%) patients with PFS or OS ≥36 months, respectively, remained on D+T. Of patients included in this analysis who had a complete response, 18/30 (60%) had PFS ≥36 months and 30/35 (86%) had OS ≥36 months. Baseline characteristics with ≥15% difference between patients with PFS < versus ≥36 months were LDH (normal, 60% versus 77%), number of disease sites (<3, 44% versus 81%), sum of lesion diameter (<median, 40% versus 68%), and disease stage (<IVM1c, 26% versus 55%). Differences ≥15% between patients with OS < versus ≥36 months were seen for LDH (normal, 54% versus 80%), number of disease sites (<3, 40% versus 64%), sum of lesion diameter (<median, 37% versus 59%), disease stage (<IVM1c, 26% versus 43%), and ECOG performance status (0, 67% versus 83%). These results are consistent with previous findings for PFS and OS ≥24 months.

The Wnt signaling status of melanoma cells predicts their invasiveness, autophagy activity and their response to pharmacological-mediated autophagy inhibition

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Melanoma is the most aggressive type of skin cancer and the leading cause of death from skin cancer. With increasing incidence of the disease, it is crucial to investigate the cellular and molecular mechanisms that lead to invasion and metastasis. High autophagy correlates with melanoma tumor aggressiveness and poor survival in patients, as it is a common mechanism of resistance to therapy. Autophagy inhibition reduces Wnt5A levels in a breast cancer model, suggesting a cross-talk between Wnt5A and autophagy in cancer. We previously showed that Wnt5A is a driver of invasion and metastasis in melanoma. β catenin, has been shown to negatively regulate autophagy in a colorectal cancer model. Given that Wnt5A downregulates β catenin, we hypothesized that autophagy promotes melanoma tumor aggressiveness through the regulation of Wnt signaling and that in turn the Wnt signaling status of melanoma cells affects their autophagy activity. Our results demonstrate that melanoma cells with high Wnt5A and low β catenin have higher autophagy levels compared to melanoma cells that have low Wnt5A and high β catenin. To determine whether there is a feedback loop between Wnt signaling and autophagy activity, we inhibited autophagy in invasive melanoma cells by shATG5 knockdown; this resulted in a significant decrease in invasion which correlated with a decrease in Wnt5A and an increase in β catenin. Moreover, we found that increasing Wnt5A in melanoma cells decreases their sensitivity to pharmacological-mediated autophagy inhibition while increasing β catenin increases their sensitivity both *in vitro* and *in vivo*. Further dissection of the mechanisms that are involved in the interaction between autophagy and Wnt signaling in melanoma will enable the development of novel therapeutic strategies that will improve patient outcome.

Transcriptomic evidence for primary melanoma subgroups with distinct immune micro-environments, and for a role for b catenin signaling in the suppression of immune responses

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Transcriptomic signatures were used to classify the immune response in 702 formalin fixed primary melanomas from the Leeds Melanoma Cohort, using a modification of the approach described by Bindea et al 2013. Unsupervised hierarchical clustering of transcripts from 380 genes for which evidence suggests expression uniquely in immune cells identified six tumor groups. One showed little evidence of an immune response and this was associated with a significantly poorer prognosis. In the remaining groups, the one with the strongest evidence for imputed T cell cytotoxic activity also showed strong expression of all checkpoint molecules tested: *PD1, PDL1, PDL2, CTLA4, TIM-3, LAG3, VISTA* and *BTLA4*. There was variation in inferred immune cell infiltration and some different patterns of

expression of checkpoint molecules in the remaining 4 groups but a similar prognosis. The group lacking evidence of an immune response demonstrated a distinct upregulation of genes in the b catenin signaling pathway, providing evidence for activation of this pathway as a possible therapeutic adjunct target in immunotherapy. Survival was strongly associated with evidence T cell infiltration in wild type, but less so in *BRAF* and not at all in *NRAS* mutated tumors.

A phase II clinical trial on rechallenging BRAF V600-mutant melanoma patients who previously experienced progression on BRAF(+MEK)-inhibition (BMi) and immune checkpoint inhibitors (ICi) with dabrafenib plus trametinib (DT)

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Patients (pts) with BRAFV600mut melanoma can be successfully treated with BMi but tumor progression (PD) develops in a majority of pts. This phase II trial addressed the anti-tumor activity of dabrafenib (D; 150 mg BID) and trametinib (T; 2 mg QD) in pts with BRAF V600mut melanoma who are documented with PD $\geq$ 12 weeks following the last day of BMi therapy, and also experienced PD on subsequent ICi. ORR% (RECISTv1.1) served as the primary end point. According to a two-stage Simon Minimax design DT-rechallenge was considered sufficiently active for further clinical investigation if a confirmed OR was documented in at least 4/25 pts. BRAFV600mut ctDNA was measured on plasma using the Idylla-platform. Between APR 2014 and FEB 2016, 25 pts initiated study treatment. Baseline characteristics: 15M/10F; med age 54y (range 29–72); AJCC stage IV-M1a/-M1b/-M1c: 1/1/23 pts. Med FU- time: 6.8 months (range 1–26). Prior targeted therapy consisted of: 6 vemurafenib (V), 3 D, 16 DT; prior immunotherapy consisted of ipilimumab (10 pts) and nivolumab or pembrolizumab (14 pts). A PR was documented in 8 pts (32%; 2 had failed prior BRAF-inhibitor monotherapy and 6 DT), SD was observed in 10 pts (40%). Median PFS was 4.9 mths (95% CI: 3.6–6.2), median OS was not reached. DT-rechallenge was well tolerated; pyrexia was the most common treatment related AE (gr 1/2 in 10 pts and gr3 in 1 pt), 1pt experienced an additional gr3 AE (panniculitis). Absence of BRAF V600mut ctDNA at week 2 correlated significantly with PFS. Rechallenging BRAFV600mut melanoma patients with DT following prior progression on BMi and ICi has sufficient activity to warrant further investigation. BRAFV600mut ctDNA levels is an early surrogate biomarker for the effectiveness of DT-rechallenge.

The impact of BRAFV600i-triggered endoplasmic reticulum stress on apoptosis induction by MEKi in NRAS mutated melanoma cells

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Activated NRAS stimulates a number of intracellular signaling pathways including the RAF/MEK/ERK pathway. Overall survival for NRAS-mutant melanoma patients (15–25%) is worse than for their wild-type counterparts. MEK inhibitors showed activity in patients with NRAS-mutant melanoma but the overall response rates were only up to 20%. In a previous study, we showed that vemurafenib induces apoptosis in BRAFV600-mutant melanoma cells through a mechanism involving induction of endoplasmic reticulum stress (ER). ER stress induction appeared to be an off-target effect of vemurafenib that remarkably enhances its pro-apoptotic activity in BRAFV600-mutant melanoma.

In this study, we investigated whether it is possible to take advantage of ER stress induction to enhance the antitumor activity of MEK inhibitors in patients with NRAS-mutant melanoma.

BRAF-mutant and NRAS-mutant metastatic melanoma cell lines were treated with the BRAF inhibitors vemurafenib, dabrafenib and encorafenib and were subjected to electron microscopy. All of the three substances were able to induce morphological features of ER stress, including a significant dilation of the ER in both BRAF-mutant and NRAS-mutant melanoma cell lines. As expected, the BRAF inhibitors inhibited the phosphorylation of ERK and growth inhibition and induced apoptosis in BRAF-mutant but not in NRAS-mutant melanoma cells in monolayer and spheroid culture. However, the BRAF inhibitors significantly enhanced growth inhibition and apoptosis induced by the MEK inhibitors. Moreover, the expression of the ER stress-related factors p8, ATF4, ATF3 and CHOP was induced. siRNA inhibition of ATF4 reduced melanoma cell apoptosis induced by the combinations.

These data suggest that BRAFV600 inhibitors induce endoplasmic reticulum stress and potentiates the antitumor activity of MEK inhibitors in NRAS-mutant melanoma.

Transcription of the migration-promoting oncogene ILEI is regulated by *BRAF* V600E

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ILEI is a secreted factor that contributes to the epithelial-to-mesenchymal transition (EMT), a cell biological process that confers metastatic properties to a tumor cell. While ILEI's role in metastatic breast cancer is established, herein we sought to identify the contribution of ILEI to other cancer types. Using data from the Human Protein Atlas we learned that ILEI is highly expressed in melanoma but not in normal melanocytes, so we hypothesized that ILEI contributes to melanoma progression. We began our study on melanoma and ILEI by observing that there are distinct ILEI high and low cell lines, and that these cell lines show an inverse correlation with the master melanocyte differentiation marker MITF. While we found that modulation of MITF did not affect ILEI expression, we felt that ILEI could still contribute to the invasive phenotype of MITF low cell lines. Thus, we conducted microarray analysis on ILEI knockdown cell lines to discover broad biological effects of ILEI knockdown. These results showed us that ILEI affects the transcription of migration-related genes. Furthermore, we used *in vitro* assays to show that knockdown of ILEI attenuates migration. Next, we wanted to understand the molecular mechanism underlying ILEI expression in melanoma cells, and we observed that inhibition of BRAF/MAPK signaling decreases ILEI expression at the mRNA level. We have used luciferase reporter assays to show that a 130 bp fragment of the *ILEI* promoter activity is critical for ILEI expression. In summary, we have shown that BRAF/MAPK signaling regulates *ILEI* transcription, and in turn ILEI leads to a migratory phenotype.

Transcriptomic profiling the microenvironment in primary melanomas

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The quantity of stroma is reported to be hazardous in many cancers (e.g. colon cancer)[1] whilst it is protective in others (e.g. oestrogen-receptor positive breast cancer).[2] We previously reported the protective effect of stromal content in 246 primary melanomas.[3] Here we report its transcriptomic correlates.

The percentage of stroma (POS) was recorded for 702 primary melanomas recruited in a population-based cohort, using RandomSpot©[4] and ranged from 0 to 98%, median 33%. ESTIMATE was used to calculate tumour, immune and stromal cell scores.[5] Cox proportional hazards models were used to evaluate factors predictive of melanoma-specific death. Linear regression was used to assess the association between POS and the whole-genome transcriptome. Pathway enrichment analysis was performed using Metacore™.

Age, sex, AJCC stage and POS were significant prognostic factors in univariable analyses. Multivariable analysis of time to melanoma-specific death confirmed that POS was an independent prognostic factor, adjusting for age, sex and AJCC stage (HR 0.98 per percentage of stroma, $P < 0.0005$, 95%CI 0.98–0.99). POS was positively correlated with ESTIMATE's stromal score. Metacore™ analysis revealed upregulation of immune pathways in stromally rich tumours, including *LCK* and genes associated with MHC Class II and ICOS pathways and downregulation of cell cycle pathways.

We have shown that for every percentage increase in stroma there was a 2% decrease in melanoma-specific death. Transcriptomic profiling revealed that increasing stroma was associated with increased immune responses and reduced cell division.

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Integrative toxicogenomic and system biology platforms to target melanoma genes by DM-1

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Melanoma is a cancer highly invasive and metastatic, with high mortality rates and chemoresistance. The MAPK pathway is frequently overexpressed, and represents a potent target-specific chemotherapeutic, as BRAF inhibitors. The DM-1 compound, sodium 4-[5-(4-hydroxy-3-methoxy-phenyl)-3-oxo-penta-1,4-dienyl]-2-methoxy-phenolate, a curcumin analog, has potentially useful antitumor activity in melanoma, but the molecular pathways involved with its action remain unclear. The use of toxicogenomic models for determining cellular responses to compounds has been shown to be effective in the initial screening for molecular targets. In this study, we seek to gain insights into the molecular aspects of DM-1 toxicity by the screening of a genome-wide deletion set of individual genes in S. cerevisiae library. Systems biology tools were applied to obtain a comprehensive view on the role of these genes in human melanoma progression. From the primary data collected, 211 sensitive genes were identified from which, 74 genes were selected according to human homologs. Enrichment analysis of the genes corresponding to the sensitive mutants reveals the key features of DM-1 toxicity, which include insulin, iron and RNA metabolism, and oxidative phosphorylation. Pathway analysis highlighted 7 target genes (ADK, ATP6V0B1, PEMT, TOP1, ZFP36, ZFP36L1, ZFP36L2) that are frequently altered among normal skin, dysplastic nevus, primary and metastatic melanomas. They are related to regulation of tumor progression, cell differentiation, and epithelial-mesenchymal transition. Taken together, these findings provide meaningful details into the core understanding of DM-1 toxicity regarding molecular mechanisms and encourage further studies in the development of combinatorial treatments for melanomas.

Novel fluorescent compounds for the early detection of melanoma

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With more than 50 000 cases of death worldwide per year, melanoma belong to the most dangerous types of skin cancer. While healing chances are promising when the disease is detected in early stages, they are rapidly decreasing for the detection in advanced cases. In addition to an increasing incidence during the last years, this circumstance emphasizes the significance of an efficient early detection. However, the methods are limited. A visual inspection of the skin is usually performed by a dermatologist. In case of suspicion, degenerated tissue is removed surgically and examined histologically. This comes along with a lot of effort and is often limited by lacking compliance of high risk patients facing regular invasive interventions.

The coincidental discovery of a fluorescent compound that was formerly used to label cell surface glycans to be selectively taken up in melanoma cells, promises new efficient possibilities for diagnostics. The idea is to develop a method of application treating suspicious areas of the skin with a spray or ointment containing the fluorescent compound. After short incubation the skin should be cleaned and an uptake of the compound should have taken place selectively in melanoma cells. By excitation of the fluorophore using light of a specific wavelength melanoma should be detectable even in very early stages by the fluorescence signal which represents a new, cost-efficient and non-invasive method of early detection. Furthermore it is conceivable in a later stage to use the core of our compound as a molecular tranporter to melanoma cells. The coupling of cytostatic drugs to the transporter molecule would enable an external treatment of melanoma and thus prevent patients from systematic chemotherapy.

Control of brain metastases with anti-PD-1 therapy in patients with melanoma post-SRS/craniotomy

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While anti-PD-1 antibodies have shown significant clinical benefit in patients (pts) with advanced melanoma, a majority of these pts develop brain metastases for which standard treatments remain radiation therapy and/or surgery. We examined outcomes in pts

who were diagnosed with melanoma brain metastases (MBM) and received upfront locoregional therapy, to determine how well their subsequent anti-PD-1 therapy could control their MBM. We retrospectively reviewed 146 pts with advanced melanoma who were treated with anti-PD-1 therapy, to identify 23 pts who had received prior stereotactic radioasurgery (SRS) or craniotomy for MBM.

There were 13 men and 10 women, with median age 56 (27–85). Most common site of metastases was in the frontal lobe (n = 13, 57%). Primary treatment for the MBM was SRS in 17 (74%), and craniotomy in 6 (26%) pts. Median follow-up was 2 years post-locoregional therapy. Eleven pts subsequently received pembrolizumab and 12 pts received nivolumab; median duration of therapy was 5 months. Eight pts received these drugs as their first-line systemic therapy post-SRS/craniotomy, 13 as second line therapy, and 2 as third-line; other first-line therapies included ipilimumab, chemotherapy or BRAF-targeted therapies. While receiving their anti-PD-1 therapy, five (22%) pts had no recurrence of any brain metastases (n = 2 with pembro, n = 3 with nivo) and eight (35%) pts had stable MRI brain imaging with no new or growing lesions. However, ten pts (44%) had progession of their brain metastases while on anti-PD-1 therapy (n = 3 with nivo, n = 7 with pembro; P = 0.1). Median overall survival from time of SRS or craniotomy was 24 months (5.6–32) for pts treated with pembro and 36 months (1.7–84) with nivo (P = 0.03).

While there may be a suggestion of improved outcome with nivolumab in MBM after locoregional therapy, larger analyses are needed for definitive conclusions.

BRAF- NRAS mutations in a series of nevi with architectural and cytological atypia

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Role of mutations in *BRAF* and *NRAS* oncogenes among nevi with architectural and cytological atypia is not fully clear. We here assessed any correlation between *BRAF-NRAS* mutations and phenotipic features in such nevi.

A consecutive sample of 44 nevi with architectural and cytological atypia was analyzed for mutations in *BRAF* and *NRAS* genes through automated sequencing assays on genomic DNA from tissue sections. Univariate and multivariate analyses were carried out using clinico-epidemiological data and dermoscopic features from patients.

Overall, 18/44 (41%) patients carried an activating *BRAF* or *NRAS* mutation: 13 (30%) in *BRAF* and 5 (11%) in *NRAS*. The *NRAS* mutations were statistically significant among patients with low nevus density and low amount of local atypical characteristics as well as in nevi with lack of structure-less areas. The *BRAF* mutations were not related to any clinical and/or dermoscopic feature. No significant correlation between histological parameters and presence of *BRAF-NRAS* mutations was observed.

The occurrence of *BRAF-NRAS* mutations in melanocytic nevi seems to be quite common. Acquired melanocytic nevi harbor oncogenic mutations in *BRAF*, whereas congenital melanocytic nevi and blue nevi frequently harbor *NRAS* mutations. In melanomas, the rate of *BRAF* mutations is about 40–45% while *NRAS* is mutated in about 16–20% of cases. In our series of nevi with architectural and cytological atypia, *BRAF-NRAS* mutations were found with a frequency more similar to that of melanomas than that of benign nevi. In literature, the dermoscopic parameter significantly associated with the *BRAF* mutations is the presence of globular patterns. In our series, the absence of asymmetric structures seems to instead predict the presence of *NRAS* mutations, suggesting that these mutations may select a subset of such atypical nevi.

NGS-based mutation analysis indicates that *BRAF* is the mostly mutated gene in primary sinonasal melanomas from South Italy

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Primary sinonasal melanoma (SNM) is a rare disease, accounting for 0.3% to 2% of all malignant melanomas. Primary SNMs present a more aggressive oncologic behavior and a poorer prognosis than other subsets of melanomas. Using a next-generation sequencing (NGS) approach, we here tried to define the spectrum and distribution of mutations in main genes involved in melanomagenesis among patients with SNM.

Twenty-fifth primary SNM samples were collected among consecutively diagnosed melanoma patients from South Italy, after obtaining their written informed consent for tissue sampling. Genomic DNA was isolated from macrodissected tumor tissues containing at least 80% neoplastic cells and analyzed for mutations in 25 most common melanoma-associated oncogenes and tumor suppressor genes, using the IMI Diagnostic Melanoma Panel on the Ion Torrent platform (Life Technologies, USA).

Overall, *BRAF* and *cKIT* genes were found mutated in 11/25 (44%) patients. The highest prevalence (9/25; 36%) was observed for *BRAF* mutations, whereas *cKIT* mutations were detected in four (16%) SNM cases; surprisingly, two patients presented coexistence of *BRAF* and *cKIT* mutations. Among others, mutations were more frequently found in *CCND1* (7 cases; 28%), *ARID2* (4; 16%), and *NF1* (3; 12%) genes. About one third (9/25; 36%) of SNM cases presented no pathogenetic mutations in candidate genes.

Mutations in *cKIT* are less frequent than those in *BRAF* among primary sinonasal melanomas from South Italy. As a confirmation of several previous studies in various malignancies from our group, genetic background may determine mutation heterogeneity in different populations.

A non-quiescent *idling* state in BRAF-mutated melanoma cells

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Understanding the dynamics of cancer cell response to anticancer drugs is a significant basic science need with profound clinical implications. In recent years, BRAF-mutated melanoma has seen a surge of research that have changed the paradigm of treatment. Small molecule inhibitors of BRAF oncogene in clinic show remarkable, short-term efficacy, however, clinical responses are variable and short-lived. While tremendous amount of work has been done in understanding resistance mechanisms to these inhibitors, most of our knowledge still comes from post-resistant tumors, and very little is known about response dynamics before tumors develop resistance. Using high throughput microscopy and mathematical modeling, here we show that BRAF-mutated melanomas exhibit a complex, non-linear response dynamics and an entry into a hitherto unrecognized idling state that precedes acquisition of resistance by (epi)genetic mechanisms. For BRAF-mutated melanoma cell populations treated with PLX4720, we observe four distinct phases of drug response: (i) drug equilibration, (ii) regression, (iii) rebound, and (iv) *idling*. Our data on single cell-derived clonal lineages suggest that these dynamics are the result of the combined effects of clonal competition and non-genetic phenotypic state transitions in response to drug. The idling state is a non-quiescent, partly-reversible state of zero net-growth with reduced metabolism and is likely a fertile ground for resistance mechanisms to unfold. We postulate that idling cancer cells may have clinical significance as reservoirs from which genetic mutations, and ultimately tumor recurrence arises. If so, this phenotypic bottleneck could be targeted, and perhaps eliminated with appropriate secondary treatment(s). We are actively pursuing molecular underpinnings of this idling phenotype which may inform rational treatment strategies for durable responses.

Randomized phase IIb trial of an autologous tumor lysate + yeast cell wall particles + dendritic cell vaccine in advanced stage melanoma to prevent recurrence

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Cancer vaccines aim to raise tumor-specific immunity and may be best suited for disease-free patient (pts) at risk of recurrence. Here, we present an update on the ongoing prospective, randomized, multi-center, double-blinded, placebo-controlled, phase IIb trial of the tumor lysate (TL), particle loaded (PL) dendritic cell (DC) vaccine in stage III/IV (resected) melanoma pts to prevent recurrence after standard therapy.

Target enrollment is 120 pts randomized 2:1(vaccine:control). The vaccine is autologous DC loaded with TL (≥1 mg) in yeast cell wall particles (YCWP). The vaccine is injected monthly x3 (primary vaccine series, PVS), then boosters at 6, 12, and 18 months. Control pts receive autologous DC containing empty YCWP. Primary endpoint is 24-months disease-free survival.

Though data is still blinded, tumor has been obtained from 133 pts, with 28 screen failures (21%), 30 pending, and 75 randomized; median f/u is 3.1 months in randomized pts. 68% are male; 94% white; median age is 61. 80% are stage III and 20% stage IV. Vaccines were created from 50 cc blood draw (BD) after Neupogen in 34 pts (mean yield 7.7 doses with 92% viability) and 120 cc BD in 36 pts (mean yield 6.9 doses with 90% viability). Vaccine production was 100% successful but required an additional BD in 12% and 14% of pts, respectively. There have been 26 related adverse events (AE; all ≤ grade 3) and 5 serious, but unrelated AE. The overall recurrence rate (RR) is 26% (n = 17), median time to recurrence is 3 months; 53% recurred prior to completion of PVS.

We have shown the TLPLDC vaccine is efficient to create with small volumes of autologous tumor and blood. The trial is nearing the end of enrollment, but early RR will prompt over-enrollment to preserve power. The pre-specified interim analysis will occur 6 months after enrollment is complete.

Systematic genetic perturbations to reveal melanoma vulnerabilities

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For a long time, advanced stage melanomas were refractory to the available therapeutic options, but recent developments have begun offering better perspectives. The small molecule inhibitor vemurafenib, specifically targeting the mutant $BRAF^{V600E}$ kinase, was the first standard of personalized care for patients diagnosed with mutant BRAF metastatic melanoma. Although this compound initially reduces tumor burden dramatically, eventually most melanomas become resistant and progress while on treatment. This occurs by acquisition of additional mutations or other alterations most of which reactivate the mitogen-activated protein kinase (MAPK) pathway.

Therefore, in spite of these new perspectives, there is a dire need to identify additional targets amenable to therapeutic intervention, to be used in combination with vemurafenib or other specific inhibitors (e.g., immune checkpoint blockade) to overcome or prevent drug resistance and achieve more durable clinical responses.

We are studying (lack of) sensitivity to targeted treatment using patient biopsies, patient-derived xenografts (PDX) and low-passage cell lines. With our PDX platform we have discovered a new resistance mechanism to BRAF inhibitors, while it also offers a robust system to study melanoma heterogeneity. We use these and other systems also for systematic function-based genetic screens to identify factors that are required for proliferation and survival of melanoma cells. Similar screens are done to modulate the response to targeted agents. The results from these and related studies will be discussed.

miR-211 functions as a metabolic switch in human melanoma cells

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The microRNA (miRNA) miR-211 negatively regulates genes that drive invasion of metastatic melanoma. Compared to normal human melanocytes, miR-211 expression is significantly reduced or absent in non-pigmented melanoma cells and lost during human melanoma progression. To further investigate its tumor suppressor function, miR-211 was ectopically expressed in non-pigmented melanoma cells. Ectopic miR-211 expression destabilized hypoxia-inducible factor 1α (HIF-1α) and increased cell death during hypoxia. HIF-1α destabilization was correlated with downregulation of a previously unidentified miR-211 target gene, pyruvate dehydrogenase kinase 4 (PDK4). miR-211 mediated regulation of PDK4 sensitized melanoma cells to hypoxia-induced cell death via HIF-1α. miR-211 acts as an important tumor suppressor by acting as a metabolic switch, and its loss likely promotes cancer hallmarks in human melanomas.

Long noncoding RNA *SPRIGHTLY* an intra-nuclear organizing hub in human melanomas

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Little is known about how long noncoding RNAs (lncRNAs) are regulated and how they regulate other genes and proteins. We previously reported that the lncRNA *SPRIGHTLY* is highly upregulated in human melanoma cells and mediates proliferation, invasion, and apoptosis. *To gain a better mechanistic understanding of SPRIGHTLY, here we identified SPRIGHTLY domain-specific RNA interacting partners. SPRIGHTLY* has three topological domains, each of which having unique and common RNA interaction partners. *SPRIGHTLY* interacts with the intronic regions of several coding genes including *SMYD3*, *SND1*, *MEOX2*, *DCTN6*, *RASAL2,* and localize to the intronic regions of pre-mRNA to coordinately express functionally-related RNA molecules. We believe that *SPRIGHTLY* functions as a transcriptional regulator in melanoma by interacting with pre-mRNAs in tumor-associated genes.

Screening of the neoantigens determinant for Anti-CTLA4 response in mouse melanoma models

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The treatment of metastatic melanomas with Immune Checkpoint inhibitors (ICi), such as CTLA4 and PD-1/PD-L1 antibodies has rendered unprecedented durable responses in a subset (20–30%) of patients. Still, the molecular determinants of the heterogeneity in the response are unknown. Recent studies in patients have correlated the mutational load and specific neoantigens expressed by tumors with their immunogenicity and sensitivity to ICi, however, mechanistic studies are very limited in humans. To recapitulate human melanoma heterogeneity, three syngeneic mouse models harboring distinct genetic alterations and carcinogenic mechanisms were employed: (i) UV-induced $Braf^{V600E}$ mutant and Pten deficient melanomas (UV-Braf/Pten), (ii) DMBA-induced Hgf and $Cdk4^{R24C}$ mutant melanomas (DMBA-Hgf/Cdk4), and (iii) UV-induced Hgf melanomas (UV-Hgf). While DMBA-Hgf/Cdk4 and UV-HGF melanomas demonstrated partial or high-degree sensitivity to anti-CTLA4, respectively, UV-Braf/Pten tumors exhibited intrinsic resistance. Tumor immunogenicity determined by vaccination assays *in vivo* was associated with anti-CTLA4 response, even though the three models were able to activate cytotoxic T-cells *in vitro* when expressed known melanoma antigens. We hypothesized that the particular mutations expressed by UV-Hgf cells could account for their enhanced sensitivity to anti-CTLA4 treatment. Mutations found by exome and RNA sequencing in the three models were analyzed *in silico* for their potential to bind MHC-I and/or MHC-II, identifying putative neoantigens. A 'neo-epitope' library based on UV-Hgf selected mutations will be expressed in the resistant UV-Braf/Pten cells; which will be treated with anti-CTLA4 in mice to determine the neoantigens required for the response. Overall, we expect that our study will provide insight into the role of mutational load and neoantigens on melanoma response to immunotherapy.

Isolation of cytotoxic compounds against melanoma cells from *Epicoccum nigrum*, an endophyte Isolated from *Ferula sumbul* plant

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Owing to the importance of endophytes for the production of biologically active secondary metabolites, current research establishes the chemical characteristics of the cytotoxic compounds (active against melanoma cells) of *Epicoccum nigrum* isolated from Ferula sumbul plant.

In the course of work aimed at the discovery of new Cytotoxic compounds from endophytes of medicinal plant, Ferula sumbal, a lipophilic extract of the endophyte *Epicoccum nigrum* displayed significant cytotoxicity against two human melanoma cell lines, SK-MEL-28 and A375P. Bioassay-directed fractionation of this extract followed by LC-MS/MS resulted in the isolation of important compounds including 2-methyl-3-nonyl prodiginine, Bis (2-ethylhexyl) phthalate, and a meroterpenoid, Preaustinoid A.

This study reveals the potential of *Epicoccum nigrum* as an important source of colored, cytotoxic compounds. Prodiginines is a large family of pigmented oligopyrrole antibiotics. Prodigionines are of potent clinical interest because these are reported to have anti fungal, anti bacterial, anti protozoal, anti malarial, immunosuppressive and anti cancer activities. This is the first report of isolation of prodiginines as well as meroterpenoid and Bis (2-ethylhexyl) phthalate from *Epicoccum nigrum*.

In conclusion, assays in current research using the crude extract and fractions of secondary metabolites of Epicoccum nigrum showed a significant cytotoxic activity against melanoma cells.

Thus strain could be further exploited for various applications in pharmaceutical, for its secondary metabolites.

Analytical validation of a clinical test for PRAME gene expression status in primary uveal melanomas

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Class 1 and 2 uveal melanoma (UM) gene expression profiles are associated with low and high risk of metastasis, respectively. Detectable mRNA expression of *Preferentially Expressed Antigen in Melanoma* (*PRAME*) was recently shown to be a biomarker for increased metastatic risk in Class 1 UMs and for shorter time to metastasis in Class 2 UMs. As PRAME-directed therapies are being investigated in clinical trials, PRAME status could also be important for trial enrollment in UM patients. For routine incorporation of PRAME into patient management, a robust clinical assay with an objectively defined threshold for positive PRAME expression (PRAME+) is critical. We developed a clinical assay for PRAME mRNA expression based on RT-PCR and defined the threshold (S2) for calling PRAME+ using a LOESS model with data from 958 UMs in a blinded fashion. This threshold was then validated by its concordance with a previously reported threshold (S1) and clinical outcomes in 119 of the UMs. The S2 threshold called 647 (68%) samples PRAME- and 311 (32%) PRAME+, whereas the S1 threshold called 673 (70%) samples PRAME- and 285 (30%) PRAME+. The S2 threshold classified 27%, 29%, and 43% of Class 1A, 1B, and 2 tumors, respectively, as PRAME+. By comparison, the S1 threshold classified 25%, 26%, and 40% of Class 1A, 1B, and 2 tumors as PRAME+. 932 of 958 (97.3%) cases were concordant for PRAME status by both thresholds (kappa = 0.9368). In 119 patients with available outcome data, both thresholds called 7/7 Class 1 tumors that metastasized as PRAME+ (100% sensitivity). All Class 1/PRAME- patients were disease-free (100% NPV). For Class 1, the S1 and S2 thresholds had comparable specificities (70% and 68%) and PPVs (29% and 28%). These data validate our PRAME assay for use in primary UM.

Molecular response patterns to MEK inhibition, but not NRAS mutation status correlate with the efficacy of combined MEK/CDK4,6 targeting in melanoma

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Treatment of NRAS mutant melanoma is challenging. The discovery that co-targeting of MEK/CDK4,6 has antitumor activity created excitement for patients and clinicians; however, first clinical results have not met pre-clinical expectations. In this study we investigate the response patterns of NRAS mutant melanoma cells *in vitro* and *in vivo* when challenged with inhibitors of MEK, CDK4,6 and their combination. Data revealed, that *in vitro* growth response patterns to MEK/CDK4,6 inhibition can be used to predict the *in vivo* efficacy of MEK/CDK4,6 co-targeting in a xenograft model of NRAS mutant melanoma. In addition, signaling changes after single MEK inhibition also correlated with efficacy of the MEK/CDK4,6 combination: Cells displaying activation of the cell cycle pathway after MEK inhibition evidenced by elevated pRb levels, showed more effective growth reduction with MEK/CDK4,6 co-targeting compared to single MEK inhibitor treatment. In contrast, MEK/CDK4,6 and single MEK inhibitor treatment were equally effective in cells that responded with unchanged or decreased protein levels of pRb after single MEK inhibition. Cells sensitive to MEK/CDK4,6 co-targeting, defined by these criteria, showed a significant reduction of tumor size and induction of apoptosis *in vivo*. This pattern is not limited to NRAS mutant cells, but can be applied to BRAF mutant cells and cells that are wild-type for these mutations. Findings suggest that mutant NRAS alone is insufficient to predict effective growth reduction with MEK/CDK4 targeting. Further, the efficacy of the MEK/CDK4,6 combination can be predicted by *in vitro* viability assays and by the changes of pRb levels of cells after single MEK inhibition. Findings might ultimately help to more precisely define melanoma patients that are most likely to benefit from MEK/CDK4,6 targeting.

Radiation therapy in melanoma patients with poor clinical characteristics–a collaborative experience with 107 patients

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Radiation therapy (RTX) is an important part of melanoma management. Even though it is effective for local disease control, the lack of improved overall survival often dampens enthusiasm for this therapy. We describe our collaborative experience treating 107 stage III/IV melanoma patients managed at a dermatology department and a radio-oncology center. Our cohort comprises a subset of melanoma patients with unfavorable clinical characteristics (median Breslow thickness: 2.8 mm (0.5–18 mm); clinical stage III/IV at diagnosis: n = 95). The median age at diagnosis of melanoma was 60.3(13.6–90.0) years. The axillary area was the most frequent anatomical site for irradiation (n = 30) followed by bone irradiation (n = 20). Therefore the majority of patients received additional systemic therapy including immunotherapy, chemotherapy and targeted therapy. Average overall survival (OS) was calculated with 57.3(SD±52.1; 1.6–423.9) months. Average overall survival after RTX (OSaRTX) was 17.1(SD±18.7; 0.7–100.8) months. Loco-regional recurrence (LRR) of melanoma was observed in 17 irradiated areas. No difference in OS or OSaRTX and loco-regional recurrence was found: OS without LRR 57.6 (SEM 5.7) years; OS with LRR 55.6(SEM 9.2) years; OSaRTX without LRR 16.2(SEM 1.8) years, OSaRTX with LRR 20.6(SEM 3.8) years (all $P > 0.05$). LDH serum levels did not correlate with local recurrence ($r = 0.07$). Subgroup analyses revealed that OSaRTX for axillary irradiation was 26.3 (SD±24.6; 0.7–100.8) months suggestive for irradiation being specifically useful for the adjuvant treatment of the axillary area. The majority of acute adverse events and all late irradiation adverse effects (n = 11) were grade 1 (CTCAE v4.02; RTOG/EORTC). From our data we conclude that RTX is a well-tolerated therapeutic modality with the potential for high local disease control, particularly in the axillary region.

Eleven years of melanoma patient management–observations and trends from a single-center study in Austria

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Recent studies indicate a constant increase of melanoma incidence rates and largely unchanged mortality ratesover the last decades. The aim of this study was to characterize the cohort of melanoma patients managed at a single-center institution in Vienna between 2000 and 2010. The mean age of patients at diagnosis was 59.1 ± 16.7 years. Women were significantly younger than men at the time of diagnosis (♀57.2 ± 17.8 versus ♂61.0 ± 15.2 years; $P < 0.001$). Superficial spreading melanoma (39.5%) was the most frequent histological subtype, followed by nodular melanoma (14.9%), lentigo maligna melanoma (5.2%) and acral melanoma (2.6%). In 25.8% the histological subtype could not be determined. The mean Breslow thickness (BT) was 1.81 mm and consistently increased with the age of the patients (31–40 years: 1.21 ± 1.42 mm; 71–80 years: 2.32 ± 2.63 mm). The vast majority of tumors were detected at early stages (T_{MIS}:7.4%; T1a/b:41.5%). None of the T_{MIS} and 3.1% of patients with tumor stage IA progressed. Out of all SLN biopsies, 17.3% of patients had a positive SLN; of those, 38.3% progressed. In total, 11.3% of all patients (including 12.9% of sentinel negative patients) experienced progressive disease of which 70.7% succumbed to melanoma. The number of patients with progressive disease increased with the clinical stage at diagnosis (IA: 3.1%; IIIC: 50.0%). Women had a better 5-year overall survival compared to men (75.8% versus 63.6%; $P = 0.025$). The findings of this study highlight that early detection is effective for preventing metastatic spread. We did not observe a decrease of median BT at diagnosis during the study period of 11 years. This might be explained by the high number of clinical stage IB patients, which require hospitalization for SLN biopsy. Alternatively, this could indicate that melanoma awareness campaigns of the recent past need to be refocused.

Cost-comparison analysis of nivolumab + ipilimumab regimen (NIVO + IPI) and dabrafenib + trametinib (DABRA + TRAME) utilizing clinical trial and real-world data

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In CheckMate 067, NIVO+IPI improved median progression-free survival (mPFS) and objective response rate (ORR) versus IPI in BRAF V600 mutant-positive advanced melanoma (AM) patients. mPFS for NIVO+IPI and IPI were 15.5mo and 4.0mo, respectively; ORRs were 66.7% and 22%, respectively. The aim of this study was to compare the total melanoma healthcare costs incurred by first-line (1L) AM patients treated with NIVO+IPI, DABRA+TRAME, and other drugs. Due to lack of real-world data, the costs for NIVO+IPI were estimated using patient-level resource utilization data from CheckMate 067 and unit costs for these resources taken from the Truven Health MarketScan® databases (MarketScan). MarketScan was used to identify total costs ($2015) for DABRA+TRAME. All melanoma-specific healthcare costs (including drug, inpatient, outpatient, procedures, tests, and emergency room costs) incurred during the first year of treatment, including before and after disease progression, were aggregated for each treatment and adjusted for censoring. The total melanoma healthcare costs per patient during the first year of treatment were $234K and $230K for patients treated with NIVO+IPI (n = 313) and DABRA+TRAME (n = 119), respectively. Patients' pre- and post-progression costs were $200K and $34K for NIVO+IPI and $155K and $75K for DABRA+TRAME. Total melanoma healthcare costs during the first year of treatment were found to be similar between NIVO+IPI and DABRA+TRAME. The higher pre-progression costs seen with NIVO+IPI were compensated by a reduction (56%) in the post-progression costs, reflecting delayed or reduced use of subsequent melanoma treatments in NIVO+IPI patients due to progression. These results suggest that 1L AM patients may realize significant clinical benefit with NIVO+IPI while maintaining melanoma-specific healthcare costs.

Our experiences with anti-PD1 antibody tharapy at patients with metastatic melanoma

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Anti-PD1 treatment is recently indicated in patients with non-metastatic melanoma. Nivolumab and pembrolizumab therapy is indicated at patients, where we see a disease progression after the CTL44 antibody (ipilimumab) treatment or as a first line therapy at our department.

The poster describes two case reports of our patients – first patient before the anti PD1 antibody treatment has been treated by ipilimumab, the second one has been treated by anti PD1 antibody as a first line therapy. Treatment regimen, side effects, CT and photo documentation are discussed.

Immunotherapy is recently new hope for patients with generalized malignant melanoma. There is new anti CTLA4 antibody a anti PD1 antibody combination therapy study up and running at our department as well. Our learning follows the similar ongoing studies results so far.

The effects of titanium dioxide (TiO_2) and octocrylene (OCT) on the proliferation activity of metastatic melanoma cells and on their ABCB5 mRNA expression

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There is no clear evidence on whether sunscreens and personal care products containing UV filters like OCT and TiO_2 are protective against or may be a contributing factor in melanoma development. A transmembrane protein ABCB5 is involved in tumor progression, disease recurrence and in melanoma clinical drug resistance. Our aim was to investigate the influence of OCT and TiO_2 on the proliferation activity of melanoma cells and on their ABCB5 mRNA expression.

Metastatic melanoma cell line WM 266-4 (ATCC) was used and treated with selected concentrations (from range 1 to 250 μg/ml) of OCT or TiO_2 (in the form of nanoparticles: nano-TiO_2 or with the particle size of ≤ 5 μm: micro-TiO_2) and incubated for up to 144 h. We used the MTT and LDH assays to measure cells' proliferation activity and cytotoxicity, respectively. RT-qPCR

using TaqMan® chemistry was performed and relative gene expression ratios were calculated for the target (ABCB5) and endogenous control (LDHA) gene.
OCT group resulted in increased ABCB5 mRNA expression at 24 h and 48 h of exposure when compared to 2 h ($P < 0.01$). The increase was 2-fold at 250 μg/ml and 5–6 fold at lower OCT concentrations after 48 h. Concomitantly, reduced cell number for 1.3% to 11.6% at 48 h, increased proliferation activity at 8 h and thereafter decreased cytotoxicity, and morphological changes (including cannibalistic activity) were observed.
On the other hand, our results suggest that TiO_2 might open a new window in the treatment modalities of melanoma. Micro-TiO_2 is progressively decreasing the ABCB5 mRNA expression, however nano-TiO_2 has a rebound increase at 48 h of exposure at all but one concentrations ($P < 0.05$) and then a significant decrease after 120 h of exposure ($P < 0.01$). This increase raises questions which should be answered before any potential use in medicine.

AURORA A overexpression (*AURKA*) is driven by *FOXM1* and MAPK/ERK activation in melanoma cells harbouring BRAF or NRAS mutations

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The identification of druggable targets to improve anti-melanoma therapeutic responses still remains a challenge.
We show that *AURKA*- and *FOXM1*- cell cycle related genes are consistently expressed in melanoma. We found that expression of *AURKA mRNA* and protein is associated with worst prognosis in two melanoma patients cohorts. *AURKA* overexpression correlates with the presence of $BRAF^{V600E}$ mutation in primary melanomas. The knockdown of $BRAF^{V600E}$ by small interfering RNA (si*BRAF*) resulted in a decrease of both *AURKA* promoter activity and AURORA A levels in $BRAF^{V600E}$ melanoma cell lines. Whereas treatment with BRAF inhibitors (PLX4720) restrictively reduced the AURORA A expression of the $BRAF^{V600E}$ cells, ERK inhibition by PD98059 drastically decreased both *AURKA* promoter activity and protein levels of melanoma cells irrespective of their *BRAF/NRAS* status. *AURKA* inhibiton by si*AURKA* or MLN8237 treatment reduces cell proliferation and induces senescence in all cell lines including two vemurafenib-resistant $BRAF^{V600E}$ cells.
FOXM1 and AURORA A expression levels are correlated in $BRAF^{V600E}$ melanoma biopsies and the silencing of $BRAF^{V600E}$ reduced *FOXM1* expression in those mutant cell lines. FOXM1 depletion by si*FOXM1*, sh-*FOXM1* or Bortezomib treatments impairs *AURKA* expression, cell proliferation and the growth of melanoma-derived xenograft tumors. AURORA A and *FOXM1* inhibitors treatment impaired melanoma tumour growth compared with tumours that received vehicle alone.
The study highlights AURORA A as a prognostic factor in melanoma and reveals that *AURKA* overexpression is driven by *FOXM1* and the activation of MAPK/ERK signalling in $BRAF^{m}$ or $NRAS^{m}$ melanoma cells. In addition, the study suggests that AURORA A and *FOXM1* are candidate targets for melanoma therapies that may benefit a broad repertoire of molecular signatures

Inflammatory-induced nitrosative stress alters the SNO-proteome profile in human melanoma

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Inflammatory microenvironment plays a crucial role in driving resistance to treatment, and is associated with poor patient prognosis in melanoma. The nitric oxide synthases are responsible for generating nitric oxide (NO), a major reactive oxidant reported in various tumor microenvironments including melanoma. We hypothesize that NO and nitrosative stress lead to S-nitrosylation (SNO) modifications of intracellular proteins, which directly alters protein function and relevant signaling pathways in melanoma cells. To date, the SNO-proteome profile of human melanoma cells has not been systematically characterized. Thus, we developed a model to deliver varying [NO] levels derived from the donors GSNO or DETA NONOate for known exposure times. A novel cysteine saturation fluorescence assay (SNOFlo), in combination with mass spectrometry (MS) was applied to quantitatively identify potential SNO proteins in melanoma A375 cells treated with 100 μM GSNO for 24 h. The SNO levels of 25 proteins showed significant increase after treating with GSNO. An Ingenuity Pathways Analysis (IPA) categorized these 25 proteins into 2 major networks and 5 canonical pathways, which involve in cell death and survival, and cancer. We further applied biotin switch assay and LC-MS/MS analysis to identify the specific SNO-modified sites of these 25 proteins. For the first time, the SNO sites of fructose-bisphosphate aldolase A, elongation factor 2, pyruvate kinase, and 14-3-3 protein gamma were clearly identified by our MS studies in response to nitrosative stress. This is the first study to characterize SNO-proteome profile in melanoma cells. Such a system could be useful to provide insight for identifying crucial proteins that undergo post-translational modification in response to local nitrosative stress in melanoma, which may be amenable to reversal through antioxidant therapies.

Real-world treatment patterns of systematic combination therapy in patients with metastatic melanoma

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Systemic therapy for metastatic melanoma (MM) includes targeted therapy for patients with BRAF-mutation and immunotherapy (irrespective of BRAF status). Targeted therapy combinations such as dabrafenib/trametinib (dab/tra) or vemurafenib/cotellic (vemu/cobi) and immunotherapy combination ipilimumab/nivolumab (ipi/nivo) are currently the most advanced treatment options. This two-part study is the first to describe real-world treatment patterns of systemic

combination therapies. Part 1 of this study uses the COTA electronic health record (EHR), while Part 2 uses the Flatiron Oncology EHR. Data from both parts will be presented at the SMR conference.

Part 1 included stage IV MM patients who were treated with either dab/tra or ipi/nivo from 2013 to 2016. Vemu/cobi was not considered due to its recent approval. Index therapy was the first dab/tra or ipi/nivo use. Patients were followed from start of stage IV (baseline) to death, or the end of index therapy. Patient characteristics, treatment patterns, and adverse events (AEs) were descriptively assessed.

Among the 19 patients included, mean baseline age was 67.6 years (SD±15.5), 14 were male, and 17 were Caucasian. All patients were tested for BRAF V600-mutation and 9 were BRAF-positive. 5 had brain metastasis, and 10 had received other prior systemic therapies. Of the 19 patients, 8 received dab/tra and 11 received ipi/nivo. Mean treatment duration was 157.94 (SD±137.9) and 95.5 (SD±76.7) days, respectively. By month 3 and 6, 50% and 63% of dab/tra patients discontinued treatment, versus 64% and 82% of ipi/nivo patients. While on treatment, 3/8 dab/tra patients experienced a total of 17 Grade 2 or above (Gr2+) AEs, and all ipi/nivo patients experienced a total of 29 Gr2+ AEs. The most common Gr2+ AEs were decreased platelet count and increased lipase among dab/tra patients, versus dyspnea and nausea among ipi/nivo patients.

Variance between experts and community practitioners in the use of immune checkpoint inhibitors for advanced melanoma

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Immune checkpoint blockade has revolutionized the care of patients (pts) with melanoma; however, treatment guidelines lack specific recommendations on the use of these agents for individual pt cases. We developed an interactive, online treatment (Tx) decision tool to assess whether expert Tx recommendations based on specific disease and pt characteristics would affect the planned Tx decisions of community practitioners. In January 2016, 5 experts provided Tx recommendations for 90 case variations based on key factors they considered important to guide Tx choice and then an online tool was developed to provide this guidance to clinicians. To use the tool, specific pt and disease characteristics along with the intended Tx for that pt were entered. Then Tx recommendations from 5 experts for that scenario were provided and users were prompted to indicate whether this online consultation would change their Tx plan. An analysis of 432 cases entered into the tool found variation between the intended use of immunotherapy among tool users versus Tx recommendations from the experts. For example, compared with the experts who all chose immune checkpoint blockade as frontline Tx for BRAF wild-type disease, 44% of tool users planned to use a different Tx or were unsure. For $BRAF^{V600}$-mutant disease with a good PS and normal LDH, all of the experts recommended frontline immunotherapy whereas 55% of users selected combination BRAF/MEK therapy for this scenario. In total, 59% of tool users whose planned Tx differed from the experts indicated that the expert recommendations from the tool would subsequently change their Tx plan. An analysis of specific first- and second-line agent and combination choice along with Tx variance in other case scenarios will be presented. Supported by Bristol-Myers Squibb, Genentech, Novartis Pharmaceuticals Corporation, and Prometheus.

New therapies for the treatment of *BRAF/NRAS* wild type melanoma

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Melanoma causes more than one death every hour in the US and, despite promising advances, improved therapies are still required to increase patient survival.

We screened for sensitivity to 180 combinations of clinically relevant drugs in a collection of 20 *BRAF/NRAS* wild type melanoma cell lines, a sub-type representing 30% of the human disease for which targeted therapies are not available.

We found that 20% of cell lines are highly sensitive to a combination of nilotinib plus trametinib and validated this combination using 2 independent experimental approaches. We confirmed the drugs synergy firstly using an independent collection of *BRAF/NRAS* wild type melanoma cell lines (n = 7), then a collection of *BRAF/NRAS* wild type patient derived xenotransplant cultures (n = 3), and finally a collection of BRAFV600E melanoma cell lines (n = 9).

We generated a gene expression signature of synergistic cell lines for nilotinib/trametinib combination, and used it to classify human melanomas from Leeds (N = 171) and TCGA (n = 470) cohorts. Tumors classified as synergistic-like (27.9 and 36.7%, respectively) are associated to decreased overall and recurrence free survival ($P < 0.05$), suggesting that our combination might be effective in a relevant fraction of aggressive tumors.

We performed a genome-wide CRISPR/Cas9 screen to identify drug resistance genes. We found that loss of tuberous sclerosis complex can confer resistance to nilotinib plus trametinib. Since tuberous sclerosis complex genes are mutated in 10% of melanomas, this approach can help to identify patients potentially refractory to the treatment.

Finally, we tested *in vivo* nilotinib/trametinib combination in a patient derived xenotransplant mouse model and showed that the combination is well tolerated and significantly more effective than the 2 drugs alone ($P < 0.01$). These data suggest a strong clinical translation potential for nilotinib/trametinib combination.

Partners in crime – signaling through Raf heterodimers

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The three Raf kinases (A-Raf, B-Raf and Raf-1) are key players in the EGFR signalling network and frequently mutated in cancer, with the activating B-Raf V600E mutation being present in about 60% of melanoma. While targeted therapies towards mutated Raf exist in form of small molecule inhibitors, many patients develop resistance to these therapies and relapse. Resistance is mainly due to network adaptations that bypass the drug blockade, with Raf heterodimerization being one of the main resistance mechanism in melanoma.

In order to determine whether signalling via Raf heterodimers is not only quantitatively but also qualitatively different compared to monomers or homodimers, we have established an inducible dimerization system to generate stable Raf homo- and heterodimers. Using co-immunoprecipitation in combination with quantitative mass spectrometry, we were able to identify dimer-specific interaction partners of Raf proteins, either in the absence or presence of Raf inhibitors. We analysed a total of 22 conditions in 6 replicates, identifying ~2000 bona fide protein interactions including components of several novel molecular processes associated with Rafs, e.g the mediator complex, the RISC complex, and chromatin remodellers. Focussing on interactions that change with dimerization status and drug treatment, we found many proteins which are altered in cancer or have been linked to drug resistance.

The identification of dimer-specific interaction partners strongly suggests that Raf heterodimers affect different targets compared to monomers. We found novel crosstalk between the MAPK pathway with other important cellular pathways that may play a role in tumour resistance to targeted therapies.

Unexpected UVR and non-UVR molecular signatures in acral and cutaneous melanomas with implications for treatment

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It is well established that ultraviolet radiation (UVR) is the predominant cause of mutagenesis in cutaneous melanoma, whilst the cause of melanoma in sun-protected sites such as acral skin and mucosa is poorly understood. To further investigate mutational processes in melanoma, whole genome sequencing was performed on 183 melanomas with detailed histopathological assessment. The clinicopathological characteristics of mucosal, acral and cutaneous melanomas with/without UVR mutation signatures were compared. As expected, all mucosal melanomas were found to harbour a non-UVR signature. However four (of 140 = 2.9%) cutaneous melanomas showed non-UVR signatures and surprisingly three (of 35 = 8.6%) acral melanomas demonstrated a UVR mutational signature. Compared with UVR dominant cutaneous melanoma, those with a non-UVR signature had lower total mutations and their primary tumors were thicker ($P > 0.05$) and had more mitoses ($P > 0.05$). Acral melanoma with UVR signature showed a predisposition for subungual sites, occurred in younger patients ($P = 0.008$), had a larger total number of mutations and UVR proportion of mutational burden more similar to melanomas on intermittently UVR-exposed skin when compared with non-UVR acral melanoma. No histopathological features predicted a UVR / non-UVR signature in acral melanomas or cutaneous melanoma. Our finding of acral/subungual melanomas with predominant UVR mutagenesis suggests that the nail plate and acral skin do not provide complete protection from UVR. We also confirm that cutaneous melanomas not caused by UVR occur infrequently. An understanding of the ever expanding heterogeneous nature of melanoma is imperative when treating advanced metastatic melanoma patients as the tumor's molecular profile (and not necessarily the primary tumor anatomical site) will likely best determine the most appropriate treatment options.

Antimelanoma activity of a single agent that concurrently inhibits mTORC1 and autophagy

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Although viewed as a purely degradative organelle, the lysosome enables mTORC1 signaling by housing mTORC1 regulators such as the Ragulator-Rag and TSC2-Rheb complexes. Thus elevated autophagy and mTORC1 activity, cardinal features of therapy-resistant melanoma, are linked to the lysosome. Clinical trials testing combinations of rapalogs with the lysosome inhibitor HCQ show promising activity in melanoma patients, however limited potency of HCQ and the onset of protective catabolism in response to PI3K/mTOR-targeting agents pose hurdles. We report a novel lysosome inhibitor, DQ661, capable of concurrently inhibiting mTORC1 and autophagy by piercing the lysosome membrane. RPPA analysis and lysosomal fractionation reveal DQ661 inhibits mTORC1 (decreased S6, S6K, 4E-BP1 activity) by ejecting mTORC1 regulators (e.g. RagA, RagC, p18) from the lysosome surface. Unlike catalytic mTOR inhibitors, DQ661 breaks the critical mTORC1-Rheb bond. Similarly, no other class of lysosome inhibitor tested broke the mTORC1-Rheb bond. BRAFi increases lysosomal mTORC1-Rheb interactions, which are blocked by DQ661. DQ661 is exquisitely cytotoxic to PD-1 blockade-resistant, *BRAF-*, *NRAS-*, *BRAF/NRAS*-mutant and *BRAF/NRAS*-WT cell lines (IC_{50} 74 -290 nM), abolishes rebound autophagy following BRAFi, blunts rebound macropinocytosis following mTORi, and potentiates the activity of BRAF/MEK inhibition. DQ661 has significant single-agent activity in HCQ-resistant melanoma xenograft and anti-CTLA-4-resistant pancreatic syngeneic mouse models with *in vivo* evidence of mTORC1 and autophagy inhibition. The ability of DQ661 to abolish autophagy and macropinocytosis, while also inhibiting mTORC1 activity, underscores the unique advantage of specifically targeting the lysosome membrane as a novel therapeutic strategy to achieve multi-faceted inhibition of both catabolic and anabolic programs utilized by melanoma cells.

CD73 expression marks a bivalent cell state in melanoma and is regulated via MAPK signaling

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Cancer cell plasticity is a key driver of tumor immune escape and tumor progression. However still little is known about the mechanisms that shape cancer cell states. CD73 is a cell surface 5′ ectonucleotidase expressed by melanoma and immune cells that converts extracellular AMP to immunosuppressive adenosine and a recently described component of tumor immune escape mechanisms.

Here we identify CD73 as a marker of an intermediate cell state recording an early inflammatory memory. Methylation of CpG islands in the 5′ regulatory sequence of CD73 is only found in $MITF^{high}$ EMT-negative cell lines. We show that inflammatory mitogenic stimulation induces CD73 in a MEK-ERK signaling dependent manner. Consistently, many melanoma cell lines with activating mutations in BRAF or NRAS exhibit high basal CD73 expression, which is robustly suppressed by the treatment with BRAF or MEK inhibitors.

Previously, we could show that melanoma plasticity is regulated by a MITF / c-JUN antagonism. In line we find that c-JUN strongly induces CD73 expression in melanoma cells. Using ChIP-qPCR analysis and an arrayed functional CRISPR-Cas9-based genome editing screening approach we identify a first intron enhancer in the CD73 gene with a canonical AP-1 binding site to be the key genomic region for c-JUN dependent regulation of CD73 expression.

Finally, also in our mouse model inflammation-induced tumor cell plasticity leading to immune escape is accompanied by upregulation of CD73.

In summary, our findings link immunosuppressive CD73 expression in melanoma cells to oncogenic MEK-ERK signaling and shed light on the induction of CD73 in a regenerative microenvironment, suggesting a novel bivalent melanoma cell state.

Elevated baseline serum lactate dehydrogenase (LDH) does not preclude durable responses with pembrolizumab

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Elevated LDH is a well-recognized adverse prognostic factor in patients (pts) with melanoma and is associated with poor ORR, duration of response, PFS, and OS with chemotherapy and BRAF inhibitors. Pts with elevated LDH treated with the anti–PD-1 antibody pembrolizumab (pembro) also have lower response rates than pts with normal LDH (Robert et al. NEJM 2015, Ribas et al. JAMA 2016), but it is unknown if responses are less durable in pts with elevated LDH. 655 pts with metastatic melanoma were enrolled in the phase 1b KEYNOTE-001 study (NCT01295827) and treated with pembro 2 or 10 mg/kg Q3W or 10 mg/kg Q2W in ipilimumab-naive and -pretreated cohorts. Response was assessed by RECIST v1.1 every 12 weeks. LDH was measured at baseline. As of the Sep 18, 2015, data cutoff date, median follow-up duration was 32 months (range 24–46). 643 pts had baseline LDH recorded, including 394 (61%) with normal LDH and 249 (39%) with elevated (ie, >1 × ULN) LDH. Among pts with measurable disease per central review at baseline, ORR was 43.2% (37.9%-48.8%) for normal LDH (n = 333) and 20.6% (15.6%-26.3%) for elevated LDH (n = 238). Median time to response was 3.1 months (2.4–33.1) for normal and 2.8 months (1.7–16.8) for elevated LDH. Among the 155 responders with normal LDH, 118 (76%) had not progressed, and median duration of response had not been reached (1.3+ to 38.5+ months). Among the 50 responders with elevated LDH, 33 (66%) had not progressed, and median duration of response was 34.6 months (1.6+ to 38.8+). The shape of the KM curves for duration of response were similar for elevated and normal LDH, with a high rate of censoring starting at ~20 months. The majority of pts with elevated baseline LDH who respond to pembro have a long duration of response, which numerically overlaps that of pts with normal baseline LDH. This suggests that pts with elevated LDH should not be excluded from treatment with pembro.

Correlation of BRAF mutation status between liquid and solid biopsies of patients with advanced melanoma disease

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The analysis of *BRAF* mutations in circulating cell-free DNA (cfDNA) is an interesting tool to monitor patients with advanced melanoma disease. The amount of mutant cfDNA seems to correlate with tumor burden, thus the detection of *BRAF* mutant cfDNA could be a valuable predictive biomarker of progression or clinical response in patients treated with both $BRAF^{V600}$ inhibitors and immune checkpoint therapies. One of the issues in evaluating cfDNA in patients with cancer is the sensitivity of the technique used for cfDNA isolation and mutation detection. The main goal of this study is to determine the correlation of *BRAF* mutation status between plasma and tissue samples of

melanoma patients. To the date, 42 patients with stage III and IV melanoma disease were enrolled in this study. Plasma extraction was performed before initiating treatment. The *BRAF* mutation status in plasma was analyzed using a new molecular test called Idylla™ ctBRAF Mutation Assay. Tissue samples were analyzed by cobas® 4800 $BRAF^{V600}$ Mutation Test or Idylla™ BRAF Mutation Test. Of the 42 analyzed patients, *BRAF* mutations were detected in 52.4% of tissue samples (22/42) and 47.6% of plasma samples (20/42). cfDNA determinations showed concordance in 85.7% of the cases (33/42) compared with tissue analysis. There were 14.3% of discordant results (6/42), including 4 cases with *BRAF* mutations detected only in tissue biopsies and 2 cases detected only in plasma samples. In conclusion, this study demonstrates a high rate of concordance of *BRAF* mutation status between liquid and tissue biopsies in advanced melanoma patients, which corroborates the clinical utility of cfDNA mutation analysis in patient management.

Revisiting the prognostic value of proliferation markers for thick primary melanoma

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Patients with thick (≥4 mm) primary melanomas have highly variable outcomes. Current staging criteria for these patients are based primarily on the presence of nodal disease, which often serves as the basis for adjuvant trial eligibility. Identification of novel biomarkers could help identify patients who may benefit from promising, new adjuvant therapies or, alternatively, spare patients with good prognoses the cost and potential toxicity of these drugs. We examined patients with thick primary melanoma to determine whether proliferation markers (mitotic index and Ki-67) and other clinicopatholgical features were associated with survival. We studied 171 patients with thick primary melanomas; median thickness was 6.0 mm (median follow-up, 3.0 years). In clinically node-negative patients, Ki-67 expression was an independent predictor of worse RFS (HR 2.19, P = 0.024) and OS (HR 2.49, P = 0.028). In a separate model, moderate (≥1 to <5 per mm^2) and many (≥5 per mm^2) mitoses were each significantly associated with RFS (HR = 9.97, P = 0.035 and HR = 11.93, P = 0.025, respectively); and OS (HR = 12.79, P = 0.033 and HR = 18.68, P = 0.017, respectively). In the same model, natural log-transformed tumor thickness was also significantly associated with worse OS (HR 2.37, P = 0.009). In sum, we identified cell proliferation markers Ki-67 and mitotic index as independent predictors of survival in clinically node-negative patients with thick primary melanoma. Greater tumor thickness was also an independent predictor of survival in this cohort. With further investigation, these measures may improve risk-stratification for patients with thick primary melanoma.

Germline *MC1R* status influences somatic mutation burden in melanoma

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The major genetic determinants of cutaneous melanoma risk in the general population are disruptive variants (*R* alleles) in the melanocortin 1 receptor (*MC1R*) gene. These alleles are also linked to red hair, freckling, and sun sensitivity, all of which are known melanoma phenotypic risk factors. Here we report that in melanomas and for somatic C>T mutations, a signature linked to sun exposure, the single nucleotide variant count associated with the presence of an *R* allele is estimated to be 42% (95% CI 15%-76%) higher than that among persons without an *R* allele. This figure is comparable to the expected mutational burden associated with an additional 21 years of age. We also find significant and similar enrichment of non-C>T mutation classes supporting a role for additional mutagenic processes in melanoma development in individuals carrying *R* alleles.

New anti-melanoma compounds targeting endoplasmic reticulum stress

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Melanoma has a capability of rapid metastasis to other organs. Even if recently encouraging results were obtained with the inhibitors of BRAF, these responses remain transitory.with development of drug resistance. Another therapies (anti-CTLA4 or anti PD1) which reactivates the immunity response of the patient, were recently developed. However, these therapies give an objective response in only 15 to 30% of patients. Thus, it appears necessary to develop new drug candidates for specific biotherapy treatment of melanoma. Using structure/activity relationship studies, we developed and selected candidates (Thiazole Benzensulfonamides) exhibiting a strong death-promoting effects in melanoma cells with HA15 as the lead compound of this series. Interestingly, HA15 is active molecule on all melanoma cells independently of mutational status and on melanoma cells freshly isolated from patients sensitive or resistant to BRAF inhibitors. HA15 exhibited also a strong efficacy in xenograft mice models performed with melanoma cells sensitive and resistant to BRAF inhibitors without any sign of toxicity in mice. We next performed pan-genomic, proteomic and biochemical studies to decipher the signaling pathway, the mechanism of action and the target of the best candidates. We identified BIP, an endoplasmic reticulum protein, as the specific target of our compound. We demonstrated clearly that the interaction between our compound and BIP increases Endoplasmic Reticulum Stress and leads to melanoma cell death by concomitant induction autophagy and apoptosis mechanisms. This molecule was also found to be active against other liquid and solid tumors. Our data suggest that our molecule has an important impact on inhibition of melanoma growth by targeting ER stress, and may therefore be developed for treatment of patients with melanoma in particular and other cancers in general. (Cerezo el al.,Cancer cell, 2016, june)

Down-regulation of RNA-binding protein CRD-BP inhibits melanoma metastasis

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Coding region determinant-binding protein (CRD-BP) is a multifunctional mRNA-binding protein overexpressed in human melanomas, which was shown to modulate the stability, localization and translation of several RNAs. In this study, we investigated the role of CRD-BP in melanoma metastasis, considered one of the most difficult diseases to treat in medical oncology. For that two different model to evaluate spontaneous metastasis development were used, syngeneic mouse model and knockout mouse. Knockout mice were developed by crossing Tyr::CreER^{T2}; PTEN$^{loxP/loxP}$;BRAF$^{F\text{-}V600E/+}$ with CRD-BP$^{loxP/loxP}$ mouse in a C57BL/6J background. Cre/lox site-specific recombination system allows the control of gene activity in a specific space, time and tissue following topical application of tamoxifen. For syngeneic study, mouse melanoma cell lines were prepared with pINDUCER lentiviral system that enables efficient doxycycline-inducible knockdown of CRD-BP. Inhibition of CRD-BP resulted in a significant reduction in lung metastases in both models. Down-regulation of CRD-BP was also effective in inhibition of melanosphere forming capacity as well as in suppressing migration and invasion in *in vitro* assays. Mechanisms of involvement of CRD-BP in melanoma metastasis will be discussed.

Impact of radiation resistance on JARID1B expression following different therapy sequences

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Melanoma is one of the most heterogeneous cancers described and although cure rates are high if melanoma is caught early; once it metastasizes, survival rates dramatically drop. This is mainly due to the emergence of resistance to current therapies. Long-term therapy responses fail for many reasons, one of which is the failure to induce cell death in all heterogeneous cell subpopulations. Recently, we have shown the existence of a surviving subpopulation that is multi-drug-resistant and shows high expression of the H3K4 demethylase JARID1B/KDM5B. In this study, we attempted to explore the role of JARID1B^{high} melanoma cells after a combination therapy of ionizing radiation (IR) and targeted therapy (TT). We designed two therapy sequences; I. IR followed by targeted therapy and II. Targeted therapy followed by IR. We show that there is more JARID1B induction in sequence I (IR à TT) compared to sequence II (TT à IR) indicating a mechanistic link between JARID1B enrichment and IR. We also show that some signaling pathways are activated differently according to the sequence of therapy. We also demonstrate that these JARID1B^{high} melanoma cells are in a reversible slow cycling state that has the ability to proliferate and repopulate the tumor. This highlights the need to a better understanding of therapy-induced proliferation arrest to be able to evaluate compounds that truly stop melanoma growth and/or eliminate these cells.

Computer-assisted image analysis demonstrates tumor area and width as prognostic factors in stage IB melanoma

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Patients with stage IB melanoma have a 10% risk of melanoma-specific mortality within five years. The current prognostic paradigm, however, is insufficient to predict which of these patients are most likely to recur. Additional prognostic characteristics of stage IB melanoma are needed to identify this patient subset who are at highest risk of recurrence, and may benefit from closer follow-up. We evaluated a prospective cohort of stage IB patients (n = 655) treated at NYU Langone Medical Center. In a research subset (n = 149) composed of patients with recurrent (n = 63) and nonrecurrent (n = 86) disease matched for age, sex, thickness, ulceration and mitoses, primary tumors were independently reviewed for digitally calculated area, manually calculated area (depth x width), width, and conformation (contiguous versus non-contiguous) using computer-assisted histopathological analysis (Aperio, Vista, CA USA). We tested the association between histologic variables and recurrence-free survival (RFS) using Cox univariate analysis. Increasing digital area (HR 1.08, $P < 0.01$), tumor width (HR 1.17, $P = 0.01$), and non-contiguous conformation (HR 0.57, $P = 0.05$) were independently prognostic of RFS. Linear regression analysis showed a significant correlation between the manual and digital area (estimate 0.64, $P < 0.01$), which became even stronger when restricted to patients with contiguous tumors (estimate 0.75, $P < 0.01$), suggesting manually calculated tumor area may also provide useful prognostic information for providers without access to similar software. Computer-assisted measurement of cross-sectional tumor area, width, and contiguity may help risk-stratification in stage IB patients. Independent validation of these primary tumor characteristic is needed to fully comprehend their prognostic role in stage IB melanoma.

RANTES: a genetic risk marker for malignant melanoma

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Cytokines, chemokines and their receptors have varied biological functions including inflammatory response, immune-cell trafficking, angiogenesis and metastasis. Chronic or recurrent inflammation is known to play a causative role in the promotion and progression of many tumors. Chemokines, identified on the basis of their ability to induce chemotaxis, have a fundamental role not only in inflammation and immune surveillance but also in cancer progression. Genetic polymorphisms of cytokine encoding genes are known to predispose to malignant disease. The RANTES (Regulated upon activation, normal T-cell expressed and secreted) gene lies on chromosome 17q11.2-q12 is one of the natural ligands for the chemokine receptor CCR5. The two polymorphisms -403G/A and -28C/G are located in the upstream noncoding region of the RANTES gene, which contains cis-acting elements involved in RANTES promoter activity. To date no association studies have been reported that have examined the possible relation to RANTES genotypes and Melanoma. Considering its potential role in cancers, we screened two

functional polymorphisms in the promoter region of the RANTES in Melanoma patients and Controls. We examined 102 melanoma subjects with (Males -59 & female-43) and age matched controls (N = 158) and all subjects are non-Hispanic Caucasians. Melanoma cases differed from controls showing significant association with -403 G/A polymorphism ($P < 0.002$) and -28C/G ($P < 0.004$). Studies correlating RANTES levels with varied distribution of RANTES genotypes and effects upon melanoma susceptibility and disease progression will be discussed.

Survival outcomes in patients (pts) with advanced melanoma with CNS metastases

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Presence of CNS metastases (CNS mets) is common in pts with advanced melanoma. With recent expansion of available treatments (tx), overall prognosis for pts with melanoma has improved, but limited information is available for those with CNS mets. The goal of this study was to investigate overall survival (OS) of pts with advanced melanoma with CNS mets in the SEER-Medicare linked database. Retrospective analysis of SEER-Medicare, a population-based linked database, was undertaken. Demographic and clinical characteristics of the pts diagnosed with stage IV melanoma in 2004–2011 with/without CNS mets (CNS mets+/CNS mets-) around the time of diagnosis were evaluated. OS was assessed by the Kaplan–Meier method. The study identified 938 pts with melanoma. Mean age was 75.2 years; 67.7% were males. CNS mets were reported in 44.9% (291/648) of the study population. The majority of pts (69.1% CNS mets+; 66.4% CNS mets-) did not receive any melanoma-specific tx. Among those who received tx, a significant proportion received chemotherapy (84.4% CNS mets+; 80.7% CNS mets-). OS was significantly lower (3.86 months [95% CI 3.56–4.43]) for the CNS mets+ group versus the CNS mets- group (9.2 months [95% CI 8.17–10.53]). Among pts who received melanoma-specific tx, OS was higher with chemotherapy tx (6.60 months CNS mets+; 13.53 months CNS mets-) versus those who did not receive any tx (3.13 months CNS mets+; 6.52 months CNS mets-). In the advanced melanoma pt population in SEER-Medicare, OS for pts with CNS mets+ was significantly lower versus CNS mets- pts. However, melanoma-specific tx demonstrated significant improvement in survival in both groups. Introduction of targeted therapy and immunotherapies may continue to bring advances for these pts; thus, continued monitoring of outcomes in a population traditionally excluded from clinical trials is essential for research and clinical practice.

Treatment patterns for patients (pts) with melanoma receiving vemurafenib (VEM) in the real-world setting

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With treatment (tx) options for metastatic melanoma (mM) expanding rapidly, information on the use of various regimens beyond clinical trials is limited. The goal of this study was to investigate characteristics of a US-managed care population of pts with mM treated with VEM. Retrospective cohort analysis of a claims database was undertaken of pts with an initial VEM claim from June 2011–June 2014. A tx episode was defined as time from index date (claim for VEM) to the addition of or switch to a different drug. Prior or subsequent therapy was defined by presence/absence of any mM tx prior to VEM initiation or switch to another mM tx with a gap of >30 days. The study population consisted of 811 pts with mM. Median age was 57 years; 63.4% were male. Overall mean duration of VEM tx was 166 days (SD 121 days). VEM was mostly used as first-line tx (72.3%). Among those who had prior tx (n = 226), 45.9% received chemotherapy; 29.3% ipilimumab and the rest combination tx. Subsequent tx was reported for 32.6% of pts; 43.6% ipilimumab, 24.6% chemotherapy, 18.9% combination of dabrafenib + trametinib, and 12.5% dabrafenib or trametinib monotherapy. Use of various tx regimens differed over time. For patients who received prior tx, use of chemotherapy decreased dramatically (60% in 2011 to 6.3% in 2014), while use of ipilimumab increased (8% in 2011 to over 40% in 2013 and 2014). Similarly, there was a decline of chemotherapy use for subsequent tx (36% in 2011 to 11% in 2014). Targeted therapies, especially combination regimens, continued to play an increasing role in subsequent tx (8% in 2011 to 38% in 2014 for all targeted therapies). With an evolving tx landscape, sequencing may present challenges in evaluation of efficacy. Continuous monitoring of tx patterns and duration may assist clinicians, policy-makers, and managed-care organizations with tx strategies in mM.

Progesterone restores melanocytes and neural crest tissue in a zebrafish mutant in PAF1, a regulator of transcription

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Multipotent neural crest cells give rise to different cell types, including melanocytes. A chemical screen designed to look for repressors of neural crest gene expression revealed that the anti-arthritis drug leflunomide, an inhibitor of dihydrooratate dehydrogenase (DHODH) involved in *de novo* pyrimidine synthesis, modulates transcription elongation in neural crest and melanoma cells. Leflunomide is currently being tested in combination with a BRAF and a MEK inhibitor in a clinical trial for metastatic melanoma. We performed a chemical suppressor screen in zebrafish embryos using whole mount *in situ* hybridization for the neural crest marker crestin, and looked for compounds that rescued crestin expression after leflunomide treatment. We identified 13 chemicals and, using metabolite profiling by mass spectrometry in human melanoma cells to evaluate nucleotide precursors and nucleotides levels, we classified chemicals into 3 distinct classes. The first chemical class specifically affects mitochondrial import or leflunomide activity, the second increases nucleotide pools. The third class activates transcription and includes progesterone. In support of this observation we found that progesterone rescued neural crest gene expression in several RNA polymerase II associated factor (PAF) mutants. To better understand the mechanism via which progesterone regulates transcription elongation in neural crest we performed RNA sequence analysis on isolated neural crest cells. The analysis revealed that progesterone can revert leflunomide gene signature. We found that tfap2e is down regulated in leflunomide treated embryos and in paf morphants. Upon progesterone treatment its expression is restored, suggesting a critical role for tfap2e. Our studies establish that

the progesterone pathway regulates transcriptional elongation in the neural crest.

The AMPK- OGT axis prevents acquired drug resistance through inhibition of drug induced epigenetic remodelling in melanoma

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Drug resistance is a major hindrance for prolonged survival in melanoma patients. We recently identified chronic stress induced multi-drug tolerant melanoma cells (IDTCs), which were generated by exposing parental cells to sublethal concentrations of Dabrafenib and/or Trametinib. IDTCs show distinct epigenetic changes, specifically an increase in H3K4me3 and H3K27me3 marks after 30 to 50 days of continuous drug exposure forming colonies. Activation of the 5′ adenosine monophosphate-activated protein kinase (AMPK), as tested by increased phosphorylation of acetyl coA carboxylase a direct downstream target of AMPK, delayed IDTC colony formation. Prolonged AMPK activation by acetylsalicylic acid (Aspirin) prevented the upregulation of H3K4me3 and H3K27me3 in a state of transition from IDTC colonies to permanent drug resistance. Increased AMPK resulted in downregulation of O-GlcNAc transferase (OGT), which was found to be increased in IDTCs, *in vivo* xenografts and RNA-seq matched patient data, concomitantly with a decrease in H3K4me3 and H3K27me3. Silencing of AMPK reversed these changes. TET1 and EZH1, both downstream mediators of OGT function were increased in IDTCs and melanoma patients but transcript levels decreased in permanent resistant cells. *In vivo* OGT knockdown was as effective as AMPK activation with Aspirin in inhibiting tumor growth which downregulated H3K4me3 and TET1 expression. ChIP-seq analysis showed a differential occupancy of H3K4me3 in promoters and gene bodies in parental versus IDTCs after 45 days of exposure to Dabrafenib. AMPK activation prevents the occurrence of H3K4me3 and H3K27 marks in IDTC colonies through a signalling network comprising OGT, TET1 and EZH1. Targeting AMPK or elements of the AMPK nexus will increase the efficacy of current therapies and inhibit the emergence of permanent resistance.

Differences in the value that patients and physicians place on durable survival: implications for the treatment of advanced melanoma

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Cancer patients value therapies that offer long-term durable survival gains, but it is unknown whether physicians place similar value on durable survival. To address this issue, we surveyed patients with advanced stage melanoma and oncologists. We measured the share of respondents who selected a therapy with a fixed survival duration (ie, nonvarying therapy) versus one with a variable survival profile, with some patients experiencing long-term durable survival and others experiencing below-average survival. Initially, both therapies had the same average survival, calibrated to ipilimumab long-term survival data. We then applied parameter estimation by sequential testing to calculate the length of nonvarying survival that would make respondents indifferent between it and the therapy with durable survival. We also tested whether patient preferences were sensitive to adverse event (AE) severity. In our sample of 81 patients and 91 physicians, 63% of patients preferred the therapy with durable survival compared to 30% of physicians ($\Delta = 33\%$, $P < 0.001$). The average patient preferred the therapy with durable survival even if the nonvarying treatment had 13.6 months longer average survival. The presence of more severe AEs did not change these preferences (15.4 versus 13.6 months, $P = 0.369$). In contrast, the average oncologist preferred treatments with fixed survival unless the survival had 7.5 months shorter average survival compared to the treatment with durable survival gains. These findings suggest patients value therapies that provide a chance at durable survival, and this result holds even when compared to therapies with more severe AEs. To reinforce the tenets of patient-centered care, physicians should take into account melanoma patient preferences for treatments with durable survival gains.

FundingFunding provided by Bristol-Myers Squibb.

Feasibility of primary care provider education and screening for skin cancer detection: pilot study within a Veterans Affairs (VA) health care system

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Some evidence suggests that skin cancer screening may improve melanoma outcomes, but little is known about feasibility of skin cancer education and screening by primary care physicians (PCP) in large health care systems. The objectives of this pilot study were to assess the effects of skin cancer training and screening by PCPs on dermatology referral patterns and rates of skin surgeries within the Palo Alto VA Medical Center. In this prospective study, 5 volunteer PCPs underwent web-based training through INFORMED (INternet course FOR Melanoma Early Detection). Patients aged 35 years and older who were offered a clinical skin examination (CSE) by these PCPs from June 2015 to August 2016 were included for analysis. Preliminary outcomes included rates of dermatology referrals and subsequent skin surgeries and referral accuracy of PCPs for presumptive skin cancer or precancer diagnosis, compared with that by the dermatologist. Among 264 patients offered a CSE (99% male, median age 70 years), 155 (59%) received a full body skin exam, 40 (15%) a partial skin exam, and 69 (26%) declined. Of the 64 examined patients referred to dermatology, 47 (73%) were for presumptive skin cancer; 5 referred patients underwent biopsy for skin cancer and 4 for other issues. The positive predictive value (PPV) of PCP referral for skin cancer and precancer was 39.4%. Further analysis of change in referral patterns by PCP, estimated costs of screening, and acceptance by clinicians and patients is underway. In this

preliminary analysis, we describe a relatively high PPV of skin cancer referral and low rates of dermatology referrals (24%) and skin surgeries (3%) following routine skin cancer screening by PCPs. Larger studies are needed to determine whether the benefits outweigh the harms before screening is widely implemented in the United States.

Combining a GSI with BCL-2 inhibition to overcome melanomas resistance to current treatments

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Major limitations of the current melanoma treatments are the relapse and the lack of therapeutic options for BRAF wild type patients who do not respond to immunotherapy. Many treatment strategies stress on killing resistant subpopulations, such as Melanoma Initiating Cells (MICs) to prevent relapse. GSIs (Gamma Secretase Inhibitors) have been found to target cancer stem cells in pre-clinical studies and have been tested or are in clinical trials for cancer treatment. Here we examined whether combining a GSI with ABT-737 (a small molecule BCL-2, BCL- XL, and BCL-W inhibitor) can effectively kill the non-MICs (bulk of melanoma) and MICs. To be clinically relevant to the present landscape of melanoma treatments, we used melanoma cell lines and multiple melanoma tumor samples of patients relapsed from current treatments, with a diverse genetic background (with or without the common BRAF, NRAS or NF1 mutations). The combination treatment reduced cell viability and induced apoptosis in the non-MIC population; disrupted primary spheres, decreased the percentage of ALDH positive cells (marker of MICs), and inhibited the formation of secondary spheres ($P < 0.05$) in the MIC-enriched population of multiple melanoma cell lines and relapsed patient samples regardless of the status of common melanoma mutations. Using a low-cell-number mouse xenograft model, we also demonstrated that the combination reduced the tumor initiating ability of MIC-enriched cultures from relapsed patient samples ($P < 0.05$). Mechanistic studies indicated that the combination therapy caused cell death by up-regulating NOXA and down-regulating MCL-1 expressions, resulting in the NOXA-dependent death. In summary, this combination may be a promising strategy for treating melanoma and overcoming resistance to therapy.

Genotype and standard clinical features in 2793 melanomas

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Purpose Racial differences determine the intrinsic molecular basis. To obtain a comprehensive view of a genomic landscape in Asian melanoma patients, we sequenced a huge melanoma samples.

Patients and methods Melanoma samples (n = 2793) were analyzed for mutations in cKIT, BRAF, NRAS, PDGFRA and TERT genes in genomic DNA by PCR amplification and Sanger sequencing. Mutations were correlated to clinicopathologic features and prognosis of the patients.

Results The incidence of somatic mutations within the CKIT, BRAF, NRAS, PDGFRA, TERT-228 and TERT-250 genes was 13.5% (376/2793), 23.9%(646/2706), 7.7%(178/2325), 5.1% (118/2325), 5.9%(32/545) and 5.5%(30/545) respectively. Acral and mucosal Melanomas were less likely to show BRAF mutations (19.1% and 9.3% respectively) while CKIT mutation frequency was unbiased between melanoma subtypes. Melanomas harbored TERT-250 mutation had the greatest impact on the prognosis, the risk ratio increased 2.43 times ($P = 0.016$); then followed by CKIT mutation ($HR = 2.32$, $P < 0.001$) and NRAS mutation ($HR = 1.96$, $P < 0.001$). Multivariate analysis showed that CKIT, BRAF and NRAS mutations were independent prognosis factors ($P < 0.001$); TERT-250 mutation was also associated with poor prognosis ($P = 0.06$); the prognosis of acral and mucosal melanoma was similar but better than melanoma without chronic sun-induced damage. Among acral and mucosal melanoma, NRAS and CKIT mutation had more influence on prognosis (HR were 3.26 and 2.60, 1.57 and 2.64 respectively).

Conclusion Acral and mucosal melanomas were predominant subtypes in Asia. Mutations of CKIT and BRAF are the most common genetic alterations, and they can be therapeutic targets for these patients. TERT-250 may be a potential new target on melanoma.

National management trends and outcomes in advanced melanoma in a contemporary cohort

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The approval of targeted therapies and immune-modulating therapies (IM) has resulted in major strides in the management of advanced melanoma. With this evolving therapeutic landscape, prognosis in advanced disease has become less well-defined. Here we use contemporary data from the National Cancer Data Base (NCDB) to describe national trends in management and outcomes of patients with stage IV disease. Patients diagnosed with clinical stage IV disease from 2004 to 2013 were identified using the NCDB. Trends in administration of chemotherapy, IM, radiation, and metastatectomy were identified. The NCDB categorizes targeted therapies as chemotherapy. The year 2011 was used to dichotomize patients diagnosed before and after this point. Overall survival (OS) was compared using Cox proportional hazards and Kaplan-Meier modeling. Patients diagnosed in 2013 had incomplete follow up so were excluded from survival analysis. From 2004 to 2013, 14 138 patients were identified with stage IV disease in the NCD; 5725 (40.5%) were diagnosed from 2011 to 2013. IM was used as first course therapy (FCT) more frequently in patients diagnosed in 2011 to 2013 (18.2% versus 8.6%, $P < 0.001$). In 2013 IM was administered as FCT in 22.5% of new diagnoses. Multiagent systemic therapy was less frequently FCT in new cases from 2011 to 2013 (5.9% versus 13.1%). There was no difference in the use of single agent systemic therapy as FCT (20.5% versus 19.6%), beam radiation therapy (37.0% versus 37.1%), or metastatectomy (18.7% versus 18.3%). Stage IV patients diagnosed in 2011–2012 had longer OS than those diagnosed 2004–2010 (HR 0.87, $P < 0.001$), despite older age and higher comorbidity score. Among patients treated with any systemic therapy, 3 years OS was 19.8% for the 2011–2012 cohort versus 13.9%, and among those with M1B disease 3 years OS was 23.4% versus 13.7%. These national trends confirm changing practice patterns translating into improved survivability.

Have we underestimated adjuvant interferons efficacy in node-positive melanoma?

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Multiple clinical trials show adjuvant interferon (IFN) delays recurrence, and metaanalyses show a benefit for overall survival (OS) as well. Many studies, however, were done before sentinel lymph node biopsy (SLNB), and often had suboptimal regional control. In an institutional analysis of node-positive melanoma patients (pts), we found that postoperative radiation improved regional control, but adjuvant IFN did not. A secondary aim of that analysis was to assess the impact of adjuvant IFN on distant metastasis-free survival (DMFS) and OS. We retrospectively reviewed 385 node-positive pts. Surgery was either therapeutic lymph node dissection (LND, n = 86) or SLNB ± completion LND (n = 252). 128 pts (33.2%) received adjuvant IFN. Kaplan-Meier analysis, log-rank tests and Cox multivariate models were used to compare outcomes. Median follow-up was 70 months (range 13–180 months). Pts treated with and without IFN were closely matched with respect to all pre-treatment variables except age: IFN was used more often in younger pts (median 50 versus 65 years; $P < 0.001$). IFN was associated with improved DMFS on univariate (5-year estimate 47.9% versus 35.4%; $P = 0.003$) and multivariate analysis (hazard ratio [HR] 0.59 [95%CI 0.44–0.79]; $P < 0.001$). IFN was associated with improved OS on univariate (5-year estimate 56.9% versus 40.6%; $P < 0.001$) and multivariate analysis (HR 0.54 [95%CI 0.40–0.73]; $P < 0.001$). In an exploratory age-matched comparison of pts treated with (n = 67, median 61 years) and without (n = 233, median 63 years) adjuvant immunotherapy, IFN still showed improved DMFS (5-year estimate 43.9% versus 37.5%; HR 0.55 [95%CI 0.38–0.81]; $P = 0.002$) and OS (5-year estimate 57.3% versus 42.5%; HR 0.53 [95%CI 0.36–0.78]; $P = 0.001$). Adjuvant IFN appears to improve overall survival among node-positive melanoma pts in a modern single-institution experience, strongly supporting the use of IFN as the control arm in clinical trials.

Immune gene expression in cancer cells support both inhibition and activation of anti-tumor T cells

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T cell-based immunotherapies have brought great progress for cancer patients. However, only little is known about how cancer cells react to T cell attack. Therefore we setup a well-controlled co-culture system to study the dynamic T cell – cancer cell interplay. Low passage melanoma cell lines were cultured with MelanA/MART1-specific CD8+ T cells and characterized using differential gene expression analysis and flow cytometry. As expected, significant fractions of melanoma cells died in presence of melanoma-specific CD8+ T cells. However, still many melanoma cells persisted. They showed increased mRNA levels of genes associated with antigen processing and presentation: HLA Class I-encoding genes (*HLA-A* and *HLA-B*), HLA Class 2-encoding genes (*HLA-DRA* and *HLA-DRB*) and *CD74*, the invariant chain that stabilizes the complex of HLA Class II α and β chains until it is loaded with a peptide, and *TAP1* and *TAP2* (encoding transporter associated with antigen presentation), responsible for peptide transfer from the cytosol to the endoplasmic reticulum. Expression of HLA-Class I and HLA-DR was also strongly increased at the protein level as assessed at 48 h of co-culture. These gene and protein expression changes were dependent on antigen-specific interaction of CTLs with melanoma cells and were mediated by IFNγ and TNFα, two cytokines secreted by CTLs upon antigen recognition. We also found upregulation of IDO1 and PDL1. Together, our data show that CTLs and/or their cytokines IFNγ and TNFα not only induce known immunosuppressive molecules, but also genes involved in antigen presentation, and further interesting changes that will be presented at the congress. These findings likely play a role in immunotherapy, and might explain why immune reactions in cancer patients are often promoting both pro- and anti-tumor immune mechanisms.

MITF regulates cell adhesion and subcompartment-specific distribution of differentially cycling melanoma cells

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Differential tumor cell behavior caused by environmental conditions, termed dynamic heterogeneity, is a prime source for drug resistance. We utilize real-time cell cycle imaging (FUCCI) to study melanoma heterogeneity. As distinct proliferative and invasive capabilities reflect variable drug sensitivities, identifying and characterizing these different responses is crucial to design effective therapies. Mouse xenograft tumors generated from cell lines with high microphthalmia-associated transcription factor (MITF) level displayed a homogeneous distribution of cycling cells throughout. In contrast, tumors generated from cell lines with low MITF levels were composed of clusters of cycling cells and clusters of G1-arrested cells. The proliferating areas were in close proximity to blood vessels, presumably characterized by oxygen/nutrient availability. Melanoma spheroids recapitulated the *in vivo* cycling behavior, considering that here oxygen and nutrients are supplied by diffusion. MITF was undetectable within the hypoxic G1-arrested spheroid core, indicating hypoxia-induced MITF downregulation. Finally modulation of MITF expression impacted spheroid density, with overexpression giving rise to less compacted structures and *vice versa*. We conclude that MITF protects from cell cycle arrest induced by oxygen/nutrient deprivation. We hypothesize that high MITF levels prevent cell cycle arrest by reducing the cell-intrinsic propensity to arrest in response to low oxygen/nutrient and concurrently by allowing sufficient supply of oxygen/nutrients to cells. The latter may be achieved through decreased cell-cell/matrix adhesion resulting in the generation of looser tumors that allows more efficient oxygen/nutrient diffusion. These data outline how MITF-regulated dynamic heterogeneity could

influence therapy efficiency, making MITF an important marker for drug design.

Can melanoma treatment be guided by a panel of predictive and prognostic microRNA biomarkers?

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Melanoma progression markers have been used for many years with varying levels of success but in many centres world-wide, they are infrequently used due to their lack of adequate precision to detect disease relapse. Therefore a sensitive and specific blood test that could detect melanoma with regional spread, prior to clinically evident distant metastasis, could have the potential to improve treatment and outcomes for melanoma patients. To address this clinical unmet need, a panel of melanoma-related microRNAs (miRNAs) that when measured in melanoma tissues, were found to be predictors of stage, recurrence, and survival. Additionally, in a minimally-invasive blood test, the panel detected the presence of melanoma (relative to controls) with high sensitivity and specificity and were found to be superior to currently used serological markers (LDH and S100B) for melanoma progression, recurrence, and survival. At the time of discovery, it was envisaged that this panel could be ideally suited to monitor tumour progression using serial sampling over time which may prompt further intervention by the treating clinician. Currently, in steps toward this goal, the panels efficacy has been assessed in serially collected blood draws from Stage IV patients being treated with either MAPK inhibitors or immunotherapies. In this interim analysis, these data are supportive for the use of the miRNA panel to show early signs of relapse prior to clinical RECIST criteria (Response Evaluation Criteria in Solid Tumors) which could prompt a change in treatment modalities. These data will be further validated in a prospective multi-centre clinical trial.

Patient and provider relations and perceptions regarding melanoma treatment decisions

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New melanoma treatments (tx) are extending survival. To evaluate the patient (pt)-provider (pvdr) relationship and stakeholders' preferences about melanoma, a survey was distributed to adult pts with melanoma, physicians, pharmacists, and nurses at the Huntsman Cancer Institute. The response rate was 41.9% (N = 220) for pts (Stage 1 or 2, 79.1%; Stage 3, 16.4%; Stage 4, 4.5%) and 37.7% (N = 20) for pvdrs. Descriptive and comparative statistics were used. Regarding pts' expectations from tx, more pvdrs than pts chose 'Feeling less pain' (65% versus 28.6%, $P < 0.001$). Most pvdrs perceived pts were 'very anxious' about their melanoma, whereas most pts reported feeling 'not anxious' ($P < 0.0001$). Almost half of pts 'strongly agree' they have enough time to discuss melanoma tx with their pvdr, while one pvdr felt similarly ($P < 0.001$). Approximately 70% of pts 'always' trust their pvdr to make the best tx decision compared to 15% of pvdrs ($P < 0.0001$). Regarding how often pvdrs share their opinions about melanoma, most pts and pvdrs selected 'always' and 'rarely', respectively ($P < 0.0001$). Conversely, most pts reported 'always' sharing their concerns and most pvdrs felt this was 'sometimes' true ($P < 0.0001$). Most pvdrs would recommend melanoma tx that would be effective for ≥ 6 to 11 months, while most pts would undergo treatment effective for ≥ 24 months ($P = 0.0070$). Almost 30% of pts would receive tx that may cause job loss, yet 90% of pvdrs indicated ambivalence ($P = 0.0142$). In conclusion, there was discordance in the pt-pvdr relationship and perceptions about quality of life expectations, degree of anxiety, sharing of opinions, and tx-related progression free survival. Elucidating pt-pvdr preferences may enrich shared decision-making especially as more efficacious txs become available. Thus, further studies to examine stakeholder differences are needed to help align across patient-provider expectations.

Factors influencing treatment decisions for BRAF mutation positive advanced melanoma: an evidence review and Delphi study

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With the advent of new therapies for the treatment of advanced melanoma, first-line treatment decisions are becoming increasingly complex. Guidelines provide little direction on selecting between immunotherapies (IMT) and targeted agents. A Delphi study was conducted to understand how disease burden and tempo determine a treatment strategy for BRAF mutation positive advanced melanoma.

Twelve European melanoma specialists with substantial experience in using IMTs and targeted agents participated in a double-blind two-phase Delphi study. In Phase 1, participants completed a questionnaire developed based on a literature review of stratification and subgroup factors from clinical trials identified in a systematic review conducted in July 2015, a review of published clinical guidelines, and health technology appraisals. Phase 2 was a face-to-face meeting to explore the outstanding issues from Phase 1 and seek consensus (defined as 80% agreement) where possible.

There was consensus that tumor burden (83% clinicians) and disease tempo (83%) were important; however, no consensus was reached regarding a specific definition, measurement, or ranking of these factors. As least 10 different components are considered in a definition of tumor burden. Consensus was reached that brain metastases (82% of clinicians) and location of metastases (89%) are important in assessing tumor burden; brain metastases (83%) and lesions near critical organs (92%) were deemed essential when advising a less experienced clinician about how to determine first-line treatment class.

Clinicians agreed that choosing a treatment class for first-line treatment of advanced melanoma is a complex, multifactorial process. Until further evidence is available, clinical judgment will remain the most important element of first-line decision-making.

The new dawn of melanoma textbooks: interactive multimedia ebooks that remain up-to-date

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BackgroundProgress in melanoma patient care and research is advancing so rapidly that it is no longer possible for textbook production to provide current information for clinicians, students

and researchers. The prevalence of tablet computers has facilitated the development of etextbooks that can be continually updated and facilitate multimedia content, unlike a traditional printed textbook. We present here the advantages of two new such ebooks.

Methods A pair of melanoma ebooks were developed and published for two audiences (specialist and non-specialist), based on a review of the published literature on ebooks and the use of tablet computers in medical settings. The edition intervals of conventional melanoma textbooks were also analysed. The textbooks were developed and distributed using the Apple iBooks Author software and store.

Results Through use of the iBooks Author publishing format, the books were able to incorporate vast galleries of corresponding clinical, dermoscopic and histopathology images, together with bespoke animated graphics, procedure atlases and videos. Interactive instructional modules, search, bookmarking and annotation functionalities also enhanced the utility of the etextbooks. This layered interactive multimedia content distinguished them from all previous melanoma textbooks. Improved learning experience and marked preference for the use of ebooks on tablets, particularly for anatomy study, have been shown in medical students and clinicians. Unlike conventional textbooks, which can have over 5 years between editions, ebooks can be updated very easily. Furthermore, ebooks are readily portable, and can be sold inexpensively compared to conventional textbooks.

Conclusions Based on price, portability, richness of multimedia content possible and ease of updating information, etextbooks are the future for melanoma clinicians and researchers and trainees.

Germline variants in individuals of low and high nevus count

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We report on a prospective case-control study of melanoma and nevi in South-East Queensland, combining an assessment of pigmentation characteristics, dermoscopically-assessed nevus subtypes together with genotype comparisons and melanoma risk for 1032 individuals. Saliva-derived DNA was collected from volunteers and has been subject to genotyping at over 500K SNPs using an Illumina CoreExome Chip. Currently we have genotype information for 493 melanoma patients, 146 individuals with familial association and 393 non-melanoma/healthy controls. In this first-in-kind study, we have captured dermoscopic images of ~25 000 nevi >5 mm in diameter and in an interim analysis, the dominant nevus subtype pattern was 'nonspecific' followed by 'reticular', with a minority having a 'globular' pattern. Interestingly, we have found 17 participants in our cohort harbour the SUMOylation deficient *MITF* E318K mutation. Using our combined phenotypic data, it is clear that these carriers are not only affected with melanoma (70%) or have a family history of melanoma (18%) but they also have significantly higher total nevi counts (TNC) on average than the controls. Additionally, of the six genes identified in a recent meta-analysis of nevus GWAS, we have replicated four including *IRF4, MTAP, PLA2G6* and *LMX1B*. The most striking of these was the *IRF4* SNP rs12203592*C/T ($P < 10^{-7}$), which was associated with a decrease in globular nevus counts ($P < 10^{-6}$). To discover additional rare variants influencing nevus traits, we have conducted an analysis by performing whole exome sequencing (WES) on 62 individuals with extremes of nevus phenotypes. In this pilot WES study, we compared the allele frequencies of 30 individuals with low TNC (Mean of 0.4 > 5 mm) with 32 high TNC (Mean of 120.8 > 5 mm). Thus far we have discovered a 10-fold excess of variant alleles in the high TNC group, including a novel *TERT* non-coding flanking variant.

Safety and clinical activity of atezolizumab + cobimetinib + vemurafenib in $BRAF^{V600}$-mutant metastatic melanoma

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Targeted inhibition of MEK with cobimetinib (C) and BRAF with vemurafenib (V) in $BRAF^{V600}$-mutant melanoma can result in both anti-cancer immune activation and direct tumor cell killing. Atezolizumab (A), an anti-PDL1 monoclonal antibody, inhibits PD-L1/PD-1 signaling. Combining C+V with A may enhance anti-tumor activity, potentially leading to improved clinical responses and durability. In this Phase Ib study, patients (pts) with untreated $BRAF^{V600}$-mutant unresectable/metastatic melanoma received A+C+V after a 28-d run-in with C+V. Doses were A at 800 mg q2w, C at 60 mg QD for the first 21 d of each 28-d cycle and V at 960 mg BID during d 1–21 of run-in and 720 mg thereafter. Safety and efficacy were evaluated in 14 pts who had ≥1 dose of A. Median safety follow-up was 5.6 (range, 1.5–12.8) months. All-grade (G) AEs occurring in >20% pts and reported as A and/or C and/or V related included arthralgia, elevated ALT/AST and bilirubin, nausea, fatigue, flu-like symptoms, maculopapular rash, mucosal inflammation and photosensitivity. C- and/or V-related G3–4 AEs were seen in 6 pts during the 28-d run-in; A- and/or C- and/or V-related G3–4 AEs were seen in 5 pts with the triple combination. All were manageable and reversible. No unexpected AEs, G5 AEs or A-related SAEs occurred. Elevated ALT/AST led to treatment discontinuation in 1 pt. Responses (unconfirmed, RECIST v1.1) were seen in 13/14 pts (93%; 1 CR, 12 PRs); 100% SLD reduction occurred in 1 pt with PR. mDOR and mPFS were not estimable due to limited follow-up (data cut: Feb 15, 2016). 11/13 pts continue in response. Biomarker evaluation showed an increase in CD8+ T-cell infiltration post C+V treatment. Overall, preliminary data show that A+C+V has manageable safety and promising anti-tumor activity in pts with $BRAF^{V600}$-metastatic melanoma, supporting continued exploration of this combination. NCT01656642

Combined loss of epidermal AMBRA1 and Loricrin identifies high risk AJCC stage I melanomas

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AJCC stage I melanomas are considered at low risk of metastasis. However, up to 10% of patients develop distant disease, emphasising the need for novel biomarkers to stratify this group for longer clinical follow-up and earlier instigation of targeted adjuvant therapies. We have recently identified AMBRA1 expression (a pro-autophagy regulatory protein involved in epidermal differentiation) in the epidermis overlying primary AJCC stage 1 melanomas as a prognostic biomarker. To increase assay specificity, dual analysis of AMBRA1 and Loricrin (a terminal epidermal differentiation marker) expression was performed in an independent cohort of 206 AJCC stage I melanomas by automated immunohistochemistry and image analyses with a Leica SCN400 digital slide scanner.

Visual comparison of epidermal AMBRA1 (as no, some or complete loss) with semi-quantitative expression (derived using Slidepath companion software) in 120 AJCC stage I melanomas revealed a significant ($P < 0.0001$) stepwise increase in overall % AMBRA1 loss (compared with % expression in adjacent normal epidermis) in line with decreased AMBRA1 expression as scored by eye, confirming visual scoring as a robust and reliable method. Subsequent combined analysis of AMBRA1 and Loricrin revealed high risk tumours (defined as those with complete loss of epidermal AMBRA1 and Loricrin) were associated with a significant decrease in disease free survival (DFS, 70.8%) compared to 98.5% DFS in low risk tumours (ie those without complete loss of AMBRA and Loricin) with 87.5% sensitivity, improved specificity to 81.1%, and a negative predictive of 98.7%. Collectively, these data highlight combined epidermal AMBRA1 and Loricrin expression as a novel independent prognostic biomarker and as a simple reliable means of identifying high-risk AJCC stage I melanomas, enabling prolonged clinical follow up and earlier therapeutic intervention.

Costs associated with adverse events (AEs) with systemic therapies in metastatic melanoma

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Systemic therapies for metastatic melanoma (MM), including immunotherapy, targeted therapy, high-dose IL-2, and chemotherapy, are associated with different AEs. A thorough understanding of AE costs will enable more accurate comparisons among treatments and better cost management. Two retrospective cohort studies were independently conducted using IMS PharMetrics Plus databases and MarketScan commercial and Medicare Supplemental databases. Patients (pts) were aged ≥ 18 years and had ≥ 1 metastatic melanoma diagnosis and ≥ 1 claim for any systemic therapy for melanoma from July 1, 2004, to June 30, 2015. AEs were identified based on ICD-9-CM diagnosis/procedure codes. Incremental cost per AE was determined by comparing the 30-day expenditures between pts with and those without the AE event using a generalized linear model. Propensity score with inverse probability of treatment weighted method was used to adjust for baseline demographic and clinical differences. 1654 pts from PharMetrics were included. Mean age was 61.2 years (SD ± 11.4 years), mean baseline Charlson Comorbidity Index was 8.0 (SD ± 2.3), and 59% were male. 1329 pts included from MarketScan had similar characteristics. Adjusted 30-day incremental costs by AE category in 2015 US$ (95% CI) were: PharMetrics/MarketScan: cardiovascular – 16 083 (15 640–16 526)/15 430 (15 052–15 809); CNS, psychiatric – 21 277 (20 748–21 806)/18 739 (18 255–19 222); gastrointestinal – 18 534 (18 061–19 007)/15 648 (15 134–15 941); hematologic, lymphatic – 14 997 (14 652–15 342)/15 538 (15 134–15 941); metabolic, nutritional – 12 340 (11 851–12 829)/17 251 (16 825–17 677); pain – 12 928 (12 553–13 303)/16 104 (15 691–16 518); skin/subcutaneous tissue – 11 016 (10 717–11 315)/10 597 (10 319–10 875); respiratory – 17 338 (16 850–17 826)/17 064 (16 620–17 508). Incremental AE costs associated with systemic therapies of MM are substantial.

Safety of nivolumab (NIVO) plus ipilimumab (IPI) in patients with advanced melanoma (MEL) metastatic to the brain: initial results from phase 2 CheckMate 204

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Brain metastases (BMts) are a major cause of treatment failure, morbidity and death in MEL patients (pts). IPI is active in BMts and NIVO+IPI has substantially greater antitumor activity than IPI in MEL pts without BMts, but with increased toxicity. CheckMate 204 is an ongoing, multicenter, phase 2 trial evaluating NIVO+IPI in MEL pts with ≥1 measurable, unirradiated BMts 0.5–3.0 cm without neurologic symptoms or steroid requirements. Pts received NIVO 1 mg/kg + IPI 3 mg/kg Q3W x 4, then NIVO 3 mg/kg Q2W until progression or limiting toxicity. Pts with severe adverse events (AEs) during NIVO+IPI induction could receive NIVO when toxicity resolved; radiosurgery was allowed for progression of ≤3 BMts if ≥1

remained unirradiated for assessment. Between March 2015 and April 2016, 60 pts were enrolled and 41 had been treated, with 24 (59%) still on treatment and 17 (41%) off treatment (7 due to toxicity, 7 due to disease progression, 2 did not meet study criteria and 1 died). With a median time on study of 4.4 months, 12 pts (29%) received 4 NIVO+IPI doses and 16 of 41 (39%) began NIVO maintenance. Any grade and grade 3/4 treatment-related AEs occurred in 88% and 37% of pts, respectively; 8 pts had grade 3/4 AEs requiring discontinuation and one had fatal treatment-related myocarditis. Clinically significant neurologic AEs of any grade occurred in 12 pts (29%): headache (8 pts, 1 grade 3/4), aphasia (1), brain edema (1), seizure (1) and dizziness (1) were considered disease-related; carpal tunnel syndrome (1), paresthesia (1), peripheral motor neuropathy (1 grade 3/4) and syncope (1 grade 3/4) were considered treatment-related. In this initial report from CheckMate 204, NIVO+IPI in MEL pts with BMts had a safety profile similar to that previously reported in MEL pts without BMts, with no increased neurologic AEs.

In vivo E2F reporting on efficacious dosing schedule of MEK plus CDK inhibition in melanoma

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Pharmacological targeting of cyclin dependent kinases 4 and 6 (CDK4/6) could represent a viable therapeutic option in combination with FDA-approved targeted therapies such as BRAF inhibitors and/or MEK inhibitors (MEKi). Indeed, continuous and concurrent dosing of MEKi plus a CDK4/6 inhibitor (CDK4/6i) leads to melanoma regressions in *in vivo* models and delays the onset of MEKi resistance. In patients, continuous treatment leads to myelosuppression especially neutropenia; thus, palbociclib is intermittently dosed in breast cancer patients. Furthermore, it is unclear what schedule of CDK4/6i would be most effective and safe in combination with a MEKi. Utilizing an *in vivo* E2F reporter system, we analyzed the efficacy of different CDK4/6i plus MEKi schedules in a quantitative and temporal manner. Intermittent dosing (3 weeks on/1 week off) of both CDK4/6i and MEKi combination therapy resulted in tolerant tumors and rapid reactivation of E2F activity during drug holiday. Continuous MEKi with intermittent CDK4/6i led to more complete responses as compared to continuous CDK4/6i with intermittent MEKi. Weight loss of mice was also evident in the continuous CDK4/6i plus intermittent MEKi arm suggesting adverse events related to continuous CDK inhibition. Importantly, functional proteomic analysis revealed distinct mechanisms of acquired resistance/drug tolerance that arose from the three scheduling arms. Taken together, *in vivo* reporting allows for quantitative measurement of pathway activity associated with inhibitor resistance and can be utilized to optimize combination schedules to improve the therapeutic index in patients.

MM-141, a novel bispecific antibody targeting IGF-1R and ErbB3, inhibits cell proliferation in metastatic uveal melanoma cell lines

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Uveal melanoma is the most common primary intraocular cancer in adults. Despite advances in the treatment of primary uveal melanoma, there are no successful treatment options for systemic metastasis.

Insulin-like growth factor 1 receptor (IGF-1R) is a transmembrane receptor with tyrosine kinase activity and its expression in primary uveal melanoma tumors correlates with poor prognosis. ErbB3, a member of the epidermal growth factor receptor (ErbB) family, is known to form active heterodimers with other ErbB family members as well as with IGF-1R, and is associated with resistance to targeted therapies in numerous cancers including metastatic uveal melanoma.

MM-141 is a fully human bispecific antibody co-targeting IGF-1R and ErbB3. We have investigated the effects of MM-141 on cell proliferation and receptor activation in metastatic uveal melanoma cell lines (TJU-UM001, TJU-UM002B, and TJU-UM004) established at Thomas Jefferson University. Flow analyses indicated all 3 cell lines express IGF-1R and ErbB3 on their cell surface. Exposure to MM-141 inhibited cell proliferation in the presence of IGF-1 and NRG-1, the ligands for IGF-1R and ErbB3 respectively. In the absence of ligands, MM-141 inhibited the proliferation of TJU-UM001, TJU-UM002B and TJU-UM004 cells by 72.4%, 31% and 45.3%, respectively. In contrast, a specific antibody against ErbB3, MM-121, did not inhibit cell growth of these cell lines. Furthermore, MM-141 inhibited ligand-induced phosphorylation of IGF-1R and ErbB3.

These data support further investigation of the role of IGF-1R and ErbB3 signaling in metastatic uveal melanoma; experiments with MM-141 and other agents, including MAPK- and PI3K inhibitors as well as chemotherapy, are ongoing. Findings warrant further investigation of MM-141 as a potential therapeutic option for metastatic uveal melanoma patients.

Characterization of MEK mutations conferring resistance to the allosteric MEK inhibitor trametinib and a novel ATP-competitive inhibitor

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Nearly 50% of melanoma tumors harbor BRAF mutations that result in constitutive MAPK pathway activation. Dabrafenib and trametinib are FDA-approved for the treatment of BRAF V600E/K melanoma, targeting BRAF and MEK1/2 kinases of the pathway, respectively. Despite robust initial responses most patients relapse, most often due to MAPK reactivation resulting from secondary mutations in the pathway. To investigate if a novel ATP-competitive MEK inhibitor can overcome resistance acquired by MEK mutations following treatment with the allosteric MEK1/2 inhibitor trametinib, we carried out a comparative resistance study with both compounds using

MEK1 and MEK2 random mutagenesis screens. Our study identified resistance mutations in three regions of MEK1/2: the allosteric drug binding pocket (helix C), which was an exclusive hotspot for trametinib; the gatekeeper residue M143 of the ATP binding pocket, which arose only with the ATP-competitive inhibitor; and the N-terminal negative regulatory helix (helix A), which is shared by mutants of both compounds. Over-expression or knock-in of MEK mutant alleles in A375 (BRAF mutant) melanoma cells showed allosteric mutations exhibited strong resistance to trametinib, but remained sensitive to the ATP-competitive inhibitor. The ATP-competitive inhibitor also demonstrated sustained efficacy in a PDX model harboring the MEK2 allosteric-pocket mutation Q218P, supporting the utility of such an inhibitor against drug-resistant mutants arising from resistance to allosteric MEK inhibitors. The expanded use of allosteric MEK inhibitors beyond the MEKi/BRAFi combination setting, including in NRAS mutant melanoma, may lead to a more diverse spectrum of resistance mutations in MEK1/2. Thus further investigation into whether an ATP-competitive inhibitor could be a second-line therapy in this setting is warranted.

Investigating the role of different 5′ partners in *BRAF* gene fusions in melanoma

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Melanoma development and progression is often driven by activation of the MAP kinase pathway though aberrant activation of the BRAF kinase. *BRAF* is activated by mutations, amplifications, alternative splicing, or recently discovered gene fusions. *BRAF* fusions involving different 5′ gene partners have been described in cutaneous melanomas and Spitz nevi, however, oncogenic potential and treatment response between *BRAF* fusions with different 5′ gene partners has not been assessed. We used fluorescence *in situ* hybridization to analyze a cohort of 59 melanoma patient tumors and identified two different *BRAF* gene fusions, *AGK-BRAF* and *ARMC10-BRAF*. The *ARMC10* 5′ gene partner has not been described in melanoma and treatment response of *ARMC10-BRAF* has not been reported in any cancer type. We generated patient-derived xenograft (PDX) models for the two *BRAF* fusions and treated them with MAP kinase pathway inhibitors specific to MEK1/2 and ERK1/2. Both PDX models responded to the inhibitors, however, the *ARMC10-BRAF* fusion was more sensitive and showed stronger tumor regression. To further understand differences between *BRAF* fusions we used computational modeling to predict the protein structures of wild type BRAF, mutated $BRAF^{V600E}$, and six *BRAF* gene fusions with 5′ partners including, *AGK*, *ARMC10*, *KIAA1549*, *TRIM24*, *PPFIBP2*, and *ZKSCAN1*. We found large variations in the MEK1/2 kinase binding domain between different 5′ partners, and we are currently overexpressing these *BRAF* fusions in melanocytes to perform additional experiments and determine the tumorigenic potential and treatment response between these different 5′ partners. Patients harboring gene fusions should only be treated if the fusion demonstrates actionable driver potential. Understanding differential responses in *BRAF* gene fusions will improve clinical treatment of patients with *BRAF* gene fusions.

Subpopulations of melanoma cells with distinct biological properties exhibit varying levels of ZEB proteins

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The expression patterns of ZEB1 and ZEB2 are tightly balanced and regulated in the physiological homeostasis of melanocytes. ZEB2 is strongly expressed in differentiated melanocytes where it maintains a proper melanocyte differentiation, while ZEB1 expression is restricted to the melanocyte stem cell compartment. Compromising ZEB1 and ZEB2 expression in adult melanocytes affects melanocyte differentiation or stemness *in vivo*. In pathological conditions, this balance is disturbed and phenotype-switching can be triggered by appropriate micro-environmental growth factors and (epi)genetic changes. We challenge this predicted oscillation pattern of ZEB proteins throughout the melanocytic spectrum in melanocyte homeostasis and melanoma models driven by $BRAF^{V600E}$ and $NRAS^{Q61K}$ oncogenic signaling. Coinciding with the phenotype-switching model, we show that subpopulations of melanoma cells that have distinct biological properties exhibit varying and oscillating levels of ZEB1 and ZEB2, regulated by microenvironmental growth factors. High levels of ZEB2 facilitates tumor cell proliferation and differentiation during both primary and metastatic outgrowth whereas transient ZEB2 downregulation allows reversible phenotype switching. Modulating ZEB1 expression *in vivo* indicates that ZEB1 expression can be regarded as a driver of melanocyte transformation and melanoma initiation, next to its ability to favor melanoma cell invasion and dissemination.

Patients (pts) with BRAF V600–mutated metastatic melanoma (MM) receiving dabrafenib + trametinib (D+T) treatment in the D+T Named Patient Program (NPP) benefited by switching from monotherapy to combination therapy

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In a retrospective chart analysis of 271 pts with BRAF V600 MM receiving D+T in the NPP (36.5% had brain metastases, and 10.8% of those with a known ECOG PS had a score ≥ 2), D+T was well tolerated/manageable, and effectiveness results (ORR, PFS, and OS) confirmed significant clinical activity in this compassionate-access population with advanced disease and poorer health relative to pts in phase 3 combination D+T clinical studies (COMBI-v and COMBI-d). Median D+T treatment duration was 8.5 months (95% CI, 7.3–10.8 months). In this analysis, we investigated whether NPP pts who were receiving BRAFi monotherapy benefited by switching to D+T combination therapy. Tumor assessments were performed by the treating physician using clinical judgment per standard clinical practice. We analyzed outcomes in NPP pts who received ≥ 1 dose of a BRAFi monotherapy for > 30 days and were switched to D+T before disease progression (n = 84). Prior to D+T treatment, these NPP pts received a BRAFi for a median of 6.1 months (range, 0.4–31.3 months). All 7 NPP pts with a complete response (CR) on BRAFi maintained a CR after switching to D+T. Of 36 NPP pts with a partial response (PR) on BRAFi, 12 pts improved to CR, 12 pts maintained PR, 4 pts had stable disease (SD), 7 pts progressed (PD), and 1 pt was unevaluable after switching to D+T. Of 20 NPP pts with SD on BRAFi, 10 pts improved (2 CR, 8 PR), 3 pts maintained SD, and 7 pts had PD on D+T. The effect of switching could not be assessed for 21 of the 84 NPP pts due to insufficient information in their monotherapy medical charts at the time of chart review.
Improvement in disease status achieved by NPP pts switched from BRAFi monotherapy to the D+T combination indicates potential benefit for pts switching to D+T prior to progression.

A chemical therapeutic screen identifies new targets for wt/wt melanoma

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Treatment of melanoma is often based on driver mutation. While targeted therapies are available for BRAF or NRAS mutant melanomas, no effective targeted therapeutic options are available for BRAF wild-type NRAS wild-type (wt/wt) melanomas. To identify target therapies that may be effective against wt/wt melanomas, we have performed a chemical screen of clinically relevant compounds towards 8 wt/wt melanoma cell lines. The combination of trametinib and ceritinb, both FDA-approved compounds, is particularly effective against these cell lines. Cell signaling analysis demonstrates synergistic reduction of MAPK and AKT pathway activity in a polypharmocological manner. Reduction of phosphorylated S6 is apparent within 3 h when ceritinib and trametinib is applied in combination. Using a chemical proteomics approach, we have identified binding targets of ceritinib within melanoma cells that may be affected during ceritinib dosing. We conclude that ceritinib's activity rests in the inhibition of multiple targets instead of its canonical lung cancer target ALK. We also observe reduced *in vitro* cellular proliferation and clonogenic survival. Furthermore, treatment with this combination drastically reduced tumor growth in rodent models. These data suggest an exciting new therapeutic strategy towards melanoma that lack BRAF or NRAS driver mutations.

Large-scale chromosomal changes dominate the genomic landscape of end-stage melanoma

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Elucidation of genetic changes linked to melanoma progression is hampered by limited access to tissues from visceral metastases. In this study, we analyzed genomic changes occurring in melanoma patients (n = 13) through progression from primary cutaneous disease to visceral metastases, including via multi-site sampling post-mortem, using whole exome and genome sequencing. Gain of single nucleotide variants (SNV) and small insertions/deletions (indel) in visceral metastases was generally limited, although in some sites massive SNV/indel acquisition was associated with mutations in DNA repair genes. In contrast, changes in chromosomal copy number and large allelic imbalances (AI) dominated the landscape of visceral metastases, with extensive loss of heterozygosity decreasing mutational load in end-stage disease in some cases. In one case, multicore sampling of a primary tumor revealed spatial heterogeneity in copy number. These findings were validated by fluorescence *in situ* hybridization. Increased ploidy and AI were identified in treatment-naïve non-visceral metastases and primary tumors in a subset of cases. Genes associated with chromosomal instability via cell cycle dysregulation, mitotic checkpoint defects and merotely were mutated in most cases. We hypothesize that extensive acquired AI and ploidy change sculpt mutational profiles that are strongly selected for during melanoma progression in most patients. Data will be presented describing the impact of such events on predicted tumor neoantigenicity and on signaling pathway activity. Mechanisms that drive ploidy change and AI and/or that permit the proliferation of melanoma cells in the face of vast genomic structural derangement are potentially promising and specific therapy targets.

Inhibition of autophagy reduces survival of trametinib-resistant, CD271 expressing melanoma subpopulations

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Acquired resistance of BRAF mutant melanoma to targeted BRAF/MEK inhibition is associated with increased expression of the neurotrophin receptor CD271 and the activation of pro-survival mechanisms including autophagy. Genetic silencing of CD271 promotes melanoma invasion, while also sensitizing tumour cells to BRAF inhibitor-mediated apoptosis, indicating

CD271 promotes survival signaling at the expense of melanoma progression. The present study aimed to determine the contribution of autophagy to invasion and survival of MEK inhibitor-resistant, CD271-expressing melanoma subpopulations. Treatment of $BRAF^{V600E}$ mutant melanoma cell lines with trametinib for 9 days increased CD271 expression, which correlated with increased LC3 II accumulation and reduced p62 expression, both hallmarks of increased autophagy. Additionally, trametinib-resistant cells were less invasive than untreated BRAF mutant cells in 3D spheroid collagen-invasion assays, indicating the acquisition of a drug-resistant phenotype is associated with reduced tumour invasion. Knockdown of autophagy-regulatory ATG5 using a doxycycline-inducible shRNA, as well as treatment with the lysosomal inhibitor chloroquine, resulted in significantly reduced viability of isolated CD271+ compared to CD271- cells, indicating autophagy is required for the survival of CD271 expressing subpopulations. Furthermore, ATG5 knockdown or chloroquine treatment of trametinib-resistant melanoma cells for 48 h significantly inhibited cell viability compared to trametinib-resistant cells alone, indicating autophagy is essential for the continued survival of melanoma cells that acquire resistance to MEK inhibitor-induced cytotoxicity. Collectively these data suggest, targeting autophagy modulation represents a viable therapeutic strategy through which to overcome MEK-induced drug-resistance.

Serine 729 is required for BRAF splice variant mediated resistance to RAF inhibitors

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BRAF V600E melanomas are highly sensitive to RAF inhibitors but the expression of aberrantly spliced BRAF V600E (BRAF V600E ΔEx) isoforms are associated with resistance in 13–30% of progressing patients. Compared to full-length BRAF V600E, BRAF V600E ΔEx exhibit enhanced dimerization and signaling via ERK1/2 during RAF inhibitor therapy; however, much remains unknown regarding their mechanisms of regulation and resistance. The 14-3-3 protein binding sites, serine 365 (S365) and serine 729 (S729), play a complex role in regulating the activity and dimerization of RAF isoforms. All reported BRAF V600E ΔEx isoforms lose the N-terminal 14-3-3 binding site (S365) but retain the C-terminal site (S729); therefore, we analyzed the requirement of S729 for BRAF V600E ΔEx mediated resistance. We measured increased phosphorylation on S729 on BRAF V600E ΔEx during RAF inhibitor treatment. Mutation of S729 to alanine does not alter BRAF V600E ΔEx basal activity but renders BRAF V600E ΔEx sensitive to RAF inhibitor as measured by reduced ERK1/2 phosphorylation and cell growth *in vitro* and *in vivo* using an ERK reporter system. This increased sensitivity is associated with decreased BRAF V600E ΔEx dimerization and binding to MEK1/2 and 14-3-3. These data highlight the importance of BRAF V600E 14-3-3 binding sites in coordinating signaling complex formation and mediating targeted therapy resistance in mutant BRAF-driven melanoma.

Modeling phenotype switching *in vitro*: MITF knockdown by means of CRISPRi

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Phenotype switching is an integral part of the evolution that melanoma cells undergo when challenged with inhibitors targeting the MAPK pathway. This switch from a proliferative to an invasive state is epitomized by the marked downregulation of the master transcription factor of the melanocytic lineage: MITF. Our lab recently demonstrated that, upon decreased expression of MITF, melanoma cells acquire the ability to resist and escape from MAPK inhibition, while also endowing the newly MAPKi-resistant cells with an enhanced invasive capacity. Phenotype switching is not solely induced by MAPKi, but may equally be induced by T cell checkpoint inhibitor therapies such as nivolumab. Phenotype switching is also not unique to melanoma cells as, for example, it is also observed in erlotinib-treated lung tumors. As phenotype switching may thus have far-reaching pan-cancer clinical consequences, we deem it essential to understand fully the processes underlying phenotype switching and the sequences of events that lead to a multi-drug resistant state in tumor cells. While it is known that a vast number of genes are differentially expressed in phenotype-switched cells compared to their parental counterparts, it is not known which genes act causally in this regard, and whether there is a temporal component to the relative contribution of potential driver genes.

To investigate in detail the process of phenotype switching in melanoma cells, we have established a model system *in vitro,* which relies on the efficient knockdown of MITF by means of CRISPR interference. We have shown that we can reliably knock down MITF in melanoma cells using this system, with the affected cells showing the traditional hallmarks of phenotype switching. We intend to use this system to further elucidate the mechanism by which cells may phenotype switch, with the ultimate goal of preventing a multi-drug resistant state in melanoma.

Tumor associated B cells in cutaneous primary melanoma and improved clinical outcome

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B cells often infiltrate the microenvironment of human tumors. B cells can both positively and negatively regulate anti-tumor immune responses. In several human cancers, higher numbers of $CD20^+$ tumor associated B cells (TAB) are associated with a favorable prognosis, whereas in human primary melanomas this association is contentious. In this study, we determined the association of TAB numbers in cutaneous primary melanoma tissue samples and patients' overall survival. The CD20 immunohistochemistry on archival non-metastasized and metastasized cutaneous primary melanoma tissues from two independent patient cohorts was performed. One cohort was used in class comparison for metastasis, the most important prognostic factor for overall survival, and the other cohort for a subsequent survival analysis. Survival association was further validated with RNA data from a third independent cohort. Whole tissue sections were read automatically via quantitative digital imaging and analysis. Survival data was analyzed by Cox

proportional hazard modelling. We discovered that cutaneous primary melanomas without metastasis contain significantly more TAB than primary melanomas that had metastasized. At time of first diagnosis, a higher number of TAB is associated with a significant better overall survival in patients with cutaneous primary melanomas of >1 mm Breslow depth. Also higher CD20/CD19 tumor mRNA level are correlated with a significant better overall survival. Thus, our data support TAB numbers as a prognostic biomarker in cutaneous primary melanoma patients with a tumor of >1 mm Breslow depth. For a survey in larger studies, whole tissue section analysis seems to be key to accurate assessment of TAB numbers.

Clinical features of acquired resistance to anti-PD-1 therapy

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Anti-PD-1 therapy has improved clinical outcomes in advanced melanoma. However, most pts manifest intrinsic or acquired resistance. Here, we investigated the clinical features that characterize acquired resistance to anti-PD-1 therapy.

We collected retrospective data from 39 pts with acquired resistance to anti-PD-1 therapy after screening 742 pts who received anti-PD-1 monotherapy across 5 academic centers. Acquired resistance was defined as pts who achieved initial partial or complete response (PR/CR) measured by RECIST v1.1 criteria followed by progression after response. From this cohort, median age was 63, 85% had cutaneous melanoma, 67% had ≥1 feature of poor prognosis (stage M1c, elevated LDH, or brain metastasis) and 59% received prior ipilimumab. PR and CR were achieved in 87% and 13% respectively, and median PFS was 11 months (m). At progression, most pts had isolated sites of progression (79%). Only 10% of pts were off therapy at progression. Of the pts with isolated progression, 17 received localized therapy (12 with surgery and 5 with radiation). Twenty-three pts received systemic therapy post-progression including continued anti-PD-1 (n = 13), BRAFi and/or MEKi (n = 5), ipilimumab (n = 4), clinical trial (n = 4) or chemotherapy (n = 1). The median post-progression survival (PPS) was 14.0 m, and the median overall survival was 33.7 m. There was a trend for improved PPS in pts with isolated progression compared to those with systemic progression (14.0 m versus 5.7 m, P = 0.056) and an initial PFS ≥ 15 m (not reached versus 8.6 m, P = 0.051).

We observed acquired resistance to anti-PD-1 was often associated with an isolated site of disease progression amenable to local therapies (surgery or radiation). Many pts experienced excellent clinical outcomes, with continued benefit following local therapy or anti-PD-1 reinduction. Further studies are needed to characterize the molecular mechanisms underpinning these relapses.

Overall survival in patients with advanced melanoma (MEL) who received nivolumab (NIVO) versus investigators choice chemotherapy (ICC) in the phase 3 CheckMate 037 trial

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In CheckMate 037, patients (pts; n = 405) with MEL who progressed on/after anti-CTLA-4 therapy (plus a BRAF inhibitor if *BRAF* V600 positive), were randomized 2:1 to NIVO 3 mg/kg IV Q2W or ICC (dacarbazine 1000 mg/m^2 Q3W or carboplatin AUC6+ paclitaxel 175 mg/m^2 Q3W). At the first analysis, objective response rate (ORR) for NIVO was 32% versus 11% for ICC. Here we report for the first time the co-primary endpoint of overall survival (OS), updated ORR, and the secondary endpoint of progression-free survival (PFS) assessed by independent radiology review.

Of 272 pts randomized to NIVO and 133 to ICC, 99 and 77%, respectively, received study treatment. A larger proportion of NIVO versus ICC pts had brain metastases (20 versus 14%) and increased lactate dehydrogenase levels (52 versus 38%) at baseline. At ~2 years' minimum follow-up, ORR (27 versus 10%) and median duration of response (32 versus 13 months) were notably higher for NIVO versus ICC. Median OS was 16 versus 14 months (NIVO versus ICC) in all randomized pts (HR: 0.95 [95.5% CI: 0.73, 1.24]) and 16 versus 12 months (0.81 [95% CI: 0.59, 1.1]) in all treated pts censored at the start of subsequent PD-1/PD-L1 therapy, as 41% of ICC pts (11% NIVO) received subsequent anti-PD1 agents. Median PFS (NIVO versus ICC) was 3.1 versus 3.7 months (1.0 [95% CI: 0.78, 1.4]). Fewer grade 3/4 treatment-related AEs were observed in NIVO pts (14 versus 34%) and most NIVO-related AEs were low grade and manageable. In summary, in pts with MEL who progressed after IPI, NIVO demonstrated higher, more durable responses and did not show statistical significance in survival difference versus ICC. OS should be interpreted with caution because of the open-label study design and increased dropout rate prior to treatment initiation, subsequent ICC arm anti-PD-1 therapy, and increased proportion of pts with poor prognostic factors in the NIVO arm.

Combating NRAS-mutant melanoma brain metastases through PI3K-AKT pathway inhibition?

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NRAS mutations in melanoma occur in up to 25% of patients and are associated with aggressive disease, increased incidence of brain metastases and poorer prognosis. There are no specific therapies for NRAS-mutant melanoma. Treatments are currently limited to slower-acting immune checkpoint inhibitors or chemotherapy. Thus, there is a need for fast-acting targeted drugs for patients with rapidly progressing NRAS-mutant melanoma, in particular for those with brain metastases.

Identifying the signaling pathways that NRAS-mutant tumors depend on for growth and survival is a major aim in the search for successful treatments. In an *in vivo* experiment, we compared the tumor growth of human NRAS- *versus* BRAF-mutant metastatic melanoma cells injected into the brain of nude mice and treated with the PI3K inhibitor buparlisib. Although buparlisib could not induce sufficient apoptosis to achieve complete clearance of the tumors, it completely inhibited proliferation and growth of both the BRAF- and NRAS-mutant tumors. Interestingly, closer evaluation suggested clear differences between these two tumor groups. For instance, necrotic areas highlighting the aggressiveness of the tumor were only found within the sham treated NRAS- but not BRAF-mutant tumors. Despite this aggressiveness, the inhibitory effect of buparlisb was much more prominent in the NRAS-mutant tumors, as only this tumor group showed clear signs of tumor regression. Furthermore, evaluation of the tumor volumes revealed a clear survival benefit of the buparlisib treated compared to the sham treated NRAS-mutant tumors.

These results indicate that NRAS-mutant melanoma brain metastases are more sensitive to PI3K pathway inhibition than BRAF-mutant brain metastases. As there is currently no effective therapy for patients with NRAS-mutant melanoma brain metastases, PI3K inhibitors may be particularly beneficiary to this patient group.

Adipocytes in the melanoma microenvironment

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The success of immunotherapy in melanoma underscores the capacity for the tumor microenvironment (TME) to have a dominant effect on tumor progression. The role of other TME cell types in melanoma are relatively unexplored but could provide novel therapeutic targets. Using the BRAF;p53 zebrafish melanoma model, we have uncovered a role for microenvironmental adipocytes as mediators of melanoma progression. Melanoma cells in subcutaneous sites show a 50% increase in lipid content compared to parental cells. This is accompanied by increased expression of the fatty acid transporter proteins FATP2 and FATP6, suggesting that the increase in lipids is derived from uptake from the surrounding TME. The melanoma cells growing at subcutaneous sites grow in direct contact with adipocytes, raising the hypothesis that TME adipocytes are the source of these exogenous lipids. Consistent with this, both fish and human melanoma cells co-cultured with 3T3-L1 adipocytes also have a 50% increase in total lipid content, and fluorescently labeled fatty acids can be transferred directly from adipocytes to melanoma cells. Co-culture of melanoma cells with adipocytes is accompanied by a significant growth advantage measured by pH3 staining, along with an increase in invasiveness. These effects can be blocked by the small molecule FATP-transport inhibitor lipofermata, which inhibits both lipid uptake as well as melanoma cell viability. RNA-seq of melanoma cells in the presence of adipocytes reveals a striking dysregulation of lipid metabolism accompanied by increased synthesis of extracellular matrix-associated genes, suggesting that the melanoma cells reshape the local microenvironment in the presence of adipocytes. Taken together, these data suggest that TME adipocytes play an important role in melanoma growth, acting as a rich source of lipids for melanoma, and that uptake of these lipids is mediated by the FATP fatty-acid transport proteins.

A subset of cutaneous melanomas contains high expression of genes related to oxidative phosphorylation determined using consensus clustering

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Despite new treatment options, prognosis of many melanoma patients remains poor and new treatment strategies are still needed. Stratification in molecular subclasses may aid therapeutic development. Therefore, we performed consensus clustering of 405 RNA microarray profiles obtained from the Gene Expression Omnibus (GEO) and 469 RNA sequencing profiles obtained from The Cancer Genome Atlas (TCGA), all from tumor samples of patients with cutaneous melanoma of any stage. We biologically annotated each cluster based on the enrichment of pre-defined Hallmark gene sets as assessed by Gene Set Enrichment Analysis (GSEA). To evaluate the concordance between both datasets Spearman's rank correlations between the Z-transformed P-values of matched genes as obtained by pair-wise class comparison were calculated. Consensus clustering revealed four molecular clusters in the GEO dataset. These clusters were biologically annotated and labeled 'immune' (27% of samples), 'estrogen response/p53-pathway' (estrogen/p53) (34% of samples), 'cell cycle' (15% of samples) and 'oxidative phosphorylation' (24% of samples). In the TCGA database we also identified four clusters and annotated them 'immune' (36% of samples), 'estrogen/p53' (7% of samples), 'cell cycle' (24% of samples) and 'oxidative phosphorylation' (32% of samples) according to the GSEA results. Spearman's rank correlations were 0.61 for the 'immune', 0.47 for the 'estrogen/p53', 0.60 for the 'cell cycle' and 0.68 for the oxidative phosporylation clusters. These data indicate four robust molecular subclasses in cutaneous melanoma that may guide future research and development of treatment strategies. Inhibition of mitochondrial function is of

interest as a potential treatment strategy for tumors in the oxidative phosphorylation cluster.

Pelvic lymph node dissection follwing clearance of the pirmary inguinal nodal basin: The Victoria Melanoma Service experience 1994–2009

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The management of Stage III lower limb melanoma remains highly controversial. Consensus has not been reached regarding optimal extent of regional lymph node dissection. In this report, we have taken a novel approach towards answering the question of what is most appropriate regarding regional lymph node dissection. We have asked 'how many patients with lower limb primary melanoma require second tier lymph node dissection following clearance of the primary nodal basin?'. To answer this question The Victoria Melanoma Service database, was retrospectively queried from 1994 to 2009 for all patients treated for primary lower limb melanoma who underwent lymph node dissection with our service. A total of 88 patients met the study group criteria. Patients were excluded for: evidence of disseminated disease, lack of adequate follow up data, or clinically evident (radiologic or palapable) pelvic dissease at the time of initial lymph node dissection. Of the study group 19 patients underwent completion lymph node dissection (CLND) after sentinal node biopsy, and the remaining 69 patients underwent therapeutic lymph node dissection (TLDN) for clinically evident groin disease. A total of 19.3% (17/88) of patients demonstrated recurrence in the second tier nodal basin during follow up, and 4.5% (4/88) of patients were managed with pelvic lymph node dissection. Our data are not consistent with current clinical recommendations including the Australian National Health and Medical Research Council (NHMRC) guidelines that would suggest a much higher rate of pelvic lymph node dissection for our study group. In conclusion, we support the idea that beyond conventional clinical endpoints of survival and recurrence, the rate of re-operation for second tier nodal recurrence should factor into the management algorithm for stage III lower limb melanoma.

Comprehensive DNA methylomic study identifies novel, independent prognostic markers for cutaneous melanoma

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Novel screening techniques facilitate the comprehensive description of molecular alterations in tumors, enabling a better understanding of mechanisms underlying tumor progression, as well as providing new biomarker candidates and therapeutic targets. Here, we performed an unbiased genome-wide DNA methylation analysis using Infinium HumanMethylation450 BeadChips on a discovery cohort of 14 benign nevi and 61 human melanomas with balanced distributions and detailed clinical annotation, to interrogate the epigenetic events characterizing tumor progression and prognosis. Inactivation of cell-adhesion and differentiation programs unleashes malignant dissemination, and subsequent activation of inflammatory and immune system programs impairs anti-tumoral defense pathways. Using an integrative approach comparing our data with publically available gene expression profiles from benign nevi, primary and metastatic melanomas, we were able to identify, and validate by pyrosequencing in an independent cohort, several biomarkers of tumor progression previously unrelated with melanoma. In addition, we determined a prognostic signature with potential clinical applicability. These findings were validated by pyrosequencing in a further independent clinical cohort and shown to be independent of tumor thickness and ulceration. Currently, we are testing the prognostic value of several biomarkers of the prognosis-linked differentially methylated genes at the protein level, in an independent melanoma cohort via immunohistochemistry. In conclusion, our data underscores the power of DNA methylation profiles to characterize different progression stages and the importance of epigenetic regulation in triggering metastatic dissemination through the inactivation of central cancer-related pathways.

Function of SDHD in MITF regulation and melanocyte growth

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Mutation in *SDHD*, a mitochondrial complex II gene, has been reported in hereditary paraganglioma, suggesting its function as a potential tumor suppressor gene. Recent genome-wide analysis of non-coding mutations in multiple cancers identified recurrent *SDHD* promoter mutations exclusively in melanoma at a frequency around 5%. Further genomic analysis indicates that *SDHD* promoter mutation is partially exclusive with *BRAF* mutation but co-occurring with loss of function mutation of *NF1* in melanoma samples, indicating a possible link between SDHD and OIS-induced senescence. We knocked down *SDHD* expression in primary melanocytes and observed the down-regulation of MITF both at RNA and protein level. Depletion of SDHD in melanocytes also leads to G0/G1 cell cycle arrest, consistent with the essential function of MITF in melanocyte growth. Furthermore, both MITF down-regulation and G0/G1 cell cycle arrest led by SDHD depletion are independent of and additive to the senescent phenotype caused by BRAFV600E overexpression. CHIP experiments performed with antibodies against histone markers at *MITF* promoter have shown a dramatic enrichment of signals marking inactivated chromatin, such as H3K9Me3, as well as a decrease of signals marking active transcription, such as H3K4Me3, in BRAFV600E overexpressing melanocytes depleted of SDHD. These observations imply a role of SDHD in regulating BRAFV600E induced senescence and thus, a clear involvement of SDHD in melanoma development. Since *SDHD* mutation is known to cause an accumulation of metabolites that inhibit α-KG-dependent dioxygenases, including the JMJD family KDMs, we are searching the downstream effectors of SDHD depletion that

target the chromatin structure at *MITF* promoter. In summary, this study links SDHD, an essential metabolism gene, to the regulation of a key melanoma oncogene, highlighting the importance of cellular metabolism in cancer development.

Progression-free and overall survival with cobimetinib (Cobi) + vemurafenib (Vem) + treatment is independent of PD-L1 expression

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Targeted therapies and immune-checkpoint inhibitors have revolutionized treatment for patients with metastatic melanoma. While PD-L1 expression has been associated with improved outcomes in patients receiving treatment with PD-L1/PD-1 inhibitors and with better prognosis, its effect on outcomes in patients receiving targeted treatment is less well understood. We performed a retrospective analysis to explore the association of PD-L1 expression with progression-free (PFS) and overall survival (OS) in patients treated with Cobi + Vem or Vem alone. Archival or baseline tissue samples were collected from 210 patients in the coBRIM trial (Vem + Cobi, n = 102; Vem + placebo, n = 108), and PD-L1 expression was measured by immunohistochemistry (SP142, Ventana Medical Systems, AZ, USA). The association of PD-L1 expression with survival was analyzed using a univariate Cox proportional hazards modeling.
Fifty-nine percent of patients had >1% PD-L1–expressing tumor-infiltrating immune cells (PD-L1+). For patients treated with Vem, there was a trend of increased PFS and OS in those with PD-L1+ melanoma, with hazard ratios (HRs) (PD-L1+ versus −) of 0.67 (95% CI, 0.42–1.06) and 0.70 (95% CI, 0.43–1.15), respectively. However, in patients treated with Cobi + Vem, a similar trend was not observed with HRs (PD-L1+ versus −) of 1.33 (95% CI, 0.79–2.32) and 1.08 (95% CI, 0.61–1.96) for PFS and OS, respectively.
PD-L1 expression has previously been shown to be a favorable prognostic factor for patients with metastatic melanoma. This association appears to hold true for patients treated in the Vem arm of coBRIM. However, the addition of Cobi to Vem appears to overcome the poor prognosis associated with a lack of PD-L1 expression.

The role of MNK/eIF4E axis in melanoma progression

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eIF4E is a rate-limiting factor responsible for the mRNA translation of multiple oncogenes and its activity can be regulated via changing its phosphorylation. eIF4E phosphorylation at serine 209 is critical for inducing oncogenicity due to the active translation of specific mRNAs that encode proteins to promote metastasis and proliferation. Not surprisingly, increased phospho-eIF4E is associated with metastasis and decreased survival in melanoma patients. MAP kinase interacting kinases 1/2 (MNK1/2) are solely responsible for eIF4E phosphorylation, therefore, therapeutics that target MNK1/2 have the potential to be employed to treat melanoma. We hypothesize that hyperactivation of the MNK/eIF4E axis can promote the progression of mutant BRAF melanomas to advanced disease. In support of our hypothesis, we have shown that pharmacologic inhibition of MNK1/2 decreases eIF4E phosphorylation and consequently, a decrease in proliferation and invasion *in vitro*. Furthermore, combination treatment with a novel MNK1/2 inhibitor and vemurafenib led to an increase in cleaved-PARP, a marker of apoptosis. To validate our *in vitro* studies in animal models, we used eIF4E$^{S209A/S209A}$ mutant knock-in (KI) mice, in which eIF4E phosphorylation is deficient. BRAF-mutant melanoma murine cells were injected into both the wild type (WT) and KI mice. The results showed that tumors grown in the KI mice were significantly smaller than those in WT mice. This suggests that a deficiency in eIF4E phosphorylation in the host can reduce tumor progression. In order to validate the effect of host phospho-eIF4E levels on melanoma metastasis, invasive melanoma cells were injected into both WT and KI mice and the results showed a significantly lower number of lung nodules in the KI mice as compared to the WT mice. The potential of therapeutically targeting the MNK/eIF4E axis in melanoma using MNK inhibitors can potentially move towards use in the clinic.

Improving early detection of melanoma: development of a patient centered survey to identify obstacles to diagnosis and treatment

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Oregon has the 3rd highest incidence and highest mortality from melanoma for women in the US. We hypothesize that there are specific obstacles to early detection and treatment of melanoma in Oregon that could be targeted to improve outcomes. Currently no instrument explicitly identifies patient's perceived barriers to their melanoma care. We completed a three-stage process to create and validate a patient experience survey to identify possible obstacles to diagnosis and treatment of melanoma. Stage 1: develop a conceptual framework and determine relevant content for the survey with the aid of dermatologists. In Stage 1, 7 categories and 25 questions related to obstacles were identified. Stage 2: confirm the content validity of the categories and questions. In Stage 2, a focus group of 11 melanoma survivors pinpointed issues related to survey content and structure including (i) understanding the questions, e.g., removing technical language, (ii) a desire to write in answers, e.g., more open ended options, and (iii) administration concerns, e.g., unclear skip patterns. The final survey has 9 categories and 31 questions. Stage 3: incorporate the survey into REDCap, a web based survey application. In Stage 3 a custom survey path was generated for each participant that varies based on respondent's prior answers. Collaborating with survivors resulted in a survey that is meaningful for them and provides clinicians the ability to identify obstacles to care. The survey will

be distributed through the OHSU Melanoma Community Registry to nearly 3000 melanoma survivors. The information from this survey regarding obstacle identification, categorization and prioritization will provide the basis for a targeted educational campaign to educate people about melanoma with a long term goal of decreasing the incidence and mortality from melanoma in Oregon.

Next-generation sequencing of acral melanoma

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Acral melanoma (AM) is a rare subtype of melanoma that typically has a low single nucleotide mutation burden and high numbers of focal amplifications in comparison with non-acral cutaneous melanoma (CM). A significant number of AMs do not carry BRAF, NRAS or KIT activating mutations.

To identify novel oncogenes in AM, we sequenced 500 melanoma- and cancer-related genes in 127 primary AM using hybrid-capture based next-generation sequencing. The prevalence of activating BRAF (20%), NRAS (27%) and KIT (11%) mutations was in keeping with prior studies. KRAS or HRAS activating mutations were identified in 4% of AM. Interestingly, mutations of residues 12 or 13 of RAS isoforms were associated with amplification of the mutant RAS allele and more common in AM than in CM, in which codon 61 mutations predominate. Kinase fusions were identified in 6% of AMs, occurring in ALK, BRAF, or NTRK3 in a mutually exclusive pattern with RAF/RAS/KIT mutations. Collectively, gain of function alterations affecting MAPK signaling were identified in 68% of cases. Of the remaining wild-type tumors, 10 (8%) demonstrated bi-allelic loss of either NF1 or SPRED1, alternative mechanisms to increase MAPK signaling.

TERT promoter mutations and TERT amplification were mutually exclusive of each other and affected 7% and 21% of AMs, respectively. Additional recurrent amplicons identified in AM include 11q13 (CCND1, 24%), 22q13 (SOX10, EP300, 14%), 12q14 (CDK2, CDK4, 10%), and 11q22 (YAP1, 10%).

In summary, we identified activating genetic alterations of the MAP-kinase pathway in 76% of cases, adding bi-allelic loss of function of NF1 or SPRED1 (8%) and kinase fusions (6%) to the panel of oncogenic alterations in AM. Additionally, we confirmed gene amplifications as a predominant mechanism to activate genes including CCND1, EP300, CDK4 and YAP1. The expanded panel of oncogenic alterations in AM suggests therapeutic strategies for clinical investigation.

BRAF mutation in acral melanomas: clinicopathological correlation and VE1 immunostains

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BRAF mutation is frequently detected in non-CSD melanomas, and those melanomas usually occur on the trunk and extremities. Acral melanomas on the palms soles, and nails are known to have low frequency of BRAF mutation. Recently, VE1 immunostain is reported to have good correlation with *BRAF* mutation status. Therefore, in this study, we analyzed 32 patients of acral melanomas. BRAF mutation was detected in 16 cases of melanomas, and 18 cases didn't have BRAF mutation. We also reviewed all clinicopathologic features, and analyzed anatomic site, melanoma cell morphology, pagetoid scatter, and pigmentation grade. We performed VE1 immunohistochemical stains of all cases. Results showed that 3 patients had melanomas on the thumbnail, 11 melanomas on the sole, and 2 melanomas on the toe. Histopathologic features revealed melanoma cells had large epithelioid cells, many pagetoid scatter, and lots of melanin pigmentation. The VE1 immunostain results showed positive stains in all 16 cases of acral melanomas, and negative in 18 cases without BRAF mutation. This study suggests that histopathologic features of BRAF mutated acral melanomas have epithelioid cells with pigmentation, and pagetoid scatter. VE1 immunostains have quite good correlation with BRAF mutation status, and can substitute mutation sequencing analysis.

NLRP1 promotes tumor growth by enhancing inflammasome activation and suppressing apoptosis in metastatic melanoma

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Inflammasomes are mediators of inflammation, and constitutively activated NLRP3 inflammasomes have been linked to IL-1β-mediated tumorigenesis in human melanoma. Whereas NLRP3 regulation of caspase-1 activation requires the adaptor protein ASC, caspase-1 activation by NLRP1 does not require ASC because NLRP1 contains a C-terminal CARD domain that facilitates direct caspase-1 activation via CARD-CARD interaction. We hypothesized that NLRP1 has additional biological activities besides IL-1β maturation, and investigated its role in human melanoma tumorigenesis. NLRP1 expression in melanoma was confirmed by analyzing 216 melanoma tumor data and 13 melanoma cell lines. Unlike immune cells with nuclear localization of NLRP1, melanoma cells expressed NLRP1 mainly in cytoplasm. Knocking down of NLRP1 revealed a tumor promoting property of NLRP1 *in vitro* and *in vivo*. Mechanistic studies showed that NLRP1 inflammasomes are active in metastatic melanoma cell lines 1205Lu and HS294T. However, unlike previous reports showing that NLRP1 induces pyroptosis in macrophages, melanoma NLRP1 behaved differently in the context of cell death. NLRP1 silencing increased caspase-2, -9, and -3/7 activities and promoted apoptosis in melanoma cells. Immunoprecipitation revealed interaction of NLRP1 with CARD-containing caspase-2 and -9, whereas NLRP3 lacking a CARD motif did not interact with the caspases. Consistent with these findings, NLRP1 activation by anthrax lethal toxin but not NLRP3 activation by monosodium urate reduced caspase-2, -9, and -3/7 activities and provided protection against apoptosis in melanoma cells, suggesting a suppressive role of NLRP1 in apoptosis. In summary, we showed that NLRP1 promotes melanoma growth by enhancing inflammasome activation and suppressing apoptotic pathways. Our study demonstrates a tumor-promoting role of NLRP1 in cancer cells.

Roles of histone demethylase KDM5B in mouse melanoma

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Epigenetic states are critical for the maintenance of cancer stem cells, which drive tumor initiation, relapse, and metastasis.

Recent studies have suggested histone demethylase KDM5B is crucial for the plasticity of melanoma stem-cell like subpopulations. Utilizing a series of human-relevant mouse melanoma models, we previously identified two types of mouse melanoma propagating cells (MPCs). Based on these findings, we hypothesized that KDM5B is critical for the maintenance of MPCs. We found that knockdown of KDM5B decreased MPC populations and clonogenicity of mouse melanoma cells. These phenotypes were linked to decreased PI3K-AKT-mTOR signaling. These results strongly suggest that KDM5B regulates melanoma formation and progression, and its inhibition could be combined with immune checkpoint blockade to treat melanoma.

Expression quantitative trait loci (eQTLs) analysis of human primary melanocytes

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Melanoma is the deadliest form of skin cancer that originates in the melanocyte. In addition to the well-known contribution of UV irradiation, a strong genetic component has been implicated in melanoma. To date, genome-wide association studies (GWAS) have implicated more than 20 common susceptibility loci associated with melanoma risk either independently or in concert with melanoma-associated pigmentation/nevi phenotypes. However, the majority of potential functional risk variants identified by GWAS fall in non-coding regions of the genome, and the identity of the gene or genes through which functional variants at these loci influence risk remains unestablished. Recent studies have demonstrated that trait-associated SNPs are more likely to be expression quantitative trait loci (eQTLs) and that application of this information can enhance discovery of trait-associated SNPs and the genes they regulate for complex phenotypes. While patterns of gene regulation are overwhelmingly tissue-specific, normal melanocyte-specific eQTL database is still lacking. To explore multiple layers of genetic mechanisms underlying natural variation in gene expression in human primary melanocytes and their relationship to melanoma risk variants, we are conducting genome-wide expression/methylation/microRNA quantitative trait loci analysis in a panel of 109 human primary melanocyte cultures. Moreover, we are preforming the same analysis for TCGA melanoma samples, a panel of >100 melanoma cell lines, as well as skin QTL databases (including GTEx and MuTHER projects), comparing across tissues. These results will highlight the potential importance of utilizing tissue-specific data for unraveling genetic association of common diseases including melanoma.

Loss of cohesin complex components STAG2 or STAG3 confers resistance to BRAF inhibition in melanoma

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The protein kinase V-Raf murine sarcoma viral oncogene homolog B (BRAF) is an oncogenic driver and therapeutic target in melanoma. Inhibitors of BRAF (BRAFi) have shown high response rates and extended survival in melanoma patients bearing tumors that express BRAF Val600 mutations, but a vast majority of these patients develop drug resistance. Here we show that loss of Stromal antigen 2 or 3 (STAG2 or STAG3), which encode subunits of the cohesin complex, in melanoma cells results in resistance to BRAFi. We identified loss-of-function mutations in STAG2 as well as decreased expression of STAG2 or STAG3 proteins in several tumor samples from patients with acquired resistance to BRAFi and in BRAFi-resistant melanoma cell lines. Knockdown of STAG2 or STAG3 decreased sensitivity of BRAF V600E-mutant melanoma cells and xenograft tumors to BRAFi. Loss of STAG2 inhibited CCCTC-binding factor (CTCF)-mediated expression of dual specificity phosphatase 6 (DUSP6), leading to reactivation of ERK signaling. Our studies unveil a previously unknown genetic mechanism of BRAFi resistance and provide new insights into the tumor suppressor function of STAG2 and STAG3.

Phenformin inhibits myeloid-derived suppressor cells and enhances the anti-tumor activity of PD-1 blockade in melanoma

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Biguanides, such as the diabetes therapeutics metformin and phenformin, have demonstrated antitumor activity both *in vitro* and *in vivo*. However, their potential effects on the tumor microenvironment are largely unknown. Here we report that phenformin selectively inhibits granulocytic myeloid-derived suppressor cells (G-MDSCs) in spleens of tumor bearing mice and *ex vivo*. Phenformin induces production of reactive oxygen species in G-MDSC, whereas the antioxidant N-acetylcysteine attenuates the inhibitory effects of phenformin. Importantly, co-treatment of phenformin enhances the effect of anti-PD-1 antibody therapy on inhibiting tumor growth in the BRAF V600E/PTEN null melanoma mouse model. Combination of phenformin and anti PD-1 cooperatively induces $CD8^+$ T cell infiltration and decreases levels of proteins that are critical for immune suppressive activities of MDSCs. Our findings demonstrate a selective, inhibitory effect of phenformin on G-MDSCs-driven immune suppression and support that phenformin improves the anti-tumor activity of PD-1 blockade immunotherapy in melanoma.

Interferon-gamma promotes melanoma tumor metastasis through STAT1-mediated signaling

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Interferon-gamma (IFNg) is the sole Type II cytokine that orchestrates the innate immune response. It is conventionally thought to play a critical role in the anti-tumor immune surveillance mechanisms. We previously reported a counter-dogmatic pro-melanomagenic role of IFNg in the context of UVB radiation-induced skin microenvironmental effects and resulting activation of melanocytes. However, it was unclear whether these effects resulted from direct IFNg-melanocyte interaction, or were mediated via indirect means. Here we show that treatment of mouse melanoma cells with physiologic levels of IFNg in culture significantly enhances melanoma cell tumorigenicity *in vivo*. Mouse melanoma cell lines were treated with IFNg in culture followed by subcutaneous or tail-vein

inoculation in syngeneic mice. No effect was seen on subcutaneous tumor growth. In contrast, IFNg treatment of the mouse melanoma cell lines consistently and dramatically enhanced tail-vein-inoculated lung metastatic colonization, independent of the transgene and mutational status of the cell lines. Interestingly, IFNg activated both Stat1 and Stat3 phosphorylation in the cell lines. We generated Stat1 knockout and Stat3 knockout melanoma cell lines. The IFNg-treated knockout cells did not show any differences in colony formation assays and subcutaneous growth in syngeneic mice as compared to the untreated knockout cells. The Stat1 knockout cells showed significant reduction in lung colonization after IFNg treatment, but Stat3 knockout cells were not affected. These results suggest that the pro-tumorigenic effects of IFNg signaling are mediated by Stat1 but not Stat3.