SMR Abstract Booklet
Cautious Addition of Targeted Therapy to PD-1 Inhibitors after Initial Progression of BRAF Mutant Metastatic Melanoma on Checkpoint Inhibitor Therapy
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Virtually all metastatic melanoma patients who progress after initial treatment with PD-1 or CTLA-4 directed antibodies will die of their disease. Salvage options are urgently needed. It is theoretically attractive to combine immunotherapy with targeted agents in progressing patients with BRAF mutation positive melanoma, but the toxicity of combined treatment has proven challenging. We have observed striking responses with the cautious addition of low doses of BRAF±MEK inhibitors to PD-1 antibody treatment at the time of disease progression following initial immunotherapy. This allowed conversion of some rapidly progressing patients to durable complete responses in BRAF mutant melanoma. We therefore performed a retrospective analysis of our patient database and identified 23 patients who progressed on initial checkpoint inhibitor treatment, who subsequently had cautious addition of BRAF±MEK inhibitor therapy to PD-1 antibody treatment at the time of disease progression following initial immunotherapy. This allowed conversion of some rapidly progressing patients to durable complete responses in BRAF mutant melanoma. We therefore performed a retrospective analysis of our patient database and identified 23 patients who progressed on initial checkpoint inhibitor treatment, who subsequently had cautious addition of BRAF±MEK inhibitor therapy to PD-1 antibody treatment. We found an objective response rate of 55% in second line therapy, with a median progression-free survival of 33.4 months and overall survival of 34.1 months, with 40% of patients in remission over 3 years. Ten of 12 responding patients were able to discontinue all therapy and continue in unmaintained remission. Toxicity of this approach was generally manageable (21.7 % grade 3-5 toxicity). There was 1 early sudden death for unknown reasons in a responding patient. These remarkable results suggest further evaluation be performed of sequential checkpoint inhibitor therapy with cautious addition of targeted therapy to appropriate patients. The strategy of 2nd line addition of targeted therapy to ongoing PD-1 treatment decreases the potential for toxicity from concurrent targeted therapy in patients who may achieve durable complete remissions with an initial checkpoint inhibitor regimen.

Final results of the BAMM Trial: BRAF, Autophagy and MEK inhibition in Metastatic Melanoma: A phase I/II trial of dabrafenib, trametinib and hydroxychloroquine in advanced BRAF mutant melanoma
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Autophagy promotes resistance to BRAF+MEK inhibition in BRAF mutant melanoma. The BAMM trial, conducted in 4 centers, tested hydroxychloroquine (HCQ) as an autophagy inhibitor in combination with dabrafenib and trametinib (D+T) in Stage IV BRAF mutant melanoma. Primary outcomes were the recommended phase II dose (RP2D) of HCQ, D+T, and 1-year progression-free survival (PFS) rate. Results: 50 patients were screened, 38 enrolled, and 34 were evaluable for 1-year PFS rate. Patient demographics: ECOG PS 1: 29%, elevated LDH: 47%; M1c/M1d: 52%; prior immunotherapy: 50%. There was no dose limiting toxicity in phase I, and HCQ 600 mg po bid + D+T was the RP2D. The 1-year PFS rate was 48.2% (95% CI = 31-66%), median PFS was 11.2 months (mos), and overall response rate (ORR) was 85% (95% CI=64-95%). The complete response rate was 41% and median overall survival (OS) was 26.5 mos. In patients with elevated LDH (n=16), the ORR was 88%, and median PFS and OS were 7.3 and 22 mos, respectively. Serial ophthalmological exams established the safety of HCQ+D+T. Tumor RNA seq identified the TGFB...
pathway as a potential mechanism of resistance to this combination. **Conclusion:** The combination of D+T and HCQ was well tolerated and produced a high response rate, but did not meet the prespecified criteria for success of 60% of patients achieving 1-year PFS. There was a high number of pre-treated and elevated LDH patients in this study compared to previous frontline targeted therapy studies. In patients with elevated LDH, the ORR and PFS were superior to previous studies of D+T alone. Based on these results, a randomized placebo controlled trial of D+T +/- HCQ in *BRAF* mutant melanoma patients with elevated LDH and prior immunotherapy (EA6191) is being conducted.

**Artificial Intelligence for Risk Classification of AMBLor in the melanoma microenvironment**

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We have previously identified the combined immunohistochemical expression of Autophagy and beclin 1 regulator 1 and loricrin (AMBLor) as a prognostic biomarker for early stage melanoma. However, although analysis comprises simple binary scoring, manual histopathological analysis of this epidermal biomarker in the tumour microenvironment remains labour intensive and subjective. Artificial intelligence (AI) has improved the accuracy of several pattern recognition tasks and has the potential to decrease the histopathologist’s workload, eliminate repetitive and routine tasks, and improve patient risk stratification and care.

To evaluate the potential of deep learning ad AI for the analysis and interpretation of AMBLor, we used the Visiopharm image analysis platform to develop a dedicated training algorithm to quantify expression. Application of this algorithm to a pilot cohort of AJCC Stage I/II non-ulcerate melanomas revealed a strong correlation between mean tumour epidermal AMBRA1 density and groups with Lost or Maintained AMBRA expression (Pearson r Correlation = 0.8312, p value= 0.0029), with similar results found for peritumoural epidermal Loricrin expression (Pearson r Correlation = 0.9437, p value<0.0001). Furthermore, data derived from this training cohort also identified those tumours at genuinely low risk of progression, suggesting Visiopharm as a viable methodology for AMBLor analysis.

Collectively these data propose the use of artificial intelligence for digitalised immunohistochemical images of tissue samples stained for epidermal AMBLor expression in the tumour microenvironment as a novel robust cost effective method for risk stratification and disease subtyping for early stage I/II melanoma to aid the clinical decision-making.

**Melanoma secretion of TGFβ2 induces loss of epidermal AMBRA1 threatening epidermal integrity and facilitating tumour ulceration**

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The combined loss of the autophagy protein AMBRA1 and the terminal differentiation marker loricrin (AMLo) in the peritumoural epidermis overlying early stage melanomas has recently been identified as a prognostic biomarker (Ellis et al Brit J Dermatol 2020). Here we evaluated the contribution of melanoma TGFβ2 secretion to the loss of AMBRA1 in the epidermal microenvironment overlying early-stage melanomas and whether this is associated with disruption of epidermal integrity. Immunohistochemistry was used to analyse AMBRA1 and TGFβ2 in a cohort of 109 all AJCC
stage melanomas, as well as TGFB2 and the gap junctional protein claudin-1 in a cohort of 30 or 42 AJCC stage I melanomas with known AMBRA1 and loricin (AMLo) expression. Evidence of pre-ulceration was analysed in a cohort of 42 melanomas, with TGFB2 signalling evaluated in primary keratinocytes.

Increased tumoural TGFB2 was associated with significant loss of peritumoural AMBRA1 ($P < 0.05$), ulceration ($P < 0.001$), AMLo high-risk status ($P < 0.05$) and metastasis ($P < 0.01$). TGFB2 treatment of keratinocytes resulted in downregulation of AMBRA1, loricrin and claudin-1, while knockdown of AMBRA1 was associated with decreased expression of claudin-1 and increased proliferation of keratinocytes ($P < 0.05$). Importantly, we show loss of AMBRA1 in the peritumoural epidermis was associated with decreased claudin-1 expression ($P < 0.05$), parakeratosis ($P < 0.01$) and cleft formation in the dermal-epidermal junction ($P < 0.05$). Collectively these data suggest a paracrine mechanism whereby TGFB2 causes peritumoural loss of AMBRA1 and reduced epidermal integrity thereby facilitating tumour erosion of the epidermis and tumour ulceration and/or metastasis. TGFB blockade may therefore represent an attractive treatment strategy for early stage melanomas stratified as high-risk by loss of epidermal AMLo.

**THE IMPLICATIONS OF A DERMATO-PATHOLOGISTS’ REPORT ON MELANOMA DIAGNOSIS AND TREATMENT**

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**Background:** A precise and comprehensive pathology report for cutaneous melanoma provides a more accurate staging and risk estimation, and therefore dictates the appropriate surgical approach. A review of all melanoma biopsy specimens by an expert dermatopathologist, hence, is mandatory in our institution.

**Objectives:** This study aims to investigate the impact of these reviews and to determine the critical primary pathology Breslow score in which a pathology revision would be most beneficial.

**Methods:** We compared the available outside pathology reports of patients referred to our institute between January 2011 and September 2019 with our in-house review, done by an expert dermatopathologist, looking at fundamental histologic and clinical prognostic features.

**Results:** Pathology reviews were performed on 177 specimens. A change in Breslow index was made in 103 cases (58.2%). In the vast majority of the cases (73.2%) the revised Breslow was higher than initially reported. Consequently, the T-stage was changed in 51 lesions (28.8%). The lowest concordance was seen in Tis (57%), in T1b (59%), in T3a (67%) and in T4a (50%). The revised report led changes in the surgical plan in 15.2% of the cases.

**Conclusions:** Our findings support the recommendation that all routine pathologies of pigmented lesions referred to a dedicated cancer-center be reviewed by an experienced dermatopathologist. Especially in decision-changing instances, such as melanoma in-situ and thin melanomas 0.6-2.2mm

**Immunophenotyping of primary melanoma to predict patient outcomes**

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**Introduction**

Immune profiling of metastatic melanoma has revealed associations between immune cell infiltrates and patient responses to immunotherapy. However, few studies have phenotyped immune cells in primary cutaneous melanomas and correlated the findings with tumour recurrence.
Aims
This study aimed to characterise the immune microenvironment of primary melanoma and identify immune correlates of patient outcome.

Methods
Two cohorts of stage I and II melanoma patients were evaluated. The first comprised patients with viable tumour dissociates (n=6), upon which flow cytometry was performed to assess the expression of CD39, CD103 and PD-1 on CD8+ T cells. Formalin-fixed paraffin-embedded tissue was collected from a second cohort (n=64) and divided based on patient 5-year survival. Fluorescent multiplexed immunohistochemistry (mIHC) was used to assess the presence of the aforementioned CD8+ T cell populations as well as B cells, NK cells, Langerhans cells and expression of Class I MHC.

Results
Flow cytometry found that CD8+ T cells in primary melanoma are primarily CD39- (79.7% ±7.027). Further phenotyping identified enriched PD-1 expression in CD39+ CD8+ T cells compared to CD39- CD8+ T cells (76.28% in CD39+ vs 38.90% in CD39-, p = 0.0022), and particularly so in a CD39+CD103+ population. mIHC revealed PD-1-CD8+ T cells to be associated with improved overall survival. Similar investigations of B cells, NK cells, Langerhans cells and Class I MHC expression coupled with spatial analysis indicated a high degree of heterogeneity in the melanoma immune microenvironment.

Conclusion
The immune composition of the tumour microenvironment of primary melanomas are highly heterogeneous. Understanding of the influence of these immune cell infiltrates and their associations with patient outcomes may help better stratify high risk stage II melanoma patients for adjuvant anti-PD-1 therapies.

Loss of Histone 2A Ubiquitylation in Uveal Melanoma
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Uveal melanoma (UM) is the most common primary intraocular cancer in adults. Once UM metastasizes, median patient survival is less than 12 months. The molecular mechanisms driving metastasis are not well understood.

We performed single cell RNA sequencing of six enucleated specimens. Individual tumor cells were assigned to a prognostic class based on their average imputed expression of GEP1 and GEP2 discriminate genes. Strikingly, there was a significant level of intratumor heterogeneity, whereby individual tumors harbored cells from both prognostic classes. To characterize the different tumor phenotypic states we applied archetypal analysis for unbiased, genome-wide transcriptomic analysis. Archetypes with high-risk features were distinguished by de-repression of Polycomb Repressive Complex 1 (PRC1) target genes. Next, we performed immunostaining for ubiquitylated Histone 2A, which is mediated by PRC1 through its ubiquitin ligase activity. Tumor samples with low-risk features exhibited significantly lower H2AK119Ub staining levels compared to those with low-risk features.

Our results demonstrate a significant level of intratumor heterogeneity in UM, and suggest a model of UM progression that is driven by loss of PRC1 activity and H2A ubiquitylation.
Connection between Malignant Melanoma and Rheumatoid Arthritis: Evidence from the NHANES dataset
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Chronic exposure to immunosuppressive medications may increase the chance of getting skin cancer. Additionally, skin cancer can be a result of the skin manifestations from rheumatoid arthritis. However, it is unknown if melanoma is associated with rheumatoid arthritis.

From the 2005-2010 National Health and Nutrition Examination Survey, we analyzed data on adults (≥20 years). We assessed Rheumatoid Arthritis (RA) and melanoma status by using the arthritis question and cancer question respectively. Complex samples logistic regression was used to assess if melanoma is influenced by arthritis in both genders.

In the population, the percentage with positive melanoma history were higher among males (13.8%) than females (12.6%). The overall crude odds ratio (OR) of increased Melanoma for RA to no RA was 2.72 (95% confidence interval [CI], 0.90-8.20, p = 0.07). When stratified by gender, OR was 5.92 (CI 1.40-25.05, p = 0.02) among male participants and was 1.09 (CI 0.21-5.51, p = 0.92) among female participants.

In this diverse cohort, our research shows that positive Melanoma status may be associated Rheumatoid Arthritis. In addition, males develop melanoma more strongly than females among those individuals with RA. Increased melanoma screening and improved sun-protective behavior may improve health outcomes.

Multi-omics profiling shows BAP1 loss is associated with upregulated cell adhesion molecules in uveal melanoma
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The National Health and Nutrition Examination Survey (NHANES) is a survey completed by non-institutionalized population of the United States. All respondents from the NHANES survey, who were 20 years or older between the years 2005-2010 were included in the analysis with follow-up through 2015. Self-reported information was used for diagnosis to assess positive history of melanoma. Analysis was performed using complex samples Cox regression to determine the relationship of history of melanoma on all-cause mortality and CVD mortality.

Percent mortality among melanoma survivors was 14.2% among Caucasians and 12.8% among African Americans with mean follow-up of 5.8 years. In age-adjusted analysis, individuals with positive melanoma history had 3.9-fold higher overall mortality than those without melanoma history (HR 3.94, 95% CI 1.21-12.84, p = 0.02). Controlling for physical health (cardiovascular disease and obesity) and socio-demographic risk factors (age, ethnicity, and gender) the relationship remained strong (HR 3.91, 95% CI 1.27-12.10, p = 0.02) for overall mortality. Findings were not significant for CVD mortality.

In a multi-ethnic population, a significant association between positive history of melanoma and overall mortality was found. These findings underscore the importance of improving screening of melanoma and increased attention to potential long-term treatment side effects and overall health of melanoma survivors.

Association between Melanoma and All-Cause Mortality in a Multi-Ethnic Population
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Melanoma is a deadly and aggressive skin cancer often diagnosed at late stages. Incidence of melanoma has been increasing significantly over the past decade in the United States. However, there is a paucity of research in the mortality outcomes of melanoma survivors or documentation of long-term side effects of newer targeted therapies against melanoma. In this study, we explore the relationship between history of melanoma and mortality.
**BRCA1-associated protein 1 (BAP1)** is a tumor suppressor gene that is mutated in cancer, including uveal melanoma (UM). Loss-of-function BAP1 mutations are associated with UM metastasis and poor prognosis, but the mechanisms underlying these effects remain unclear. Upregulation of cell-cell adhesion proteins is involved with collective migration and metastatic seeding of cancer cells. Here, we show that BAP1 loss in UM patient samples is associated with an upregulated gene expression profile of multiple cell adhesion molecules (CAMs), including E-cadherin (CDH1), cell adhesion molecule 1 (CADM1), and syndecan-2 (SDC2). Similar findings were observed in UM cell lines and single cell RNA sequencing data from patient samples. BAP1 re-expression in UM cells reduced E-cadherin and CADM1 levels. Functionally, knockdown of E-cadherin decreased spheroid cluster formation and knockdown of CADM1 decreased growth of BAP1 mutant UM cells. Together, our findings demonstrate that BAP1 regulates the expression of CAMs which may regulate metastatic traits.

**Identifying novel mechanisms of resistance to cancer immunotherapy**

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**Introduction:** The development of immune checkpoint blockade (ICB) has changed the way we treat various cancers. While ICB produces durable survival benefits in a number of malignancies, a large proportion of treated patients do not derive clinical benefit. Recent clinical profiling studies have shed light on molecular features and mechanisms that modulate response to ICB. Nevertheless, none of these identified molecular features were investigated in large enough cohorts to be of clinical value.

**Method:** Literature review was performed to identify relevant studies including datasets of ICB-treated patient (anti-PD1/L1, anti-CTLA4 or the combo) and available sequencing data. Tumor mutational burden (TMB) and 38 previously reported gene expression (GE) signature were computed with respect to the original publication. Biomarker association with ICB response (IR) and survival (PFS/OS) was investigated within each study and combined together for meta-analysis.

**Results:** We gathered a cohort of 3,500 ICB-treated patients from 25 studies. We hypothesized that a **de novo** pan-cancer GE analysis would bring forth critical pan-cancer ICB resistance mechanisms and unravel novel therapeutic targets. The predictive value of this **de novo** signature (PredictIO_100), composed of the 100 genes most significantly associated with IR, was greater to TMB and other GE biomarkers. Within PredictIO_100, 2 genes, F2RL1 and RBFOX2 were associated with worse outcome, T-cell dysfunction in ICB-naive patients and resistance to dual PD1/CTLA4 blockade in preclinical mouse cancer models.

**Conclusion:** Altogether, this study demonstrates the potential impact of PredictIO_100 in pan-cancer ICB-treated patients and identifies F2RL1, previously involved in tumor immune regulation, and RBFOX2, a critical regulator of epithelial-to-mesenchymal transition, as potential therapeutic targets to overcome ICB resistance.

**Elucidating the genomic profile of acral lentiginous melanoma in Mexican patients**

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Acral lentiginous melanoma (ALM) is considered a rare melanoma subtype globally, but it is the most common type of melanoma in some Latin American countries, such as Mexico. From previous studies, it is known that the genomic alterations in ALM tumors differ significantly from the ones found in other UV-induced subtypes; however, our knowledge of ALM genomics has been derived mostly from populations of European and Asian descent. In this study, we performed whole-exome sequencing on 143 tumors
from 109 patients that were treated in the National Cancer Institute of Mexico. Ninety-seven tumors from 70 patients passed quality controls and were included in subsequent analyses. SNVs were identified using Mutect, VarScan and CaVEMan programs, taking forward variants called by two out of three. Frequently mutated genes were identified using the dNdScv tool. Identification of copy number alterations (CNA) was performed using Sequenza and GISTIC2 programs on 47 tumors where ploidy and copy number could be confidently defined. The most frequently mutated genes were \(\text{BRAF} \), \(\text{KIT} \) and \(\text{NRAS} \), which were present in 16, 14 and 13 samples respectively. More than half of the samples do not display driver mutations commonly seen in other melanoma subtypes. CNA analysis identified 26 regions as commonly amplified and 4 regions were identified as frequently deleted. Genes affected by amplifications include \(\text{CCND1} \) and \(\text{CRKL} \), while deletions affect genes such as \(\text{CDKN2A} \). CNApp revealed that there is a negative correlation between mutations in \(\text{BRAF} \), \(\text{KIT} \) and \(\text{NRAS} \) and the number of CNAs. The alterations identified in ALM tumors from Mexican patients are similar to those reported previously in other populations. Our future work will investigate the genomic determinants of prognostic characteristic such as ulceration and the proportion of tumours that respond to small molecule inhibitors through the use of PDX models.

**Overall survival benefit from tebentafusp in patients with best response of progressive disease**

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**Background:** Tebentafusp (tebe) is the first T cell receptor (TCR) therapeutic to demonstrate an overall survival (OS) benefit in a randomized Phase 3 (Ph3) study [NCT03070392]. In Ph2, 42% of pts with best overall response (BOR) of progressive disease (PD) survived > 1 year (yr), suggesting RECIST-based radiographic assessments underestimate OS benefit of tebe. Here we analyzed OS in the Ph3 study in a cohort of pts with BOR of PD by comparing tebe to the control arm of investigator’s choice (IC).

**Methods:** 378 pts were randomized in a 2:1 ratio to tebe vs. IC. BOR was assessed by investigators using RECIST v1.1. Treatment beyond first disease progression (TBP) was permitted for both arms. On the IC arm, only patients receiving pembrolizumab (pembro) continued with TBP and were included in the TBP-related analyses. No crossover to tebe was permitted; investigators were free to choose subsequent therapy. This analysis was conducted on the first interim analysis (data extracted Nov-2020). Kaplan-Meier estimates of OS were based on Day 100 landmark to eliminate immortal time bias and to capture majority of the PDs.

**Results:** By Day 100, PD as BOR occurred in 52% (130/252) of tebe pts (PD-tebe) vs. 60% (76/126) of IC pts (PD-IC). Key baseline characteristics including lactate dehydrogenase, alkaline phosphatase, ECOG performance, age, and sex were similar between PD-tebe vs PD-IC. The proportion of pts with PD due to progression of target lesions (TL), non-TL, or new lesions were also similar between the two groups.

More pts received TBP among PD-tebe 53% (69/130) vs PD-pembro 16% (10/61). Median duration of TBP was longer for PD-tebe (7 weeks) vs PD-Pembro (3 weeks). The safety profile of PD-tebe pts during TBP was similar to all tebe-treated pts.

OS was superior for PD-tebe vs PD-IC, HR = 0.41 (95%CI 0.25-0.66), even when considering key
baseline covariates. While some pts had regression of TL despite diagnosis of PD (<10% of pts), the OS benefit remained even when limited to pts with best change of tumor growth of TL, HR 0.46 (0.29, 0.73). 58% (75/130) PD-tebe and 52% (40/76) PD-IC pts received subsequent therapies. In a landmark OS analysis of these pts beginning on 1st day of subsequent therapy, prior tebe was associated with better OS vs. prior IC, HR 0.59 (95%CI 0.36-0.96).

Conclusion: Tebe is the first TCR therapeutic to demonstrate an OS benefit in a solid tumor. Surprisingly, a strong OS benefit from tebe is observed even in pts with BOR of PD, suggesting that RECIST-based radiographic assessments do not capture the complete benefit from tebe. The safety profile of tebe during TBP was consistent with that for long-term tebe treatment. © 2021 American Society of Clinical Oncology, Inc. Reused with permission. This abstract was accepted and previously presented at the 2021 ASCO Annual Meeting. All rights reserved.


Metastasis to central nervous system correlates with higher plasma burden of circulating tumor DNA in melanoma
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Background: The role of ctDNA based assays in estimating prognosis in melanoma is being investigated. It is unknown whether shedding patterns of ctDNA vary with different sites of metastasis in melanoma. We aimed to correlate clinical sites of metastasis with patterns of ctDNA detection in patients with stage IV melanoma.

Methods: Serial blood samples from patients diagnosed with BRAF mutation-positive stage IV metastatic melanoma were tested in a rapid real-time PCR-based point-of-care mutation detection system (Idylla; Biocartis, Belgium). The ctDNA mutation detection rate was analyzed relative to sites of metastasis.

Result: Of the total 37 patients with stage IV BRAF mutation-positive metastatic melanoma, 22 (59%) had metastasis to central nervous system (CNS) including brain parenchyma, meninges and spinal cord (CNS group); the remaining 15 (41%) had metastasis to other visceral organs (Non-CNS group). Median follow-up time in the CNS group was 15 weeks and that in the Non-CNS group was 36 weeks. Overall circulating BRAF mutation was detected in 24 of 37 (65%) patients. 17 of 22 (77%) patients in the CNS group had detectable ctDNA whereas 7 of 15 (47%) patients in the non-CNS group had detectable ctDNA. The Non-CNS group has not reached median overall survival with only 2 deaths; whereas the median survival in the CNS group was 21 weeks with 17 deaths. In patients who had more than one longitudinal sample tested, the median percentage of detectable ctDNA tests in each individual’s follow-up was 20% in non-CNS group and 80% in the CNS group; p<0.0005.

Conclusions: Persistently positive BRAF-mutant ctDNA correlates with greater odds of metastasis to central nervous system. Longitudinal testing of plasma for ctDNA maybe a useful adjunct to scheduled imaging surveillance in detecting metastasis to the CNS.

Immunotherapy administered concomitantly with radiation increases the risk of immune related adverse events
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Background: Radiation therapy is used to treat unresectable melanoma often as a supplement to systemic immunotherapy. The latter may result in immune related adverse events (irAEs). We investigated the development of irAEs in patients receiving concomitant radiation with immunotherapy.

Methods: We assessed 513 patients receiving systemic immunotherapy (IO) to identify those receiving radiation therapy (RT) as well. The treatment was defined to be concurrent if the IO or RT was initiated within a month of one another. Types of irAEs and their time of appearance with respect to initiation of the treatments were analyzed.
Results: The cohort of 513 patients receiving IO included 494 treated for melanoma and 19 for Merkel cell carcinoma. Of the patients treated for melanoma, 194 patients (71/194; 37% female) received both IO and RT; of which 127 patients (median age 66yrs) had received IO and RT concurrently while 67 patients (median age 66yrs) received those non-concurrently. In the concurrent group, the most common IO was with ipilimumab and nivolumab (48, 38%), followed by ipilimumab alone (31, 24%), nivolumab alone (26, 20%), and pembrolizumab alone (22, 17%). In the concurrent group 46% (59/127) experienced irAEs after radiation whereas in the non-concurrent group 33% (22/67) had irAEs after radiation. The common irAEs in the concurrent group were 43% dermatological, 31% gastrointestinal and 10% endocrine. In the concurrent group 70% (89/127) patients had experienced irAEs, with 46% (59/127) developing irAEs after RT, of which 11% (14/127) developed irAEs within a month of RT.

Conclusion: Our results indicate that when radiation is initiated within a month of starting immunotherapy it is associated with increased incidence of irAEs, especially in the immediate post radiation period.

Human melanocyte site-specific development and decoding of melanoma dedifferentiation patterns at single cell resolution
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In humans, epidermal melanocytes are responsible for skin pigmentation, defense against ultraviolet radiation, and the deadliest common skin cancer, melanoma. While there is substantial overlap in melanocyte development pathways between different model organisms, there are known species dependent differences and the conservation of these processes in human skin remains unresolved. Thus, the biology of developing and adult human melanocytes remains largely uncharacterized. Here, we used a single-cell enrichment and RNA-sequencing pipeline to study human epidermal melanocytes isolated directly from skin, capturing transcriptomes across different anatomic sites, developmental age, sexes, and multiple skin tones. We uncovered two distinct subpopulations of melanocytes exhibiting anatomic site-specific enrichment that occurs during gestation and persists through adulthood. The volar-enriched subpopulation transcriptional signature is retained in acral melanomas. In addition, we identified human melanocyte differentiation transcriptional programs that are distinct from gene signatures generated from mammalian model systems. Finally, we used these programs to define patterns of dedifferentiation that are predictive of melanoma prognosis and response to immune checkpoint inhibitor therapy.

Radiotherapy may increase response to immunotherapy in Uveal Melanoma patients
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Introduction
Treatment of cutaneous melanoma has undergone a revolution in recent years with median overall survival reported at 6 years.
Uveal melanoma is a rare subtype of melanoma that has not seen great success with standard immunotherapy as best reported response rates stand at around 10% only, resulting in dismal prognosis. Radiotherapy is known to potentiate immunotherapy by various mechanisms, however its role today in uveal melnoma is limited to palliative therapy only. Here we sought to understand whether the addition of radiotherapy to immunotherapy in uveal melanoma can improve response to therapy.

Methods
Medical records at the Ella Lemelbaum Institute for immuno-oncology & melanoma were screened for metastatic uveal melanoma patients and were divided based on whether they were concomitantly treated with radiotherapy. Patients records were analyzed for baseline parameters, immunotherapy regimen, response to therapy and progression-free survival. Data were collected and analyzed in accordance with Sheba Medical Center IRB approval. Statistical analyses were done with Stata v.17.

Results
Thirty-eight patients were found to have been treated with immunotherapy between the years 2015-2020. Eight patients received combination of immunotherapy & radiotherapy (group A) and 30 patients were treated with immunotherapy.
alone (group B). Eighteen patients were treated with Ipilimumab-Nivolumab combination and 20 were treated with single agent anti PD-1. Baseline parameters were comparable between the two groups. Overall response rate was significantly better in the radiotherapy + immunotherapy group compared to immunotherapy alone (50% vs 10%, p = 0.01). Progression-free survival did not differ between the groups (5 months vs 4 months, p = NS).

**Discussion**
Addition of radiotherapy to standard immunotherapy in metastatic uveal melanoma patients may increase response rates. Prospective studies warranted.

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**Efficacy and toxicity of Ipilimumab-Nivolumab combination therapy in elderly metastatic melanoma patients**
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**Introduction**
Immunotherapy has revolutionized metastatic melanoma therapy. The most active regimen is combination therapy of Ipilimumab-Nivolumab (Ipi-Nivo) with response rates (RR) of almost 60% and median overall survival (OS) of 6 years. Serious immune-related adverse events (IrAE) are common with Ipi-Nivo at 60% grade 3-4, and pose a challenge when treating older patients. We sought to examine whether Ipi-Nivo therapy is feasible in elderly metastatic melanoma patients.

**Methods**
Electronic medical records of patients treated at the Ella Lemelbaum Institute for Melanoma treated with Ipi-Nivo between the years 2017 - 2020 were screened for age. Elderly patients were defined as age 75 and older (group A) and were matched with records of patients age <75 (group B). Records were analyzed for baseline parameters, immunotherapy regimen, response rate, toxicity and progression-free survival (PFS).

**Results**
Twenty-eight relevant patients age >75 (median 77) were identified and were matched to 35 younger patients (median age 55). No statistically significant differences were noted in terms of baseline parameters except for BRAF mutation status (group A 15%, group B 48%, p = 0.008). RR was numerically, but not significantly, higher in the elderly patients (group A = 41%, group B = 57%, p = NS) while a trend was noted for longer PFS in the younger patients (group A = 5.5 months, group B = 9.5 months, p = 0.085). Treatment was similarly tolerated: 54% of the younger patients completed 4 cycles of therapy compared to 50% of the elderly patients (p = NS). Median number of cycles was 3 for both groups. Grade 2-4 IrAE were noted in 62.5% of the younger patients compared to 57% in the elderly patients (p = NS).

**Conclusion**
Ipilimumab-Nivolumab combination therapy in elderly metastatic melanoma patients is well tolerated and is a feasible treatment option for this particular age group.

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**Risks and benefits of re-induction ipilimumab/nivolumab (ipi/nivo) in melanoma patients (pts) previously treated with ipi/nivo**
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In melanoma pts who progress after prior ipi/nivo, limited treatment options often lead to consideration of later reinduction with checkpoint inhibitors. There is little information regarding the risks and benefits of re-induction ipi/nivo. We conducted a retrospective review of 26 melanoma pts treated at MSKCC since 2012 who received re-induction ipi/nivo at least 6 months following completion of an initial course of ipi/nivo. We collected data on demographics, genetics, immune-related adverse events (irAEs), best overall responses (BOR), time to treatment failure (TTF), and overall survival (OS).

The BOR rate (CR + PR) was 74% (95% CI 52-90) after the first course of ipi/nivo but only 23% (95% CI 8-45) after re-induction. Response to re-induction did not correlate with response to the initial course. Among the 16 pts who had an objective response to the first course, only 4 (25%) responded to re-induction. Of 5 pts who did not respond to the first course, 1 responded to re-induction (PR). For all pts, median TTF was 5.3 months after re-induction; TTF was shorter for re-induction than for the first course in 85% of pts. Median OS from re-induction was 8.4 months; estimated 2 yr OS was 18%. Although re-induction was associated with fewer irAEs than the initial course of ipi/nivo (58% of pts vs 85% of pts in the initial course), 8 (31%) pts experienced at least one new irAE after the second course. We conclude...
that BOR rate and TTF were markedly less favorable after re-induction with ipi/nivo than after the initial course of ipi/nivo. Re-induction ipi/nivo was associated with frequent irAEs although less frequent than for the initial course. Response rates to re-induction ipi/nivo are disappointingly low, and alternative treatment strategies should be considered for these patients.

Non-canonical tumor suppressive functions of PTEN in melanoma
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Genomic losses of the tumor suppressor PTEN occur frequently in melanoma and result in hyperactivation of the PI3K/AKT pathway. However, targeting AKT in melanoma patients and mouse models provoked limited therapeutic effects. In addition to opposing PI3K/AKT activation as a lipid phosphatase, PTEN has protein phosphatase activity and scaffold functions and we found that reactivating endogenous PTEN in PTEN-deficient melanomas halted tumor growth. Since PTEN reactivation restores both its canonical and non-canonical functions. To determine whether PTEN suppresses melanoma through both its canonical and non-canonical pathways, we expressed activated AKT in PTEN-restored melanoma cells. Activated AKT didn’t rescue the tumor suppression by PTEN, adding to previous evidence suggesting that AKT activation alone is insufficient to promote melanoma upon PTEN loss. Thus, PTEN deficiency may promote melanoma through PI3K-dependent effectors other than AKT and/or through lipid phosphatase-independent functions. To test the role of PTEN protein phosphatase activity in melanoma suppression we compared the tumor suppressive potential of PTENWT, lipid- and protein-phosphatase-dead PTENC124S, lipid-phosphatase-dead PTENG129E, and protein-phosphatase-dead PTENV138L in Pten-deficient mouse melanoma cells. Both PTENV138L and PTENVG123E decreased melanoma cell growth in vitro and tumor formation but neither was as potent as PTENWT. Thus, both lipid and protein phosphatase activities suppress melanoma. Our findings suggest that PTEN suppresses melanoma through non-canonical pathways, involving AKT-independent lipid phosphatase as well as protein phosphatase functions. Thus, combinatorial inhibition of PTEN-regulated pathways may be a promising treatment strategy for PTEN-deficient melanoma.

Extending intratumoral therapeutic durability with a multivalent immunotherapy platform
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There is a critical need for a safe and effective melanoma immunotherapy that expands on checkpoint inhibition, which has limited efficacy in some patient populations. Immune activators are a promising alternative, but their development has been limited due to severe systemic side effects. We have developed a long-acting, locally-delivered immunotherapy platform that could benefit melanoma patients by stimulating an anti-tumor immune response within the primary site that, once activated, could initiate systemic surveillance against metastases.

We synthesized multivalent protein (MVP) conjugates by conjugating multiple copies (i.e. valency) of immune stimulating proteins (e.g. IL-15) to long-chain biopolymers. We can reproducibly make MVPs with 20-120 protein copies (±10%) per polymer. At high valencies, MVP binding affinity was >100X unconjugated protein controls. Additionally, the MVP hydrodynamic radius was >10X larger than unconjugated therapeutics. We injected fluorescently modified MVPs or unconjugated counterparts directly into SK-MEL-28 tumors in mice. The intratumoral (IT) half life was measured using in vivo fluorescence. The large MVP size slowed diffusion from the tumor and exhibited a higher IT signal gradient within the tumor compared to the unconjugated controls, resulting in an IT half-life extension by >5X.

The MVP platform could modulate the potency and therapeutic durability of a wide range of immunotherapies. Since MVPs stay focused after IT injection, they could generate a sustained anti-tumor immune response with minimal systemic exposure. Therefore, we expect MVPs to have a better safety profile than other smaller immunotherapies. We will continue to develop our internal MVP pipeline to finalize a candidate for IND-enabling studies. We are also seeking collaborations for co-development of additional immunotherapies that could benefit from the MVP platform.
Chronically sun damaged fibroblasts remodel the microenvironment of primary melanoma

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The ageing, sun-exposed dermis accumulates ultraviolet radiation (UV) damage, and older patients develop more melanomas at UV-exposed sites. As fibroblasts play key roles in both the stromal response to UV and in cancer progression, we investigated whether long term UV modifies dermal fibroblast function and how this affects melanoma invasion.

Chronic UV fibroblasts persistently upregulate MMP1, resulting in persistent degradation of collagen, and a disorganised matrix. Collagen degradation decreased melanoma invasion in vitro and inhibiting MMP1, or higher collagen expression restored the invasion. We validate high collagen deposition in the dermis is a robust predictor of poor melanoma-specific survival in 3 international cohorts of primary cutaneous melanoma. Thus, melanomas arising over UV-damaged, collagen-poor skin are less invasive, and this improves survival. However, we discovered a subset of melanomas arising over collagen-poor, UV-damaged dermis have a poor outcome, and found that increased new collagen synthesis at the invasive front in these cases restores melanoma cell invasion and drives poor outcome. Invasive front collagen also correlated with tumour infiltrating lymphocytes, with dense collagen limiting infiltration.

Fibroblasts damaged by chronic UV show overlapping features with cancer associated fibroblasts. They have a glycolytic metabolism, producing lactate driving OXPHOS in melanoma cells. UV fibroblasts are sensitised to TGFβ signalling, inducing a greater production of collagen and myofibroblast markers than non-UV fibroblasts. Melanoma cells producing more TGFβ induced higher collagen production in fibroblasts, driving invasion of melanoma cells.

Therefore, melanoma cells arising in collagen poor UV skin can recruit local damaged fibroblasts to produce new collagen allowing for invasion and modification of the immune landscape, leading to poor outcomes.

Monitoring 1q21.3 amplification in circulating cell-free DNA from melanoma patients receiving immune checkpoint inhibitors

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The 1q21.3 amplification is a frequent event occurring in metastatic melanoma. This prospective study aims to determine the clinical utility of 1q21.3 amplification detection in circulating cell-free DNA (cfDNA) to monitor immune checkpoint inhibitor (ICI) treatment response in melanoma patients. Melanoma cell lines, tissues, PBLs, plasma, and serum were assessed using a multiplex digital droplet PCR (ddPCR) assay targeting four genes allocated in 1q21.3 region and two reference genes in one reaction. Over 200 blood samples obtained from 50 melanoma patients receiving ICI treatment (nivolumab; ipilimumab-nivolumab; or pembrolizumab) were assessed for 1q21.3 amplification. The multiplex ddPCR assay was initially optimized using DNA from normal and melanoma cell lines. The multiplex ddPCR assay was applied to genomic DNA isolated from skin tissues to determine the optimal cutoff. Any value ≥1.23 copies was the cutoff value to consider 1q21.3 region amplified in tissues. The 1q21.3 detection was significantly increased in 111 melanoma tissues compared to normal skin (p<0.0001). A significantly higher number of 1q21.3 copies were observed in stage III and IV compared to stage I/II (p<0.01). In metastatic melanoma tissues, 1q21.3 amplification was a prognostic factor for shorter overall survival (OS, p=0.022). The ddPCR assay was adapted to a blood assay. CfDNA 1q21.3 amplification was analyzed in blood samples from 50 patients receiving ICI treatment. CfDNA from melanoma patients who had progressive disease had a higher frequency of 1q21.3 amplification. The amplified 1q21.3 region in metastatic melanoma and specific genes allocated in the 1q21.3 region may represent a prognostic
biomarker for OS in melanoma patients. The ddPCR cfDNA assay allowed for predicting response to ICI therapy in melanoma patients.

**Contribution of the epithelial-to-mesenchymal transition transcription factor PRRX1 to melanoma plasticity and progression.**
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Melanoma is a very aggressive skin cancer, known for its high degree of plasticity and heterogeneity that confers high metastatic capabilities and resistance to therapies. Increasing evidences indicate that melanoma progression is not only regulated by mutation-driven mechanisms but also by non-genetic mechanisms that play an important role in melanoma phenotypic plasticity. The reactivation of the expression of neural crest genes in melanoma is associated to progression and resistance to therapies. Among those, epithelial-to-mesenchymal transcription factors (EMT-TFs) have been proposed to regulate reversible switches between phenotypic states driving melanoma progression. However, the role of Prrx1, an important EMT inducer also expressed during neural crest development, has not been investigated in detail. We have found that Prrx1 is expressed in melanoma and that Prrx1 down-regulation induces cell cycle retention and reduces cell proliferation and cancer stem-like properties in melanoma cells. We have also generated a clinically relevant melanoma mouse model that cannot reactivate Prrx1 expression in melanocytes to address the contribution of this EMT-TF to melanoma initiation, progression and responses to therapies.

**Genome-wide screen identifies PDPK1 as a synergistic target to enhancing the efficacy of MEK1/2 inhibitors in NRAS mutant melanoma**
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Melanomas frequently harbor activating NRAS mutations; however, there has been little advance in targeted therapy options for NRAS mutant melanoma patients. MEK inhibitors (MEKi) showed modest efficacy in clinic, which is insufficient to be approved by FDA. In this study, we performed a genome-wide CRISPR/Cas9-based screening, identified PDPK1 (Phosphoinositide-dependent kinase-1) as a therapeutic target to enhancing the efficacy of MEKi, and validated it in various NRAS mutant melanoma cell lines via pharmacological and genetic approaches. Combined inhibition of PDPK1 and MEK (PDPK1i + MEKi) profoundly inhibited NRAS mutant tumor growth in a xenograft model. Notably, the combinatorial treatment induced pyroptosis, and increased ratio of intratumoral CD8+ T cells, delayed tumor growth and prolonged survival in an immune competent allograft model whereas it showed a significantly weaker potency in an isogenic immune deficient model. These data suggest PDPK1i + MEKi is an efficient strategy against NRAS mutant melanoma in an immune-response-dependent manner. Our discovery rationalizes the clinical development of PDPK1i plus MEKi in NRAS mutant melanoma patients and further synergy of targeted therapy and immunotherapy.

**Targeting SOX10-deficient cells to reduce resistance to targeted therapy in melanoma**
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Cellular plasticity contributes to intra-tumoral heterogeneity and phenotype switching, traits that enable tumor cell adaptation to metastatic microenvironments and resistance to targeted therapies. Despite these wide-ranging effects, the key mechanisms that underlie tumor cell plasticity remain poorly understood. We studied the role of SOX10, a neural crest lineage transcription factor, and its role in cutaneous melanoma plasticity. SOX10 was heterogeneously expressed in melanoma patient samples. Loss of SOX10 was sufficient to induce a slow cell cycling phenotype in vitro and in vivo, which was associated with invasive properties including expression of mesenchymal genes and
extracellular matrix, as well as tolerance to BRAF and/or MEK inhibitors. Long-term exposure of co-mixtures of SOX10-proficient and SOX10-deficient cells to targeted therapy selected for SOX10 knockout cells. Furthermore, cell lines generated from BRAF-MEK inhibitor resistant xenografts showed dramatic reductions in SOX10 expression. To identify synthetic lethal interactions with SOX10 loss, we screened a drug compound library and identified the class of cellular inhibitor of apoptosis protein-1/2 (cIAP1/2) inhibitors as selectively inducing cell death in SOX10-deficient cells. Combining cIAP1/2 inhibitor with BRAF/MEK inhibitors delayed the onset of acquired resistance in melanomas

**Tumor Lysate Particle Only Vaccine (TLPO) vs. Tumor Lysate Particle-loaded, Dendritic Cell (TLPLDC) Vaccine to Prevent Recurrence in Resected Stage III/IV Melanoma Patients: Results of a Phase I/IIa Trial**

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**Background:**
The autologous tumor lysate, particle-loaded, dendritic cell (TLPLDC) vaccine is produced from dendritic cells (DC) loaded *ex vivo* with autologous tumor lysate (TL). TLPLDC has been shown to decrease recurrence in resected Stage III/IV melanoma patients in a Phase 2B trial. The TL particle only (TLPO) vaccine is produced by loading of yeast cell wall particles with autologous TL and direct injection allowing for *in vivo* DC loading. Circumventing DC processing reduces production costs and time. We have compared the TLPLDC and TLPO vaccines in an embedded portion of a randomized, double-blind trial.

**Methods:**
Patients (pts) rendered clinically disease-free after surgery were randomized 2:1 to receive the TLPO or TLPLDC vaccine and followed for recurrence and death. Patients had scheduled intradermal inoculations at 0, 1, 2, 6, 12, and 18 months after enrollment. Kaplan-Meier and log-rank analysis were used to compare 36-month disease-free survival (DFS) and overall survival (OS) in an intention-to-treat (ITT) analysis.

**Results:**
Sixty-five pts were randomized, 43 TLPO and 20 TLPLDC. Pts randomized to the TLPO arm were more likely to be female (37.2% vs. 10.0%, *p* = 0.026), but otherwise no significant clinicopathologic differences were identified. At a median follow-up of 20.5 months, the 36-month DFS (64% vs. 58.7%, *p* = 0.821) and OS (94.8% vs. 93.8%, *p* = 0.936) were equivalent between the TLPO and TLPLDC groups, respectively, shown in Figure 1.

**Conclusions:**
In a randomized, double-blind phase 2 trial, there were no differences in DFS or OS in clinically disease-free melanoma pts receiving TLPLDC versus TLPO vaccines. Given prior efficacy shown with TLPLDC, further testing for efficacy of these two vaccines is warranted in a phase 3 trial.

**Acquired resistance to BRAF inhibitor sensitizes melanoma cells to Chk1 inhibition-induced replication stress**

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Melanoma cells with acquired resistance to BRAF inhibition (BRAFiR) gain a range of genetic and functional alterations that give them abilities to escape alternative pharmacological agents. The search for these alterations is critical to elaborate new
treatment approaches for resistant tumors. Here, we investigated Chk1, a kinase of the DNA Damage Response (DDR) pathway, as a therapeutic target in BRAFiR melanomas. We demonstrated that most BRAFiR cells are hypersensitive to Chk1 inhibitor (GDC0575 and AZD7762) compared to their respective treatment-naïve cells both in vitro and in vivo. Monitoring of cell cycle progression showed that S phase is crucial for Chk1i-induced cytotoxicity in BRAFiR cells, in which they partially failed to incorporate nucleotides. BRAFiR cells also showed a greater increase in phospho-RPA and yH2AX levels than treatment-naïve, indicating that DNA replication stress induced by Chk1i is exacerbated in BRAFiR cells. Interestingly, DNA Fibers assay demonstrated that, in the absence of any drug, BRAFiR cells show an increase in replication origin firing. We therefore set out to investigate the factors responsible for the increased replication stress and hypersensitivity to Chk1i. Exome sequencing data revealed mutations in RAS family genes in all BRAFiR cell lines classified as hypersensitive to Chk1i. Using an independent melanoma cell line panel, we confirmed that the presence of RAS mutations is associated with higher Chk1i sensitivity, in line with the literature showing a synthetic interaction between RAS mutations and inhibition of DDR pathway members in several tumors. In summary, we show here that enhanced sensitivity to Chk1i-induced replication stress is common among BRAFiR melanoma cells that harbor RAS mutations. Further work is ongoing to test the effect of different RAS mutations on Chk1i sensitivity in melanoma cells.

The role of dystroglycan receptor glycosylation in melanoma progression.
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The interaction of tumor cells with extracellular matrix in the basement membrane (BME) is critical in various steps of melanoma progression. Laminin in the BME binds to the alpha subunit of Dystroglycan (DG) receptor complex, which is highly glycosylated. α-DG and its associated glycosyltransferases are mutated in muscular dystrophy, but little is known about their importance in melanoma spread. Bioinformatics analysis to study the implication of mutations in DAG1 (DG receptor gene) and its associated genes in cutaneous melanoma patients indicated that POMT1 mutations had a lower significant median month survival (27 months) compared to those with no POMT1 mutations (61 months) and high levels of expression of POMT1 and FKTN but no high LARGE1 level negatively impacted melanoma patient overall survival. Immunofluorescence analysis of tissues from the Dct-Grm1/K5-Edn3 metastatic mouse model showed POMT1 and α-DG expression in primary tumor, dormant disseminated tumor cells (DTCs) and metastatic foci in the lungs. We screened various human melanoma cell lines (A-375, SK-MEL-5, A2058, WM115, YUWERA-H) and observed that they express different levels of α-DG glycosylation that are correlated with levels of POMT1 expression. The levels of α-DG glycosylation and proliferation rates of them were modified by growing the melanoma cells on BME. Our results suggest that glycosylation of α-DG and its binding to BME may be important in melanoma progression and could be used as a potential therapeutic target.

Blockade of dendritic cell differentiation and function by melanoma-driven secretion of MIDKINE
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Immune checkpoint blockers are becoming a standard of care for metastatic melanoma. However, a significant fraction of patients fail to respond in a sustained manner. Discovering biomarkers of resistance to these agents is a main pending question in the field. Antigen presentation via dendritic cells (DCs) is key for an effective immune surveillance. However, aggressive cancers such as malignant melanomas impair DC differentiation and function, but the underlying mechanisms are not well understood. We have previously reported the growth factor MIDKINE (MDK) as a melanoma-secreted factor that acts in a systemic manner “educating” the lymphatic vasculature at premetastatic niches. We have also found a new immune suppressive role of MDK on macrophages that ultimately promotes T cell dysfunction. Now, we have performed a more comprehensive characterization of immunomodulatory functions of MDK. Using a combination of transcriptomic analyses, animal models and functional assays we have unveiled an unexpected three-way action of MDK on DCs: First, MDK reduces DC infiltration in melanoma and
lymphoid organs by blocking the differentiation of these cells at the bone marrow. Second, MDK inhibits DC-mediated antigen presentation. Third, MDK educates DCs to a tolerogenic transcriptome with increased resistance to immunotherapy. Finally, we have identified an MDK-educated DC gene signature that stratifies melanoma patients with differing overall survival and response to immune checkpoint blockers. In conclusion, MDK hinders anticancer immune responses by a multipronged interference with DC biology, facilitating resistance to immunotherapy. Together our results may hold translational relevance for therapeutic intervention in melanoma.

Dietary omega-3 fatty acids as adjuncts to anti-PD-1 immunotherapy in metastatic melanoma
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Less than half of metastatic melanoma patients experience objective responses to first line anti-PD-1 immune checkpoint inhibition (αPD-1 ICI). Dietary omega-3 fatty acids and their metabolic products elicit downstream effects on macrophage and T-cell differentiation, abrogating murine melanoma and human breast cancer progression. Cost-effective adjuncts (i.e. diet) which alter immune effectors may improve ICI response rates. In a synchronous, heterogenous model, C57-BL6/J mice were injected with YUMM 1.7 melanoma cells in left flanks and YUMMER 1.7 (irradiated, immunogenic strain of YUMM 1.7) in the right, fed control diets or omega-3 rich fish oil (FO) chow (10% w/w, 30%kcal/kcal), at 12 days post tumor implantation. Intraperitoneal αPD1 or IgG2a vehicle were injected q3-4 days starting day 12. In a comparable experiment, solo YUMM 1.7 was implanted, diets initiated at day 7, and all mice received αPD-1 starting day 12, as above. Tumors were assessed for growth, harvested and characterized via flow cytometry. All significant results (p<0.05) assessed by 2-way ANOVA or t-test. In the heterogenous model, FO decreased tumor volume at day 26 in vehicle (28%) and αPD-1 (38%) treated mice, but did not synergize with αPD-1. In the solo model, FO decreased tumor volume (21%) by day 32. In YUMM 1.7 tumors of heterogenous mice, FO increased monocytes (CD45+, CD19-, CD11b+, Ly6C+, Ly6G -) and trended towards increasing CD8+ T cells (p=0.07). In solo tumors, FO increased total CD3+ T-cells, monocytes, and PD-L1 + CD4+ T-cells. Fish oil impaired in vivo melanoma tumor growth, increasing tumor microenvironment monocytes. Fish oil appears to abrogate melanoma growth independent of αPD-1 ICI. Despite a lack of “synergy”, dietary omega-3 may prove beneficial when paired with αPD-1 immunotherapy due to independent metabolic effects on tumor growth before or during treatment.
**Melanoma brain metastasis in previously treated patients by tumor mutation type**

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Metastatic melanoma patients with brain metastases have poor prognosis and represent a population with substantial unmet medical need. We sought to quantify the risk of developing brain metastasis among advanced melanoma patients previously treated with cancer immunotherapy (CIT) or BRAF and MEK-targeted therapy (TT). Using the nationwide de-identified Flatiron Health electronic health record (EHR)-derived database, we included patients diagnosed with metastatic or unresectable melanoma between 1/1/2011-11/30/2020, who had received CIT or TT if positive for $BRAF$ $V600$ mutation and had subsequently initiated another line of treatment (index treatment). We evaluated patients with known $BRAF$ or $NRAS$ status who were free of brain metastasis at the index, comparing patients with these mutations to $BRAF$ or $NRAS$ wild type. Patients were followed from the index to the earliest occurrence of death, end of study (11/30/2020) or 6-months of no EHR activity. Outcomes were incident brain metastasis and overall survival (OS). Hazard ratios (HR) and 95% confidence intervals (CI) were estimated by the Fine-Grey proportional hazard (PH) model, accounting for death as a competing risk for brain metastasis, while the Cox PH model was used for OS. All models were adjusted for age, sex, index treatment line, and ECOG. Patients with $BRAF$ $V600$ mutation ($n=603$) had a higher brain metastasis risk [1-year cumulative incidence 25% vs. 15%, HR: 2.0, 95% CI (1.5, 2.8)] compared to those without $BRAF$ mutation ($n=407$). Brain metastasis risk [1-year cumulative incidence 13% vs. 16%, HR: 0.7, 95% CI (0.3, 1.3)] and OS [HR: 1.1 (0.8, 1.7)] were similar by $NRAS$ status ($n=79$ and 204 for positive and negative, respectively). These findings highlight the high disease burden of brain metastasis in $BRAF$-$V600$ patients who have received standard of care therapies.

**Early Melanoma Metastasis Through the Walls of Vessels is Regulated Through Synergistic Interaction between Macrophage Inhibitory Cytokine-1 and Inflammatory Factors**

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Very little is known about the interactions of proteins produced by cancer cells, with others present in cancer patients to control the very earliest stages in melanoma metastasis. This research suggests that microphage inhibitory cytokine-1 (MIC-1), which is secreted by cancer cells trapped in capillaries, can cooperate with the inflammatory factors VEGF, BK, and PAF present in cancer patients. This occurs through the synergistic cooperation between these proteins to create gaps in vessel walls through which cancer cells can transit and metastasize. MIC-1 and the inflammatory factors synergistically enhance changes in myosin light chain (MLC) phosphorylation in the endothelial cells to cause contraction of these cells that create gaps in vessel walls. Knockdown of MIC-1 eliminates the synergism with the inflammatory factors to prevent gap formation and the movement of cancer cells through the endothelial layers. Tightening the junctions between the endothelial cells by activating Sphingosine-1-Phosphate Receptor 1 (S1PR1) using the drug ASR396, can inhibit MLC phosphorylation to prevent gap formation mediated by MIC-1 and the inflammatory factors. The result is a decrease in the development of lung metastases since there are fewer holes through which they can pass. The vascular leakiness mediated by the cooperation between MIC-1 and the inflammatory factors also aids the creation of larger blood-filled tumors because of the leaky vasculature. Knockdown of MIC-1 can shrink the tumors up to 73% by causing a 10-fold reduction in the underdeveloped leaky vessels and blood-lakes. This study is significant as it identifies the cooperation between MIC-1 and systemic inflammatory factors to create gaps in the endothelial lining of vessels, which can aid the earliest stages of metastasis.
Sexual dimorphic effects of tumour microenvironment dictate melanoma progression and therapy resistance

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Melanoma is a disease of ageing with elderly patients showing poor prognosis than younger patients. Across several epidemiological studies age and sex have emerged as the two key factors underlying melanoma incidence, development, and survival. Overall, females have a significantly lower risk and better prognosis than males when adjusted for age. While role of ageing microenvironment in melanoma progression is now recognized the mechanisms underlying the sex disparity are not well understood but attributed to sex-specific behaviors as well as to the biologically intrinsic differences. Dermal fibroblasts (dFs) reside in close proximity to melanoma cells and have been shown to have profound impact on tumor progression, we examined if both sex-dependent and age-related changes in dFs can alter the course of melanoma tumor growth, visceral metastasis, and variable responses to targeted therapy. We observed that age-matched female dFs show reduced proliferation and undergo early replicative senescence. Despite being senescent, age-matched female dFs show reduced SASP characteristics confirmed by reduction in melanoma spheroid invasion and resistance to BRAF/MEK inhibition. Further analysis of melanoma cells subjected to dFs derived conditioned media showed sex and age-based differences in proliferation and concomitant changes in key redox effector, APE1 and therapy resistance driver, AXL supporting elevated metastasis in aged male host. The age-matched female dFs also showed reduced endogenous reactive oxygen species and lower DNA damage as well as expedited repair when subjected to oxidative stress. Furthermore, melanoma cells grown on aged male dFs derived matrices exhibit increased migration and resistance to BRAF/MEK inhibition. Our data provides an integrated view of how age and sex of the tumor microenvironment host contributes to melanoma progression.

Primary Ipilimumab/Nivolumab followed by adjuvant Nivolumab in patients with locally advanced/oligometastatic melanoma

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We investigate the efficacy of Ipilimumab/Nivolumab combination as primary treatment of locally advanced or oligometastatic melanoma pts within an open label, single arm study. Treatment schedule consists in 4 cycles of Ipilimumab 1 mg/kg and Nivolumab 3 mg/kg every 3 weeks, followed by surgery and adjuvant Nivolumab 480 mg every 4 weeks for 6 cycles. Primary objective is pathological complete remission (pCR) rate, according to Neoadjuvant Melanoma Consortium criteria. Secondary objectives are: safety, feasibility and efficacy; QoL; identification of molecular and immunological biomarkers of response and resistance; degree of immune activation; evaluation of microbioma.

From March 2019 to April 2021, 35 pts were treated within the trial. 4 pts withdrew during primary phase for progression (2), toxicity (1) and consent withdrawal (1). Two pts are waiting for surgery. 29 pts underwent to surgery after neoadjuvant phase: pCR was reached in 16 (55%), pCR/near pCR was reached in 18 (62%), pathological partial remission in 4 (14%) and pathological no response (pNR) in 7 (24%) pts. 22 pts concluded the adjuvant therapy. With a median follow-up of 13 months, 33/35 pts are alive. Relapses occurred in 2 pts after neoadjuvant and in 7 pts (1 pCR and 6 pNR at surgery) during/after adjuvant phase. 6 pts (17%) developed related G3-4 adverse events (AE): 3 transaminitis, 1 pneumonitis, 1 myocarditis, 1 CPK increase and 1 miositis; all of them but two underwent to surgery after toxicity resolution. One patient died 5 months after the end of therapy due to ischemic stroke and one other six month after progression.

Primary Ipilimumab/Nivolumab is feasible and able to achieve a pCR/near pCR rate of 62%. Toxicity was lower than that already observed with this schedule. Translational data evaluated longitudinally during therapy on each patient will be presented.
Identifying immunoregulatory stromal cell populations in the tumor microenvironment of a zebrafish model of melanoma
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T cell infiltration is a known predictor of immunotherapeutic response in melanoma, but the mechanisms within the tumor microenvironment that govern T cell infiltration are not well understood. Our work aims to identify tumor-specific stromal cell populations that regulate T cell infiltration in a transgenic zebrafish model of melanoma. Using fluorescent reporters for T cells (CD8) and stromal cells (cxcl12a) in a BRAFV600E;p53NULL model, we visualized the localization of stromal cells relative to infiltrating CD8+ T cells in the tumor microenvironment and observed that CD8+ T cell clusters are found proximal to stromal cells and vessels in mitfa-negative spaces along the surface of the tumor. Single-cell RNA-sequencing data of sorted stromal cells from tumors, pre-tumorous lesions, and normal skin revealed that tumor-derived stromal cells clustered separately from normal skin-derived cells, indicating that there is a tumor-activated transcriptional state. Tumor-derived stromal cells also were enriched for genes which have previously been shown to be expressed in human melanoma and were proposed as candidates for stromal regulation of T cell infiltration. We are now characterizing the effect of genetic knockout and overexpression of these genes on T cell infiltration. Understanding how stromal cells inhibit or promote T cell infiltration, and what stromal cell genes regulate this interaction, would have implications for determining which tumors would best be treated by immunotherapies and suggest new therapeutic co-targets to increase the proportion of responders to immunotherapy.

Evaluating lipid droplets as a prognostic marker in suspicious melanocytic lesions
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Recent studies suggest that lipid and pigment may play an important role in melanoma progression and proliferation; however, the prognostic significance and complete clinical implication of these findings are unclear. In this study, we evaluate the intracellular lipid and melanin content in benign melanocytic nevi and malignant melanoma. We obtained 14 benign melanocytic lesions, classified as Melanocytic Pathology Assessment Tool and Hierarchy for Diagnosis (MPATH-Dx) Class 1, and 22 malignant melanomas, classified as MPATH-Dx Class 4 & 5, from patient biopsies collected between October 2016 and October 2019 at Boston University Medical Center. The malignant melanomas have an average greatest thickness of at least 1.8 ± 2.1 mm with 7/22 biopsies showing the presence of ulceration. Biopsies were obtained from 20 male patients (median age, 59.5 years; age range, 27-82 years) and 16 female patients (median age, 55 years; age range, 28-66 years). We excluded re-excision specimens and biopsies from unknown anatomic sites and from patients of unknown gender. Slides were prepared from paraffin-embedded blocks and are stained for melanin using the Fontana Masson stain. Staining for adipophilin, the main protein component of lipid droplets, and SOX-10, a melanocyte marker, was optimized for use in a multiplexed assay prior to initiation of the study. All specimens were evaluated for adipophilin and SOX10 immunohistochemically and scored for percent of tissue staining and intensity of staining. The purpose of this study is to evaluate melanocytic lesion lipid and pigment content with clinical correlations, which may improve the clinical detection of malignant melanoma in patients. We hypothesize that higher MAPTH-Dx Class status of melanocytic lesions correlates with a higher adipophilin expression and lower melanin content compared to benign melanocytic lesions.

The impact of epigenetics on mucosal and uveal melanoma transcriptomics and immune signaling.
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Mucosal melanoma (MM) and uveal melanoma (UM) have worse response rates to immunotherapy compared to cutaneous melanoma (CM) and acral melanoma (AM). CM and AM are transcriptionally similar despite substantial genomic differences, whereas MM and UM are transcriptionally distinct from CM and AM. MM and UM demonstrate a
melanocytic cell state with low levels of innate immune and inflammatory gene expression. Given the discrepancy between the genomic and transcriptomic profiles across melanoma subtypes, we evaluated the epigenetic state of MM and UM as a potential mechanism underlying gene dysregulation. We performed ATAC-seq to characterize the epigenetic state of 21 melanoma cell lines (8 CM, 4 AM, 4 MM, and 5 UM). In agreement with RNA-seq, there were many gene loci with differential chromatin accessibility (p<0.01) in UM (n=14,159) and MM (n=4,669) compared to CM, with fewer differences in AM (n=496). DAVID pathway analysis identified 40 pathways enriched in MM and 81 enriched in UM (p<0.05), and these included immune response (CIITA, IL15, IL10, ICOS, IFIHI) MAPK signaling (KRAS, SOS1), and cellular phenotype and differentiation (DCT, T7, KIT, MC1R). We treated 2 UM (MP-41 and 92-1) and one MM (MB 2141) cell line using DNMT1 inhibitors (5'-Azacytidine, Decitabine) and several HDAC inhibitors. We found Decitabine and Class I HDAC inhibitors (Romidepsin and Mocetinostat) to most effectively re-express epigenetically-silenced genes, with Romidepsin having the greatest effect. In summary, the epigenetic landscapes of MM and UM were vastly different from CM and AM and account for a significant portion of the transcriptomic differences in these subtypes, including suppression of immune-related pathways. Epigenetic modifying drugs Decitabine and Romidepsin, particularly in combination with immunotherapy, may be successful treatments for patients with MM or UM.

Exploring the Role of CADM1 in Melanoma Circulating Tumor Cells
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Despite the availability of targeted therapies, melanoma remains to be a threat due to its propensity to metastasize to distant organs. Melanoma metastasis is dependent on the survival of circulating tumor cells (CTCs) to colonize distant organs. Thus, inhibiting CTC viability would have clinical benefits. Previously we found that expression of the adhesion molecule, CADM1 reduced metastatic potential and improved overall survival of melanoma patients. Here, we show CADM1 expression may have a role in melanoma CTCs as it associates with non-adherent cell death. We demonstrate that non-adherent melanoma cells undergo parthanotic cell death, illustrated by increased PARP1 activity and poly-ADP ribose (PAR) expression, and PARYlation of apoptosis inducing factor (AIF) and hexokinase I (HKI). Using mutational analysis we found that extracellularly truncated CADM1 was unable to elicit cell death. Furthermore, inhibition of PAR chain recycling by poly-ADP ribose glycohydrolyase (PARG) enhanced parthanotic death in non-adherent cells, implicating clinical relevance for PARG inhibitors to limit metastatic spread in melanoma patients. Together, our results highlight a role for CADM1 in a novel cell death pathway and offers insights to how PARG inhibitors may be used to augment this response to reduce metastatic spread.

Melanoma-risk variants associated with primary melanoma tumor prognostic characteristics and melanoma-specific survival
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Low-penetrant genetic variants associated with cutaneous melanoma susceptibility have been identified in genome-wide association studies (GWAS) and candidate pathway studies. Our aim was to investigate whether these variants could influence tumor aggressivity and outcome. We examined the association of melanoma-risk variants with primary melanoma tumor prognostic characteristics and melanoma-specific survival in the Genes, Environment, and Melanoma (GEM) Study. GEM enrolled 3,285 participants of European origin with incident invasive primary melanoma. For each...
of 47 melanoma-risk single nucleotide polymorphisms (SNPs), we used linear and logistic regression modeling to estimate, respectively, the per allele mean changes in log of Breslow thickness and odds ratios for presence of ulceration, mitoses, and tumor-infiltrating lymphocytes (TILs). We also estimated the per allele hazard ratios for melanoma-specific survival using Cox proportional hazards regression modeling. Passing the false discovery threshold \( P = 0.0026 \) were associations of \( IRF4 \) rs12203592 and \( CCND1 \) rs1485993 with log of Breslow thickness, and association of \( TERT \) rs2242652 with presence of mitoses. \( IRF4 \) rs12203592 also had nominal associations \( P < 0.05 \) with presence of mitoses and melanoma-specific survival, as well as a borderline association \( P = 0.07 \) with ulceration. \( CCND1 \) rs1485993 also had a borderline association with presence of mitoses \( P = 0.06 \). \( MX2 \) rs45430 had nominal associations with log of Breslow thickness, presence of mitoses, and melanoma-specific survival. Our study indicates that further research investigating the associations of \( IRF4 \) rs12203592, \( CCND1 \) rs1485993, \( TERT \) rs2242652, and \( MX2 \) rs45430 with underlying biologic pathways related to tumor progression is warranted.

Characteristics and Outcomes of Pregnancy-Associated Melanoma

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Melanoma diagnosed within one year of pregnancy is defined as pregnancy-associated melanoma (PAM). There is no robust data on how pregnancy influences melanoma and no guidelines for PAM management. With IRB approval, 82 female patients (median age 31.2 years) with a pathology-confirmed melanoma diagnosis within one year of pregnancy treated at our institution 2005-2020 were identified. 26.8\% of patients were diagnosed with melanoma prior to, 31.7\% during and 41.5\% within one year after pregnancy. 69 women had early stage disease (ESD) and 13 late stage disease (LSD): 80.5\% AJCC V8 Stage I, 3.7\% Stage II, 13.4\% Stage III, 2.4\% Stage IV. Anatomic site was head/neck in 7.3\%, trunk in 47.6\%, lower extremity 34.1\% and upper extremity 11\%. Tumor characteristics were as follows: median Breslow thickness 0.75mm (IQR 0.85 mm – 0.35 mm), mean mitotic rate 0.76/mm2 (54.9\% <1 mit/mm2, 22.0\% ≥ 1 mit/mm2, 23.2\% unreported), 4.9\% ulcerated. 67 of 69 ESD patients (97.1\%) and 100\% of LSD patients received standard of care (SOC) therapy including wide local excision (WLE), sentinel lymph node biopsy (SLNB), complete lymph node dissection (CLND) and adjuvant or systemic treatments (including BRAF-targeted therapy and/or immunotherapy) per NCCN Guidelines. No significant delays in therapy were noted. No patients received systemic therapy during pregnancy. 8 of 26 patients (31\%) who underwent SLNB were SLN-positive; 2 of 6 (33\%) proceeding to CLND had non-SLN metastasis. The Kaplan-Meier estimates of reoccurrence were 0\% and 15.4\% at both 1 and 3 years than 2.1\% and 15.4\% at 5 years for ESD and LSD respectively. Kaplan-Meier 5-year overall survival estimates for the entire PAM cohort was 98.6\% (LSD: 90\% and ESD: 100\%). In conclusion, we found clinical-pathologic features and outcomes of PAM following SOC treatment at a highly specialized center for melanoma care were comparable to what is known about non-PAM.

Interaction between developmental status and immune responses drives responses to immune checkpoint blockade in melanoma

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It has been shown that therapeutic responses of melanoma to immune checkpoint blockade (ICB) depend on either developmental status or immune microenvironment of the tumor. How these two factors regulate each other to determine the overall responses is not clear. In this study, we analyzed published developmental markers (Perez Guijarro et al. 2020) and immune signatures (Bagaev et al. 2021) in transcriptomic data from pre-ICB-treatment human melanoma and mouse melanoma models to investigate the interaction of the two mechanisms. In both human and mouse melanoma, subtypes based on either developmental markers or immune signatures are associated with distinct responses to ICB. However, no association existed between subtypes of the two categories, suggesting the multifactorial nature of the interaction. Further analysis showed “dedifferentiation” markers Axl and Ngfr were associated with signatures of immune checkpoint, MHC II, and Treg, while “melanocytic
differentiation” markers Sox10, Erbb3, and Mitf were associated with signatures of tumor proliferation, Th1, and Th2 responses. Interestingly, Axl and Ngfr are associated with extracellular matrix signatures and a myeloid cell traffic signature, respectively. These results suggest that each developmental subtype of melanoma can modulate multiple immune functions. We have also shown that classification of inter-dependent mechanisms will be required when combining developmental and immune markers to reach better predictive power. Our results also implied the developmental pathways as the therapeutic targets in the combination with ICB.

**18F-FDG-PET/CT response assessment in patients with advanced melanoma treated with combination of low-dose ipilimumab and anti-PD1: A real-world experience.**
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Background: Combination therapy with anti-PD1 and low-dose ipilimumab has shown reduced rate of immune-related adverse effects compared with standard dose used in the Checkmates studies 067 and 204. However, the discussion whether low-dose ipilimumab may hamper the response rate in advanced melanoma is still open.

Methods: We conducted a retrospective analysis of response evaluation based on RECIST 1.1 response criteria and 18F-FDG-PET/CT for patients with advanced melanoma treated with combination of nivolumab 3mg/kg plus ipilimumab 1mg/kg for 4 cycles (N3+I1) followed by anti-PD1 maintenance therapy.

Results: Between December 2017 and August 2020, 45 patients with advanced melanoma treated with N3+I1 in first-line setting were identified. Unresectable stage III/stage IV were 2/43 patients, respectively. Among stage IV patients, 60.5% were M1c, 23.3% had elevated LDH and 28% had brain metastasis. At a median follow-up of 16.7 months, 11 patients (24.4%) had G3/G4 toxicity. Review of response evaluation by RECIST was possible in 36 patients and showed an objective response of 50%. Complete response (CR):11% and partial response (PR): 39%. Eight percent presented progressive disease (PD). In 37 patients, review of response evaluation using 18F-FDG-PET/CT was possible. Twenty-four patients (65%) achieved metabolic CR, 5 (13.5%) PD and 8 (21.5%) were classified as non-CR non-PD. 12-month PFS and OS were: 72.5 and 89%, respectively. During the study follow-up, only 1 patient with metabolic complete response relapsed and 3 out of 8 with non-CR non-PD progressed.

Conclusions: Using low-dose ipilimumab combination does not hamper the response rates and, possibly due to fewer protocol interruptions, these patients may achieve more complete responses as showed by 18F-FDG-PET/CT evaluation.

**Increasing evidence for the application of FAK inhibitors in melanoma therapy**
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Despite the successful application of immune and targeted therapies, there are still, as yet, no consistently reliable treatment regimens for advanced melanoma, emphasising the further need for
translational studies to define the molecular mechanisms underlying melanoma development and progression. Recently, we investigated the multifaceted role of AMBRA1 (Activating Molecule in Beclin-1-Regulated Autophagy) in melanoma development and explored its potential therapeutic relevance in melanoma treatment (Di Leo et al., Nat Commun 2021). Using preclinical mouse models of melanoma carrying BrafV600E and Pten deletion (tamoxifen-inducible and syngeneic models) and a panel of human melanoma cells, we showed AMBRA1 is crucial for melanoma development. Deletion of AMBRA1 induced melanomagenesis and the promotion of tumour growth and metastasis by hyperactivating the FAK1 (Focal Adhesion Kinase 1) signaling pathway, both in vivo and in vitro. Strikingly, both tumor growth and invasion were significantly reduced by FAK1 inhibition in AMBRA1-low or -null melanoma models, suggesting the targeting of oncogenic FAK1 signaling as a novel therapeutic strategy. Clinical studies to evaluate the potential correlation between AMBRA1 expression and FAK1 activation in a cohort of AJCC stage I and II primary melanoma are ongoing. Overall, this study identifies AMBRA1 as a novel tumor suppressor in melanoma and the potential for the inhibition of oncogenic FAK1 signaling as a novel therapeutic approach for tumours stratified by low AMBRA1 expression.


Targeting drug-resistant persister cells having high levels of ALDH activity
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Cancer drug resistance is thought to be the end of cellular evolution, in which drug-resistant persister cells remain and lead to disease relapse. Here, we explore the Aldehyde Dehydrogenase(ALDH) expressing drug-resistant persister subpopulations. ALDHs are enzymes that convert toxic aldehydes into less harmful carboxylic acids that can be excreted and removed from the cells. Notably, cells with higher ALDH activity may be able to function as drug-resistant persister cells. Higher levels of cellular ALDH appear to modulate tumor-reactive oxygen species (ROS) levels and related signaling, important for cancer cell survival during drug therapy. ROS levels are higher in a sensitive cancer cell population, and as drug resistance develops, the amount of ROS required for drug resistance development decreases, resulting in a shift in cellular communication and metabolism, driving this process. This study shows that employing a specific ALDH inhibitor to target ALDH+ve cells can limit ALDH+ve cell growth while having minimal effect on ALDH-ve cell survival. However, targeting both ALDH+ and ALDH- subpopulations with combination therapy can more effectively prevent drug resistance by disrupting the critical ROS balance mediated by the ALDH expressing persister cells. The clinical significance of this project shows that targeting ALDH+ve persister cells in combination with agents inhibiting other tumor cells can more effectively prevent drug resistance.

Loss of Ambra1 affects metabolic pathways in melanoma
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Reprogramming of cellular metabolism, a recently re-emerged hallmark of cancer, results from both tumorigenic mutations and adaptation to harsh tumor environment. Despite the recent (re-)arisen interest towards metabolic rewiring, the decoding of metabolic plasticity and the subsequent therapeutic applications in melanoma still remain poorly defined. Recently, we have provided evidence indicating that the Activating Molecule in Beclin-1-Regulated Autophagy (AMBRA1), which in years has been found to regulate several biological processes spanning from autophagy to cell proliferation, has an antitumorigenic role in melanoma, with its loss entangling increased proliferation and high metastatic potential. Preliminary data from our lab are now convincingly adding another layer of complexity to the processes affected by Ambra1 in melanoma. Indeed, transcriptomics analyses and high-quality
metabolic models revealed that metabolic pathways are significantly affected in Ambra1-depleted melanomas, specifically lipid and fatty acids metabolism. Interestingly, exposure to exogenous lipids, such as oleic acid, dramatically altered the high cell migratory capacity typical of AMBRA1-silenced human melanoma cells without, however, compromising cell viability. A higher sensitivity was instead observed upon exposure to mitocans, i.e. drugs targeting mitochondria, in AMBRA1-silenced human melanoma cells, indicating that alterations of mitochondrial metabolism may play a major role in Ambra1-depleted tumors. Altogether, these results implicate that adaptations of metabolic pathways actively occur in Ambra1-deficient melanoma and that their deep understanding may contribute to the development of personalized targeted therapy in AMBRA1 null or low-expressing tumors.

Anchored multiplex PCR custom melanoma next generation sequencing panel for analysis of circulating tumor DNA

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Detection of melanoma mutations using circulating tumor DNA (ctDNA) from plasma is a potential alternative to using genomic DNA from invasive tissue biopsies. In this study, we have developed a custom melanoma next-generation sequencing (NGS) panel which encompasses the top 15 gene mutations in melanoma including the TERT promoter. To date, mutations in the GC-rich TERT promoter region, which is commonly mutated in melanoma, have been technically difficult to detect using NGS panels. We analysed 21 stage III and IV melanoma patients who were treatment-naïve or on various therapies. A BRAF or NRAS mutation was detected in the ctDNA of 62% of patients while TERT promoter mutations (C250T, C228T or CC242TT) were detected in 48% of patients. Co-occurrence of TERT promoter mutations with BRAF or NRAS mutations was found in 9/10 patients. The detection of a TERT C250T mutation in one BRAF and NRAS mutation negative sample increased the detection rate of the custom panel to 67% (based on BRAF/NRAS/TERTpromoter). The custom ctDNA panel showed a concordance of 76% with tissue based-detection and included 12 BRAF/NRAS mutation positive and 4 BRAF/NRAS mutation negative patients. The ctDNA mutation detection rate for stage IV was 75% and for stage III was 20%. Based on BRAF, NRAS and TERT promoter mutations, the custom melanoma panel displayed a limit of detection of ~0.2% mutant allele frequency (MAF) and showed significant correlation with droplet digital PCR. For one patient, we detected a novel MAP2K1 H119Y mutation in an NRAS/BRAF/TERT promoter mutation negative background at a MAF of 0.18%. To increase the detection rate to >90% of stage IV melanoma we plan to expand our custom panel to 50 genes. This study represents one of the first to successfully detect TERT promoter mutations in ctDNA from melanoma patients using a targeted NGS panel.

Circulating tumor DNA based molecular residual disease detection for treatment monitoring in advanced melanoma patients

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Circulating tumor DNA (ctDNA) is an emerging non-invasive biomarker for molecular residual disease (MRD) detection that can inform treatment decision-making. This 3-cohort pilot study used a personalized, tumor-informed ctDNA-assay (Signatera™) for MRD detection and treatment response monitoring in advanced melanoma patients (n=57). In the stage III adjuvant setting (n=25), with median follow up of 13.3 months, 16% (n=4) of patients were ctDNA+ after surgery. Of MRD+ patients with serial testing, 1 of 3 patients cleared ctDNA post-treatment, while 2 patients did not clear ctDNA and eventually relapsed. During surveillance,
3 more patients became ctDNA+, one with subsequent imaging has had confirmed progression. ctDNA-positivity was significantly associated with distant relapse free survival (HR: 16.33, 95%CI:1.5-181, p<0.05), with mean lead time over imaging of 3 months. Among the stage IV patients (n=22) given anti-PD-1 as 1st line therapy, 95% had detectable baseline ctDNA. Serial ctDNA analysis (21/22 patients; median follow up 8.3 months) showed 3 patients with stable/progressive disease had ctDNA detected across all time points;14 patients with tumor response to therapy cleared ctDNA and remained without disease on imaging. All patients (3/3) who initially cleared ctDNA and became ctDNA+ later had confirmed progression. ctDNA-dynamics 2 months post-treatment was significantly associated with PFS (p<0.05). In the stage IV (n=10) post-PD-1 therapy cohort, 80% patients remained ctDNA- after treatment and have not relapsed. One patient who became ctDNA+ subsequently died from suspected disease relapse. Our findings suggest that ctDNA-detection strongly correlates with response to anti-PD-1 therapy and/or disease relapse in advanced melanoma. Prospective studies are needed to determine clinical utility of ctDNA monitoring in this setting.

Efficacy, safety, pharmacokinetic (PK) and pharmacodynamic (PD) data from a phase 1 study of a novel therapeutic peptide, ST101, targeting the oncogenic transcription factor C/EBPβ, in patients (pts) with advanced and metastatic solid tumors

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CCAAT/enhancer-binding protein β (C/EBPβ) promotes tumor survival and proliferation while inhibiting differentiation. ST101 antagonises C/EBPβ, in pre-clinical models of melanoma glioblastoma, breast and prostate cancer (PC). The primary objective of this first phase of a phase 1-2 study was to evaluate safety/tolerability of ST101 in pts with refractory solid tumors. Secondary and exploratory objectives included PK, preliminary efficacy, and PD. We used a 3+3 design, dosing ST101 IV at 0.5, 1, 2, 4, 6, 9 mg/kg weekly (QW). As of August 6, 2021, 21 pts had received a median of 5 weeks’ treatment (range 1 – 50). There were no DLTs, dose modifications, or SAEs related to ST101. The only AEs of note were G1-2 histaminergic IRRs, largely pruritis and urticaria, managed with antihistamines, montelukast, and interruption/slowing of infusion. IRRs affected 92% pts on 1st dose at ≥4mg/kg. Intensity and frequency of IRRs decreased with repeat dosing. No other AEs were consistently reported. PK was dose-proportionate, with continued exposure with no evidence of accumulation and no anti-drug antibodies. Tumor immunohistochemistry showed dose-proportionate staining for ST101 and an inverse relationship with Ki67, suggesting decreasing tumor proliferation with increasing dose. There is one confirmed partial response to date lasting >23 weeks in a pt with multi-metastatic cutaneous melanoma refractory to all standard therapy, and 4 pts with varied histologies had stable disease lasting 18-45 weeks (2 ongoing).

ST101 was safe at all doses explored with evidence of efficacy, particularly at higher doses. PK and PD support a dose relationship for efficacy and with safety will guide selection of a QW dose to open phase 2 expansions by September 2021.
Validation of AMBLor as a prognostic biomarker for non-ulcerated cutaneous AJCC stage I/II Melanoma

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Melanoma incidence is predicted to increase by +60% by 2040. Although AJCC Stage I and II tumours represent 91% of all melanomas, all patients diagnosed with early stage tumours are managed as high risk, even though fewer than 20% progress. Furthermore, the 8th edition of AJCC staging criteria are unable to identify subsets of patients with stage I/II melanomas with low risk of disease progression, emphasising the acute need for credible prognostic biomarkers to stratify patient follow-up based on personalised risk.

The combined immunohistochemical expression of AMBRA1 and Loricrin (AMBLor) in the epidermis overlying non-ulcerated AJCC stage I melanomas has recently been identified as a robust prognostic biomarker and valuable pre SLNB test (Ellis et al. Brit J Dermatol 2020). In the present multicentre validation study, retrospective analysis of AMBLor was performed in a mixed cohort of 334 AJCC stage I and 77 non-ulcerated AJCC stage II cutaneous melanomas derived from the Roswell Park Cancer Centre, Buffalo, USA (n=241) and The Peter McCallum Cancer Centre, Melbourne, Australia (n=170). Clinical follow up data ranged from 60 to 287 months and each cohort was powered to represent rates of metastasis of 10% for AJCC stage I or up to 20% for stage II disease. Results revealed retention of AMBLor was associated with significantly increased disease free survival of 97% compared to 87% for patients with melanomas in which AMBLor was lost (P=0.01; HR 0.20, 95% CI 0.09-0.42), and with a negative predictive value of 97.14%.

Collectively these data suggest AMBLor as a marker to identify genuinely low risk subsets of AJCC stage I/II melanomas. Inclusion of AMBLor into clinical pathways may therefore aid the stratification of patients for reduced follow up and/or SLNB, with the potential for significant reduction in patient anxiety and savings on healthcare resources.

Variability in Response to Combination Immunotherapy of Metastatic Melanoma as a Function of Sex

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We report a sex bias in 5-year progression free and overall survival (PFS and OS) for n=340 patients treated for metastatic melanoma with immune checkpoint inhibitors (ICI). When restricted to a front-line immunotherapy, male patients treated with combination ipilimumab (IPI) plus nivolumab (NIVO), had prolonged PFS relative to female patients. No sex bias was observed in patients treated with single agent ICI. Site of origin (cutaneous, mucosal, acral or uveal) (p<0.001), vitiligo (p<0.001), and an adverse event requiring steroids (p=0.003) were all significant predictors of patient outcome, while BRAF status (p=0.265), cancer stage (M1a/b vs M1c/d) (p=0.525), age (p=0.073), and presence of other cancers (p=0.119) were not. The sex bias persisted (p = 0.028) following data restriction to include only cutaneous patients receiving simultaneous frontline IPI plus NIVO (HR=0.47, 95% CI: 0.24-0.92) (n = 60). The MGH IPI+NIVO cohort was combined with a similarly restricted cohort from Vanderbilt University Medical Center (VUMC). This larger cohort (n=127) retained the statistically significant sex bias (p=0.031) (HR=0.61, 95% CI: 0.38-0.96). A multivariate analysis was used to adjust for all relevant variables: age, stage, number of treatment cycles, and adverse event (requiring steroids) presence and showed that none of these variables accounted for the significant sex bias. To identify biological pathways underlying the observed sex bias, we are analyzing paired RNA expression of sex-specific tumor
suppressors PPP2R3B, STAG2, DDX3X, KDM6A, ATRX, IPTPR3, STAT1, FZD7, MGMT and IDG1 using RNASeq from a subset of the initial cohort (n = 85) and examining protein expression using IHC on available tumoral FFPE sections for markers implicated in ICI response: CD8, PD-L1, SOX10, MITF, AXL, NGFR and CD163 to assess any differences in the distribution of these markers between the sexes.

Astrocyte Reactivity Promotes Melanoma Brain Metastasis

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Melanoma Brain Metastases (MBM) are a substantial clinical challenge for patients with advanced melanoma. Approximately 50% of advanced melanoma patients develop brain metastases, resulting in significant morbidity and mortality. MBM are often refractory due to difficulties in targeting therapeutic agents to the CNS and an attenuated immune response compared to extracranial tumors. While peripheral tumors are known to benefit from the local microenvironment, the role of the unique brain microenvironment in promoting MBM growth, immune response, and treatment resistance is a critical gap in our knowledge. Astrocytes, the most abundant brain cell, regulate brain metabolism and support neuronal function. Astrocytes are also in situ regulators of the neuroinflammatory response. They undergo functional and morphological changes, termed reactive astrocytosis, including upregulation of cytoskeletal genes, secretion of growth factors, and inflammatory cytokine production. Post-mortem analyses of MBM patients reveal perilesional reactive astrocytosis and invasion of astrocytes into tumors. Despite many observations of reactive astrocytosis in MBM, the role of reactivity is poorly understood.

Here we evaluate the role of astrocyte reactivity in promoting MBM growth, using in vitro, ex vivo, and in vivo model systems; demonstrating that astrocyte secreted factors increase tumor outgrowth and oncogenic signaling, furthered by secreted factors derived from reactive astrocytes. In parallel, we investigate how MBM growth influences astrocyte reactivity and signaling. Using transcriptomic and proteomic technologies, we dissect the relationship between melanoma and reactive astrocytes. Our findings provide evidence that astrocyte reactivity promotes MBM, potentially providing a new therapeutic avenue yet to be explored for treating MBM.

Perspectives on melanoma diagnosis, treatment, and unmet needs as assessed by a social media (SM) listening study

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Using SM platforms to examine patient (pt) experiences reveals clinical insights and patterns of behavior in the pt journey. This study explored the diagnosis, treatment, and unmet needs of pts with melanoma.

A SM listening (SML) approach using melanoma-specific terminology from publicly-available blogs, forums, and SM sites, collected data retrospectively over 2 years (Nov 2018–Sep 2020), from across 15 European countries. Manual and automated relevancy approaches filtered the extracted data for content that provided pt-centric insights. This contextualized data was then mined for insights on melanoma diagnosis, treatment and unmet needs.

Of 182.4K mentions of melanoma, Twitter was the primary channel used (71% of conversations) and pts the predominant contributors to conversations (62%). Of 864 insightful conversations recorded, where the top two topics discussed were melanoma treatment (n=437) and melanoma diagnosis (n=255). Surgery was the most frequently discussed treatment (67% of conversations), followed by immunotherapy (12%), radiation therapy (5%), targeted therapy (4%), and chemotherapy (3%). Surgery had the highest number of positive mentions
compared with other treatments, while 31% of conversations about immunotherapy were positive. Chemotherapy was often associated with negative sentiments; this was chiefly due to treatment side effects but may also result from the non-curative approach of this treatment.

Most diagnosis discussions were about confirmatory tests (36%), where biopsy was frequently mentioned. Key unmet needs were discussed in 121 conversations, including availability of effective treatments (25%) and access to good healthcare practitioners (25%).

SML studies provide pt perspectives on melanoma treatment that are not usually available from the published literature.

**MacroH2A limits melanoma progression by inhibiting chemokine expression in cancer-associated fibroblasts**

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Deregulation of epigenetic states promotes melanoma progression. MacroH2A, a histone variant associated with transcriptional repression, is downregulated in melanoma vs. benign nevi, where it suppresses proliferation and metastatic potential. However, its role as a barrier to tumorigenesis has not been investigated *in vivo*. We found that mice constitutively lacking macroH2A variants exhibit accelerated melanoma growth compared to their wild-type counterparts. MacroH2A-deficient tumors display impaired cytotoxic T cell function and increased monocyte infiltration, consistent with a compromised anti-tumor immune response, as well as upregulation of Ccl2, Cxcl11 and Il6 chemokines. Through single-cell transcriptomic profiling of the entire melanoma microenvironment, we identified cancer-associated fibroblasts (CAF) as the source of these pro-tumor myeloid chemotactants. MacroH2A loss led to increased CAF abundance and activation, accompanied by increased chromatin accessibility at binding sites of signal response transcription factors AP-1 and NF-kB, as well as EBF2, an emerging regulator of the fibroblast inflammatory phenotype. Mechanistically, both Ebf2 and AP-1 family member Fosl2 are located in large macroH2A-bound chromatin domains in CAFs, and are upregulated upon macroH2A loss. Together with increased accessibility, this suggests their increased binding at inflammatory gene promoters and enhancers, which we have confirmed to date for FOSL2. Altogether, our data supports a novel tumor suppressor role for macroH2A through repression of pro-inflammatory signaling within the melanoma stromal compartment.

**Epigenetic regulation of phenotype switching in in vivo melanoma models**

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Tumor metastasis is largely driven by epigenetic changes leading to aggressive cellular invasion phenotypes which are transient and reversible. While the process of epithelial-to-mesenchymal transition (EMT) is known to be associated with acquired metastatic phenotypes in epithelial malignancies, the precise epigenetic landscape controlling these transitions remains unclear. Moreover, as melanoma cells are of neural crest origins, phenotype switching within the context of EMT is less certain. Here, we investigate the role of specific epigenetic complexes in the phenotype switch associated with melanoma cell migration and metastasis using novel in vivo model systems. *Xenopus laevis* and zebrafish embryo developmental systems are explored within the context of melanoma phenotype switching given the rapid developmental programs, large numbers of progeny, and ease of in vivo imaging. As a proof-of-concept experiment, we focus on CoREST, an epigenetic-modifying complex with both lysine-specific demethylase 1A and histone deacetylase activity. In an inducible *Xenopus* melanocyte migration/metastasis model, we find that a small molecule CoREST inhibitor increased the LSD1
histone methylation target, H3K4me1, indicating inhibition of endogenous *Xenopus LSD1*. To further validate these results, we have established human tumor xenograft models in zebrafish as well as dTAG targeting systems for critical epigenetic regulatory proteins. Given the high sequence homology of epigenetic enzymes in humans, Xenopus, and zebrafish, including HDACs and EZH2, these systems may provide an opportunity to evaluate epigenetic pathways and small molecule inhibitors in a high-throughput fashion within the context of both endogenous and tumor xenograft melanocyte/melanoma cells and the processes of cellular migration, invasion and metastasis.

**Targeting mRNA translation to effectively treat uveal melanoma**

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Uveal Melanoma (UM) is a highly metastatic subtype of melanoma that arises in the eye. UM differs from other types of melanoma with a propensity to metastasize to the liver. Specifically, two mutually exclusive mutations in GNAQ/GNA11 are present in more than 85% of patient tumors, leading to the constitutive activation of the PI3K/mTOR and MAPK signaling pathways. These pathways ultimately converge to activate the eIF4F complex through the regulation of the mRNA cap-binding protein eIF4E. Regulating the levels and availability of eIF4E is a critical step in maintaining cell homeostasis. Phosphorylation of eIF4E (Ser209) is also important as increased levels of phospho-eIF4E have been reported in several cancers. The phosphorylation of eIF4E by its kinases, MNK1/2, leads to an increase in the translation of a subset of mRNAs that encode for proteins with roles in cell survival and invasion. We have screened a panel of UM-derived cell lines to determine the expression status of the two axes that regulate translation: mTOR/4EBP-1 and MNK1/2-eIF4E. Some UM cell lines had increased levels of total/p-eIF4E, MNK1, and hyperphosphorylated 4E-BP1. Our data lead us to hypothesize that uveal melanomas are driven, at least in part, by dysregulated mRNA translation.

Concerns of patients (pts) with melanoma: gaining insights from social media listening (SML) research on symptoms and quality of life (QoL)

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Social media (SM) provides a platform to learn from pts and the public to better understand pts' needs. This research examined SM discussions on symptoms, impact of the COVID-19 pandemic, and QoL of pts with melanoma.

A SML approach using melanoma-specific terminology from publicly-available blogs, forums, and SM sites, collected data retrospectively over 2 years (Nov 2018–Sep 2020) across 15 European countries. Manual and automated relevancy approaches filtered the extracted data for content that provided pt-centric insights. This contextualized data was then mined to gain insights on symptoms, QoL, and the impact of the COVID-19 pandemic.

Of 182,4K mentions of melanoma, Twitter was the primary channel used (71% of conversations), followed by blogs (17%), and forums (11%). Pts were the predominant contributors to conversations (62%), with caregivers (22%), and family/friends (12%) also contributing. The top 5 regions where conversations took place were the UK (38%), Spain
(16%), Italy (13%), Germany (11%), and France (11%). Female-led conversations were more common (55%), and malignant and metastatic disease accounted for 77% of the types of melanoma discussed.

Of the 864 insightful conversations identified, QoL was mentioned in 255 (30%); emotional burden was the most frequent topic mentioned (70%), followed by physical (24%), social (17%), and financial (4%) impacts. Symptoms were mentioned in only 2% of conversations, with pain (36%), hardened nodules under the skin (21%), and itchy skin (14%) the most common. 5% of discussions highlighted treatments being postponed, rescheduled, or cancelled, which was often attributed to the COVID-19 pandemic.

Emotional burden was the main impact on QoL identified in this study. SML is a useful tool to understand concerns surrounding the QoL of pts with melanoma.

**ROLE OF STANNIOCALCIN 2 IN MELANOMA TUMOUR MICROENVIRONMENT AND METASTASIS**
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Stanniocalcin 2 (STC2) is a secreted protein involved in biological processes such as calcium regulation and stress responses like Endoplasmic Reticulum stress, oxidative stress and apoptosis. It is predicted to exert its biological function in an autocrine and/or paracrine manner. However, the precise physiological functions and signaling pathways in which STC2 is involved remain largely unknown. In cancer, STC2 expression has been shown to promote tumor cell proliferation, invasion and migration in a variety of cancer models. Importantly, STC2 levels associate with poor prognosis, including higher potential of progression and distant metastasis, and specifically lymph node metastasis, in multiple tumour types, such as colorectal cancer, hepatocellular carcinoma, pancreatic cancer, among others.

In melanoma, we identified STC2 as a differentially secreted factor in several metastatic cell lines. However, its specific role in melanoma has not been addressed. By analysing TCGA data for melanoma patients, we observe that STC2 highest levels correlate with poorer prognosis. Additionally, deconvolution analysis of the bulk RNA-seq data from TCGA shows significant changes in the tumour microenvironment of melanoma samples with the highest STC2 mRNA levels. In particular, our analysis shows that high levels of STC2 correlate with a significant decrease in infiltration of T cells and increased number of macrophages. Additionally, high STC2 also correlates with increased endothelial cell content and especially, significant increase in the number of CAFs. We therefore focus on studying the influence of STC2 in the tumour microenvironment in melanoma.

**Triphasic melanocytic tumors: A case series.**
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Melanocytic tumors can be categorized into conventional nevi, melanocytomas, or melanomas, and these designations often correspond to having one, two, or three genetic or chromosomal “hits,” which reflects oncogenic progression. To date, three categories of melanocytoma have been described, in which secondary populations emerge based upon BAP1 inactivation, PRKAR1A inactivation, or beta catenin mutation (deep penetrating melanocytoma). Herein, we report a series of two triphasic tumors.

A 20-year-old man presented with a pigmented papule on the cheek. Biopsy demonstrated an atypical compound melanocytic proliferation comprised of a conventional melanocytic nevus component, an epithelioid dermal component with plasmacytoid melanocytes, and a heavily pigmented component with fusiform melanocytes at the junction and focally in the dermis. Immunohistochemical analysis confirmed loss of BAP1 in the epithelioid/plasmacytoid component and loss of PRKAR1A in the pigmented component with preserved p16 expression throughout. These findings indicate a triphasic melanocytoma with BAP1 inactivation and PRKAR1A inactivation occurring in parallel.

A 74-year-old woman presented with a solitary hyperpigmented papule on the left shoulder. Biopsy revealed a compound melanocytic proliferation including conventional melanocytic nevus, a peripheral dermal component with eccentrically nucleated plasmacytoid melanocytes, and a nodular compound component of large irregular epithelioid melanocytes with pagetoid scatter, consistent with melanoma. Immunohistochemistry confirmed loss of BAP1 in the plasmacytoid melanocytes, with p16
loss restricted to the melanoma component. These findings suggest sequential BAP1 and p16 loss, elucidating the evolution of a melanocytic nevus into a BAP1-inactivated melanoma.

Taken together, these striking cases nicely illustrate oncogenic progression. Melanocytomas may develop in parallel or may give rise to melanoma.

**Targeting Replication Stress Using CHK1 Inhibitor Promotes Innate and NKT Cell Immune Responses and Tumour Regression.**

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Drugs selectively targeting replication stress have demonstrated significant preclinical activity, but this has not yet translated into an effective clinical treatment. Here we report that targeting increased replication stress with a combination of Checkpoint kinase 1 inhibitor (CHK1i) with a subclinical dose of hydroxyurea targets also promotes pro-inflammatory cytokine/chemokine expression that is independent of cGAS-STING pathway activation and immunogenic cell death in human and murine melanoma cells. In vivo, this drug combination induces tumour regression which is dependent on an adaptive immune response. It increases cytotoxic CD8⁺ T cell activity, but the major adaptive immune response is a pronounced NKT cell tumour infiltration. Treatment also promotes an immunosuppressive tumour microenvironment through CD4⁺ Treg and FoxP3⁺ NKT cells. The number of these accumulated during treatment, the increase in FoxP3⁺ NKT cells numbers correlates with the decrease in activated NKT cells, suggesting they are a consequence of the conversion of effector to suppressive NKT cells. Whereas tumour infiltrating CD8⁺ T cell PD-1 and tumour PD-L1 expression was increased with treatment, peripheral CD4⁺ and CD8⁺ T cells retained strong anti-tumour activity. Despite increased CD8⁺ T cell PD-1, combination with anti-PD-1 did not improve response, indicating that immunosuppression from Tregs and FoxP3⁺ NKT cells are major contributors to the immunosuppressive tumour microenvironment. This demonstrates that therapies targeting replication stress can be well tolerated, not adversely affect immune responses, and trigger an effective anti-tumour immune response.

**DELTA-1: A global, multicenter phase 2 study of ITIL-168, an unrestricted autologous tumour-infiltrating lymphocyte (TIL) cell therapy, in advanced cutaneous melanoma**

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Made from each patient’s digested and cryopreserved tumor, ITIL-168 is an autologous TIL cell therapy manufactured to offer an unrestricted T-cell receptor repertoire. DELTA-1 is a global, multicenter phase 2 study to evaluate efficacy and safety of ITIL-168 in pts with melanoma relapsed after or refractory to PD-1 inhibitor (PD-1i), pts intolerant to PD-1i, and pts whose best response to PD-1i was stable disease. Pts aged ≥18 years with histologically confirmed advanced cutaneous melanoma and ECOG PS 0–1, and adequate organ function will be enrolled in 1 of 3 cohorts. Cohort 1 (n=80) will include pts who relapsed after or were refractory to ≥1 prior line of systemic therapy, including a PD-1i and, if BRAF-
mutated, a BRAFi ± MEKi. Cohorts 2 and 3 (n=25 each) will include pts intolerant to PD-1i and those with stable disease after ≥4 doses of PD-1i, respectively. After tumor resection for TIL harvest, pts must have ≥1 remaining measurable lesion per RECIST 1.1. Pts with uveal, acral, or mucosal melanoma, prior allogenic transplant or cell therapy, and with central nervous system (CNS) disorder or symptomatic and/or untreated CNS metastases are ineligible. Pts will receive 5 d of lymphodepleting chemotherapy (cyclophosphamide ×2 d overlapping with fludarabine ×5 d) followed by a single ITIL-168 infusion (≥5×10⁹ cells) and short course high-dose IL-2. The primary endpoint is objective response rate (ORR) per central review. Key secondary endpoints include duration of response, progression-free survival, overall survival, disease control rate, TIL persistence, and safety. Hypothesis testing of ORR will be performed for cohort 1. The primary analysis will occur when all pts in the cohort 1 modified intent-to-treat population have been followed for ≥6 mo after the first posttreatment disease assessment.

Metastatic uveal melanoma (mUM) is an aggressive form of cancer. Until the recent reports about tebentafusp, no treatment has improved survival. Unlike primary uveal melanoma (pUM), little is known about the molecular landscape of mUM. Here, in the PEACE (Posthumous Evaluation of Advanced Cancer Environment) study we performed tumour sampling after death in 9 mUM patients (pts). In total, 378 post-mortem tumour samples were collected (12-68 per pt) from up to 15 metastatic sites per pt. 7/9 and 4/9 pts received immune checkpoint inhibitors and tebentafusp, respectively. The last dose of tebentafusp was administered up to 2 years prior to death. We performed detailed histopathological analysis of metastases (mets) and archival primary tumours in 9 pts (328 H&E slides) and targeted panel sequencing in a subset of 4 pts to examine the morphological and molecular landscape of lethal mUM. Epithelioid cells are the most frequent cell type at death. Nodular growth pattern is present in 57.5% of liver metastases and is associated with small epithelioid cells and tumour capsules. mUM mostly presents 'cold' and T-cell altered excluded phenotypes. Notably, treatment with tebentafusp was associated with the presence of T-cell infiltrate within metastatic samples. Clonal GNA11 (Q209L) mutations (mut) were found in all 4 pts. SF3B1 mut (2 pts) were associated with prolonged latency between primary and metastatic disease, while BAP1 mut (2 pts) was associated with disease relapse within 1 year. Our study suggests that lethal mUM is associated with specific histopathological characteristics. It also supports the hypothesis that tebentafusp facilitates T-cell trafficking to the tumour, which persists after tebentafusp discontinuation, although this may be confounded by subsequent lines of treatment. Results from the full cohort (9pts) and phylogenetic analyses will be presented at SMR.
Skin cancer surveillance behaviors and attitudes among hair professionals and melanoma survivors: A cross-sectional study

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Studies suggest that hair professionals may play an important role in the detection of head, neck, and scalp melanoma (HNSM), but the attitudes and behaviors of HNSM survivors regarding skin cancer surveillance in haircare settings are unknown. Between August 2017 and January 2018, surveys were administered to 229 practicing hair professionals in the metro Atlanta area and 141 HNSM survivors seen at Emory University Hospital. Discordance between hair professional and patient opinion was assessed using a chi-squared (Χ²) test.

Fifteen percent of hairdressers and 51% of HNSM respondents were male. The majority of hairdressers (75%) and melanoma survivors (79%) agreed that hair professionals should be trained in skin cancer detection (Χ² P = 0.34); additionally, 41% of hairdressers and 44% of melanoma survivors believed training should be mandatory (Χ² P = 0.56).

A greater percentage of melanoma survivors (81%) compared to hairdressers (71%) agreed that hairdressers should refer clients to a physician if they notice an 'abnormal mole' regardless of training (Χ² P = 0.04). Fifty-two percent of hairdressers reported previously referring a client to a physician for an abnormal mole and 20% of HNSM survivors reported being previously referred to a physician by a hairdresser. Knowledge of the ‘ABCDE’ rule for melanoma was reported by 20% of hairdressers and 40% of melanoma survivors. These findings suggest a concordance of opinion among HNSM survivors and hairdressers in support of training hairdressers in skin cancer detection, and that a large proportion of hairdressers already refer clients for abnormal moles despite prior training or proper knowledge of common melanoma warning signs. The outcomes of hairdresser referrals and the potential public health impact of training hairdressers in skin cancer detection deserve further study.

Implementation of the Personalised Immunotherapy Platform in a Pilot Study to Predict Immunotherapy Response in Metastatic Melanoma

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Immune checkpoint inhibitors targeting CTLA-4 and PD-1 have significantly improved the outcomes of many patients with metastatic melanoma, however approximately 50% of patients demonstrate no benefit. There is an urgent need to identify patients who are unlikely to respond to standard immunotherapies and require a novel clinical trial agent. To address this, we have developed the Personalised Immunotherapy Platform; a predictive model of response utilising the clinical, immune and molecular properties of tumours. The pilot study involves the assessment of baseline formalin-fixed paraffin-embedded tumour biopsies from patients with Stage III unresectable or Stage IV metastatic melanoma. Tumour mutation burden is assessed following DNA sequencing. Immune and transcriptomic profiles are characterised via multiplex immunofluorescence (including CD8, PD-L1, CD68, CD16 and SOX10) and targeted-RNA sequencing. These data are entered into a machine-learning-derived classification model to identify patients at high risk of disease progression on standard anti-PD-1-based treatment (<6 months progression-free survival) and determine optimal alternative therapies for these patients. Predictive model development was performed on a retrospective cohort of 271 patients treated with anti-PD-1/-anti-
Uveal Melanoma: a miR-16 disease?
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Chromosome 3p monosomy is associated with a poor clinical outcome of patients with uveal melanoma. Since a copy of the tumor suppressor miR-16 gene is lost for these patients, we postulated that a 3p loss may reduce the miR-16 amount and activity, promoting RNA derepression and tumor burden (loss of brake effect) as observed in chronic lymphocytic leukemia. Unexpectedly, we found that miR-16 expression level is not decreased despite the 3p monosomy. In contrast, our results suggested that miR-16 activity is impaired in uveal melanoma. Here, we investigated the molecular mechanism explaining the sequestration of miR-16 by RNAs. By defining the miR-16 interactome, two genes sets have been highlighted, suggesting two divergent miR-16 functions. In addition to the canonical miR-16 targets such as CCND3 and CDC25A, we identified another set of miR-16-interacting RNAs called thereafter miR-16 sponges. miR-16 binds to these RNAs sponge without inducing their decay. Mechanistically, the miR-16/RNA non-canonical base-pairing promoted stability of mRNAs involved in cancer cell proliferation. The biological relevance has been challenged in uveal melanoma. We showed that patients with poor overall survival expressed higher levels of miR-16 sponges and canonical miR-16 targets. These results strongly suggested that miR-16 is no longer able to repress its targets and, in contrast, promotes RNA stability and protein expression of oncogenes. miR-16 activity assessment using our Sponge-signature discriminates the patient’s overall survival as efficiently as the current method based on copy number variations and driver mutations detection. To conclude, miRNA loss of function due miRNAs sequestration seems to promote cancer burden by two combined events – “loss of brake and an acceleration”. Our results highlight the oncogenic role of the non-canonical base-pairing between miRNAs/mRNAs in uveal melanoma.

Real-world outcomes in patients with melanoma brain metastases (MBM): a multisite retrospective chart-review study of systemic treatments
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Patients with MBM have a poor prognosis. Nivolumab plus ipilimumab (NIVO+IPI), anti–PD-1 monotherapy (anti–PD-1), and BRAF/MEK inhibitors (BRAFi/MEKi) are first-line (1L) systemic therapies for MBM, but real-world efficacy data remain limited. This US, multisite, retrospective chart review study assessed outcomes in patients diagnosed with MBM (June 2017–June 2019) and treated with 1L NIVO+IPI (n=246), anti–PD-1 (n=112), or BRAFi/MEKi (n=114). Patients treated with whole brain radiotherapy or surgical resection were excluded. Patient characteristics in the NIVO+IPI, anti–PD-1, and BRAFi/MEKi groups, respectively, were: mean age, 60/66/61 years; ECOG performance status (PS) 3/4, 7%/12%/4%; symptomatic MBM, 55%/60%/65%; and BRAF-mutant disease (in evaluable patients), 32%/23%/100%. Median follow-up since systemic treatment initiation was 15.4, 13.3, and 14.0 months, respectively. Patients treated with BRAFi/MEKi or anti–PD-1 vs NIVO+IPI were more likely to have more extensive and larger MBM, poorer ECOG PS, and more comorbidities at baseline. In the NIVO+IPI group, median progression-free survival (PFS) was 22.6 months and median overall survival (OS) was 35.8 months. In the anti–PD-1 group, median PFS was 16.7 months and median OS was 18.7 months. In the BRAFi/MEKi group, median PFS was 15.3 months, and median OS was not reached. In the BRAF–wild-type subgroup, NIVO+IPI provided OS benefit vs anti–PD-1 (HR, 0.43; 95% CI, 0.28–0.64). In the BRAF-mutant subgroup, NIVO+IPI provided numerical OS benefit...
Development of Membrane Lipid Transporterhe pan-RAF inhibitor DAY101 in a patient with an NRAS-mutated acral lentiginous melanoma

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Genomic alterations resulting in deregulation of the MAPK pathway, including activating mutations of NRAS and BRAF alterations, have been shown to occur in many adult and pediatric malignancies, especially melanoma. We report a >8-year complete response to monotherapy with the oral, highly selective, CNS-penetrant, type II pan-RAF inhibitor, DAY101, in a heavily pretreated patient with NRAS-mutant melanoma. The female patient was initially diagnosed with an acral lentiginous melanoma in 1997 at 57 years of age. The tumor was BRAF and KIT wild-type but had an NRAS exon 2 mutation (p.G12D). In Oct. 2003, she received 2 cycles of neoadjuvant chemotherapy comprising cisplatin, vinblastine, dacarbazine and interferon-alpha. Recurrent nodules and lymph node metastases were excised on multiple occasions between 2003–2011. In Feb. 2011 she underwent hyperthermic limb perfusion with melphalan and eventually progressed. She subsequently started ipilimumab in Nov. 2011 but developed rash, oral lesions, and colitis, necessitating steroid treatment. She then started low-dose temozolomide early in 2012 but progressed in Apr. 2012. The patient received one dose of nivolumab plus peptide vaccine on a phase 1 trial but stopped due to severe toxicity. In May 2013, she was enrolled on a phase 1 trial of DAY101 (NCT01425008). Initial dosing was 200 mg every other day, but rash, thrombocytopenia, and peripheral edema developed. DAY101 dose was decreased to 140 mg weekly and was tolerated well. In a remarkable response, all skin lesions resolved and a biopsy in Nov. 2017 revealed no evidence of disease. DAY101 treatment was maintained long-term under a single-patient IND and the patient continues to do well on-treatment (>8 years), with only a mild intermittent grade 1 rash as residual toxicity. A phase 1/2 trial (NCT04985604) of DAY101 is currently ongoing.

Extracellular vesicle-derived RNA profiling predicts melanoma response to immunotherapy

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Biomarkers that can predict long-lasting responses to immune checkpoint inhibitors (ICI) are critical to improving melanoma clinical outcomes. Plasma-derived extracellular vesicles (EVs) and their capacity to carry tumour-specific molecular cargo could serve as a relevant biomarker. We carried out a retrospective discovery study in 17 melanoma patients with plasma samples collected prior to commencing ICI with the anti-PD-1 antibody pembrolizumab. Transcriptome analysis of circulating EVs identified a set of 21 genes associated with response to treatment. The gene signature was validated in an independent cohort of 28 melanoma patients treated with ICI, using custom qRT-PCR assays. Notably, this gene signature was associated with B-cell activation by gene ontology analysis. Similar genes have been previously reported to be upregulated in tumour tissues of melanoma, lung cancer and renal carcinoma patients responsive to ICB and found associated with the presence of intratumoral tertiary lymphoid structures. Overall, our preliminary results highlight EV-derived RNA profiling as a new opportunity for a liquid biopsy to predict response to ICI and potentially guide treatment decision-making in melanoma.
Profiling SWI/SNF complex mutations in melanoma initiation

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The SWI/SNF complexes are multimeric chromatin remodelers with a recognized role in controlling chromatin architecture and gene expression. Nevertheless, the individual contribution of each SWI/SNF component remains difficult to dissect, given the complex modular nature of these assemblies. The BAF, PBAF and ncBAF SWI/SNF conformations present in fact variable components that provide each complex with a distinct identity and functions. Notably, SWI/SNF subunits are mutated in > 20% of all cancers with alterations in specific SWI/SNF genes enriched in distinct tumor types. In particular, the PBAF-specific components (ARID2, PBRM1 and BRD7) are highly mutated in melanoma; yet, it remains unclear how these mutations affect SWI/SNF activity and promote oncogenesis.

Preliminary data from our lab showed that ARID2 deficiency in melanoma cells disrupts PBAF subcomplex assembly while inducing a redistribution of core subunits to the BAF subcomplex. However, screening of different PBAF-mutant melanoma cell lines revealed that differential mutations have distinct consequences on PBAF assembly, leading to the formation of aberrant and potentially oncogenic complexes.

To systematically investigate the role of SWI/SNF mutations, I am taking advantage of genome editing techniques to model the most frequent SWI/SNF mutations as well as the complete loss of individual SWI/SNF components in primary melanocytes and melanoma cell lines. I will perform a gene-editing screen to pinpoint cancer relevant SWI/SNF mutants and I will analyze their role in promoting melanoma initiation by employing cellular and epigenetic methods. I will finally explore the mechanism of action of mutant SWI/SNF remodelers to discover vulnerabilities in clinically relevant models.

Single cell-derived melanoma clonal sublines uncover heterogeneity-driven resistance to immunotherapy

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Immune checkpoint blockade (ICB) is currently the first-line treatment for unresectable or metastatic melanoma; however, disease resistance and relapse remain a clinical issue. Intratumoral heterogeneity and phenotypic plasticity have been identified as major players underlying therapeutic resistance and treatment failure, but the regulatory programs that control these features are still unknown. We developed the M4 cutaneous mouse melanoma which represents UV-induced RAS-mutated human melanoma with a transitional developmental state, between neural crest-like and differentiated melanocytic cells. This model is ideal for investigating intratumoral dynamics due to its mixed response to α-CTLA-4 treatment and phenotypic heterogeneity. To understand the specific mechanisms of ICB resistance, we generated 24 single cell-derived clonal sublines from M4 melanoma and characterized them by genomic and single cell transcriptomic analyses, followed by implantation of the sublines into syngeneic immunocompetent and nude mice. A wide range of tumor growth kinetics were observed in vivo, which was in part associated with mutational profiles and dependent on T cell-response. Further inquiry into melanocytic differentiation states, tumor microenvironment (TME) subtypes, and immune cell infiltration of untreated tumors from 11 clonal sublines demonstrated correlations between highly inflamed and differentiated phenotypes with the response of sublines to α-CTLA-4 treatment. Our results evidence that M4 sublines generate intratumoral heterogeneity at both levels of intrinsic differentiation status and extrinsic TME profiles and this may impact tumor evolution during therapeutic treatment. These 24 clonal sublines proved to be a valuable resource to study the complex determinants of response to ICB and specifically the role of melanoma plasticity in immune evasion mechanisms.
Development of Membrane Lipid Transporter Knockdowns in Melanoma Cells Using the dTAG System
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Melanoma cells often develop resistance to MAPKi targeted therapies, which poses a challenge when developing treatments. Previous research showed that melanoma cells occupy several phenotypic states; the extreme “differentiated” and “dedifferentiated” states are associated with drug resistance. Both states are associated with changes in lipid content; “differentiated” cells have lower levels of lipid droplets than “dedifferentiated” melanoma cells, with changes in lipid levels due to differential uptake. We hypothesize that the cells’ ability to alter lipid metabolism is linked to their capacity to switch between phenotypic states and develop drug resistance.

While current small molecules can effectively inhibit lipid uptake in melanoma, they are unspecific and have unknown off-target effects. So, we implemented specific, reversible knockdowns of two transport proteins that have previously been implicated in melanoma cell lipid uptake, Fatty Acid Transport Protein 1 and 2 (FATP1, FATP2). We used the degradation TAG (dTAG) system, where FATP1 or 2 is fused with a mutant FKBP protein. Heterobifunctional dTAG molecules bind to mutant FKBP and recruit proteasomes to degrade the fusion proteins. We exogenously overexpressed the FATP1 or 2 fusion proteins in six melanoma cell lines: 451Lu, 1205Lu, SkMel5, WM793, WM1552C, and WM852. The dTAG13 molecule was most effective, degrading 50-95% of FATP1 or 2 within six hours of treatment. The 451Lu-overexpression line also migrated 20-24% more and proliferated 30-40% less than wild-type cells. Further experiments aim to knock-in these fusion proteins to recapture wild-type expression and generate FATP1/2 knockdowns in phenotypically distinct melanoma cells. This will improve our understanding of how the FATPs influence phenotype switching in melanoma, which could lead to new therapies to arrest phenotype switching and MAPKi resistance.

Adipocyte age dependent metabolic programming dictates metastasis in melanoma
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The skin undergoes changes with age, and loss of subcutaneous fat is a hallmark of aged, inelastic skin. Previous work revealed the important role of aged dermal fibroblasts in melanoma progression and the role of fat in melanoma invasion. Here, we studied the distinct lipid secretory profile of young and aged human subcutaneous adipocytes. We discovered young and aged adipocytes contribute different lipids to melanoma cells, activate different signalling pathways, and modify the metabolic dependencies of melanoma cells uniquely by age.

We obtained human preadipocytes from donors of different ages and differentiated them to functional adipocytes. We studied their morphological, transcriptomic, proteomic, and lipid profile and exposed melanoma cells to either young or aged adipocyte secretome. We observed melanoma cells exposed to young adipocyte secretome increased the rate of proliferation, invasion, and migration compared to melanoma cells exposed to aged adipocyte secretome. This effect was linked to a greater amount of total lipids and a unique ratio of lipids secreted by young adipocytes, which correlated with greater uptake of lipids by melanoma cells. Critically, lipid transfer led to distinct signalling pathway activation and metabolic reprogramming of melanoma cells, with increased oxidative phosphorylation in melanoma cells exposed to young secretomes, and increased adaptability to oxidative stress in melanoma cells exposed to aged adipocyte secretome. In vivo, these differences led to a greater rate of metastasis and distinct tropism of melanoma cells exposed to aged adipocyte secretomes. Finally, we systematically tested the contribution of differentially secreted lipids by age and identified the specific lipids that function as signalling molecules to switch metabolic dependencies and fuel proliferation, invasion, and migration of melanoma cells.
Repurposing bortezomib for improved treatment of melanoma by exploiting immunogenic cell death
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Immunogenic cell death (ICD) constitutes a prominent pathway for the activation of the immune system against cancer, which in turn determines the long-term success of anticancer therapies. Only a few agents can elicit bona fide ICD, including some clinically established chemotherapeutics such as the proteasome inhibitor bortezomib, as demonstrated in malignant myeloma and mantle cell lymphoma, but not yet in melanoma. We have shown in melanoma that bortezomib induces NOXA-dependent apoptosis. Here, we show that bortezomib indeed causes ICD in vitro through induction of endoplasmic reticulum stress, autophagy and apoptosis and through translocation and/or secretion of damage-associated molecular patterns (DAMPs). Vaccination with bortezomib-treated dead melanoma cells induced tumour immunogenicity in vivo, as evidenced in a significant reduction/delay after challenge with live cells. Intralesional injection of bortezomib synergised with subsequent systemic treatment with immune checkpoint inhibition using CTLA-4 and PD-1 antagonists. Re-challenge demonstrated long-term protection through bortezomib combined with immune checkpoint inhibition. Polyfunctional T cell assays revealed that intralesional bortezomib injection generates a tumour-specific T cell response. Finally, immune checkpoint inhibitor-resistance was reverted by bortezomib-induced immunogenicity. In summary, bortezomib-induced ICD is a good strategy to recruit the inflammatory immune response. Bortezomib-induced ICD enhances response to immune checkpoint inhibitors, even in ICI-resistant tumours. We propose intralesional injection of bortezomib combined with systemic CTLA-4 and PD-1 antagonists to improve immune therapy in melanoma.

Phenotypic melanoma heterogeneity is regulated through cell-matrix interaction-dependent changes in tumor architecture
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Phenotypic heterogeneity of cancer cells plays a critical role in shaping treatment response. This type of heterogeneity is organised spatially with specific phenotypes, such as sharply demarcated clusters of proliferating and cell cycle-arrested cells, predominating within discrete domains within a tumour. What determines the occurrence of specific tumour cell phenotypes in distinct microdomains of solid cancers is poorly understood. Here, we show that in melanoma spatial organization of phenotypic heterogeneity is dictated by the expression and activity of MITF. We reveal that this lineage survival oncogene controls ECM composition and organization, and ROCK-driven mechanotransduction through focal adhesion maturation and actin cytoskeleton functionality. In turn, altered tumour microarchitecture and structural integrity impact tumour solid stress which then mediates phenotypic heterogeneity through p27Kip1. Rho-ROCK-myosin signalling is necessary to transmit the effect of the reciprocal cell-ECM regulation into phenotypic heterogeneity. Our findings place cell-ECM crosstalk as a central driver of phenotypic tumour heterogeneity. Melanoma shares these physical properties with any solid cancer underscoring the importance of our findings for therapeutically targeting this phenomenon.
**Environmentally stressed drug-naïve melanoma cell subpopulations share a molecular signature with cells undergoing induced drug tolerance**

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Dynamic tumor heterogeneity is a leading cause of melanoma drug resistance. Slow-cycling tumor cells, often located in the center of tumors, are more drug resistant than rapidly proliferating cells at the periphery. Here, we compared the gene signature of the quiescent inner subpopulation of three-dimensional melanoma spheroids to that of early persister cells (EPCs) in *vitro*. Gene set enrichment analysis (GSEA) revealed hypoxia, TNF-alpha and EMT pathways as the top upregulated pathways in both the quiescent inner and EPCs, while downregulated genes were associated with cell cycle and proliferation-associated pathways, such as E2F targets, G2/M checkpoint and MYC. Survival analysis of the Skin Cutaneous Melanoma (SKCM) patient cohort within The Cancer Genome Atlas (TCGA) with the top upregulated gene signature sets derived from both the quiescent inner cells and EPCs demonstrated poor prognosis.

**Immunotherapy for treatment of melanoma in patients with preexisting psoriasis: safety and efficacy**

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Immune checkpoint inhibitors (ICIs) are a mainstay of melanoma treatment. Retrospective analyses demonstrate safety of ICIs in most patients with autoimmune disease, although disease exacerbations may occur. Psoriasis vulgaris is an immune-mediated disease. Outcomes of ICI treatment in patients with psoriasis are not well-described; thus, we sought to define the safety and effectiveness of ICIs in this population. In this retrospective cohort study, patients from 8 centers with preexisting psoriasis who received ICIs for melanoma were evaluated. Safety outcomes were psoriasis flares and immune-related adverse events (irAEs). We also assessed progression-free survival (PFS) and overall survival (OS). Of 62 patients studied (42 [68%] male; median age 65 years), 38 (61%) received anti-PD-1 antibodies, 8 (13%) anti-CTLA-4, and 16 (26%) combination anti-PD-1/CTLA-4. With ICIs, 37 patients (60%) experienced a flare of cutaneous and/or extracutaneous psoriasis after a median 43 days of therapy. Of those had a flare, 16 patients (43%) were managed with topical therapy only while 15 (41%) needed systemic therapy. Only 5 patients (8%) required ICI discontinuation for psoriasis flare. Forty patients (65%) experienced other irAEs, 15 (24%) were grade 3/4. Of 55 patients with evaluable responses, the response rate was 56.4% (21 CR, 10 PR); an additional 6 patients had stable disease. Median PFS and OS were longer in the flare versus no flare group (43.8 vs. 5.5 months [p = 0.0175]; not reached vs. 30 months [p = 0.047], respectively). Thus, immunotherapy for melanoma was associated
with frequent psoriasis exacerbations, although mostly manageable with standard treatments, and patients with flares performed at least as well as those without. While it may require additional management, these data indicate that psoriasis should not preclude treatment of melanoma with immunotherapy.

The role of c-MYC in resistance to immunotherapy
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Immunotherapy has revolutionized melanoma treatment, however a large portion of patients still do not respond. Here, we analyzed the transcriptome of pre-treatment biopsies from 73 melanoma patients who received immunotherapy and identified elevated expression levels of c-MYC in correlation to interferon gamma resistance in samples from patients who did not respond to therapy. In vitro analysis of melanoma cell lines cultured with interferon gamma revealed that c-MYC overexpression resulted in impaired interferon gamma signaling, reflected in the reduction of pSTAT1 and JAK2 expression, along with a reduction in interferon response genes. To further examine the effect of c-MYC on the interferon gamma pathway, we conducted a reporter assay that demonstrated negative regulation of the JAK2 promoter by c-MYC. Moreover, cytokine secretion assays by antigen-specific T cells co-cultured with c-MYC transfected melanoma cell lines, resulted in decreased T cell effector functions. Importantly, the inhibitory effect was reversed by knockdown of c-MYC and its co-activator MAX, along with sensitization of melanoma cells to interferon gamma following c-MYC inhibition by small molecules, indicating an immune suppression mediated by c-MYC.

Altogether, our findings suggest an association between c-MYC expression levels and clinical resistance to immunotherapy, which is explained mechanistically by its ability to impair interferon signaling and T cell effector functions. These results suggest the potential of c-MYC targeted therapy in combination with immunotherapy approaches to improve clinical response.

Evolution of Prognostic Factors for Overall Survival (OS) in Patients (Pts) with Melanoma Brain Metastasis (MBM) Over Time
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Treatment (tx) options for MBM pts have changed significantly over the past decade. We evaluated how these changes have impacted outcomes and prognostic factors for MBM pts by collecting pt demographics, tumor features (fx), tx given (prior to and initial tx for MBM), and OS for pts diagnosed (dx) with MBMs from 2009-2013 (Prior Era; PE) and 2014-2019 (Current Era; CE). OS was computed from MBM dx to the last follow-up. Kaplan-Meier method was used to estimate OS; log-rank test assessed differences between groups; multivariable (MV) analyses were performed with Cox proportional hazards models. All statistical tests used a significance level of 5%. A total of 791 MBM pts were reviewed (PE, n=332; CE, n=459). Year of MBM diagnosis was associated with OS (p=0.008). Median and 2-yr OS were 10.3 months and 27% for PE and 14.4 months (p<0.001) and 39% for CE MBM pts, respectively. Several fx differed at MBM dx for PE vs CE pts, including mutation status, LDH, extracranial (EC) disease control, prior immunotherapy (IMT), MBM maximum diameter, and initial tx for MBM. MV analysis was performed separately for PE and CE MBM pts. Features associated with worse OS in both groups included elevated LDH, > 3 MBMs, and LMD. Other prognostic features were specific to PE (EC disease, larger MBM maximum diameter, Prior Targeted Tx, initial tx craniotomy/SRS/targeted tx) and to CE (Age, Prior IMT, Symptoms) MBM pts. Results of recursive partitioning analysis for OS for PE and CE MBMs pts also suggest different drivers of prognosis over time. In summary, our analysis of one of the largest cohorts reported to date shows how both OS and prognostic factors for MBM pts have evolved over time and highlight key areas for further improvement.
Clinical Feasibility and Treatment Outcomes With Unselected Autologous Tumor-Infiltrating Lymphocyte (TIL) Therapy in Patients (Pts) With Advanced Cutaneous Melanoma

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The intrinsic antitumor activity and broad neoantigen-specific T-cell receptor repertoire of unselected autologous TILs may provide advantages over other therapies in solid tumors, including checkpoint inhibitor–refractory melanoma where outcomes are poor. In this retrospective compassionate use clinical series, unselected autologous young TILs from digested tumor tissue were manufactured under a MHRA Manufacturing Specials license. Pts with advanced cutaneous melanoma and no other treatment options received lymphodepleting chemotherapy (Cy/Flu) followed by TIL infusion and post-TIL high-dose (HD) IL-2. Efficacy for 15 imaging-evaluable pts was reported by investigator assessment of CT/MRI per RECIST v1.1; 6 additional pts were followed using non–RECIST v.1.1 imaging (PET) and clinical monitoring. Adverse events (AEs) were reported for all treated pts. The data cutoff date was Dec 31, 2019. From Oct 2011 to Aug 2019, 21 pts with high-risk metastatic disease were treated. Pts received a mean of 3 prior therapies (any checkpoint inhibitor, 91%; PD-1i, 57%; BRAFi, 52%; MEKi, 24%). With a median follow-up of 52.2 mo, the overall response rate in imaging-evaluable pts was 53% (13% complete response rate); the disease control rate was 73%. Durable responses were also observed in the 6 pts followed by PET and clinical monitoring. For all treated pts, median survival was 21.3 mo. AEs were generally self-limited and consistent with Cy/Flu and HD IL-2. Common any-grade AEs were thrombocytopenia (62%), pyrexia (57%), rigors (43%), neutropenia (29%), and tachycardia (29%); no treatment-related deaths occurred. Overall, the high response rates observed in this series highlights the feasibility of unselected autologous TILs manufactured from digested tumors and supports further investigation in prospective trials.

Patient (pt) decisions and decisional satisfaction over time with adjuvant (adj) anti-PD1 therapy

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The clinical risks and benefit of adj anti-PD1 therapy is difficult to assess for pts. They must consider risk of relapse, risk of adverse events, magnitude of improvement in relapse-free survival, the lack of evidence for overall survival benefit so far, and the fact that immunotherapy at time of relapse can have excellent results. We conducted a study to identify factors pts consider in deciding whether to undergo adj immunotherapy and to assess quality of life, satisfaction, and decisional regret in the year subsequent to their decision. Stage IIIB, IIIC, IIID, or IV cutaneous melanoma pts who were free of disease and who had not yet discussed adj treatment options with their oncologist were eligible. Pts with BRAF V600 mutations were excluded since this added another element of decision-making. To standardize presentation of risks and benefits of adj anti-PD1 therapy, we created brief information videos using scripts tailored to disease stage and presented by a trained professional actor. After study consent, pts viewed the video, met with their medical oncologist, and made their decision about whether to pursue adj anti-PD1 therapy or observation only. All pts completed baseline assessments regarding factors that contributed to their decision, quality of life (FACT-M), and attitudes toward adj immunotherapy (adapted FACIT-TS-G). Subsequent evaluation of quality of life and immunotherapy attitudes as well as decisional regret was conducted at 3, 6, 9, and 12 months. 34 patients are evaluable (38% stage IIIB, 44% stage IIIC, 6% stage IIID, and 12% stage IV). Of the 34 pts, 20 (59%) declined adj therapy. When risks and benefits of adj anti-PD1 therapy were presented objectively and in detail, the majority of our patients declined adj anti-PD1 therapy. All data collection will be completed by September 2021 at which time we will have final results.
Changes Within the Tumor Microenvironment Following Plasmid IL-12 Gene Electrotransfer Enhances Therapeutic Effect When Combined with anti-PD1 in Metastatic Melanoma in a Murine Model
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Interleukin-12 (IL-12) is a potent immunostimulator that can stimulate the growth and survival of T cells as well as NK cells. Localized GET delivery of pIL-12 to the tumor microenvironment (TME) increased T-cell infiltrate and significantly reduced Treg and MDSC. Treatment with pIL-12 delivered with gene electrotransfer (pIL-12 GET) alone resulted in prolonged disease-free survival and induced long term immune memory protecting against challenge in a B16.F10 - C57Bl/6 mouse melanoma model. To further test the potential of this approach, we utilized a metastatic model consisting of a subcutaneous B16.F10 tumor and a B16F10 cells expressing luciferase injected via the intraperitoneal route. Treatment with pIL-12 GET while successful in reducing or eliminating subcutaneous tumors was only successful in about 50% of the mice in reducing or eliminating the peritoneal spread. When combined with anti-PD-1, results were enhanced. The combined therapy consisted of pIL-12 GET delivered to the subcutaneous tumor together with an IP injection of anti-PD-1. Observations of mice on day 60 using an IVIS Imaging System revealed background levels of luminescence in 90% of mice and was associated with long-term disease-free survival in these mice as the subcutaneous tumors in these mice also completely regressed. Mice treated with anti-PD-1 alone or with pIL-12 without GET did not result in a response at either site. Cell depletion experiments (CD4, CD8 and NK) revealed that CD8+ and CD4+ but not NK cells played a major role in inducing the observed responses. It was also observed that CD44 expression increased with time on CD8 CTLs, suggesting that the memory response was still active at 120 days. The present findings have practical implications for the combination of pIL-12 GET with anti-PD1.

Cautious Addition of MEK Inhibitors to PD-1 Antibody Treatment in Patients with NRAS or NF1 Mutant Metastatic Melanoma Failing Initial Immunotherapy: A Case Series
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There is an urgent need for improved treatment options for patients with metastatic melanoma that lack BRAF activating mutations and who have progressed on initial checkpoint inhibitor therapy. Specifically, there is limited data concerning treatment options for patients with NF-1 and NRAS mutations. MEK inhibitors have been tested to target these mutations, with minimal clinical single-agent activity. We hypothesized that combining MEK targeted therapy with checkpoint inhibitor therapy would directly slow cancer progression, while potentially enhancing immune responses. We performed a retrospective analysis that identified 12 patients with progression on initial check point inhibitor therapy, treated with subsequent continuation of PD-1 antibody treatment with the addition of low dose MEK inhibitors. Four patients had NF-1 mutations, 8 had NRAS mutations. Overall, 2 patients with NF-1 mutations achieved a complete response, 1 had progressive disease, and 1 patient was resected to CR and remains in remission using this approach. In NRAS mutant patients, 3 patients achieved a complete response, 1 had stable disease, and 4 had progressive disease with combination therapy. All complete responses have proven durable with over 18-month median follow-up from the start of combination therapy. Four patients have been able to completely discontinue treatment and remain in an ongoing remission. Two responding NRAS-mutant patients died of intercurrent disease. The majority of patients had at most grade I-II toxicity associated with combined treatment. Our results suggest a novel option for patients with NF-1 and NRAS melanoma progress on initial immunotherapy.
Inhibiting the MNK1/2-eIF4E axis impairs melanoma phenotype switching and potentiates anti-tumor immune responses
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Melanomas commonly undergo a phenotype switch, from a proliferative to an invasive state. Such tumor cell plasticity contributes to immunotherapy resistance, however, the mechanisms are not completely understood and thus are therapeutically unexploited. Using melanoma mouse models, we demonstrated that blocking the MNK1/2-eIF4E axis inhibited melanoma phenotype switching and sensitized melanoma to anti-PD-1 immunotherapy. We showed that phospho-eIF4E-deficient murine melanomas expressed high levels of melanocytic antigens, with similar results verified in patient melanomas. Mechanistically, we identified phospho-eIF4E-mediated translational control of NGFR, a critical effector of phenotype switching. Genetic ablation of phospho-eIF4E reprogrammed the immunosuppressive microenvironment, exemplified by lowered production of inflammatory factors, decreased PD-L1 expression on dendritic cells and MDSCs, and increased CD8+ T-cell infiltrates. Finally, dual blockade of the MNK1/2-eIF4E axis and the PD-1/PD-L1 immune checkpoint demonstrated efficacy in multiple melanoma models regardless of their genomic classification. An increase in the presence of intratumoral stem-like TCF1+PD-1+CD8+ T cells, a characteristic essential for durable anti-tumor immunity, was detected in mice administered a MNK1/2 inhibitor and anti-PD-1 therapy. Using MNK1/2 inhibitors to repress phospho-eIF4E thus offers a new strategy to inhibit melanoma plasticity and improve response to anti-PD-1 immunotherapy.

Spatially resolved transcriptomics reveals a novel role for cilia at the tumor-microenvironment interface
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During tumor progression, cancer cells come into contact with many non-tumor cell types, but it is unclear how tumor cells sense and adapt to these new environments. To investigate tumor-microenvironment cell interactions in vivo, we applied spatially resolved transcriptomics, single-cell RNA-seq, and single-nucleus RNA-seq to a zebrafish model of BRAFV600E-driven melanoma. Using spatially resolved transcriptomics, we identified a unique “interface” cell state where the tumor contacts neighbouring tissues. We used single-cell and single-nucleus RNA-seq to find that the interface is composed of specialized tumor and microenvironment cells that upregulate a common set of cilia genes, and used confocal microscopy to show that cilia proteins are enriched specifically where tumors contact the microenvironment. We discovered that cilia gene expression is regulated by ETS-family transcription factors, which normally act to suppress cilia genes outside of the interface. We also found the stromal cells (fibroblasts, immune cells, adipocytes), collectively referred to as the tumor microenvironment (TME). Moreover, TME-secreted growth factors, cytokines, and extracellular matrix (ECM) are vital to tumor progression and therapy resistance. Aging causes remodeling of the TME that becomes conducive to melanoma progression. In addition, extracellular vesicles (EVs) have been shown to transport lipids, mRNAs and functional proteins between different cells in vitro and across tissues in vivo. EVs have also been shown to play an important role in tumor progression in different cancer types, and aging can change their composition as well as their cargo. We performed proteomics on EVs released by young and aged dermal fibroblasts, the predominant stromal cell population of melanoma TME, to investigate how aging of the cells changes the EVs released by them. Moreover, we performed functional assays to investigate what influence this will have on the tumor progression. Our study shows that EVs secreted by dermal fibroblasts change in their composition as well as their cargo adding another dimension to age-associated increase in melanoma progression, and therapy resistance.

Influence of Age on Fibroblast Derived Extracellular Vesicles and their Role in Melanoma Progression
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Age is an important prognostic factor in cutaneous melanoma, which commonly arises in the elderly. Melanoma progression is the concerted outcome of cellular and molecular changes that occur not just within the tumor cells but also within the milieu of the stromal cells (fibroblasts, immune cells, adipocytes), collectively referred to as the tumor microenvironment (TME). Moreover, TME-secreted growth factors, cytokines, and extracellular matrix (ECM) are vital to tumor progression and therapy resistance. Aging causes remodeling of the TME that becomes conducive to melanoma progression. In addition, extracellular vesicles (EVs) have been shown to transport lipids, mRNAs and functional proteins between different cells in vitro and across tissues in vivo. EVs have also been shown to play an important role in tumor progression in different cancer types, and aging can change their composition as well as their cargo. We performed proteomics on EVs released by young and aged dermal fibroblasts, the predominant stromal cell population of melanoma TME, to investigate how aging of the cells changes the EVs released by them. Moreover, we performed functional assays to investigate what influence this will have on the tumor progression. Our study shows that EVs secreted by dermal fibroblasts change in their composition as well as their cargo adding another dimension to age-associated increase in melanoma progression, and therapy resistance.
evidence of a cilia-enriched interface cell state in human patient samples, suggesting it is a conserved feature of human melanoma. Our results reveal a novel role for cilia at the tumor boundary, and demonstrate the power of spatially resolved transcriptomics in uncovering the biology underlying cell-cell interactions in vivo.

PHGDH - A therapeutic vulnerability in melanoma
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The genomic locus containing the Phosphoglycerate Dehydrogenase (PHGDH) gene at chromosome 1p12 is frequently amplified in melanoma. PHGDH is the rate limiting enzyme that converts the glycolytic intermediate 3-Phospho Glycerate (3-PG) to serine through the serine synthesis pathway (SSP). Serine is utilized to make glycine, glutathione, one carbon metabolites, proteins and lipids. PHGDH inhibition suppresses proliferation of 1p12 amplified melanoma cells. However, the role of PHGDH in chromosome 1p12 non-amplified melanoma is poorly understood. We observed that 1p12 non-amplified melanoma cell lines have enhanced expression of the SSP enzymes as compared to melanocytes cultured in tetradecanoyl-phorbol acetate (TPA, a growth factor that stimulates MAPK pathway). Moreover, TPA starvation in melanocytes increased PHGDH expression. This led us to hypothesize that activation of oncogenic MAPK signaling leads to high expression of PHGDH which can serve as therapeutic vulnerability in melanomagenesis. Acute expression of oncogenic BRAFV600E in melanocytes resulted in upregulation of SSP enzymes, while treatment of melanoma cells with either BRAF or MEK inhibitor resulted in downregulation of SSP enzymes. Downregulation of PHGDH in 1p12 non-amplified melanoma cell lines decreased their cell proliferation and colony formation ability in vitro. We also observed that Phgdh knockdown in a BrafV600E/WT and Pten+ (BPP) melanoma mouse model significantly increased survival. Furthermore, serine-glycine starvation (external source of Serine-glycine) in BPP chimeras did not affect the tumor development or survival of these mice. These results suggest the importance of PHGDH for melanoma cell proliferation and tumor growth irrespective of its amplification status. The exact mechanism of PHGDH regulation by BRAFV600E and the therapeutic effect of PHGDH inhibition in melanoma initiation and progression are the focus of our studies.

Real-world outcomes of adjuvant pembrolizumab for completely resected stage III cutaneous melanoma
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Pembrolizumab was approved in the US for adjuvant treatment of stage III melanoma with lymph node involvement in February 2019 based on KEYNOTE-054 (KN054). Pre-specified KN054 results presented in 2020 showed that 1-year recurrence-free survival probability was 75.3% in the pembrolizumab arm. This study examined patient and disease characteristics and clinical outcomes in patients treated with adjuvant pembrolizumab for stage III melanoma in real-world community oncology practices.

The iKnowMed electronic health record database and chart review were used to identify and follow adult patients with completely resected stage III cutaneous melanoma who received adjuvant pembrolizumab within The US Oncology Network between February 1, 2019 and June 30, 2020. Eligible patients were followed from pembrolizumab initiation (index) until first of December 31, 2020 or loss of follow-up. Patient and disease characteristics were summarized descriptively. Real-world recurrence-free survival (rwRFS) and overall survival (OS) were estimated using the Kaplan-Meier method.

Seventy-nine patients were included in the study. Median age was 61 years (range: 22, 90+), 57.0% were male, 35.4% had stage IIIA melanoma, and 39.2% had 1+ comorbidities. In patients with data, 95.3% had ECOG performance status of 0 or 1 and 54.0% were BRAF wild-type. Median follow-up was 9.3 months (range: 0.0, 21.9). At end of follow-up, 11 (13.9%) had recurrence. rwRFS probability at 12 months was 81.0%. Median rwRFS was not reached. Seven (8.9%) patients died. Survival probability at 12 months was 93.0%.

The findings from this real-world study support the clinical effectiveness of adjuvant pembrolizumab in stage III cutaneous melanoma. The rwRFS are consistent with phase III studies. Studies with longer follow-up are needed to confirm these findings.
Vascular Lakes: a new prognostic parameter for Uveal Melanoma?
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Uveal melanoma (UM) is the most common primary intraocular cancer in adults. About half of UM patients will develop metastases, which are usually fatal. Currently clinical, histopathological features and genetic changes are used to predict tumour-related mortality, including alterations in copy number of Chr3 and Chr8q, and whether BAP1 mutations occur. Vascular lakes (VL) represent irregular immature blood vessels, and often contain tumour cells within their ‘lumen’. VL have never been assessed with respect to cell morphology and tumour genetics nor their potential association with UM metastasis.

A retrospective cohort of 132 haematoxylin-and-eosin (H&E) stained slides of UM were analysed on whole slide scanned images (WSI); which had previously correlated with patient data and clinical outcome by the Liverpool Ocular Oncology Group (LOORG). Each Prognostic Indicator (PI) was divided into two groups: Group 1 (PI Present) and Group 0 (No PI). The WSI were then annotated for VL in QuPath, to assess the number and area of VL; these two datasets were compared using SPSS Version 27.0 for windows, and the Mann-Whitney U Test.

We show that UM characterised by nBAP1- and Chr3 loss have significant differences between the number of VL present (p = 0.034; 0.013) and VL area within the tumour (p = 0.004; 0.035), compared to nBAP1+ and disomy 3 UM. That is, they are larger in area and have a higher incidence. Also, UM samples with Extraocular Extension (EOE) have a higher number of VL compared to UM with no EOE (p = 0.028).

Further, those UM patients with higher VL area: tumour size ratio showed significantly shorter overall survival (p = 0.033). Namely, these patients died within two years of diagnosis compared to those with longer survival times.

Taken together, our preliminary results suggest that VL is an additional prognostic parameter in UM.

The H2A.Z-specific histone chaperone complex SRCAP is a mediator of proliferation and survival in malignant melanoma
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High levels of the histone variant H2A.Z are a hallmark of melanoma proliferation and correlate with poor prognosis of melanoma patients. Our group reported that the H2A.Z.2 isoform controls expression of E2F target genes, and in concordance, loss of H2A.Z.2 attenuates melanoma cell growth. However, histones and their variants are challenging drug targets due to their flat interaction surface. By targeting their chaperones, it may be possible to control their deposition into chromatin.

Two chaperone complexes can deposit H2A.Z isoforms into chromatin: SRCAP and TIP60-P400. YL1 is a common subunit of both complexes that also directly binds to the H2A.Z-H2B dimer. Using mass spectrometry, we identified most of the members of the SRCAP, but not the P400 complex as enriched on H2A.Z nucleosomes. Further, loss of the SRCAP or YL1 subunit, but not the P400 subunit results in depletion of H2A.Z from melanoma chromatin. These findings suggest that the SRCAP complex is the main H2A.Z deposition chaperone complex in melanoma cells.

Genomic analysis shows co-localization of H2A.Z and YL1 mainly at active enhancers and promoters, marked by H3K27 acetylation. Strikingly, upon YL1 knockdown these regions show a dramatic loss of H4 acetylation. Genes that lose H4 acetylation include growth-promoting genes such as E2F1 and CDKN2D. In turn, loss of YL1 leads to suppression of these genes, which manifests in G1/S cell cycle arrest and apoptosis of melanoma cell lines of distinct genetic backgrounds. These findings provide a rationale for targeting the H2A.Z-YL1
interaction as novel epigenetic strategy for melanoma treatment.

**Randomized trial of precision prevention materials to improve skin cancer prevention activities among Hispanics**

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Hispanics experience worse morbidity and mortality from melanoma than non-Hispanic Whites. Precision prevention incorporating genetic testing for MC1R, a skin cancer susceptibility marker, may improve prevention behavior. Within MC1R higher- and average-risk groups, Hispanic participants (n=920) from Tampa, FL and Ponce, PR were randomized to receive precision or generic prevention information. We collected baseline information on demographics, phenotypic characteristics, health literacy and numeracy, family history of cancer, and psychosocial measures. Participants reported hours of weekend and weekday sun exposure, numbers of sunburns, frequency of five sun protection behaviors intentional outdoor and indoor tanning, and skin examinations at baseline, and after three and nine months. Participants also reported these outcomes for their eldest child ≤10 years old. Among MC1R higher-risk participants, precision prevention increased sunscreen use (OR=1.78, p=0.03) and receipt of a skin exam (OR=6.51, p=0.0006); and it decreased weekday sun exposure hours (β=-0.94, p=0.005) and improved sun protection behaviors (β=0.93, p=0.02) in their children. There were no significant intervention effects among MC1R average risk participants; and the intervention did not elevate cancer worry among higher- or average risk participants. Moderation analyses identified intervention effects within subgroups in average-risk and higher-risk participants. As hypothesized, receipt of MC1R precision prevention materials improved some skin cancer prevention behaviors among higher-risk participants and their children and did not result in reduced prevention activities among average-risk participants. Despite these encouraging findings, levels of sun protection behaviors remained suboptimal among participants, warranting more awareness and prevention campaigns targeted to Hispanics.

**A developmental cellular hierarchy in melanoma uncouples growth and metastatic phenotypes**

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Although melanoma is notorious for its high degree of heterogeneity and plasticity, the origin and magnitude of cell state diversity remains poorly understood. Equally, it is not known whether melanoma growth is supported by a subfraction of Melanoma Stem-like Cells (MSCs) and if so, whether MSCs and Metastasis-Initiating Cells (MICs) represent overlapping, interchangeable, or distinct cell populations. By combining single-cell gene expression profiling, multicolour lineage tracing and quantitative mathematical modelling, we developed a spatially and temporally resolved map of the diversity and trajectories of melanoma cell states during primary tumour growth and metastatic dissemination in a clinically-relevant mouse model of melanoma. We show that melanoma growth and metastatic dissemination are fuelled, respectively, by two transcriptionally and spatially distinct melanoma subpopulations. Our findings implicate a hierarchical model of tumour growth that is supported by a population of cancer stem-like cells, which reside in a perivascular niche and exhibit a transcriptomic signature of pre-migratory neural crest cells established transiently during embryonic development. Metastatic dissemination is, instead, driven by a “mesenchymal-like” subpopulation, which preferentially accumulates at the invading front of primary lesions. We identified the transcription factor Prrx1 as a driver of the
mesenchymal-like melanoma phenotype, and demonstrate that this population fuels metastatic dissemination to lymph nodes and distant organs through an EMT-MET-like continuum. These results will pave the way for the development of strategies that detect and, ultimately, intercept melanoma before its dissemination to vital organs.

**Real-World Evidence of Dual versus Single Immune Checkpoint Inhibitors in BRAF V600 Negative Advanced Melanoma**

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In this real-world retrospective study, we evaluated the efficacy and safety of first-line dual immune checkpoint inhibitors (ICI) (ipilimumab+nivolumab with or without maintenance nivolumab) versus single ICI (nivolumab or pembrolizumab) in patients with BRAF V600 wild-type advanced melanoma.

Our study had 67 patients in total. Patients of younger age (P=0.011), received baseline corticosteroid use (P=0.045), had baseline brain metastasis (P=0.023), and with non-cutaneous melanoma (P=0.019) were more likely to receive dual ICI than single ICI. Multivariable Cox analyses demonstrated dual ICI had a significant improvement in overall survival (OS) when compared to single ICI in patients with BRAF wild-type (HR 0.110, 95%CI 0.028-0.434, P=0.002). Presence of baseline brain metastasis was shown to be independent predictive factor for worse OS in BRAF V600 wild-type advanced melanoma patients (HR 8.657, 95%CI 2.319-32.316, P=0.001). Of the 35 patients who progressed on single-ICI, only 6 received second-line ipilimumab, and none achieved complete response or partial response.

Dual ICI were significantly associated with more frequent immune-related adverse events (irAEs) (93% vs. 52%, P=0.005), multiple irAEs at once (36% vs. 4%, P=0.003), and more severe irAEs (grade 2: 50% vs. 44%, grade 3-5 36% vs. 7%, P=0.026) compared to single ICI. More systemic corticosteroid use (93% vs. 30%, P<0.001) and higher prednisone-equivalent ≥1mg/kg use (29% vs. 4%, P=0.039) were required in dual ICI group than single ICI group for managing irAEs.

Our real-world study supported the use of dual ICI over single ICI in advanced melanoma patients with BRAF V600 wild-type, albeit with a more frequent irAEs necessitating systemic corticosteroid use. Future studies on better patient selection for dual ICI would be required to achieving optimal survival benefit while minimizing toxicities.

**Novel functions of CTLA4 in melanoma beyond an immune checkpoint**

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The role of Cytotoxic T Lymphocyte Antigen 4 (CTLA4) as an immune checkpoint is well established and has been targeted by immunotherapy against melanoma. Our previous studies indicated that CTLA4 was highly upregulated in mutant-BRAF/NRAS melanoma cells. Although CTLA4 is almost entirely expressed intracellularly in melanoma cells, its intracellular functions have remained ambiguous. Ectopic expression of Ctl4 in mouse melanoma cell lines substantially promoted lung colonization in allograft model systems in syngeneic mice. Intriguingly, similar results were seen in immunocompromised recipient mice as well, suggesting that Ctl4 had pro-tumorigenic effects in melanoma that were independent of its immune checkpoint function. Therefore, we investigated the intracellular functions of CTLA4 and its potential roles in melanomagenesis. We observed significant suppression of apoptosis associated with upregulation of anti-apoptotic proteins (Bnip3, Birc6, Pak1, Mcl-1, Survivin, Rac1/Cdc42, Bel-2, and Bnip3l) and downregulation of pro-apoptotic proteins (p53 and Bad) in CTLA4-expressing human melanoma cell lines. Moreover, CTLA4-expressing cells showed significantly higher invasion in Matrigel-coated transwell assay. These effects were reversed when CTLA4 was knocked out by CRISPR/Cas9. Our data also indicated that CTLA4 overexpression activated mTORC1, which is known to regulate protein synthesis, survival, and metabolism of tumor cells. We found that Ctl4 overexpression increased cap-dependent, but not cap-independent, protein translation leading to enhanced translation efficiency. We also found that CTLA4 translocated to mitochondria, where it downregulated OXPHOS and increased glycolysis. These findings lead us to conclude that CTLA4 plays a significant role in regulating apoptosis, protein translation, and mitochondrial metabolic functions, leading to the promotion of melanoma progression and metastasis.
Intermittent MEK Inhibition for the Treatment of Metastatic Uveal Melanoma (MUM)
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Background
MUM is a rare melanoma with poor outcomes. Although >90% harbor mutations of GNAQ/11 with upregulated MAPK signaling, the efficacy of MEK inhibition (MEKi) has been limited. We hypothesized that intermittent MEKi may reduce compensatory activation, allow for higher dosing and more complete pathway inhibition, and prevent resistance.

Methods
We performed a phase I trial of selumetinib on days 1-3 weekly in patients (pts) with mUM, ECOG status ≤2, and no prior MEK therapy. The primary aim was to estimate the maximum tolerated dose (MTD) and to assess safety. Secondary endpoints included RR, progression-free survival (PFS) and overall survival (OS). Tumor biopsies were collected at baseline, day 3 on treatment (tx), and day 11 off tx from 9 pts.

Results
29 pts were treated across 4 dose levels (DL) with a median age of 58 and 1 prior tx. All pts had a tx-related adverse event (AE); 5 were ≥grade 3. There were 2 dose-limiting toxicities at DL2 (125mg BID) and DL3 (150mg BID), and 1 at DL4 (175mg BID). The estimated MTD was 150mg BID with an estimated probability of toxicity of 29% (90% probability interval 16%-44%). RR was 0%; 11 pts achieved stable disease (SD) with a median duration of 5.1 months. Median PFS and OS were 1.8 mos (95% CI 1.7, 4.5) and 7.1 mos (95% CI 5.3, 11.5). Inhibition of pathway signalling was seen in 5/9 cases at day 3, with reactivation at day 11 in 4 cases, and was not associated with outcomes. Preliminary findings from single cell sequencing revealed large-scale copy number alterations, and changes in microenvironment composition that may be associated with tx resistance.

Conclusions
Intermittent administration of selumetinib permitted a 100% increase in MTD but efficacy was not enhanced compared with prior results. Further correlative analyses of single cell transcriptomes and network-based inference of protein activity are ongoing.

Consensus on optimal management of dabrafenib and trametinib-related pyrexia in patients with melanoma: a UK Delphi study
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Dabrafenib and trametinib combination therapy (DT) is indicated for the treatment of BRAF-V600 mutation-positive unresectable/metastatic melanoma, & as adjuvant treatment for patients with resected stage III disease. Pyrexia is an adverse event associated with DT which can precipitate early therapy discontinuation. A modified Delphi panel was conducted to develop consensus on the optimal management of DT-related pyrexia (DTRP) in melanoma patients.

10 practising UK melanoma oncologists participated in a 3-round modified Delphi study. Round 1 was conducted by 1:1 interview & rounds 2 & 3 by email survey. Participants rated their agreement with statements about the definition & management of DTRP. Consensus was reached on 42/66 statements. Percent agreement is reported in (parentheses). ‘Drug related pyrexia’ when presenting as a side effect of medical treatment, was agreed as being strictly an elevation of body temperature, although other symptoms may be present (89%). Participants agreed on the need for simple & generic guidance on DTRP management that does not differentiate between patient groups (100%), & that management of first & second DTRP episodes should be the same regardless of treatment intent (100%). Participants agreed that: both D&T should be interrupted (100%) without considering the use of steroids (89%); patients on DT presenting to non-oncology services with pyrexia should be directed to an oncology-specific service as soon as possible, alongside assessing for infection (100%). Statements on steroid use following DT interruption & when to restart DT did not reach consensus.
The consensus derived from this study provides a framework on the optimal management of DTRP in melanoma patients, which can inform future guidelines to support clinical management.

Molecular and immune features associated with overall survival (OS) in patients (pts) with melanoma brain metastases (MBMs)  
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MBMs are diagnosed in up to 60% of metastatic melanoma pts. Recently, we reported RNA sequencing (RNAseq) data for a large cohort of MBMs (88 tumors from 74 pts). Here, we have integrated clinical outcomes for these pts with their RNAseq data to identify genes and/or pathways that are associated with OS. The assessed MBMs were from pts who underwent craniotomy between July 1991 - October 2015; median OS from craniotomy was 11.2 months (range 0.6 – 146.9). In order to explore genes associated with OS, RNAseq data was compared for pts in the upper (n=26, median OS 22.6; Long Term Survivors, LTS) and lower (n=25, median OS 2.6; Short Term Survivors, STS) terciles of OS. Differential gene expression (DEG) analysis identified a total of six downregulated and 187 upregulated genes in the LTS compared to STS. Many upregulated genes were associated with interleukin receptors, MHC subunits, and apolipoproteins. Gene set enrichment analysis (GSEA) similarly identified enrichment of interferon-related gene sets (FDR, adj-P.val= 1.48e-08) in LTS, and enrichment of MYC-targets (FDR= 1.12e-07) and oxidative phosphorylation (OXPHOS)-related (FDR= 1.18e-03) genes in STS. Pre-selected gene signatures including Immunescore, IFN-γ Index, expanded immune gene signature, and T cell inflamed signature confirmed the GSEA results that there is significant enrichment of immune-related pathways in MBMs from LTS (p < 6e-05 for each signature). MBMs from STS demonstrated significant enrichment of OXPHOS, using our previously published OXPHOS-Index based on single sample GSEA of 8-gene sets (p= 0.0117). IHC staining for CD3 and CD8 confirmed increased immune infiltrates in LTS MBMs. These results support the clinical significance of immune infiltrates and OXPHOS in MBMs. Integration with clinical prognostic factors is ongoing to further investigate these associations.

HETEROGENEITY IN THE ENCODING REGION OF BRAF, MAP2K1 and MAP2K2 GENES IN PRIMARY AND METASTATIC MELANOMA.  
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The superficial spreading melanoma (SSM) is related to intermittent exposure to ultraviolet radiation. Acral lentiginous melanoma (ALM) does not seem to have solar radiation influence. The MAP kinase pathway is one of the most active pathways in melanomas, responsible for cellular signaling of cell growth, proliferation and differentiation. BRAF mutations appears in 50 to 70% of melanomas. BRAF-targeted therapies increased the survival of patients with metastatic melanoma, but this response may last a few months, due to several resistance mechanisms, including, but not only, other subclones with mutations in MAP2K1 and MAP2K2 genes. Genetic heterogeneity results in this genotypic variation that may result in different efficacy responses, thus to therapeutic failure. The aim of this research was to analyze the mutations and the heterogeneity in the coding region of the BRAF, MAP2K1 and MAP2K2 genes of primary and metastatic melanomas. Twenty-seven samples of primary and metastatic SSM and ALM were analyzed for BRAF, MAP2K1 and MAP2K2 mutations using new generation sequencing technique. In ALM series, 50% (7/14) rate of BRAF and MAP2K1 gene and 28.6% (4/14) in MAP2K2 mutation was found. The SSM set, had 76.9% (10/13) BRAF, 30.8% MAP2K1 (4/13) and 23.2% (3/13) MAP2K2 mutations. Primary and metastatic lesions of ALM were heterogeneous for BRAF, MAP2K1 and MAP2K2, whereas SSM were heterogeneous for BRAF and MAP2K1. In conclusion, samples of ALM and SSM showed intra- and intertumoral heterogeneous mutational profile of BRAF, MAP2K1 and MAP2K2 genes.
EVIDENCE OF VARIANTS WITH PATHOGENIC SIGNIFICANCE IN THE CONSTITUTIVE REGION OF TERT GENE
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Nowadays, molecular techniques are part of the diagnostic routine in most pathology laboratories. The identification of mutations in tumors makes it possible to determine the diagnosis, prognosis and eligibility of patients for targeted therapies. This is already a routine in the case of various types of tumors. Melanomas are heterogeneous malignant tumors that, depending on the subtype, have mutations in genes such as BRAF and/or NRAS, KIT and TERT. This study investigates variant mutations present in a conserved region of the TERT gene by NGS in FFPE samples from melanomas Acral subtype, primary and metastatic.

In primary and metastatic tumors, 16 and 11 different variants were respectively found distributed in the samples. In primary melanomas, 4/7 of the samples had more than one variant, 2/7 only one and 1/7 had no variant in the gene. In metatstastics, 2/7 had more than one variant and the others had only one variant (5/7). Surprisingly, the variant c.3327G>A (p.Gly1109fs - frameshift deletion) classified as “probably Pathogenic” by AMP, was frequent in 85% (6/7 in primaries and metastatic) of the samples, in addition, 2/7 in primaries and 4/7 in metastasis showed only this variant. Only 1 metastatic tumor sample presented other variants of “probably Pathogenic’ classification, c.930_931insC (p.Ser311fs) and c.2259delG (c.2259delG). This study illustrates that, despite the conserved constitutive region of the TERT gene, it has variants that may have pathogenic significance, and that using the “correct variant” strategy most clonal SNVs can be retrieved in an FFPE sample with high precision and sensitivity.

Expression of apoptosis pathway-associated proteins in Acral Lentiginous Melanoma (ALM)
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ALM is an aggressive type of cutaneous melanoma that arises on palms, soles, and nail units. ALM is rare (<3%) melanoma subtype in Caucasians, but it is more frequent (~50%) in dark-skinned melanoma patients (pts). There is an unmet need to develop new personalized and more effective treatments strategies for ALM. Increased expression of anti-apoptotic proteins (i.e., BCL2, MCL1) has been shown to contribute to tumorigenesis and therapeutic resistance in multiple tumor types and has been observed in a subset of ALM and mucosal melanoma cell lines in vitro. However, little is known about their expression and clinical significance in ALM pts. Thus, we assessed protein expression (by IHC) of BCL2, MCL1, BIM, and BRAF V600E in melanoma patient samples from MDACC and from INEN, including 20 ALM (16 primary tumors, 4 metastases) and 12 non-ALM (NALM; 2 primaries, 10 metastases). H-score was calculated for BCL2, MCL1 and BIM. BRAF staining was recorded as positive or negative. BCL2, MCL1, and BIM were expressed in both ALM (86.66%) and NALM (75%) tumors, and no significant difference in expression of any apoptotic pathway proteins were detected between the groups, nor primary and metastatic tumors regardless of the type. There were also no significant associations between protein expression with BRAF V600E status, OS, or ethnicity. In summary, ALM demonstrate frequent expression of apoptosis-related proteins BCL2, MCL1, and BIM. Our findings suggest that patients with this aggressive melanoma may be potential candidates for apoptosis-directed therapies. Further studies involving larger numbers of pts are warranted.

Cessation of BRAF±MEK inhibitor therapy prior to progression in advanced BRAF mutant melanoma.
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Most patients (pts) with BRAF mutant melanoma treated with BRAF ± MEK inhibitors (targeted therapy, TT) will progress, however some will have a durable response. It is unknown if TT can be safely discontinued. We sought to describe the outcomes of pts with unresectable stage III/IV melanoma who ceased TT prior to disease progression (PD).

Pts were retrospectively identified from 11 centres. Characteristics at start and end of therapy, PD, and outcomes with subsequent therapy were collected. 94 pts were identified: 45 (48%) were male; 81 V600E and 9 V600K; 88 stage 4 and 6 stage 3. Most were on dabrafenib and trametinib (53%) or vemurafenib only (22%). 9 (14%) received prior immunotherapy (IO). Median treatment duration was 29.6 months (range 0.4-77.9). Reasons for cessation were toxicity (40=43%), patient choice (5=5%), complete response (CR) (38=40%) and other (11=12%). At TT cessation 67 (74%) were in CR, 21 (23%) in partial response (PR), and 2 (2%) stable disease (SD). Median months on TT was higher in the non PD group (18.3 vs 34.6, p = 0.0004).

After median follow up from cessation of 43.6 months (range 0.0-88.7) 36 (38%) had PD; median time to PD was 4.7 months (range 0.7-56.9), 30 (88%) were asymptomatic and 24 (67%) progressed at new sites including 8(23%) in brain. There was no difference in the rates of PD by prior best response: CR (23/67 = 34%), (PR 9/21 = 43%, p = 0.66). Of the 22 pts treated with subsequent TT, 15 (68%) responded (7 CR, 8 PR), and 10 (45%) had ongoing response at a median of 22.8 months from retreatment. 11 received subsequent IO with 5 (45.5%) responses. 8 melanoma related deaths occurred in the PD group, and 1 unrelated death in a patient without PD. The median OS from cessation was not reached.

There is a significant risk of progression following cessation of targeted therapy. However, responses to retreatment with TT and IO are common.

**An emerging role for CDK4/6 inhibitors as immunomodulatory agents in melanoma**

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Inhibitors of cyclin dependent kinases 4 and 6 (CDK4/6) were developed as a cancer therapeutic on the basis of their tumor-intrinsic cytostatic potential, but have since demonstrated profound activity as immunomodulatory agents. CDK4/6 inhibitors are currently under evaluation in clinical trials in combination with melanoma standard-of-care therapies, including both MAPK-targeted therapy and immune checkpoint blockade. Using integrated single cell multi-omics, clinically applicable mouse models, and patients samples, we mapped the immunomodulatory effects of CDK4/6 inhibitors, both alone and in combination with existing melanoma therapies. We discovered that treatment with a CDK4/6 inhibitor skew T cell differentiation in the tumor microenvironment, promoting a stem-like T cell phenotype that supports long-term endogenous anti-tumor immunity and dramatically enhances the efficacy of anti-PD-1 checkpoint blockade. However, in combination with BRAF- and MEK- targeted therapies, CDK4/6 inhibitors significantly depleted tumor-associated myeloid populations, including immunostimulatory subsets such as cross-priming CD103+ dendritic cells and pro-inflammatory macrophages. The absence of these myeloid subsets in tumors correlated with poor overall survival and clinical responses to ICB in both mice and melanoma patients. Encouragingly, altering the dosing regimen of this combination protected these subsets from depletion, suggesting strategic scheduling of these therapeutics may be needed to realise their optimal clinical potential. Together, these data uncover novel translational and mechanistic insights into the prospective utility of CDK4/6 inhibitors as immunomodulatory clinical tools. Our findings have important implications for the strategic design of therapy regimens that incorporate CDK4/6 inhibitors with immunotherapy.
or BRAF/MEK-targeted therapies for the treatment of melanoma patients.

Combining Fecal Microbiota Transplantation with Immunotherapy in Treatment-Naïve Patients with Advanced Melanoma

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The gut microbiota has been shown to influence the effectiveness of immunotherapy and can be modified by Fecal Microbiota Transplantation (FMT). Furthermore, recent studies have shown that FMT with feces from anti-PD1 responders can stimulate a response to anti-PD1 re-challenge in patients with advanced melanoma who experienced immunotherapy resistance. We report early Phase 1 clinical data on 12 patients with advanced melanoma who received FMT with healthy donor stool prior to the first treatment of single-agent anti-PD1 therapy at 3 academic centres in Canada (NCT03772899). Participants had advanced cutaneous melanoma and were anti-PD1-naïve at the time of study entry. Prior targeted therapy was allowed for patients with a BRAF-mutated tumour. A single FMT was completed using 80-100 g of stool via oral capsules. The first dose of anti-PD1 therapy was administered ≥7 days after FMT to allow engraftment. The primary outcome was safety. Secondary outcomes included survival and objective response rate (ORR) by RECIST 1.1/iRECIST criteria. The median age was 76 years (51-89) and half were female. The median time on treatment was 6.5 months (4.2-23.7) and median follow-up was 7.6 months (4.8-25.0). FMT side effects were grade 1-2. There were no unexpected toxicities during anti-PD1 therapy. Out of 12 patients, there were 3 complete responses (CR), 6 partial responses (PR), 1 with stable disease (SD), and 2 with progressive disease (PD) as best response. The ORR was 75% with a disease control rate of 83%. We conclude that FMT with healthy donor stool prior to starting anti-PD1 therapy in treatment naïve patients with advanced melanoma is safe. Early results suggest that the ORR is substantially improved from what has been reported in phase 3 clinical trials with anti-PD1 therapy alone. Correlative microbiome and immunological mechanistic studies are ongoing.

Activation of the Integrated Stress Response sensitizes melanoma cells to mitoribosome-targeting antibiotics

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The ability of tumour cells to adapt to environmental stress, including therapeutic insult, contributes to tumour evolution and drug resistance. Drug-tolerant persister (DTP) cells from multiple cancer types often exhibit elevated activation of the integrated stress response (ISR), which promotes survival by dampening CAP-dependent mRNA translation, one of the most energy-demanding cellular processes. Here we show that activation of ISR in DTP cells promotes selective translation of a subset of mRNAs encoding for mitochondrial proteins. Moreover, DTP survival upon therapeutic pressure relies on the ISR-dependent concomitant upregulation of mitochondrial protein synthesis, a vulnerability that can be exploited using FDA-approved mitoribosome-targeting antibiotics. Accordingly, such agents sensitize to MAPK inhibition, thereby delaying and even preventing the development of resistance in BRAFV600E PDX models. Additionally, this treatment hampers the growth of therapy-resistant and NRAS-mutant cutaneous melanomas as well as uveal melanomas and prevents resistance to both immunotherapy and targeted therapy. Consistently, a melanoma patient exposed to doxycycline, a mitoribosome-targeting antibiotic commonly used to treat infections, experienced a complete and long-lasting response of a treatment-resistant lesion. Our study indicates that the repurposing of mitoribosome-targeting antibiotics offers a rational salvage strategy for targeted therapy in BRAF-mutant...
melanoma, and a therapeutic option for the treatment of NRAS-driven and immunotherapy-resistant cutaneous and uveal melanomas that can be easily implemented in the clinic.

**Association of Steroids and Other Immunosuppressive Agents on Efficacy of Immune checkpoint inhibitor therapy in patients with advanced melanoma.**

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**Background:** Systemic steroids (SS) and other immunosuppressive agents (ISAs) are used for the treatment of immune-related adverse events (irAEs), but the relationship of ISAs to immune checkpoint inhibitors (ICI) efficacy is not well reported. **Methods:** We evaluated patients (pts) with advanced melanoma (AM) treated with ICI from 1/1/2011 to 4/1/2018 at five Lombardi Cancer Center affiliated hospitals. CTCAE V4.03-irAEs, provider's assessed overall response rate (ORR), time to treatment failure (TTF), and overall survival (OS) were collected. **Results:** 370 pts with AM were included and among 1457 unique pts treated with ICIs: Median age 65 years (24 – 95), 87% (321) White, 64% (236) male, and 86% (315) ECOG 0-1. 37% (136) received anti-CTLA-4, 29% (109) anti-PD-1, and 30% (112) both (combo). 14, 2nd and ≥3rd Line ICI use was 55% (203), 31% (114), and 14% (51). Any grade and grade ≥3 irAEs were 57% (212) and 22% (84). 37% (137) received SS and 3% (11) other ISAs. The ORR for SS + ISAs vs. SS only vs. no SS/ISAs for the entire (E) and combo (C) cohort was 91% (10) vs. 43% (52) vs. 33% (73), p<0.002, and 100% (9) vs. 51% (27) vs. 49% (23), p=0.09, respectively. The OS (weeks) for irAEs present vs. no; SS plus ISAs used vs. SS only vs. No SS or ISAs; was NR vs. 44, p<0.001; NR vs. 361 vs. 90, p<0.001, for E cohort and NR vs. 49, p=0.002; NR vs. NR vs. 168, p=0.0048; for C cohort. Similarly, TTF for above groups 50 vs. 15, p<0.001; 224 vs. 41 vs. 18, p<0.001; for E cohort and 84 vs. 31, P=0.010; 224 vs. 67 vs. 34, p=0.034; for C cohort. A similar enhanced benefit was seen among AM pts when we restricted ICI duration to ≤3 months. **Conclusion:** We noted that irAEs, SS+/− ISAs were positively correlated with ICI efficacy. Although this is the largest reported cohort, future prospective studies are needed to confirm our findings.

**Molecular and functional landscape of immune checkpoint resistance in melanoma**

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**PD1 immune checkpoint blockade (ICB) has transformed the treatment of metastatic melanoma patients, producing an objective response rate of 45% and a 5-year overall survival of 39-44%; responses are further improved when used in combination with the CTLA4 inhibitor ipilimumab (ipi). Resistance to ICB is common with around 55% of patients showing innate resistance to PD1 inhibitor, and 40% resistance to Ipi+PD1 inhibitor combination. Moreover, 25% of responding patients acquire resistance to PD1 inhibitor within two years of treatment. Mechanisms of ICB resistance include**
defect in IFNg signaling, immune exclusion and disruption of antigen presentation. However, the spectrum and dominance of resistance mechanisms to ICB remain incompletely defined.

We comprehensively profiled 23 melanoma models derived from 19 patients progressing on PD-1 inhibitor, either alone or in combination with ipi, to dissect mechanisms of ICB resistance. Loss of tumor IFNg signaling was uncommon, with 1/23 cell lines showing a JAK2 mutation, resulting in minimal PD-L1/L2 and MHC-I/II induction. In contrast, 7/23 cell lines displayed high constitutive tumor IFNg signaling, and this was associated with elevated expression of immunosuppressive molecules and melanoma dedifferentiation. Melanoma dedifferentiation, observed in another 5/23 cell lines, diminished the antigenic repertoire and reduced immune cell recognition. Disruption in antigen presentation appeared to be the dominant ICB resistance mechanism (74%), although this occurred discretely via multiple mechanisms including melanoma dedifferentiation (52%), silencing of antigen presentation regulators B2M and/or CIITA (17%) or loss of heterozygosity across the MHC-I/II loci (9%). Given the preponderance of reduced antigen presentation as an ICB resistance mechanism, novel therapeutic strategies should be explored to overcome ICB resistance.

Investigation of the sexual dimorphic tumor suppressor role of DDX3X in melanoma
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Patient sex remains a poorly understood prognostic factor in melanoma. At all melanoma stages, men have higher incidence rates and poorer prognosis (Joosse, A. et al., J Clin Oncol 2012; Joosse, A. et al., J Clin Oncol 2013). Recently, our group performed a mutational meta-analysis of cutaneous melanoma combining 1,014 exomes from five studies, which identified new significantly mutated genes (Alkallas, Lajoie et al., Nature Cancer, 2020). Interestingly, we found that loss-of-function (LoF) mutations in X-linked DEAD-box RNA helicase, DDX3X, are solely found in male patients. Additionally, we demonstrated that DDX3X can escape from X-inactivation, which would protect females from complete DDX3X loss in the case of a single mutational event. To date, the reported functions of DDX3X include RNA metabolism, regulation of translation and mediators of important cancer signaling pathways. However, the role of DDX3X in melanoma is not entirely clear. We hypothesize that DDX3X is a sexual dimorphic tumor suppressor gene that plays a role in mediating the observed differences in incidence and outcome observed between female and male melanoma patients. To address this, we generated stable CRISPR/Cas9-mediated DDX3X knock-out (KO) in male human melanocyte and melanoma lines. Moreover, we identified DDX3X-null human lines to carryout DDX3X gain of function studies. In these models, we determined DDX3X plays a role in proliferation, migration and invasion in melanoma providing supports for its tumor suppressor role in this malignancy.

Therapy combining senogenic and senolytic agents reveals a role of proteome reprogramming in the regulation of senescence to apoptosis fate switch
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The outcome of cancer treatment depends on the type of cellular response it elicits. While therapy-induced apoptosis is associated with tumor regression and long-term remission, therapy-induced senescence may cause incomplete tumor removal and relapse. Here we investigated whether drugs that are known to kill senescent cells (senolytics) can convert the outcome of senescence-inducing therapies into cell death. Two distinct senogenic agents were used, an aurora kinase inhibitor alisertib and paclitaxel. Notably, we found that senolytic BCL-2/xL inhibitor navitoclax converted senescence-inducing therapies into apoptosis-inducing, which dramatically improved their anti-tumor activity in melanoma based on a pre-clinical trial in 19 distinct melanoma
patient-derived organoids. We also showed that this combination caused regression of highly aggressive B16F10 murine melanoma tumors in mice. Combination therapy induced caspase-dependent apoptosis accompanied by mitochondrial depolarization, caspase and PARP cleavage, and morphological signs of apoptosis, including cell blebbing, apoptotic bodies, nuclear fragmentation, and abnormal mitochondria. Furthermore, we found that the mechanism of navitoclax-mediated cell fate switch from senescence to apoptosis required p53 transcriptional activity induced by senogenic therapy. The addition of navitoclax to senogenic therapy caused a drastic remodeling in the cell proteome that affected the balance of pro-senescence and pro-apoptotic p53 targets. These findings provide a rationale for the clinical development of senogenic and senolytic therapy in melanoma and implicate p53 as a reliable biomarker for patient stratification. Furthermore, it uncovers molecular mechanisms of cell fate regulation in cancer cells undergoing treatment that can be targeted to improve therapeutic outcomes.

Epitope spreading toward wild type melanocyte-lineage antigens rescues suboptimal immune checkpoint blockade responses
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Although immune checkpoint inhibitors (ICIs), such as anti-programmed cell death protein 1 (PD-1), can deliver durable anti-tumor effects, most patients with cancer fail to respond. Recent studies suggest that ICI efficacy correlates with a higher load of tumor-specific neoantigens and development of vitiligo in patients with melanoma. Here, we report that patients with low melanoma neoantigen burdens who responded to ICIs had tumors with higher expression of pigmentation-related genes. Moreover, expansion of peripheral blood CD8+ T cell populations specific for melanocyte antigens was observed only in patients who responded to anti-PD-1 therapy, suggesting that ICIs can promote breakdown of tolerance toward tumor-lineage self-antigens. In a mouse model of poorly immunogenic melanomas, spreading of epitope recognition toward wild type melanocyte antigens was associated with markedly improved anti-PD-1 efficacy in two independent approaches: introduction of neoantigens by ultraviolet (UV) B radiation mutagenesis, or the therapeutic combination of ablative fractional photothermolysis plus imiquimod. Complete responses against UV mutation-bearing tumors after anti-PD-1 resulted in protection from subsequent engraftment of melanomas lacking any shared neoantigens, as well as pancreatic adenocarcinomas forcibly overexpressing melanocyte-lineage antigens. Our data demonstrate that somatic mutations are sufficient to provoke strong anti-tumor responses after checkpoint blockade, but long-term responses are not restricted to these putative neoantigens. Epitope spreading toward T cell recognition of wild type tumor-lineage self-antigens represents a common pathway for successful response to ICIs, which can be evoked in neoantigen-deficient tumors by combination therapy with ablative fractional photothermolysis and imiquimod.

Long-Term Follow-up of KEYNOTE-029 1B and 1C: Standard-Dose Pembrolizumab (pembro) + Alternate-Dose Ipilimumab (ipi) in Advanced Melanoma
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The phase 1b KEYNOTE-029 study evaluated standard-dose pembrolizumab (for ≤ 24 mo) + alternate-dose ipilimumab (for 4 doses) in advanced melanoma. In part 1B, study treatment was pembrolizumab 2 mg/kg (amended to 200 mg) Q3W + ipilimumab 1 mg/kg Q3W. In part 1C, study treatment was pembrolizumab 200 mg Q3W + ipilimumab 50 mg Q6W (arm A) or pembrolizumab 200 mg Q3W + ipilimumab 100 mg Q12W (arm B). Previous reports of parts 1B and 1C showed standard-dose pembrolizumab + alternate-dose ipilimumab had antitumor activity and manageable safety. Analyses with additional follow-up are presented (data cutoff, April 1, 2021). Response was assessed per RECIST v1.1 by independent review. In part 1B (N=153), median follow-up (range) was 69.0 mo (0.8-74.6). ORR was 65.8% (52 CR/44 PR). Median DOR (range) was NR (1.6+-70.1+), and 86.2% were estimated to have response duration ≥4 y. Median OS and PFS were NR. 5-y OS rate was 68.3%; 6-y OS rate was 65.3%. 5-y PFS rate was 51.9%. TRAEs occurred in 96.1% of pts (gr 3-4, 47.1%; gr 5, 0%). In part 1C (N=102; 51 per arm), median follow-up (range) was 41.3 mo (0.8-45.4) for arm A and 41.3 mo (0.4-45.6) for arm B. ORR was 69.6% (15 CR/17 PR) in arm A and 76.7% (14 CR/19 PR) in arm B. Median DOR (range) was NR (1.4+-40.9+) in arm A and NR (3.8-40.0+) in arm B, and 82.3% in arm A and 78.7% in arm B were estimated to have response duration ≥3 y. Median OS and PFS were NR in both arms. 3-y OS rate was 74.3% in arm A and 70.4% in arm B. 3-y PFS rate was 56.5% and 59.7%, respectively. TRAEs occurred in 100.0% of pts in arm A and 96.1% in arm B (gr 3-5: 27.5% in arm A; 43.1% in arm B). 1 pt in arm A died of treatment-related autoimmune myocarditis. While follow-up was longer for part 1B, standard-dose pembrolizumab + alternate-dose ipilimumab continued to show durable response, good long-term survival, and manageable safety in pts with advanced melanoma in parts 1B and 1C of KEYNOTE-029.

Vessel Co-option and Angiotropic Extravascular Migratory Metastasis: An Embryogenesis-like Continuum of Tumor Growth and Spread in Melanoma?
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Vessel co-option (VCo), a non-angiogenic mode of tumor growth, and angiotropic extravascular-migratory-metastasis (EVMM), a non-hematogenous mode of tumor migration and metastasis, are two emerging fields investigating the biology of tumor cells along the abluminal vascular surface. VCo is a mechanism by which tumor cells access blood supply by spreading along existing blood vessels in order to grow locally. Angiotropic EVMM involves “pericytic mimicry” (PM) which constitutes tumor cells continuously migrating in the place of pericytes distantly along abluminal vascular surfaces, in the absence of intra- or extravasation. When cancer cells are engaged in PM and angiotropic EVMM, they migrate along blood vessels beyond the advancing front of tumor to secondary sites with the formation of regional and distant metastases. Angiotropic EVMM has mainly been studied in melanoma but also occurs in other cancer types. Considering the re-activation of embryonic signals by “cancer stem-cells”, as well as the essential role of laminins and epithelial-mesenchymal-transition, there are striking analogies between embryonic organogenesis (and neural crest development in particular) and the melanoma metastatic process, suggesting that embryonic migratory and growth-related events recur abnormally during cancer progression. Notably, there is no blood circulation during the first trimester of embryogenesis, even though extensive migration of cells to distant (“secondary”) sites occurs during this period. Therefore, angiotropic EVMM and VCo may constitute complementary processes and represent an extravascular continuum of cancer progression from the primary tumor to metastases, analogous to embryogenesis programs. Such a perspective may greatly influence the development of new effective therapeutics for metastasis.
Lipid droplets support oxidative metabolism in melanoma
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Lipids benefit melanoma cells as a multipurpose building block to support tumor progression. However, lipid dysregulation induces cell toxicity. Melanoma demonstrates metabolic plasticity but the mechanisms regulating lipid utilization in melanoma are poorly understood. Here, we combine a zebrafish melanoma model and single-cell transcriptomics to identify a melanoma transcriptional program linking melanocytic lineage with upregulation of oxidative and lipid metabolism. We found that melanoma cells store excess lipids in lipid droplets which are organelles responsible for storing and regulating lipid release. Suppression of lipid droplet production slowed melanoma growth and transcriptionally upregulated glycolysis pathways. Our results suggest a dependency for lipid droplets to support oxidative metabolism, revealing a critical metabolic vulnerability in melanoma progression.

Single-cell transcriptomic analysis of embryonic melanoblasts uncovers lineage-specific mechanisms of melanoma metastasis and therapy resistance
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Melanoma is a cancer of the melanocyte characterized by a propensity to metastasize and therapy resistance. Immune Checkpoint Blockade (ICB) is a first-line therapy of metastatic melanoma however it is unclear why some patients do not respond to ICB. We hypothesize that during embryonic development, melanocyte precursor (melanoblast) subpopulations exist that harbor distinct characteristics and that some of these characteristics are advantageous to melanoma metastasis and therapy resistance. To query this, we used an iDct-GFP transgenic mouse model which labels embryonic melanoblasts, allowing for their isolation for single-cell transcriptomic analysis (scRNA-seq). Embryonic day (E) 11.5 and E15.5 melanoblasts were studied as these stages are enriched for transmigration across the basement membrane and colonization of hair follicles – processes that may be relevant to metastatic progression and survival. We used UMAP clustering to identify melanoblast subpopulations and generated gene expression signatures of these to establish a Developmental Gene Module (DGM) classification system. To study DGM contribution to metastatic outgrowth and ICB response, we used a syngeneic mouse melanoma model (M4), which we showed adopts heterogenous cell states during metastatic progression and has a heterogeneous response to ICB therapy. We performed scRNA-seq in both (1) lung metastases over time coupled with immunohistochemical analysis, and (2) tumors following ICB treatment coupled with analysis of patient data and mouse ICB resistant models. We find strong commonalities in the cell states that underlie ICB resistance and early metastatic colonization, including a new Extracellular Matrix-enriched lineage-derived subtype. Our work has uncovered conserved biology which drives melanoma cell states, providing potential therapeutic insight.

Removal of Soluble Tumor Necrosis Factors Receptors 1/2 in Patients with Metastatic Solid Tumors Using Immune Apheresis
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TNFα is a cytokine produced by immune cells and by tumor cells. The soluble forms of membrane TNF receptors 1/2 (sTNF-R1/2) act as decoy to neutralize TNFα, and are highly abundant in cancer patients. Elimination of sTNF-R1/2 may therefore unmask endogenous TNFα, to presumably exert anti-neoplastic effects and reverse resistance to immune checkpoint inhibitors. Immune Apheresis (IA) is a
procedure designed to specifically capture sTNF-R1/2 from plasma by passing it over an affinity column. Here we employed Immunicom’s LW-02 Immunopheresis® device for removal of sTNF-R1/2 from plasma of cancer patients. **Method:** In cohort A, patients with melanoma, RCC, NSCLC or TNBC refractory to standard therapy were treated with IA only. IA treatment of 2 plasma volumes was done x3/week up to total of 36 treatments. sTNF-Rs removal and circulating inflammatory biomarkers were measured by immunoassays. Pre- and post-treatment tumor biopsies were analyzed. **Results:** Cohort A included 6 patients (3 Melanoma and 3 TNBC): 3 completed full study regimen, and 3 were withdrawn due clinical progression. AEs included chills (4/6), fever (2/6), anemia (6/6), central line thrombosis (1/6) and pulmonary embolism (1/6) All were Grade 2 except G3 anemia (1/6). There were no treatment related SAE's. sTNF-Rs were significantly reduced, followed by enhanced detection of TNFa, and IFNg in some cases. In 2 patients, CD8 counts and PD-1 and PD-L1 expression were increased. Congruently, blood mass cytometry showed reduction in Treg subsets and differential increase of CD8 subsets following treatment. **Conclusions:** The use of LW-02 in combination with Terumo BCT Spectra Optia Apheresis System is safe and efficient in the removal of sTNF-Rs. Subsequent immunoassay analyses indicated inflammatory response which may facilitate effects of immunotherapy, yet to be investigated in cohort B.

The 31-gene expression profile test can improve sentinel lymph node positivity likelihood prediction

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Sentinel lymph node biopsy (SLNB) assesses clinically occult melanoma metastasis to the SLN. Guidelines recommend avoiding or offering SLNB if the estimated likelihood of a positive SLN is <5% or >10%, respectively. As more than 80% of SLNBs are negative, improvements are needed for the identification of which patients should undergo SLNB. The 31-gene expression profile (31-GEP) stratifies patients into low (Class1A), intermediate (Class1B/2A), and high-risk (Class2B) groups. Previous multi-center studies have shown that, regardless of age, patients with T1-T2 tumors and a Class 1A 31-GEP result had a <5% likelihood of a positive SLN and could be considered to forgo SLNB. We analyzed data from all available patients >18yo with a successful 31-GEP test and SLNB from a single surgical center from 2015-2021 (n=156). The overall SLN positivity was 20.5%. Patients with a Class 1A result were significantly less likely to have a positive SLN (1.7%, 1/58) than those with a non-Class 1A result (31.6%, 31/98, P<.001). Age is a known factor in SLN positivity and patients with T1-T2 tumors over 55 years old (n=66) had an overall SLN positivity of 6.0%. Patients with a Class 1A result were significantly less likely to have a positive SLN (0%, 0/40) than those with a non-Class 1A result (15.4%,4/26, P=.021). Integrating the 31-GEP with clinicopathologic factors can significantly improve SLN positivity risk estimates, thus focusing surgical costs on patients with a higher likelihood of SLN positivity while reducing costs and surgical risks.

Response to Talimogene laherparepvec (TVEC) oncolytic virus therapy in patients with advanced melanoma – a single institution case series
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Talimogene laherparepvec (TVEC) is an oncolytic herpes virus used as intraslesional therapy in patients with unresectable stage IIIB - IV melanoma. TVEC functions by replicating within cancer cells and increasing local production of GM-CSF, which subsequently activates anticancer immune response locally at sites of injection and remotely in the body. Multiple large-scale randomized control trials support TVEC efficacy when used as monotherapy and when used in combination with other antineoplastic agents. Here, we report a case series of eleven patients with stage IIIB to IV melanoma treated with TVEC. All patients had clinically unresectable tumors and failed systemic therapies prior to initiation of TVEC. Four patients had excellent partial or complete responses at both injected and non-injected sites. Two patients had minimal to no response to TVEC at the time of the intratumoral therapy. However, these two patients developed striking clinical clearance of cutaneous
disease upon treatment with subsequent checkpoint inhibitor therapy and remain without progression. Two patients received combined therapy with TVEC and experienced adverse events. Four patients had no response to TVEC therapy. Our series highlights the variable response to TVEC in cases of advanced melanoma.

Predictors of sentinel lymph node metastasis in patients with thin melanoma: a multi-institutional collaborative
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Consideration of sentinel lymph node biopsy (SLNB) is recommended for patients with T1b (Breslow depth 0.8-1mm or <0.8mm with ulceration) and T1a (<0.8mm without ulceration) melanomas with high-risk features. Still, even in this group, the proportion of patients with actionable results is very low. We aimed to identify clinicopathologic factors predicting SLNB positivity by examining a multi-institutional population.

Data were extracted on adult patients with thin (T1) cutaneous melanoma who underwent SLNB between 2005-2018 in 5 tertiary referral centers in Europe and Canada. Univariable and multivariable logistic regression analyses were performed to identify predictors of SLNB positivity.

676 patients were analyzed. The median age was 56 years and 54% were female. Most patients had some high-risk feature: Breslow thickness 0.8-1mm in 78%, ulceration in 8%, mitotic rate >1 (per mm²) in 43%, Clark’s level ≥4 in 34%, lymphovascular invasion in 1%, nodular histology in 3%, and absence of tumor infiltrating lymphocytes in 14%. 53 patients (8%) had a positive SLNB. Breslow depth and mitotic rate independently predicted SLNB positivity. The odds of positive SLNB increased by 50% for each 0.1mm increase in Breslow depth past 0.74mm (OR=1.50, 95% confidence interval: 1.05-2.13) and by 22% for each mitosis per mm² (OR=1.22 (1.06-1.41)). Patients who had one excised node (versus ≥2) were 3 times less likely to have a positive SLNB [3.6% versus 9.6%, OR=2.9 (1.3-7.7)].

Our international multi-institutional data confirm that Breslow thickness and mitotic rate remain independent predictors of SLNB positivity in patients with T1 cutaneous melanoma. Even with these refinements, the number needed to diagnose is 13:1 in this already highly selected population, indicating that more work is required to identify accurate predictors of sentinel node positivity.

Whole-genome CRISPR screen identifies modulators of anti-tumor immunity in murine melanoma
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The revolution of immune-based therapies has radically changed the way melanoma is treated, opening treatment paradigms leading to extended survival for patients with advanced disease. However, most cancer patients do not respond to immunotherapies, and there remains much to understand about how the immune system responds to cancer, and to identify additional opportunities to enhance these responses.

We aimed to identify tumor-intrinsic targets for augmenting anti-tumor immunity by performing a whole-genome CRISPR screen in murine melanoma tumors. Briefly, we infected YUMMER-G cells with a sgRNA library and harvested cells from plates grown in culture, or from tumors grafted into WT or Rag-/- mice. After gDNA isolation and PCR amplification, we sequenced guides to identify sgRNAs present following selection in each condition. We compared guides found in in vivo tumors to those from cells grown in culture to control for guides that are required for overall cell
survival. Furthermore, we compared tumors from wild-type hosts with tumors from Rag-/- hosts, to identify factors that make tumors particularly susceptible to adaptive immunity.

Factors comprising several major pathways dropped out of the wild-type arm of the in vivo screen, indicating that they are required for tumor cell survival in an immune-competent microenvironment. Pathways in this category included antigen presentation, interferon-gamma signaling, and H3K9-trimethylation; further studies are underway to clarify the mechanisms by which these factors are responsible for tumor cell resistance to immune attack, and whether they represent therapeutically actionable targets.

Genomics for Histologically Indistinguishable Tumors: A Case of Malignant Melanotic Nerve Sheath Tumor
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Appropriate identification of malignancy is the first step towards defining treatment plans and prognostication. Here, we describe a rare case of Malignant Melanotic Nerve Sheath Tumor (MMNST), which is histologically indistinguishable from metastatic melanoma (MM). Briefly, a 66-year-old gentleman presented for treatment recommendations for MM of unknown primary with a single site of disease in the sacrum. Despite the radiographically impressive 4.6cm lesion causing sacral canal narrowing and encasement of cauda equina nerve roots, the lesion was incidentally found, and he was largely asymptomatic. On pathological re-review, the pigmented MART1/SOX10/S100+ spindle cell neoplasm was felt consistent with MM. Molecular testing revealed low tumor mutational burden, PD-L1 negative, BRAF/NRAS/cKIT wild-type. He started standard ipilimumab+nivolumab for MM, complicated by Grade I-II colitis, hepatitis, thyroiditis, and pneumonitis. Restaging studies after 2 cycles revealed stable disease; repeat tumor board review with genomic medicine and pathology highlighted an inactivating frameshift PRKAR1A mutation [c531delT] identified on molecular testing. Interrogation of COSMIC revealed that this mutation has not been reported in melanoma. Germline inactivating PRKAR1A mutations have been reported in the context of Carney Complex and somatically in neuroectodermal cancers including nerve sheath tumors. Given the mutational profile and his clinical presentation (indolent, asymptomatic presentation with a single focus of spinal disease), the diagnosis was revised to MMNST of which the recommended treatment course is definitive radiation. This case illustrates the critical roles that genomic analysis, clinical suspicion, and multidisciplinary review play in the diagnosis and treatment of a rare cancer that is histologically indistinct from metastatic melanoma.

Genetic tools for the stable overexpression of circular RNAs in melanoma
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Circular RNAs (circRNAs) are a novel class of RNA molecules characterized by a circular structure, meaning they lack 3’ end and 5’ ends, polyadenylated tails, and 5’ CAPs. circRNAs are particularly stable molecules, with longer half-lives than the corresponding linear transcripts because they are more resistant to destabilization by miRNAs and exonucleases. Many recent studies now indicate that circRNAs have important roles in many biological processes, including in diseases such as cancer. Through RNA-seq analysis we identified 42 differentially expressed (DE) circRNAs in melanoma, 21 of which were validated through qRT-PCR and Sanger sequencing. Analyzing the circRNAs expression in Hermes1, Hermes2 and Hermes3 melanocytes cell lines, Hermes1BRAF mutated cell line and WM164, WM793 and 1205Lu melanoma cell lines, we found that several of the DE circRNAs are upregulated in melanoma compared to melanocytes. Thus, we hypothesize that these candidate circRNAs have pro-tumorigenic functions. However, the lack of sophisticated tools for stable circRNA overexpression is the main hurdle to a comprehensive characterization. To overcome this limitation, we developed and validated genetic tools for the stable overexpression of circRNAs in vitro and in various mouse models. We demonstrated that circRNA can be stably overexpressed in cultured cells via transposons. We further showed that circRNA transposons can be delivered to mouse livers via hydrodynamic tail vein injection, resulting in ectopic circRNA expression in a hepatocellular carcinoma mouse model. Furthermore, we generated genetically engineered mice harboring circRNA expression constructs to enable constitutive, global
circRNA overexpression as well as inducible circRNA expression directed specifically to melanocytes in a melanoma mouse model. We will use these tools to functionally analyze our circRNA candidates in vitro and in vivo.

DAY101-102a: A Phase 2 Subprotocol of DAY101 Monotherapy for Patients with Recurrent, Progressive, or Refractory Solid Tumors with an Activating BRAF Gene Fusion

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DAY101 is an oral, highly selective, CNS-penetrant, small-molecule, type II pan-RAF kinase inhibitor. DAY101 targets both monomeric and dimeric forms of RAF with demonstrated preclinical activity in BRAF V600E, wild-type BRAF, CRAF, and KIAA1549:BRAF fusion models. BRAF kinase activity is inhibited by DAY101 without the paradoxical activation of the MAPK pathway reported for type I BRAF inhibitors. Single-agent activity has been observed in patients with BRAF- and NRAS-mutant melanoma and low-grade glioma. DAY101-102 (NCT04985604) is an open-label, multi-center, international, phase 1b/2a study structured as a master protocol evaluating the safety and efficacy of DAY101 as monotherapy or in combination with other targeted therapies in recurrent, progressive, or refractory solid tumors harboring MAPK pathway aberrations. The first sub-study (DAY101-202a) is currently enrolling patients ≥12 years of age with recurrent or progressive solid tumors with a histologically confirmed, activating BRAF fusion to receive DAY101 as monotherapy. For eligibility, brain metastases if present, must be stable for at least 2 radiographic images taken ≥4 weeks apart. Exclusion criteria include known presence of a concurrent activating mutation and prior therapy with MEK and/or RAF inhibitors.

DAY101 will be orally administered once weekly, on days 1, 8, 15, and 22 at 600 mg for adults and 420 mg/m² for patients aged 12 to <18 years. Cycles will repeat every 28 days until disease progression or unacceptable toxicity. The primary endpoint is the overall response rate as assessed by investigators. Secondary endpoints include assessment of DAY101 safety and tolerability, duration of response, and time to response. Response assessment will be performed according to RECIST v1.1 for solid tumors or according to RANO for CNS tumors.

A computational pathology approach for segmentation of skin melanoma regression.

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Primary skin melanoma accounts for approximately 3% of all skin malignancies but contributes to most skin cancer-related deaths, being the Breslow thickness an essential histological parameter for outcome prediction. However, in the case of an early invasive melanoma with signs of regression, which develops through 3 sequential phases: “early (inflammatory),” “intermediate,” and “late” phases, with the progressive disappearance of the tumor replaced by fibrotic tissue, the assessable thickness does not reflect the actual degree of invasion of the lesion. Thus, the absolute prognostic significance of regression and its predictive role on sentinel node status is still widely debated, and the histopathological quantification of regression still represents a problem and indeed an unsolved point in managing cutaneous melanoma.

This work aimed to explore an Artificial Intelligence approach in processing histopathological images of regressed cutaneous melanomas and training a neural network to define a deep learning algorithm that can recognize the regression areas in histological preparations highlighting and quantizing them by color maps. Our analysis was carried on archived histopathological images of skin melanomas at various stages of regression, digitalized at 40x with an Aperio AT2 digital slide scanner, and manually annotated by expert pathologists. We based the training of UNet network on 51 Whole Slide Images for a full account of 22,464 tiles and validated the algorithm on 10 Whole Slide Images for a full
account of 816 tiles, achieving on the validation set a mIoU of 0.64 and an accuracy of 89%. Our algorithm is proposed as a valid computer aided diagnostic tool in the definition and quantization of the regression phenomenon in cutaneous melanomas, in order to facilitate the pathologist's work and normalize this evaluation by reducing elements of individual variability.

Preliminary results from the skin cancer cohorts from an ongoing multi-cohort phase 2 clinical trial of RP1 combined with nivolumab (IGNYTE)
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Background: RP1 is an enhanced potency oncolytic version of HSV1 that expresses human GM-CSF and the fusogenic protein GALV-GP R-. Preliminary data from the phase 1/2 trial (NCT03767348) demonstrated tolerability and anti-tumor activity for RP1 + nivolumab (nivo). Here, we present updated results from the melanoma and non-melanoma skin cancers (NMSC) cohorts from the trial. Methods: RP1 is administered via intratumoral injection Q2W, ≤10 mL/visit, first alone at a dose of 10⁶ PFU/mL and then starting with the 2nd dose at 10⁷ PFU/mL in combination with nivo (240 mg IV Q2W for 4 months then 480 mg IV Q4W up to 2 yrs) for up to 8 doses, with the option to re-initiate RP-1 if protocol specified criteria are met. Eligible patients (pts) must have at least one measurable & injectable tumor of ≥1 cm, ECOG 0-1, and no prior oncolytic therapy. Results: As of June 2021, the combination continued to be generally well tolerated with no new safety signals identified. The objective response rate (ORR) in PD1 naïve cutaneous melanoma pts (n=8) was 62.5% and 31.3% in anti-PD1 failed (n=16). The ORR in CSCC (n=15) was 60% including 46.6% durable CRs. ORR in BCC (n=4), MCC (n=4) and angiosarcoma (n=5) were 25%, 75% and 60% respectively. Responses have been observed to be durable and to deepen over time. Conclusions: RP1 in combination with nivo provides durable anti-tumor activity in pts with skin cancers, including CSCC, and anti-PD1/anti-CTLA-4 failed melanoma. Based on this data, the clinical trial has been expanded to include a registration directed cohort of pts who have anti-PD1 failed cutaneous melanoma (125 pts) and pts with anti-PD1 failed non-small cell lung cancer and NMSC.

Overcoming resistance to immunotherapy with BH3 mimetics
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Despite the remarkable improvements of immune checkpoint inhibitors in melanoma, 40% of patients will not respond to anti-PD1-based immunotherapies and 25% of responding patients will eventually progress within 24 months. Novel combination therapies are urgently sought to improve response rates and circumvent resistance. Defects in the apoptotic cell death machinery can promote tumourigenesis and impair tumour responses to anticancer therapy. The therapeutic potential of BH3-mimetic drugs targeting anti-apoptotic BCL-2 family proteins (BCL-2, BCL-XL and MCL-1) have been reported in different tumour types and are now
progressing to the clinic.

We have previously confirmed that IFNγ signalling was intact in 22/23 short-term melanoma cells derived from tumours progressing on anti-PD1 (PD1 PROGs), by demonstrating transcriptome response to exogenous IFNγ exposure. However, only 8/23 (28%) PD1 PROG cell lines responded to IFNγ treatment by undergoing marked (>15%) apoptosis.

In this study, we found that the IFNγ stimulated the transcript and protein expression of anti-apoptotic MCL1 and pro-apoptotic BH-3 only NOXA. MCL1 was complexed preferentially with NOXA at the expense of binding to other pro-apoptotic proteins BIM and BAK. To explore whether these alterations primed cells for apoptosis, we found that BH3 mimetics S63845 (MCL1 inhibitor) or navitoclax (BCL-2/BCL-XL/BCL-W inhibitor) potently restored IFNγ-mediated apoptosis in 7/8 (88%) PD1 PROG melanoma cells. Interestingly, navitoclax-induced cell death appeared to reflect the levels of IFNγ induced NOXA protein. Accordingly, NOXA knockout significantly diminished the sensitivity of cells to navitoclax, but not to S63845 in IFNγ-primed cells. We also confirmed the impact of BH3 mimetics in co-culture models of PD1 PROGs and paired autologous T cells (mixed in 1:1 ratio), with dramatically enhanced melanoma cell killing using S63845 (41%) and navitoclax (28%).

Increasing responsiveness to immunotherapy by inhibition of the MAPK pathway
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Immunotherapy has revolutionized the treatment of advanced melanoma by dramatically increasing the 5-years survival rate and thereby becoming a new therapeutic pillar. Nevertheless, the majority of the patients ultimately do not respond or fully benefit from immunotherapy. Delineation of these mechanisms is therefore of utmost importance, with direct clinical implications. A recent paper from our group, where proteomics analysis was performed on melanoma tumors obtained from responders and non-responders to immunotherapy, revealed that aerobic metabolism was associated with response. Here, we aim to understand the underlying mechanisms by pinpointing relevant pathways using FDA-approved drugs, which can be implemented immediately in clinical utility. Our preliminary results demonstrate that exposing melanoma cells to MAPK inhibitors, augments MHC and PDL-1 expression, which are known as crucial immune molecules. Functional experiments demonstrated an increase in killing rate and T cell activation when melanoma cells were pre-exposed to MAPKi. In agreement with these results, RNA sequencing showed that the IFN pathway and the lipid metabolism were positively enriched, while the glycolysis, hypoxia and cell cycle were de-enriched. Subsequently, MAPKi led to a substantial increase in the protein level of IFNγ response proteins. Inhibition of mitochondrial activity confirmed that the activation of the IFNγ pathway by MAPKi is driven by the metabolic effect. The new findings provide insights on the underlying mechanisms linking aerobic metabolism and response to immunotherapy, and pave the way to increasing the clinical benefits from the existing therapies.

First in Human Early Feasibility Study of Tumor-Treating Fields (TTF) in Combination with Nivolumab and Ipilimumab in Patients (pts) with Metastatic Uveal Melanoma (UM)
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Background: Uveal Melanoma is a rare type of melanoma that originates in the eye. Despite effective local therapies for primary tumors, around 50% of patients will develop metastatic disease (mUM), predominately to the liver. Few effective treatment options are available for pts with mUM. TTF is a new treatment modality that is FDA approved for the treatment of glioblastoma and mesothelioma. Pre-clinical data suggests that TTF may cause immunogenic cell death and may be synergistic with CPI. Therefore, we initiated a first in human feasibility study of TTF in combination with nivolumab and ipilimumab (N/I) for patients with mUM.

Methods: TTF-UM is an early feasibility study of TTF in combination with N/I in pts with mUM. 10 patients with predominant liver involvement (50% or > disease volume in the liver) and who are naïve to
anti-CTLA4 therapy, or have not received anti-CTLA-4 in 90 days, will be treated in the study; all other prior therapies are allowed. Patients will receive ipilimumab (3mg/kg) and nivolumab (1 mg/kg) for 4 cycles followed by maintenance nivolumab (480 mg) every 4 weeks, up to one year of therapy. TTF will be directed over liver metastases and will start following the first infusion of N/I. TTF will continue daily for at least 18 hours a day until confirmed disease progression. Imaging will be done prior to starting treatment and every 12 weeks. Pretreatment and pre-cycle 2 biopsies will be collected on at least 5 patients for correlative studies. Blood will be collected every cycle for ctDNA, cytokine assays, and other correlative studies. If multiple responses are seen, the study may be amended to include more patients, allowing for statistical comparison for the overall response rate of TTF in combination with N/I vs the reported response rates of 11-18% for N/I alone.

**Real-world outcomes of patients with resected stage IIIA melanoma treated with adjuvant nivolumab or observation**

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Nivolumab (NIVO) is approved in the US and other countries for the adjuvant treatment of resected stage III–IV melanoma based on results of the phase 3 CheckMate 238 trial, which included only a limited number of patients (pts) with stage IIIA disease (AJCC v8). This retrospective, observational study used the nationwide Flatiron Health electronic health record (EHR)-derived de-identified database to describe characteristics, treatment patterns, and outcomes in pts with resected stage IIIA melanoma (AJCC v8) selected from February 1, 2016 to April 30, 2021. Median recurrence-free survival (RFS) and overall survival (OS) were calculated from the date of first sentinel lymph node (SLN) biopsy on/after initial diagnosis. This study included 183 pts who were either treated with adjuvant NIVO (n=71) or followed with observation (n=112). In the adjuvant NIVO and observation cohorts, respectively, median age was 59.0 and 60.0 y, most pts were male (51% and 55%) and white (83% and 84%), and the median study follow-up period (range) was 18.8 mo (1.6–42.0) and 25.8 mo (0.03–60.0). SLN tumor burden data were not collected. Median duration of NIVO treatment was 12.0 mo. Median RFS and OS were not reached in either cohort. RFS was numerically higher in the adjuvant NIVO cohort (12-mo RFS, 97%; 18-mo RFS, 94%) than in the observation cohort (12-mo RFS, 94%; 18-mo RFS, 92%). No deaths were reported in the adjuvant NIVO cohort (12- and 18-mo OS, 100%) and 8 deaths were noted in the observation cohort (12-mo OS, 99%; 18-mo OS, 97%). Results of this real-world study appear to confirm that pts with resected stage IIIA melanoma (AJCC v8) have a favorable prognosis. Treatment with adjuvant NIVO may provide benefit over observation in pts with stage IIIA disease though further follow-up is needed.

**LUMINOS-102: PVSRIPo with or without immune checkpoint blockade in unresectable anti-PD-1 refractory melanoma**

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PVSRIPo, a novel viral immunotherapy, infects solid tumors and antigen-presenting cells (APCs) via CD155. Infection is lethal in malignant cells, but nonlethal infection of local APCs yields type I/III interferon-dominant induction with subsequent anti-tumor T-cell priming and activation. A phase 1 dose-escalation study (Beasley 2021, JITC) showed PVSRIPo was well tolerated and demonstrated anti-tumor activity in both injected and noninjected lesions in patients (pts) with αPD-1–refractory melanoma. In preclinical models, PVSRIPo-mediated immune activation upregulated the PD-1/L1 pathway, leading to greater anti-tumor response with PVSRIPo and αPD-1 combination therapy. Taken together, these data suggest PVSRIPo is active in αPD-1–refractory melanoma and that PVSRIPo±αPD-1 therapy warrants further clinical investigation.

LUMINOS-102 (NCT04577807) is an ongoing...
multi-center, open-label, randomized phase 2 study investigating the efficacy, safety, and pharmacodynamic effects in the tumor microenvironment following PVSRIPO±αPD-1 therapy in pts with αPD-1–resistant, unresectable, non-uveal melanoma. A safety run-in cohort of 6 pts to characterize PVSRIPO injection in ≤6 lesions/cycle or maximum dose of 6x10^8 TCID<sub>50</sub> has fully enrolled; 1:1 randomization of 50 participants to receive PVSRIPO (Arm 1) or PVSRIPO+αPD-1 (Arm 2) is ongoing. Stratification factors include time since last αPD-1 dose and baseline LDH level. Crossover (Arm 1 to 2) is allowed upon confirmed progression, SD at 26 weeks, or PR ≥6 mos. Primary endpoints include safety, ORR per RECIST 1.1, and change from baseline in CD8+ tumor-infiltrating lymphocytes and PD-L1 expression. Key secondary endpoints include DOR, DCR, PFS, and OS. Exploratory endpoints include ORR via iRECIST and additional biomarker analysis evaluating the immune activation phenotype of PVSRIPO.

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Regulation of the Selenocysteine Stress Response as an Antioxidant Mechanism in Cancer Metastasis
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Metastasis is the leading cause of death from melanoma and its suppression is an urgent therapeutic need. Our laboratory has shown that metastasizing cells undergo high levels of oxidative stress compared to the primary tumor. Some of the major regulators of intracellular redox biology are selenoproteins – a family of 25 proteins that contain selenocysteine (Sec), the 21<sup>st</sup> amino acid. These proteins range from antioxidant enzymes (glutathione peroxidases, thioredoxin reductases) to ER protein folding chaperones (Selenoproteins S, K, M). Sec is structurally identical to cysteine; however, it contains a covalently bound selenium in place of sulfur. Unlike other amino acids, selenocysteine is synthesized on its own unique tRNA<sup>Sec</sup> and inserted an in-frame UGA stop codon. Two isoacceptors of tRNA<sup>Sec</sup> exist, differing only by a single 2’-O-ribose-methylation at the wobble uridine (Um34) on the anticodon loop. This modification is influenced by both selenium status and oxidative stress and it induces a conformational change of the tRNA. Um34 modification is unique to tRNA<sup>Sec</sup> and is thought to selectively regulate a subset of stress response selenoproteins, however this has not been fully characterized and the methyltransferase remains unidentified. In our PDX model of metastatic melanoma, we see increased levels of selenoproteins and associated biosynthesis machinery, in metastatic nodules compared to the primary tumor. Thus, we hypothesize that tRNA<sup>Sec</sup> Um34 modification is increased in metastasizing cells due to higher oxidative stress levels and that this modification increases cell survival by regulating stress-response selenoproteins. We have identified the enzyme responsible for Um34 methylation of tRNA<sup>Sec</sup> and are currently characterizing its role in metastatic melanoma. We aim to identify novel and targetable vulnerabilities that are specific to metastatic disease.

Real-world outcomes of nivolumab in adjuvant melanoma in Belgium and Luxembourg (PRESERV MEL)
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Based on the results of the CheckMate 238 registrational trial, nivolumab (NIVO) was the first PD-1 immune checkpoint blocking monoclonal antibody reimbursed in Belgium and Luxembourg as an adjuvant treatment for melanoma (MEL) patients (pts) with lymph node (LN) involvement or with metastatic disease who have undergone complete resection. The objectives of this observational study, PRESERV MEL, are to describe the baseline characteristics of pts initiating adjuvant NIVO in routine practice, treatment disposition, treatment effectiveness and safety.

A total of 150 pts receiving ≥ 1 dose of NIVO were enrolled between Jan 2019 and Dec 2020 at 15 hospitals in Belgium and Luxembourg. Kaplan-Meier methods estimated effectiveness via relapse-free survival (RFS). Safety was described via reporting treatment-related adverse events (TRAEs).

Median follow-up time was 9.2 months. Median age was 60 years (range: 29-85), 19%/28%/33%/4%/11%/5% pts had stage IIIA/IIIB/IIIC/IIID/IV/other or missing disease, respectively. 56 (37%) had BRAF V600-mutant MEL, 74 (49%) had clinically occult only LN
involvement, and 53 (35%) had complete LN dissection. Median time from resection to start of NIVO was 1.3 months. 56% of pts had discontinued NIVO at the time of this analysis. 22%/18%/10%/1% pts discontinued NIVO due to treatment completion/AEs/recurrent disease/pt decision, respectively. 1-year RFS was 83% (95% CI: 77-89). TRAEs occurred in 81% of pts; 13% had at least one grade ≥ 3 TRAE. There were no treatment-related deaths.

Baseline pt characteristics (apart from stage IIIA pts per AJCC v8 being treated in the real world), effectiveness, and safety of adjuvant NIVO were largely consistent with those observed in CheckMate 238. This study demonstrates the real-world effectiveness of NIVO as an approved adjuvant treatment for pts with MEL.

Elucidating the role of REST in ARID2-deficient melanoma
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Sequencing studies in melanoma have unveiled frequent mutations in ARID2, a subunit specific to the SWI/SNF PBAF chromatin remodeling complex. Recurring loss-of-function mutations point towards a tumor suppressive role. Interestingly, by mining TCGA, we found that genes upregulated in ARID2 mutant melanoma patients show enrichment of REST targets. REST is a transcription factor that primarily functions to repress neuronal genes in non-neuronal cells and is important for proper melanocyte development. Previous research demonstrated that BRG1, an ATPase for all SWI/SNF complexes, facilitates binding of REST to chromatin; however, the relationship between the PBAF complex specifically and REST-mediated gene repression remains unexplored. Intriguingly, we identified a preferential association between PBAF-specific genomic regions and the REST binding motif.

Molecular and functional characterization of melanocyte subpopulations in human epidermis based on single-cell RNA sequencing
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The biological and molecular mechanisms are largely unknown that underpin malignant transformation of normal melanocytes to melanomas. In part, this is because understanding is limited of normal human melanocyte homeostasis, and of the manner in which melanocytes respond to oncogenic insults such as ultraviolet radiation (UVR). This is particularly true for interfollicular epidermal melanocytes that have the highest levels of UVR exposure and impedes development of strategies for active, targeted prevention of melanoma formation. To address this, we evaluated epidermal melanocytes transcriptionally, phenotypically and functionally after isolating them from human hairy skin. Single cell RNAseq identified transcriptionally distinct melanocyte subpopulations that RNA velocity and pseudotime analysis suggested represented different states of melanocytic differentiation. One identity, which was characterized by upregulation of genes linked to ribosome biogenesis and of neurotrophic receptor tyrosine kinase 2 (NTRK2), showed progenitor-like molecular characteristics, a distinct differentiation trajectory, and anatomical distribution throughout follicular and interfollicular epidermal
compartments. NTRK2+ melanocytes purified via flow cytometry showed increased survival and clonogenicity after UVR exposure in primary cell culture without feeder cells, as well as in ex vivo whole skin explant culture, compared to NTRK2-melanocytes. Inhibition of ribosome biogenesis or NTRK2 activity resulted in suppression of melanocytes with the identity. We thus report the discovery of human epidermal melanocyte subpopulations, of a putative melanocytic cellular hierarchy within human epidermis, and of a melanocyte subpopulation with increased proliferative response to UVR exposure that is thus a candidate and therapeutically targetable cell of origin of melanoma.

*Helicobacter pylori* is negatively associated with immune checkpoint inhibitors response in melanoma patients, independent from the gut microbiome composition

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Fecal microbiome diversity and bacterial composition have emerged as biomarkers of immune checkpoint inhibitors (ICI) response in patients with advanced melanoma. However, the impact of gastric bacteria, such as *Helicobacter pylori* (HP) on ICI activity is unclear. HP was recently shown to dampen CD8+ T cells cytotoxic activity in mice and its presence correlated with shorter overall survival in patients with non-small cell lung cancer. We aimed to determine the impact of HP seropositivity on clinical outcomes and microbiome composition in patients with melanoma treated with ICI.

We retrospectively evaluated 35 patients with advanced melanoma on ICI treatment and HP IgG was quantified in the serum, and stratified them into HP Positive (Pos) or Negative (Neg) groups. Matching fecal samples were obtained and microbiome metagenomics analysis was performed. Progression-free survival (PFS) was evaluated according to RECIST 1.1.

Nine (26%) patients were HP Pos and baseline clinical characteristics were well balanced between both groups. The HP Pos group had a significantly shorter PFS compared to the HP Neg group (10.2 mo vs 15.4 mo, p=0.01), and 75% and 95% of patients were still alive in the HP Pos vs Neg groups, respectively. When considering the entire cohort, responder patients' microbiome composition was associated with higher alpha diversity and enrichment of *Ruminococcus obeum, Alisitpes indistinctus*, and *Ruminococcus sp JC304*. However, the alpha diversity was not different between HP groups and none of the known beneficial bacteria were increased in the HP Pos patients.

Altogether, our results suggest that HP might play an important role in ICI efficacy, independent of the fecal microbiome composition. Further studies are needed to confirm this observation in patients with melanoma treated with ICI.

**What does not kill it makes it stronger:** Acquired resistance to anti-MAPK targeted therapy confers an immune-evasive tumor microenvironment and cross-resistance to immunotherapy

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Targeted therapies and immunotherapies represent main pillars of cancer treatment, yet how they shape tumours during treatment response and resistance and thereby influence subsequent therapeutic responses is poorly understood. Here, we show in melanoma patients and mouse models that when tumours relapse on targeted MAPK pathway inhibitors, they are cross-resistant to immunotherapies, despite their entirely different mode of action. We find that cross-resistance is mediated via a cancer cell-instructed, immune-suppressive tumour microenvironment that lacks functional CD103+ dendritic cells, precluding an effective T cell response. Restoration of CD103+ dendritic cell numbers and functionality can re-sensitize cross-resistant tumours to immunotherapy. Using our lineage tracing method CaTCH, which allows the retrospective isolation of founding clones prior to evolutionary selection, we demonstrate that cross-resistance is acquired during MAPKi treatment. Cross-resistance does not arise from the selective pressure of an immune response during the evolution of resistance, paradoxically it results from the MAPK pathway, which is not only reactivated during the formation of targeted therapy resistance but has gained increased transcriptional output driving immune evasion. Our work provides mechanistic evidence for cross-resistance between unrelated therapies and a scientific rationale for treating patients with immunotherapy before they acquire resistance to targeted therapy.

MOUSE MODELS TO ADDRESS THE ROLE OF THE GROWTH FACTOR MIDKINE IN MELANOMA

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Midkine (MDK) is a growth factor highly expressed at embryonic states that becomes downregulated in most adult tissues unless pathogenic situations such as cancer. Our group has recently shown that MDK is secreted by aggressive melanoma cells, and that this secretion defines poor patient prognosis and resistance to immune checkpoint blockade. We have now generated several mouse models to address the role of MDK in melanoma initiation, progression and response to therapy. Here we will present ongoing studies with (i) a reporter strain whereby the endogenous expression of murine MDK (Mdk) is coupled to bioluminescent and fluorescent proteins.

Background
Primary malignant melanoma of esophagus (PMME) is extremely rare and aggressive disease, which comprises only 0.5% of all noncutaneous melanomas. Since it is usually recognized by dysphagia and epigastric discomfort, PPME is often diagnosed in the advanced disease and prognosis is poor. In this study, we aim to investigate the treatment outcome and efficacy of immune check point inhibitor (ICI) in 51 patients with PPME.

Patients and Methods
A total of 51 patients with PMME treated in our institution between 2010 and 2020 were retrospectively analyzed and their clinicopathological parameters including treatment course and overall survival were investigated.

Results
Male/female ratio was 14/37. 17 patients had local disease, 15 patients had nodal disease and 9 patients had metastatic disease at the time of initial diagnosis. As for tumor location, 7 patients were upper esophagus, and 36 patients were middle and/or lower. The mean age at initial diagnosis was 67.3 years. In the analysis of all 51 cases, 5-years overall survival (OS) rate and median OS were 47.3% and 55.0 months. Nineteen patients (37%) died during follow up period. As for survival of each stage, median OS was not reached for local disease, 24.9 months for regional and 34.3 months for metastatic disease. There were no BRAF mutant cases. Thirty three of 51 cases were treated with ICI. The objective response rate was 21.2% and disease control rate was 33.0% (CR: 3, PR: 4, SD: 4 and PD: 21 cases).

Conclusion
In this study, the prognosis of PMME was better than previously reported. Recently, treatment of malignant melanoma has changed dramatically with the immune checkpoint inhibitors (ICI) and targeted therapy. Although response rate of ICI in PMME is lower than that of cutaneous melanoma, some patients with PPME benefit from ICI. We aim to further observe whether these new drugs improve prognosis of PMME.
(Luciferase and dTomato); (ii) an inducible gain of function model to follow the impact of Mdk in the context of activation of oncogenic Braf and loss of the tumor suppressor Pten; and (iii) Mdk deficient mice to further define the specific requirement of this protein for tumor development. Downstream effectors of MDK are being validated using computational, histological and functional studies in patient biopsies. We expect these animal models will serve as a platform for gene discovery and for pharmacological analyses of novel anticancer agents.

Safety and Efficacy of Retreatment with Ipilimumab and Nivolumab in Patients with Advanced Solid Tumors
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Background: Patients (pts) who respond to single-agent anti-CTLA-4 for a duration greater than 6 months have a high chance of repeat clinical benefit. It is not known if pts retreated with anti-CTLA-4/anti-PD-1 combination therapy have similar benefit.

Methods: We retrospectively analyzed pts with advanced solid tumors treated with 2 separate courses of ipi/nivo between March 2011 and December 2020 at UCSD. Data regarding clinical history, response, and toxicity were collected.

Results: 22 pts were included. Median age was 61 years (32-79) and 82% were male. 15 pts (68%) had advanced melanoma including 2 choroidal and 1 mucosal. 3 pts had HPV+ oropharyngeal squamous cell cancer. 2 pts each had small cell lung cancer and metastatic colorectal cancer. 8 pts (36%) received single-agent immune checkpoint inhibition (5 with anti-PD-1, 3 with anti-CTLA-4) prior to first course of ipi/nivo. 15 pts (68%) completed induction with 4 cycles of ipi3/nivo1, while 3 pts (14%) completed 2-3 cycles of ipi3/nivo1, and 4 pts (18%) completed 2-4 cycles of ipi1/nivo3. Median time to retreatment with ipi/nivo was 7 months (1.5-40). Of the 15 pts who received induction with 4 cycles of ipi3/nivo1, 8 pts (53%) were retreated with 4 cycles of ipi3/nivo1 with stable disease seen in 6 pts (75%) and progression in 2 pts (25%). Despite irAE of any grade with induction, only 2 pts (25%) had Grade 2 irAE upon retreatment. Among 3 pts with cutaneous melanoma still alive, 1 pt continues on maintenance nivolumab since retreatment in 6/2020 and 2 pts remain off therapy since retreatment in 2019, including 1 pt treated 3 separate times with 4 cycles of ipi3/nivo1.

Conclusion: Retreatment with ipi/nivo was tolerable and demonstrated moderate disease control in this small cohort. This strategy may be considered for pts who are not candidates for targeted therapy or clinical trials.

SGK compensates for AKT inhibition and may represent a novel therapeutic target in BRAF-driven melanoma
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Despite the advent of novel therapies, the five year survival rate for Stage IV melanoma remains at only 15-20%. Such poor long-term survival highlights the critical need for new therapeutics to treat this recalcitrant disease. Two key signaling systems: 1. The RAF>MEK>ERK (MAPK) and; 2. The PI3'-kinase (PI3K)>AKT pathways are frequently co-activated and play a critical role in melanoma initiation and progression. Mutational activation of BRAF is detected in ~50% of all human melanomas. However, many patients with BRAF-mutated melanoma will display either primary or acquired resistance to pathway-targeted therapies. Dysregulated PI3K>AKT signaling is frequently observed in conjunction with BRAF mutations, with the most common alterations being silencing of PI3'-lipid phosphatases such as PTEN, or mutational activation or amplification of PI3'-kinase-a or the AKT1-3 protein kinases. However, despite compelling preclinical data and clinical trials, no inhibitors of either PI3K or AKT have been FDA approved for the treatment of advanced melanoma. We have demonstrated that, while pharmacological targeting of AKT is without effect, siRNA-mediated inhibition of AKT expression promotes melanoma cell death in vitro. Thus, there is a paradox regarding the role of AKT as a potential target in melanoma. To address we noted that, when AKT is inhibited by pharmacological agents, the expression of SGK1 (serum/glucocorticoid regulated kinase), is elevated and apparently serves to rescue melanoma cell proliferation and survival. Furthermore, a combination of AKT and SGK inhibitors induced a
marked decrease in the proliferation of BRAF-driven melanoma cells through effects on downstream mTORC signaling. These findings suggest that combinatorial inhibition of both AKT and SGK may represent a novel strategy for combination therapy for BRAF-driven melanoma.

**Carbonic Anhydrase IX expression as a predictor of resistance to immune checkpoint inhibitor therapy in melanoma**

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A hypoxic microenvironment is a common feature of solid tumours. Hypoxia alters the metabolism of cancer cells, inducing a shift from oxidative phosphorylation to glycolysis for cellular respiration. Interestingly, tumours may rely on glycolysis even in the presence of oxygen (aerobic glycolysis). We recently identified upregulation of genes associated with hypoxia, including carbonic anhydrase IX (CA9) among a population of patients with melanomas resistant to anti-PD-1 therapy. CA9 encodes a transmembrane protein that is upregulated by hypoxia inducible factor 1 alpha (HIF-1α) under hypoxic conditions to maintain cellular pH. Acidosis, a consequence of hypoxia, is a potent inhibitor of T cell effector function. Our findings implicated CA9 upregulation as a potential mechanism of resistance to anti-PD-1 monotherapy by promoting cell survival and contributing to a hypoxic and acidic tumour microenvironment.

This study aimed to further characterise the metabolic tumour microenvironment of non-responders and the potential utility of CA9 immunohistochemistry (IHC) as a predictive marker of resistance to immune checkpoint blockade (ICB) therapies.

Our cohort included 25 responders and 25 non-responders to ICB therapies. We used multiplex immunohistochemistry to evaluate the expression and distribution of markers of a hypoxic/acidic microenvironment (CA9 and HIF-1α) and glycolysis (GLUT1) in relation to proximity to vessels (CD34). Melanocytes and T cells were also identified using SOX10 and CD3 respectively. We hypothesise that tumours with increased expression of CA9 reflect an acidic/hypoxic environment that promotes resistance to ICB therapies, resulting in lower response rates and decreased progression-free survival in melanoma patients. Results are pending and will be presented at the conference.

**Potentiating Immunotherapy by Targeting PI3K in Metastatic Melanoma (MM) or other Advanced Solid Tumors (ST)**

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There is an unmet need to improve the efficacy of immune checkpoint blockade (ICB) in MM. We previously demonstrated that hyperactivation of PI3K signaling by PTEN loss in MM promotes resistance to ICB. Based on these findings we conducted a Ph I/II trial (NCT01458067) to determine the safety of GSK2636771 (PI3Kβi) + pembrolizumab (P) in patients (pts) with PTEN-loss ST, including PD-1 refractory MM. The recommended Phase II dose of GSK2636771 was 200 mg in combination with P as determined by the sponsor. Among 27 treated pts, the observed clinical benefit (CB) rate was 52%, including 3 pts with >1 year on therapy. Analysis of serum proteins (n=79) showed increased levels of CXCL10, PD-L2, IL18, CD27 & IL12p70 (FDR≤0.05) after treatment. On-treatment changes in VEGF (p<0.01) and CXCL5 (p=0.03) serum levels were significantly different between patients with and without CB. CyTOF analysis of blood identified reduced Tregs (p<0.01) and increased effector CD8+
T cells (p=0.04) with treatment. Consistent with our previous studies, GSK2636771 did not inhibit AKT phosphorylation in any tested (n=20) immune cell subsets. Analysis of tumor samples from the trial is ongoing. In parallel, pre-clinically we characterized PI3K isoforms by both genetic and pharmacological inhibition for effects on tumor development and T cell function. Activation of the PI3K pathway in T cells primarily depends on PI3Kα and PI3Kγ isoforms; tumor cells rely on PI3Kα and PI3Kβ. Inhibition in tumors of either PI3Kα or PI3Kβ sensitized response of PTEN-present (B16/MC38) and PTEN-absent tumors (DM4) to in vivo anti-PD-1. Collectively, our results suggest that targeting PI3Kα or PI3Kβ isoform inhibitors can be tolerable with ICB and may overcome immune resistance in subsets of cancer patients.

Phase 3 Randomized Trial Comparing Tebentafusp with Investigator’s Choice in First Line Metastatic Uveal Melanoma
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Background: Metastatic uveal melanoma (mUM) has a poor prognosis with a 1-yr OS rate of 52%. No systemic treatment has proven an OS benefit in randomized trials. Tebentafusp (tebe), a bispecific consisting of an affinity-enhanced T cell receptor fused to an anti-CD3 effector that can redirect T cells to target gp100+ cells, has shown promising activity in previously treated mUM pts. Here, we report the primary analysis of overall survival (OS) in the intention-to-treat population (ITT) of a Ph3 trial of tebe vs. investigator’s choice (IC) as first line (1L) therapy in pts with mUM [NCT03070392].

Materials and Methods: In this randomized, open-label, Ph3 trial, 1L HLA-A*02:01+ pts with mUM were randomized 2:1 to receive tebe or IC of pembrolizumab, ipilimumab or dacarbazine, stratified by LDH. The primary endpoint was OS, defined as the time from randomization to death from any cause. Dual primary objectives were to evaluate 1) OS in the ITT population by comparing all tebe-randomized pts to all IC-randomized pts; and 2) OS in tebentafusp-treated patients with rash during week 1 versus all IC-treated patients. Secondary endpoints included safety and RECIST-defined overall response rate (ORR), progression free survival (PFS) and disease control rate (DCR). Here we present the OS in the ITT population. The study was unblinded by an independent data monitoring committee at the first pre-specified interim analysis. Investigator-reported radiographic-based endpoints were not mature at the first interim analysis. This analysis was conducted on the first interim analysis (data extracted Nov 2020).

Results: 378 pts were randomized to tebe (252) or IC, including pembrolizumab (103), ipilimumab (15) or dacarbazine (7). Tebe significantly prolonged OS compared to IC (HR 0.51; 95% CI 0.36-0.71; P<0.0001) in the ITT population, with estimated 1-yr OS rate of 73.2% (95% CI 66.3-78.9) vs 57.5% (95% CI 47.0-66.6), respectively. The OS benefit of tebe was observed in pre-specified subgroups, including by stratification variable of LDH>ULN and versus pembrolizumab IC.

Most common TRAEs were skin-related (gp100+ melanocytes) or cytokine-mediated (T cell activation) and included pyrexia, pruritus, and rash. These AEs decreased in frequency and severity after the first 3-4 doses and were generally manageable with standard interventions. In the tebe arm, the rate of treatment discontinuation due to TRAEs was low (<4%), and
there were no treatment-related deaths.

**Conclusions:** In 1L treatment of mUM pts, tebe monotherapy significantly improved OS compared to IC; the first investigational therapy to improve OS in pts with mUM. Tebe had a predictable and manageable AE profile with a low rate of related discontinuation. Tebe is the first TCR therapeutic to demonstrate an OS benefit.


**In search of an origin: defining the molecular signature of isolated pulmonary melanoma.**

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It is controversial whether primary pulmonary melanoma exists. Candidate primary pulmonary melanomas may instead be Stage IV disease from an occult primary melanoma of cutaneous or non-cutaneous origin. This distinction is crucial as it influences staging, management strategies and outcome. This study aims to characterise the molecular features of isolated pulmonary melanoma, including whether such tumours harbour an ultraviolet (UV) damage DNA signature, to clarify the histogenesis and clinical behaviour. All patients presenting with an isolated pulmonary melanoma were identified through our institutional database (from 1961-2021), excluding patients with a history of melanoma of cutaneous, mucosal or uveal origin (n=58). Patients were selected for those with formalin-fixed paraffin embedded (FFPE) tissue available. DNA extraction and targeted next generation sequencing (NGS) were performed to characterise the mutational landscape and identify whether there was a dominance of C->T base substitutions at dipryrimidine sites characteristic of UV damage signature. A total of 8 patients with isolated pulmonary melanoma with FFPE available were subjected to targeted next generation sequencing alongside 8 controls of metastatic cutaneous melanoma involving the lung, and 4 rectal primary mucosal melanomas. The results of NGS will be presented, as well as interpretation of UV damage DNA signatures. Molecular features that enable elucidation of the origin of candidate primary pulmonary melanomas will enable accurate staging of patients who present with solitary organ involvement, resulting in appropriate management.

**Specific biochemical regulation of each BRAF or RAS mutant determines adaptation to treatments and instructs drug combinations**

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Targeting MAPK signaling has shown clinical activity, but it is frequently limited by adaptive drug resistance that prevents complete inhibition of signaling in the tumor. Increasing the drug dosage is usually associated with toxicities, thus there is a need for drugs and drug combinations with increased Therapeutic Index. Therefore, for most tumors a “3-drug” combinatorial strategy is predicted to be most effective: one drug targeting MAPK signaling directly, such as a MEK inhibitor (“pathway” inhibitor), one drug targeting mutated BRAF or RAS selectively in the tumor (“therapeutic index” inhibitor) and one drug targeting components of the feedback loop that is responsible for adaptive resistance (“feedback” inhibitor).

Recent work by us and others revealed that BRAF and RAS mutant oncoproteins vary dramatically in their regulation. However, a systematic interrogation of the relative dependence of each mutant on upstream signaling and the consequences on adaptive response to therapy has been lacking. We developed isogenic models to dissect biochemical regulation of the main BRAF and RAS mutants in their native state. Our results are consistent with a model by which “Class I” mutants, such as BRAF(V600X) and RAS(Q61X) are independent of upstream signaling...
and promote maximal levels of both MAPK activation and negative feedback. In contrast, “Class II” BRAF and RAS mutants depend on upstream signaling and promote modest levels of both MAPK activation and negative feedback. RAS(G13D) was unique, as it behaves as either Class I or Class II mutant according to NF1 status. Based on this model, we propose specific 3-drug combinations based on the specific BRAF or RAS oncprotein driving the tumor. Our data enable the development of a roadmap for the treatment of MAPK-driven tumors using drug combinations tailored to the specific driver oncprotein.

Dissecting the (entire) melanoma ecosystem one cell at the time
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Melanoma is notorious for its high degree of intratumoral heterogeneity (ITH) and plasticity, which nowadays can be studied by single cell genomics to an unprecedented resolution. Leveraging single cell RNA sequencing (scRNAseq), we aim to establish a comprehensive view of the transcriptomic landscape of the entire melanoma ecosystem in human and mouse. The scRNA-seq data was generated from 17 human metastatic lesions from treatment naïve, stage III, and IV melanoma patients. More than 30K cells were identified and analyzed using standard Seurat pipelines. To overcome the challenge of integration of malignant cells across patients posed by the high degree of inter-patient genetic variability, our analysis was further guided by deep scRNAseq from Tyr::NRasQ61K/°;Ink4a−/− murine melanoma lesions with high transcriptomic, but little genetic ITH. This cross-species comparison allowed the identification of novel, shared (evolutionarily conserved) and thereby biologically relevant melanoma cell states. Intriguingly, one of the melanoma cell states expressed higher levels of genes involved in the antigen processing and presentation and was more abundant in tumors from responding to immune-checkpoint blockade patients. Moreover, our analyses suggested that ITH is not solely driven by genetic makeup, as shown by the discrepancy between transcriptional and genetic (CNV) cluster identity. Instead, we conclude that ITH is partly driven by specific cell-cell interactions with the TME, which was consistent with a non-random geographical distribution of the melanoma cell states revealed by spatial transcriptomic analyses. Summarizing, analyses of our newly generated scRNA-seq data from human and mouse melanoma lesions unraveled previously undescribed melanoma cell states and their molecular identity and further emphasized the critical role of the TME as a driver of ITH.

Inhibition of the MNK1/2-eIF4E axis enhances the anti-tumor effects of palbociclib
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Palbociclib, an inhibitor of CDK4/6, has demonstrated clinical potential in the management of HR+ breast cancer and is being investigated for efficacy in other malignancies, including melanoma. Specifically, in melanoma, palbociclib has been combined with inhibitors of Mitogen Activated Protein Kinase (MAPK) signalling such as vemurafenib and trametinib. Moreover, palbociclib and MEK inhibitors are being combined and clinically investigated in patients (NCT03981614). Downstream of the MAPK pathway are the MAP Kinase-interacting-protein kinases 1 and 2 (MNK1 and MNK2). These proteins are the only known kinases of eukaryotic translation initiation factor 4E (eIF4E). eIF4E is the 5'cap binding protein within the eIF4F complex that regulates mRNA translation, and patients whose melanomas express higher phosphorylated-eIF4E levels show lower survival rates than patients with low phosphorylated-eIF4E levels. We have discovered that, in melanoma cells, treatment with palbociclib induces the phosphorylation of eIF4E. We thus hypothesized that blocking the activity of MNK1/2, may sensitize
cancer cells to the anti-tumor effects of palbociclib. Indeed, co-treatment of melanoma cells with palbociclib and the MNK1/2 inhibitor, SEL201 significantly decreased colony formation compared to single agents alone. This effect is recapitulated when cells, deficient in MKNK1 and MKNK2, are treated with palbociclib. Quantitative proteomics analysis of melanoma cells treated with the combination shows a subset of downregulated proteins with critical roles in cell cycle progression and mitotic regulation. Furthermore, in murine models of melanoma, we observed a significant overall survival advantage in the mice treated with the combination therapy, compared to either monotherapy. Overall, these data underscore the therapeutic potential of combined CDK4/6 and MNK1/2 inhibition in melanoma.

Intertumoral Heterogeneity Generates Distinct Tumor Microenvironments and Suppresses Systemic Antitumor CD8 T Cell Function in Synchronous Melanoma
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Inherent tumor genetic instability leads to intertumoral differences and acquired immunotherapy resistance in patients with metastatic melanoma. These genetic differences generate distinct tumor microenvironments (TMEs) that contain numerous potential T cell suppression mechanisms. As these tumors share one host immune system, we investigated the effects of intertumoral heterogeneity on systemic T cell functionality and PD-1 immunotherapy response in synchronous metastatic melanoma.

To recapitulate clinical intertumoral heterogeneity, we simultaneously injected YUMM 1.7 and its more immunogenic, UVB-irradiated derivative, YUMMER 1.7, melanoma cell lines into opposite flanks of the same mouse. Identical immunodominant CD8 T cell clones were found in both synchronous YUMM and YUMMER tumors, supporting the existence of systemic T cell immunity against both tumors in the same mouse. Interestingly, the presence of the less immunogenic YUMM tumors allowed contralateral YUMMER tumors to escape CD8 T cell-mediated tumor rejection and gain resistance to anti-PD-1 treatment. Compared to control CD8 T cells, synchronous YUMMER-infiltrating CD8 T cells exhibit reduced functionality as evidenced by decreased surface CD107a, increased persistence of PD-1, and inability to respond to PD-1 blockade. Concurrently, these synchronous YUMMER tumors have increased PD-L1 (PD-1 ligand) expression on the surface of tumor cells and intratumoral macrophages, potentially creating a more immunosuppressive TME to induce irreversible T cell exhaustion.

Overall, our results show that intertumoral heterogeneity may lead to a “reverse abscopal effect,” by which immunologically “cold” tumors systemically suppress T cell function to facilitate PD-1 immunotherapy resistance and immune escape of synchronous tumors in metastatic melanoma.

Identifying and targeting novel immunosuppressive driver mechanisms in adolescent and young adult (AYA) melanoma patients
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There is a current lack of effective treatment for advanced AYA melanoma patients aged 15 to 30 years. Cancer therapies that target cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) or programmed cell death protein 1 (PD-1) pathways have improved the 5-year overall survival from 5% to 50%. However, AYA patients are less responsive to immunotherapy due to their unique genomic biology and clinicopathological differences when compared to those of older patients. The primary aim of this study was to determine the distinct immunogenic patterns of treatment response and immunosuppressive microenvironment present in the AYA patients. Whole transcriptome sequencing, advanced bioinformatics and multiplex
immunofluorescence were performed on formalin-fixed paraffin-embedded samples (n=75) taken prior to anti-PD-1 and/or anti-CTLA-4 immunotherapy. The AYA tumours demonstrated high infiltration of regulatory T cells when compared to the adult melanomas (median = 13.1 versus 0.815 cells/mm2, 95% confidence interval (CI): 7.86–28.7 versus 0.468–1.447; P-value < 0.0001). Immune deconvolution demonstrated infiltration of tumour-associated macrophages (median = 9.57%, 95% CI: 5.78–12.3%; P-value < 0.05), and lower proportion of cytotoxic CD8 T cells in AYA tumours compared to adult tumours (median = 0.031% versus 3.36%, 95% CI: 0–1.48% versus 2.32–5.80%; P-value = 0.0036). Down-regulated genes (CD3D/E/G, CD8A, CXCL9 and TIGIT; adjusted P-value < 0.05) were implicated in antigen processing and presentation signalling and chemokine pathways, suggesting AYA tumours can camouflage and hide from immunotherapy treatment by altering the tumour microenvironment or secreting immune suppressive cytokines. Novel treatment strategies focused on targeting the microenvironment may improve the effectiveness of cancer immunotherapies in advanced stage AYA melanoma patients.

Combination BRAF and MEK inhibition is effective in the treatment of BRAF non-p.V600 mutant melanomas with co-occurring NFI loss-of-function alterations.

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Genetic studies of cutaneous melanoma have uncovered activating, hotspot mutations in BRAF (p.V600), NRAS (p.G12, G13, Q61) and loss-of-function mutations of NFI in approximately 50%, 20% and 15% of patients respectively. Past studies have characterized a class of BRAF non-p.V600 mutants (class III) that require cellular dysfunctions that increase RAS to activate the MAPK pathway. These mutants co-operate with oncogenic RAS by forming BRAF mutant/ wild-type CRAF heterodimers driving MAPK pathway activation (Yao et al., 2017). Interestingly, we show BRAF non-p.V600 (class III) mutations co-occurred with NFI loss more frequently than expected by chance. However, the mechanism underlying this cooperation is unknown. We found that the class III, BRAF p.D594N mutant, found to co-occur with NFI loss in melanoma patients, was capable of signalling as a monomer and activating the MAPK pathway upon NFI loss. Interestingly, we observe that class III, BRAF non-p.V600 mutants can signal as monomers and dimers within an NFI-null context. Notably, BRAF inhibitors that inhibit both monomeric and dimeric BRAF synergized with MEK inhibition to significantly reduce cell viability in vitro and tumor growth in vivo in class III, BRAF non-p.V600 mutant melanomas with co-occurring NFI LoF mutations. Thus, patients harboring complex BRAF non-p.V600 mutant melanomas may benefit from FDA-approved BRAF/MEK inhibitor combination therapy.

Germline Alterations in Cancer Predisposition Genes Across Melanoma Subtypes

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Existing associations between melanoma and germline alterations in cancer disposition genes such as CDKN2A are largely based on analyzing few genes in small referral cohorts dominated by cutaneous melanoma. To better understand which germline alterations are associated with clinically aggressive melanomas of varying subtypes, we investigated germline alterations in 76–90 genes using MSK-IMPACT in a sequential cohort of 445 patients (pts) with melanoma undergoing billable
somatic testing of their tumors for therapeutic purposes. Likely/known pathogenic alterations were categorized based on functional pathways such as homologous recombination repair (HRR), including BRCA1/2, ATM, CHEK2, and base excision repair (BER), including MUTYH.

Within the cohort, 68/445 (15.3%) pts carried 69 total germline alterations across 26 genes. Median age at initial diagnosis was similar for pts with vs without a germline alteration (59.5 vs 61). Germline alterations were detected in 50 of 293 (17.1%) of cutaneous, 7/37 (18.9%) of unknown primary, 9/58 (15.5%) of acral melanomas. Among cutaneous and unknown primary melanomas, most alterations (30/57, 52.6%) were in the HRR pathway, most commonly CHEK2 (N=8), NBN (N=3), and FANCC (N=3); 9/57 (15.8%) were in the BER pathway, primarily monoallelic MUTYH (N=7).

Among mucosal and uveal melanomas, 8 of 12 alterations (67%) were in the HRR pathway, most commonly ATM and BRCA1 (N=3 each).

Thus, in a large sequential cohort of pts with clinically aggressive melanomas, detectable germline alterations among 76-90 cancer predisposition genes varied across melanoma subtypes from 15-19% in cutaneous, unknown primary, and mucosal melanomas to 0-5% in acral and uveal melanomas. Most alterations were in the HRR and base excision repair pathways, with CHEK2 and MUTYH detected most often.

### 7-year Follow-up of KEYNOTE-006: Pembrolizumab (pembro) Versus Ipilimumab (ipi) in Advanced Melanoma

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Pembro demonstrated improved efficacy vs ipi in advanced melanoma in KEYNOTE-006. Here, we present 7-y follow-up. All patients (pts) enrolled in KEYNOTE-006 and ongoing at study end were eligible to roll over to KEYNOTE-587 for long-term follow-up of survival, progression, and start of new anticancer therapy. Of 228 pts eligible in the pembro arm, 158 enrolled; of 105 pts eligible in the ipi arm, 52 enrolled. Pts in KEYNOTE-587 who were not on treatment were followed radiographically per local SOC; pts on pembro were followed radiographically per the KEYNOTE-587 protocol. Pts who did not enroll in KEYNOTE-587 were censored for OS and PFS at date last known to be alive. For modified PFS, pts without PD were censored at date last known to be alive. At data cutoff (Apr 19, 2021), median follow-up (range) was 85.3 (0.03-90.8) mo. 7-y modified PFS was 23.8% for pembro vs 13.3% for ipi. Median OS was 32.7 mo for pembro vs 15.9 for ipi (HR 0.70; 95% CI 0.58-0.83); 5-y OS was 39.9% vs 31.0%; 7-y OS was 37.8% vs 25.3%. 7-y OS rates from best response with pembro were 85.2% for pts with CR, 61.8% for PR, and 25.9% for SD. HRs for OS favored pembro regardless of BRAF status, prior BRAF-inhibitor therapy, LDH level, tumor size (≥ or <10 cm), or presence of brain metastases. In 103 pts who completed ≥94 wk of pembro and had SD or better, 5-y OS rate was 92.9% and 5-y PFS rate was 70.1%. 16 pts who progressed after SD or better received 2nd-course pembro (median treatment-free interval, 45 [range 29.5-66.7] mo). ORR with 2nd-course pembro was 56.3% (BOR: 4 CR, 5 PR, 5 SD, 2 PD) and 2-y PFS rate was 62.5%. At 7-y follow-up, pembro continued to show improved OS vs ipi, including in pts with poor prognostic characteristics. 2nd-course pembro provided additional antitumor activity. These results show pembro provides long-
term OS benefit and confirms pembro as SOC for advanced melanoma.

Evaluating patients (pts) with a complete response (CR) in the Phase III COMBI-i study of spartalizumab (sparta) or placebo (pbo) plus dabrafenib and trametinib (DabTram) in pts with unresectable or metastatic cutaneous BRAF V600–mutant melanoma

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COMBI-i (NCT02967692) evaluated sparta (n = 267) or pbo (n = 265) plus DabTram in pts with advanced BRAF V600–mutant melanoma but did not meet its primary endpoint of improved progression-free survival (PFS) with sparta-DabTram vs pbo-DabTram. CR rates were similar between arms (19.9% [n = 53] and 17.7% [n = 47], respectively).

We report an analysis of pts who achieved a CR. There was no significant interaction of treatment effect (sparta-DabTram vs pbo-DabTram) with key baseline covariates. More pts who achieved a CR with sparta-DabTram vs pbo-DabTram had poorer baseline prognostic factors. There was a trend toward pts attaining CR more rapidly with sparta-DabTram than with pbo-DabTram (median, 4.6 vs 8.3 mo).

Median duration of CR was not reached in either arm; 12-mo CR rates were 75.2% with sparta-DabTram and 83.5% with pbo-DabTram. Pts in both arms who achieved a CR had improved PFS vs those who did not, with a trend toward greater benefit in the pbo-DabTram vs sparta-DabTram arm (12-mo rates, 93.6% [95% CI, 81.5%-97.9%] vs 86.3% [95% CI, 73.4%-93.2%]; medians not yet reached). At the data cutoff, 7 pts (14.9%) progressed on pbo-DabTram and 14 pts (26.4%) progressed on sparta-DabTram. Among pts who achieved a CR, 30 pts (56.6%) in the sparta-DabTram arm discontinued treatment vs 16 pts (34.0%) in the pbo-DabTram arm.

Reasons included adverse events (26.4% vs 8.5%), death (2.0% vs 0.0%), physician decision (7.6% vs 4.2%), and pt decision (0.0% vs 10.6%). These data may partially explain the apparent lower progression rate in the pbo-DabTram arm. Overall, these descriptive analyses provide further insight into the role of immunotherapy when added to DabTram in pts who achieved a CR.

MB097: A clinically-defined consortium of bacteria with potent anti-tumor efficacy in vitro and in vivo

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Independent groups have demonstrated that the gut microbiome of cancer patients impacts response to Immune Checkpoint Inhibitor (ICI) therapy. However, each study identified different sets of bacteria linked to outcome.

The Cambridge (UK) MELRESIST study includes a cohort of advanced melanoma patients receiving approved ICIs. Pre-treatment stool samples from MELRESIST were analysed by Microbiotica’s platform, which comprises the leading physical Reference Genome Database to give the most comprehensive and precise mapping of the gut microbiome. A bioinformatic analysis identified a small discrete microbiome signature that was different between responders and non-responders, which we extended using three published melanoma cohorts. The resultant bacterial signature predicted
whether a patient responded to ICIs with an accuracy of 91% across all four studies. We validated the signature using a NSCLC study indicating that it has great potential as a clinical biomarker. At the core of the signature was nine species strongly associated a positive outcome, which we hypothesised to be a central driver of drug response. MB097 is a consortium comprised of all nine bacteria. In a syngeneic tumour model, MB097 demonstrated synergistic tumour growth inhibition in combination with anti-PD1. The bacteria, individually or as a consortium, strongly activate dendritic cells with many being potent inducers of IL-12. These bacteria-stimulated dendritic cells triggered Cytotoxic T Lymphocytes to upregulate格拉辛溶酶B, Perforin and IFNg, and kill tumor cells in vitro.

In summary, Microbiotica’s precision microbiome profiling and the MELRESIST study has allowed us to identify a consortium of bacteria, MB097, strongly linked to ICI response across studies. MB097 drives immune-mediated tumour killing in vivo and in vitro and is being developed as a novel co-therapy with ICIs.

**Midkine inhibits dormancy of melanoma disseminated tumor cells**

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Disseminated tumor cells (DTCs) are non-proliferative cancer cells at distant organs that undergo dormancy and can reactivate later causing disease relapses. Melanoma patients remain asymptomatic for an extended period of time after the successful removal of the primary tumors. However, patients experience disease recurrences and it is believed that the reactivation of DTCs is responsible for the formation of deadly metastases. Therefore, profiling DTCs will help in understanding the dormancy mechanisms and how to prevent DTC reactivation.

Midkine (MDK), a secreted heparin-binding protein has been associated with malignancy in several human cancers including melanoma. NR2F1, an orphan nuclear receptor, is responsible for the dormancy phase of DTCs. Here, we explore the correlation between NR2F1 and MDK functions in melanoma dormancy.

Highly metastatic SK-Mel 147 melanoma cells were depleted from MDK and s.c. injected in nude mice. Immunofluorescence analysis of the lung DTCs revealed increased percentages of dormant NR2F1+ DTCs and reduced metastasis in MDK knocked down (KD) cells when compared to shControl cells. The activation of NR2F1 expression involved upregulation of previously known targets, like SOX9 and RARβ. Further, MDK KD increased the p-p38high/p-ERKlow ratio which has been associated with dormancy and NR2F1 induction in several tumor types. Furthermore, we have identified a new agonist for NR2F1 that specifically induces NR2F1 activity and prevents metastasis by inducing dormancy of head and neck carcinoma DTCs. Our preliminary data suggest that the combination of the NR2F1 agonist and MDK inhibitor may be a potential therapeutic design to induce an NR2F1-dependent dormancy program that may extend the dormancy phase of melanoma DTCs.

**Understanding The Contribution of Brain Macrophages and Neuroinflammation to Melanoma Brain Metastases**

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Melanoma Brain Metastases (MBMs) are associated with very poor prognosis and constitute a very common clinical problem in melanoma patients. Systemic treatments including targeted therapy and immunotherapy can contribute to disease control in these patients but BMs very often behave differently to extracranial metastasis and this may be mostly due to the unique brain microenvironment. The majority of brain cells are glia, with key roles in homeostasis and in pathological conditions. In particular microglia, brain resident macrophages, as part of the innate immune system of this organ gets activated upon brain damage. However, sustained inflammatory activation of microglia is considered a pathological hallmark and an important mechanism driving neurodegenerative diseases. Our analysis of preclinical models of MBMs indicates that microglia account for the majority of immune cells within MBMs. Although macrophages influence melanoma
progression and constitute a source of resistance to therapies, very little is known about the contribution of brain macrophages and neuroinflammation to melanoma metastasis in the immuno-specialised microenvironment of the brain. We have established relevant MBMs preclinical models in glia-reporter transgenic mice that also allows gene silencing in specific populations to investigate the impact of neuroinflammation and the co-evolution of the immune system during MBMs progression and responses to therapies. Our aim is to identify novel targetable candidates that will help to design better therapeutic strategies for patients with MBMs.

C/EBPβ antagonist peptide, ST101, as a novel therapeutic for melanoma

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CCAAT/Enhancer Binding Protein Beta (C/EBPβ) is an oncogenic transcription factor that is active in many cancers, where it drives aberrant expression of factors that promote tumor survival, proliferation and metastasis. In melanoma, increased C/EBPβ expression is associated with suppressed Melanocyte Inducing Transcription Factor (MITF) expression, driving melanoma progression (Swoboda et al., Oncogene 2020). C/EBPβ dimerizes via leucine zipper domain interactions that are required for its transcriptional activity. ST101 is a novel peptide antagonist of C/EBPβ currently being evaluated in a Phase I/2 clinical study in patients with advanced unresectable and metastatic solid tumors, with a confirmed partial response in a patient with multi-metastatic cutaneous melanoma. ST101 selectively binds to the C/EBPβ leucine zipper domain, antagonizing its dimerization, thereby rendering C/EBPβ susceptible to proteosomal degradation and resulting in significantly decreased C/EBPβ transcriptional activity. Here, we describe the non-clinical anti-melanoma activity of ST101. In multiple cancer cell lines ST101 exposure led to significant decreases in the transactivation of genes involved in cell survival and proliferation including BCL2, BIRC3, BIRC5, ID1, ID2, ID3, CCNA2, CCNB1, and CDK1. ST101 induced selective cytotoxicity in tumor cell lines in vitro, with an EC₅₀ of 0.7 µM in A375 melanoma cells. In contrast, normal human white blood cells and epithelial cells demonstrated ST101 EC₅₀ values that exceeded 80 µM. ST101 anti-tumor activity was demonstrated in vivo in an A375 melanoma subcutaneous xenograft model, where 25 mg/kg ST101 resulted in tumor growth delay and a significant decrease in tumor volume (p<0.05 vs. controls). These data emphasize the therapeutic potential of ST101 and support its clinical development as a potent peptide therapeutic for patients with melanoma.

Risk factors for central nervous system (CNS) metastasis (mets) among stage IV melanoma patients (pts)

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We previously identified risk factors for CNS mets in pts diagnosed (dx) with stage I-III melanoma. We performed analyses to identify risk factors for CNS mets in stage IV pts to rationally personalize their CNS surveillance and/or treatment (tx).

Demographics, tumor features, sites of mets, tx and laboratory values were reviewed for pts dx with stage IV melanoma at MD Anderson from 2012 to 2017; pts with uveal melanoma were excluded. Incidence and timing of CNS mets (from stage IV dx) was determined for all pts. Cumulative incidence (CI) of CNS mets was determined using competing risks and univariable (UV) and multivariable (MV) associations were assessed by proportional sub distribution hazards models.

A total of 1,936 stage IV pts were identified, including 1,408 non-acral cutaneous (73%; CM), 95 acral (5%; AM), 233 mucosal (12%; MM) melanomas, and 200 melanoma of unknown primary (10%; MUP). The CI of CNS mets was 27%, 37%, and 42% at 1-, 2-, and 5-years. The median time from stage IV dx to CNS mets dx was 8 months. On UV analysis, the risk of CNS mets was associated with melanoma subtype (p≤0.006; lower for MM); mutations [higher for BRAF (p<0.001) and KIT (p=0.016)]; younger age (p<0.001); higher serum LDH (p=0.024) and higher neutrophil to lymphocyte ratio (p=0.015); ≥3 non-CNS met sites (p=0.004); and was higher for pts with lung mets (p<0.001) and lower for pts with liver mets (p=0.012). Melanoma subtype, KIT mutation, and age were significant on MV analysis. KIT mutations were associated with a higher risk of CNS mets particularly among MM pts (HR=2.6; p<0.006) compared with non-MM pts (HR=1.4; p=0.042).
Among stage IV pts MM subtype was associated with lower CNS mets risk, but KIT mutation was associated with higher risk (specifically among MM pts). Association of tx for stage IV with CNS risk is in process and will be presented.

Role of Skin Microenvironment in Melanomagenesis
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BRAFV600E is the most common driver mutation in melanoma. However, this mutation is known, after a brief burst of proliferation, to cause growth arrest of transformed melanocytes and prevent uncontrolled cell proliferation. This phenomenon is known as oncogene induced senescence (OIS). It is believed that escape from senescence leads to melanoma development. Additional genetic events such as loss of tumor suppressor and/or epigenetic changes that are thought to facilitate senescence evasion have been studied in detail. However, the role of the skin microenvironment, especially the role of epidermal keratinocytes on melanomagenesis has not been investigated. In this study, we employed keratinocyte-conditioned medium from autologous white Caucasian and allogeneic black African-American keratinocytes to investigate the role of keratinocyte derived factors on OIS in BRAFV600E transduced white and black melanocytes. Here, we show that in white melanocytes, keratinocyte conditioned medium from both autologous white and allogeneic black keratinocytes suppresses senescence development. Targeted proteomic analysis of the spent melanocyte medium for selected growth factors showed upregulation of VEGF-A production by transformed white melanocytes in the presence of autologous white but not allogeneic black keratinocyte-conditioned medium. Additionally, we found that white and black melanocytes exhibit opposite effects in VEGF-A production in the presence of allogeneic keratinocyte-conditioned medium. We also found that black melanocytes secrete higher levels of LIF in the presence of autologous black or allogeneic white keratinocyte-conditioned medium than white melanocytes under identical conditions. These data suggest that skin microenvironment influences melanomagenesis from melanocytes that acquire BRAFV600E mutation.

Immune suppressive roles of the dsRNA helicase DDX46 in melanoma
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Malignant melanoma is notoriously known for alterations in the mRNA expression profile, resulting in an extremely high cellular plasticity. However, RNA binding proteins (RBPs), the master regulators of the RNA isoform repertoire are largely understudied in melanoma. Defining the roles of these proteins is particularly challenging as they constitute a family of over 1500 members. Among RBPs, the components of the spliceosome machinery responsible for alternative splicing are particularly unknown in the context of melanoma progression, and more importantly, with respect to resistance to targeted or immune-based therapies. Here we report the RNA helicase DDX46 as a new pro-tumorigenic driver of melanoma, with an unexpected immune suppressive function. Curiously, although DDX46 has been traditionally reported with a main role in mRNA splicing, here we show that in melanoma, this protein acts as broad-spectrum controller of gene expression. Here we will show that DDX46 is overexpressed in melanomas and favors tumor progression and metastasis, acting in part by halting the recruitment of several immune cell types. Specifically, we will present genome-wide splicing and gene expression analyses that have revealed unexpected targets of DDX46 in antigen presentation. Ultimately, our data broaden the impact of this RBP in the aggressive behavior of melanoma and provides new strategies to explore in the immune therapy field.

Health-related quality of life (HRQoL) with relatlimab plus nivolumab (RELA+NIVO) vs NIVO in patients (pts) with previously untreated metastatic or unresectable melanoma:
RELATIVITY-047
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Lymphocyte-activation gene 3 (LAG-3) and programmed death-1 (PD-1) are immune checkpoints that contribute to tumor-mediated T-cell exhaustion. In the phase 2/3 RELATIVITY-047 trial, RELA+NIVO (anti–LAG-3 and anti–PD-1 antibodies, respectively) showed superior progression-free survival (PFS) vs NIVO in pts with previously untreated metastatic/unresectable melanoma; grade 3/4 treatment-related adverse event (TRAE) rates were 18.9% and 9.7%, respectively (Lipson, ASCO 2021). Median follow-up was 13.2 mo. HRQoL was evaluated using the FACT-M and EQ-5D-3L questionnaires prior to dosing in each 4-wk treatment (tx) cycle. Results from the FACT-M Trial Outcome Index; FACT-G Total; physical well-being, functional well-being, and melanoma subscales; and the EQ-5D-3L visual analogue scale (VAS) are presented here. Changes from baseline (BL) were analyzed using a mixed model for repeated measures adjusting for stratification factors and BL QoL value. Clinically meaningful changes from BL were determined using prespecified minimally important differences (MIDs). Proportions of pts “bothered by side effects of tx” were summarized using the FACT-M GP5 item. The questionnaire completion rate at each tx visit was ≥86%. Mean scores for all FACT-M scales/subscales and the EQ-5D-3L VAS were similar between RELA+NIVO and NIVO at BL and on-tx time points (with changes from BL not exceeding MIDs). The proportion of pts reporting that they were bothered “quite a bit” or “very much” by tx side effects was low at on-tx time points in each arm (highest at wk 4: RELA+NIVO, 6%; NIVO, 5%). These results suggest that HRQoL with RELA+NIVO was stable and similar to that observed with NIVO. Despite increased TRAEs with RELA+NIVO, a low proportion of pts reported being bothered by tx side effects.

Immuno-related toxicity as a possible predictor of progression-free survival. One center’s experience
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Melanoma accounts for 5.6% of all new cancer cases according to data provided by the NIH Surveillance, Epidemiology and End Results Program (SEER). Adjuvant immunotherapy is administered in order to avoid relapse disease. Unfortunately, a group of patients experimented progression disease in spite of receiving immunotherapy.

For this work, we have conducted a retrospective study reviewing patients who have received adjuvant anti-PD1 immunotherapy after surgical resection of melanoma stage III-IV (AJCC 8th classification). We included 16 patients from our hospital since 2019. Comparison of progression-free survival (PFS) of patients with or without toxicity to immunotherapy treatment has been carried out. Descriptive statistics were extracted and the Log-Rank test was applied to calculate the difference in survival between these two groups.

Our results showed that the 62.5% of the subjects (10 patients) manifested immunorelated toxicity. Cutaneous rash and pruritus were the main symptoms, followed by asthenia. Most of the population experimented grade 1-2 in toxicity, and only one grade-4 case (hypertransaminasemia). BRAF mutation was detected in the 30% of the toxicity group and in the 83.3% in the non-toxicity group; PDL-1 expression was negative in most of the cases.

At the time of the data collection in July 2021, the 43.7% of the patients presented disease progression. In those patients who manifested immuno-related toxicity, 20% presented progression disease, while in patients without toxicity, the 83.3% experienced progression disease. The median PFS was 5.8 months in the non-toxicity group, while the median PFS was not reached in the immunotherapy group. The Log-Rank test was statistically significant (p-value < 0.005).

In this study, we provide data on the PFS increase when immuno-related toxicities appear, but patient inclusion and follow-up time should be increased.
Autoantibodies as prognostic biomarkers for cutaneous melanoma
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The incidence of cutaneous melanoma, especially thin (≤ 1.0 mm) melanomas, continues to increase worldwide. Breslow thickness, ulceration and sentinel lymph node status, which are currently regarded as the most accurate prognostic markers, fail to identify patients at high risk of disseminating disease amongst those with thin melanomas. Autoantibodies reflect a biologically amplified, stable signature of the anti-tumour immune response that is produced often prior to the clinical detection of other tumour markers and prior to the first clinically detectable signs of cancer recurrence.

In this retrospective study, clinical data will be extracted from the population-based Western Australia Cancer Registry, regarding the survival outcomes of 104 early stage (in situ - stage II) melanoma patients. The potential of autoantibodies to predict the risk for progression will be evaluated by analysing the autoantibody profiles exhibited at the time of diagnosis relative to their present disease status (median follow-up of 3.83 years). Sera (n=104) were screened against a high-throughput microarray platform containing 1627 functional proteins. The ultimate selection of three potentially prognostic autoantibodies will be based on their biomarker score, which represents the frequency and strength of the signal in patients who progressed compared to the ones who did not progress, of > 10 % or < -10 %, the by iPpathwayGuide determined fold change >2 (high risk versus low risk), p<0.05, AUC > 0.7 and literature relation to melanoma.

An in-house immunoassay will be developed to measure IgG autoantibody levels of the three potentially prognostic autoantibodies identified, using the Bio-Plex® immunoassay platform. AUCs will be calculated for each autoantibody and correlated to risk of progression and survival.

Improved EV enrichment from plasma and proteome characterization in metastatic melanoma and lung adenocarcinoma patient cohort.
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Extracellular vesicles (EVs) in human blood have strong potential to be used as clinical biomarkers in various pathological conditions. In this work, we design a workflow for EV enrichment from plasma using size-exclusion chromatography and in-depth proteome analysis of EVs and albumin-depleted plasma using the high-resolution isoelectric focusing (HiRIEF) LC-MS method. We start with the optimization of a simple, scalable, and reproducible method to isolate EVs with high yield and purity. Subsequently, we characterize EVs and assess long-gradient (LG) and HiRIEF method performance for EV samples. HiRIEF demonstrated high proteome coverage, reproducibility, and potential for extensive proteomics profiling of the eluted EV fraction. Finally, the workflow was validated using a metastatic melanoma (MM) and lung adenocarcinoma (LUAD) patient cohort. Results show, despite a similarity in the EV and plasma proteomes, EVs provide unique, additional proteomics information that could not be obtained from plasma alone. While the detected EV protein cargo shares many proteins with the plasma, the majority of proteins are unique to the EVs. The EV dataset (1462 proteins) consists of 92 EV-specific proteins, including traditional EV markers (such as HSPA8, CD63), greater than 80 proteins from the Exocarta Top100 list and some novel EV markers (such as LGALS3BP, RAP1B). Majority of the EV detected proteins are universally accepted marker proteins for different EV subtypes (small EVs, large EVs and microvesicles), such as ESCRTs, tetraspanins, RNA-binding proteins, cargo selection and trafficking. Additionally, we detected several known clinical markers proteins for MM and LUAD patients in the detected EV proteins. Our study provides an optimized workflow for EV enrichment, in-depth EV and plasma proteomics for cancer biomarkers detection.

Immunotherapy responses in a mouse model of melanoma are associated with early on-treatment changes in myeloid and T cell compartments
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Immunotherapy has transformed the management of patients with advanced melanoma, with a five-year survival reaching 52% for immunotherapies blocking...
both CTLA-4 and PD-1 immune axes. Lack of treatment response and the development of resistance remain common yet leave few actionable options. Here we report a novel preclinical animal model that faithfully recapitulates immunotherapy responses observed in melanoma patients.

YUMM3.3 mouse melanoma cells (BrafV600E, Cdkn2a-/-) yield progressively growing tumors after subcutaneous injection. YUMM3.3 cells were modified by UVR-mutagenesis and cloning. Mice bearing the resulting YUMM3.3UVR-34 tumors were subjected to immunotherapy, which significantly improved median survival (parental, 21 days; anti-CTLA-4, 42 days; anti-PD-1, not reached with a 160-day follow-up). All progressing tumors were heavily infiltrated with proliferating exhausted (PD-1 high) CD8 T cells. Early-on-treatment responses to each single agent alone or in combination, were analysed by flow cytometry after two treatment cycles. Upregulation of MHC class I and PD-L1 on tumor cells, enhanced tumor immune (CD45) infiltration and an influx of MHC class II-positive monocytes were observed in all treatment groups. Anti-CTLA-4 (+/- anti-PD-1) therapy led to an increase in central memory CD8 T cells and a decrease in antigen-specific regulatory T cells in the tumor, with a corresponding increase in blood regulatory T cells. Furthermore, an increase in proliferating blood effector CD4 and CD8 T cells was observed in all treatment groups.

Our results show a striking similarity to immunotherapy responses in human cohorts, indicating that the model could be used for the dissection of molecular mechanisms of immunotherapy resistance and may also aid in selection of salvage therapies for immunotherapy-resistant tumors.

Diagnostic utility of PRAME, p53 and 5-hmC in melanoma, neurofibroma, scar and naevi.

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The use of immunohistochemistry in assessing the malignant potential of cutaneous melanocytic lesion is a low cost, rapidly performed ancillary test which is available widely. High expression of PRAME (preferentially expressed antigen in melanoma) and low expression of 5-hydroxymethylcytosine (5-hmC) have previously been described as informative in diagnosing melanoma over benign naevi. Expression of p53 has also been shown in desmoplastic melanoma. This study examined 313 cutaneous melanocytic lesions (melanoma n=260, naevi n=53) using multiplex immunofluorescence to assess the combined staining of PRAME, 5-hmC and SOX10. We also assessed a subgroup of desmoplastic melanoma (n=20), cutaneous neurofibroma (n=20) and scar (n=15) for expression of PRAME, 5-hmC and p53.

PRAME expression was significantly higher in melanomas than in naevi (p<0.0001), with the lowest PRAME expression found in low grade desmoplastic melanoma compared to the other melanoma subtypes. In non-desmoplastic melanomas, 38% showed strong, 4+ staining and 70% stained 3 or 4+. Conversely, 96% of naevi showed 0, 1 or 2+ expression. 5-hmC expression was significantly lower in melanomas than in naevus (p < 0.0001), with the exception of the acral subtype, which was not significantly associated (p = 0.84).

With respect to desmoplastic melanoma compared to scar or neurofibroma, strong PRAME or p53 staining was almost exclusively found in high grade desmoplastic melanoma. 5-hmC was not useful in distinguishing desmoplastic melanoma from neurofibroma or scar. We recommend a “positive” PRAME result be established at 3 and 4+ staining rather than the previously published 4+ threshold. While the use of 5-hmC, PRAME or p53 appears to have a limited role in the diagnosis of low grade desmoplastic melanoma, combining PRAME and 5-hmC leads to an increase in sensitivity and specificity in distinguishing melanoma from naevus.
Enterotypes underlie gut microbial associations with response and toxicity in neo-adjuvant immunotherapy

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Immune checkpoint inhibitor immunotherapies have revolutionised the treatment of melanoma, although drug resistance or concurrent immune-related adverse events (irAEs) still impact majority of patients. The gut microbiome has attracted interest as a tool for potentially modifying both response rates and irAEs since it modulates many immune functions. However, previous microbiome-immunotherapy studies have lacked agreement as to the key microbial drivers of response and resistance. We therefore sought to identify features of the gut microbiome that were associated with response and irAE development during combination anti-PD-1/anti-CTLA-4 immunotherapy using a highly homogenous stage-III melanoma trial cohort split across two continents (Australia & the Netherlands). Analysis of pre-treatment microbiomes of the Australian patients revealed that patients who both failed to respond to therapy and developed severe irAE had low microbial diversity and reduced abundance of Ruminococcaceae and methanogenic archaea. These features were associated with reduced fibre and omega-3 consumption. Although geographical variance in biomarkers predictive of response and irAE development were observed between countries, differences in the distribution of gut microbiome community types (enterotypes) explained this effect. Importantly, accounting for geographic variation in microbiome community types resulted in improved prediction accuracy for response and irAE using machine learning. Landscape-level differences in the microbiomes of human populations are a factor underpinning microbial associations with clinical outcomes and highlight the importance of considering the overall assembly of microbial communities. Incorporating gut microbiome enterotype assessment could optimise personalised predictions of patient outcomes and facilitate the design of therapeutic dietary interventions.

Single cell analysis reveals how therapy remodels the tumor microenvironment in melanoma CNS metastases

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Brain metastases (MBM) and leptomeningeal metastases (LMM) are two manifestations of melanoma dissemination to the CNS with vastly different survival outcomes. Analysis of single cell RNA-Seq data from 43 clinical specimens has uncovered a distinct, immune-suppressed T cell landscape in the LMM microenvironment that is distinct to those of the brain and skin metastases. An LMM patient with an extraordinarily long survival and response to therapy demonstrated an immune repertoire that was distinct from typical poor survivors and more similar to CSF from non-LMM donors. Analysis of serial specimens over the course of therapy demonstrated reductions in melanoma cells and macrophages, coupled with increased levels of active T cells and dendritic cells in the CSF of the extraordinary responder, whereas poor survivors showed no improvement in T cell responses. In MBM patients, targeted therapy and immunotherapy was associated with increased immune infiltrate, with similar T cell transcriptional diversity noted between skin metastases and MBM - suggestive of immune cell trafficking into the brain. Immunotherapy was associated with a more diverse lymphocyte landscape and higher numbers of antibody-producing cells. These findings were confirmed in an immune-competent mouse model of MBM. Correlation analysis across the entire immune landscape identified the presence of a rare, novel population of dendritic cells (DC3s) to be correlated with increased overall survival, regardless of disease site/treatment. The presence of DC3s positively regulated the immune environment of both patient samples and preclinical melanoma models through modulation of activated T cells and MHC expression in the tumor.
Our study provides the first comprehensive atlas of two distinct sites of melanoma CNS metastases and identifies rare populations of cells that underlie the biology of this disease.

dsRNA signalling as an inherent vulnerability in melanoma with implications in metastasis and resistance to immune modulators
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Melanomas are notorious for their altered mRNA expression profiles. Yet, the specific contribution of RNA binding proteins (RBPs) to melanoma development is unclear. Moreover, specific roles of RBPs in the resistance to targeted or immune-based therapies remain also largely unknown. A main complication in assigning individual RBPs to specific roles in malignant transformation and treatment failure is the very complex nature of these proteins (over 1500 RBPs have been described to date, most of which have yet to be functionally characterized). As melanomas have unique signaling cascades that distinguish them from other tumor types, we questioned whether specific RBPs could have lineage-specific roles in this disease. To this end, we mined genomic landscape of known mRBPs across different cancer types. In addition, we performed genome-wide transcriptomic, proteomic, and RNA-immunoprecipitation studies, as well as defined the mode of action of candidate genes that were identified with a distinct expression and functional requirement in melanoma vs other pathologies. We will present data identifying dsRNA-related stress as an intrinsic vulnerability of melanoma cells, with implications for cell proliferation, metastasis and response to immune checkpoint blockade. Specifically, we will present RBPs with an unexpected function as internal breaks to endogenous dsRNA actively produced in melanoma cells, that if not controlled, would drive potent proinflammatory and antitumoral responses. Together these data illustrate the relevance of selected RBPs as putative prognostic factors and targets for therapeutic intervention in melanoma.

Hijacking the Murine Melanoma Tumor Microenvironment by Seven Different Date (Phoenix dactylifera L.) Varieties by mitigating the Immunosuppressive Cytokines IL-6 & IL-10
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Melanoma is a hot immunogenic tumor. Yet, tumor microenvironment (TME) acts as a formidable barrier inducing resistance to recent immunotherapeutic agents for melanoma patients. Thus, a perfect therapeutic regimen should alleviate the immunosuppressive TME to decrease the risk of innate or acquired resistance. Our group recently showed the impact of nutraceuticals in improving the immune recognition capacity of cancer cells. However, the impact of nutraceuticals on TME has rarely been probed. Dates (Phoenix dactylifera L.) possess potent anticancer activity against several tumors. Yet, their activity against melanoma cells is yet to be probed. The aim of this study is to screen 7 date varieties against B16F10 melanoma cells and to unravel their role in tuning the TME.

Seven varieties of date fruits were extracted by sonication. Fractionation was performed using water and methanol. Serial dilutions of the 14 fractions were performed (1-5000µg/ml). Screening for cytotoxicity against B16F10 and RAW264.7 cells was performed using MTT. ELISA was used to assess interleukins (IL-) 6 and 10. Screening of the fractions demonstrated the anti-cancer activity of both fractions of Safawi and Sukkari varieties and the aqueous fraction of Saqai as they significantly repressed B16F10 cell proliferation. However, only the methanolic fractions of Safawi and Sukkari were selective anti-cancer agents at 10µg/ml as they were non-toxic to RAW264.7 cells. The methanolic fractions of Safawi and Sukkari repressed the immunosuppressive tumor promoting cytokines: IL-6 and IL-10.

This study sheds light on the potential role of Safawi and Sukkari dates as selective immunotherapeutic agents for melanoma. It also supports utilizing dates as adjuvant therapy for resistant patients.

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Using cutaneous immunotherapy exceptional responders and failures to inform and predict outcomes in acral, mucosal and uveal melanoma subtypes

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Treatment with immune checkpoint inhibitors (ICI) has dramatically improved the outcomes for patients with metastatic melanoma. For cutaneous melanoma (CM), there are several tumoral and immune markers that correlate with response to therapy. However, improvement in the treatment of melanomas of acral, mucosal, and uveal origin have not seen comparable improvements. These rare melanomas are less likely to respond to ICI therapy than CM, although a subset of rare melanomas do respond to ICI. To identify patients in these subgroups, we compiled demographic, pathologic, and clinical data for patient cohorts of acral (n=37), mucosal (n=40), and uveal (n=62) melanoma and compared these CM patients that exceptionally responded (ER) (n=54) or failed (EF) to respond (n=41) to ICI. CM patients were sorted into either EF or ER based on survival analysis (individuals who had either the longest (ER) or shortest (EF) of both overall and progression free survival). We stained FFPE tumor samples for patients in each of the subgroups: acral (n=21), mucosal (n=23), uveal (n=12), cutaneous EF (n=37) and cutaneous ER (n=26). The FFPE patient samples were stained for tumoral and immune markers implicated in ICI response in CM: PD-L1, CD8, MITF/AXL, NGFR/SOX10, and CD163. Analysis of the IHC is ongoing, but preliminary analysis demonstrates that CD8 T-cell expression is highest in the CM ER and lowest in the CM EF. Additionally, acral melanoma had consistently detectable PD-L1 expression while CM EF overall lacked PD-L1 expression. In this cross-histologic analysis, our goal is to identify shared and exclusive tumor-intrinsic and immunologic features of ICI response and/or resistance.

Development of a New Molecular Predictor for Risk of Melanoma Brain Metastases

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Of all common cancers, melanoma exhibits the highest propensity to metastasize to the brain. Prognoses for those who develop brain metastases are exceedingly poor, therefore treating melanomas before they are able to metastasize to the brain is of critical importance to patient survival. In recent years, advancements in adjuvant therapy have led to an increase in survival for many patients diagnosed with metastatic melanoma. These treatments, however, are not without an associated risk of toxicity and financial burden. As treatment combinations become more popular, these effects can become compounded. This creates a need for understanding individual risk.

In order to predict which patients may benefit most from adjuvant therapy, we developed a gene expression profile (GEP) that stratifies patients into appropriate risk categories for developing brain metastases from melanoma. Using RNA-seq from three distinct data sets, we have identified a group of genes that are significantly dysregulated between melanomas of patients who will later develop brain metastases versus those who will not. These genes were compared to RNA-seq data from a mouse model of melanoma that generated brain metastases, then further refined using a statistical significance threshold. The final candidate genes were used to build an ensemble machine learning classifier capable of predicting incidence of melanoma progression to brain metastasis. This classifier was validated on an independent, retrospective dataset to further evaluate performance, with results indicating the need for minor adjustments to classifier structure. Following optimization, the GEP will be translated to a lower cost sequencing platform and evaluated in a prospective study. Ultimately, this profile will be used to enhance clinical decision-making with respect to emerging treatment regimens for melanoma brain metastasis.
Regional lymph node evaluation in pediatric melanoma: A Single center 10-year review
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Background - Pediatric melanoma is the most common skin cancer in children. Because of its rarity, adult guidelines are applied to the management; however, the prognosis are different, and the management of regional lymph nodes is unclear. The objective of this study was to examine if sentinel lymph node biopsy (SLNB) and completion lymph node dissection (CLND) provide prognostic and therapeutic value in pediatric cutaneous melanoma and to examine prognostic factors associated with survival and recurrence of the disease.

Methods - We retrospectively reviewed patients < 18 years being diagnosed with localized pediatric cutaneous melanoma without regional node involvement between 2009 and 2020. Demographic data, primary tumor characteristics, nodal status, regional lymph node management, recurrence and survival rate were analyzed and compared. Univariate analysis was performed to identify factors associated with disease-free survival (DFS) and overall survival (OS). Subgroup analysis was performed for pediatric Atypical Spitzoid tumor (AST) and Spitzoid melanoma (SM).

Results - There were 111 patients (median 10 years). Seventy-five patients (66%) had AST/SM. The 5-and 10-year OS were 95% and 90%, respectively. The location of SLN of extremity lesions was found to be either the ipsilateral groin or axilla for lower and upper extremities, respectively. A positive SLN correlated with 5- and 10- years DFS (76% vs 94% at 5 years, P=0.006 and 68% vs 94% at 10 years, P=0.008) with hazard ratio (HR) 4.8 (P=0.049). There was no difference in 5 years of OS and DFS between CLND versus nodal observation (93% vs 93%, P=0.36 and 75% vs 77%, P=0.88 respectively). A SLN status (HR=4.8, P=0.04), truncal lesion (HR=4.9, P=0.01), age<10(HR 0.26, P=0.04), AST/SM subtype (HR=0.1, P=0.003), positive ulceration (P=0.048), mitosis (HR=1.1, P=0.0003), depth (HR=1.3, P=0.01) and margin < 5 mm (HR 3.6, P=0.04) were associated with recurrence. Age ≥ 10 and conventional melanoma were associated with higher mortality rate (p=0.045 and 0.04, respectively). In ATS/SM subgroup, none of the factors correlated with survival outcome, however, TERT promoter mutations (P<0.0001) and margin < 5 mm (P=0.005) were associated with higher recurrence rate.

Conclusion - ATS/SM has a better prognosis than conventional melanoma in pediatric patients. Although, SLN status is associated with recurrence of the disease in pediatric melanoma, it is not prognostic in pediatric ATS/SM unless there is a TERT promotor mutation may be candidates for more aggressive therapy. SLN location of extremity cutaneous melanoma is predictable and was consistently located either in the axilla or the groin for upper and lower extremity melanoma, respectively. CLND does not provide prognostic or therapeutic value in pediatric melanoma.

Identifying the microenvironmental targets in melanoma
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The tumor microenvironment plays a critical role in melanoma progression and metastasis. In normal physiology, melanocytes export their melanin to an adjacent cell type called a keratinocyte, through a specialized organelle called a ‘melanosome’. During oncogenic transformation, melanocytes acquire mutations most typically in the BRAF gene, and those nascent tumor cells are similarly in direct contact with keratinocytes. Several studies report that transformed melanoma cells can transfer extracellular vesicles such as exosomes into the keratinocytes, suggesting there is robust communication between these two cell types. Because melanosomes and exosomes contain abundant RNA and protein molecules, we hypothesized that transfer of RNA/protein from the melanoma cell to the keratinocyte plays an important role in melanoma progression. Using a zebrafish model of melanoma as well as human melanoma keratinocyte co-culture systems, we have engineered a Cre/LoxP based donor/recipient system to identify which keratinocytes receive cargo from the melanoma cells. Using this system, we have found that the tumor cells can transfer mRNA into a small number of neighboring keratinocytes. Using ATAC-seq and RNA-seq, we find that the switched keratinocytes undergo epigenetic reprogramming when compared to the non-switched keratinocytes. Further, we performed a small-molecule screen to identify regulators of melanoma keratinocyte communication. Our studies identify a previously unknown mechanism of microenvironment reprogramming by melanoma cells, in which oncogenic transformation of neighboring keratinocytes via melanoma derived
molecules contributes to overall tumor growth. Our approach using zebrafish as well as patient derived melanoma samples can identify the consequences of perturbing cell-cell communication in melanoma progression and metastasis as well as the role of the tumor microenvironment in mediating this process.

**Ferroptosis as a modulator of checkpoint inhibitor immunotherapy**

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Since many patients with advanced melanoma and other cancers do not benefit from immune checkpoint inhibitors or relapse after an initial response, there is a need to identify pathways that enhance responses to these treatments. Using our YUMMER-G cell line, we completed a pooled, whole genome in vivo CRISPR/Cas9 screen to identify genes that reduce or enhance anti-tumor immune responses. Data from this screen identified several ferroptosis genes as regulators of anti-tumor immunity. Based on this, we hypothesized that ferroptosis is critical for effective anti-tumor immune responses and inducers of ferroptosis will enhance the efficacy of immunotherapies. To test this, we first demonstrated via flow cytometry that treatment of mice with YUMMER-G tumors with anti-PD-1 increased the amount of intratumoral lipid peroxides compared to untreated tumors. Increased amounts of the lipid peroxide intermediates 4-hydroxynonenal and malondialdehyde were also detected via IHC and TBARS assay, respectively. This indicated that ferroptosis occurs in tumors after treatment with anti-PD-1, so we next evaluated whether treatment with ferroptosis inhibitors was sufficient to block anti-tumor immune responses induced by anti-PD-1. Treatment of mice with YUMMER-G tumors with anti-PD-1 alone resulted in 30% tumor clearance and survival, whereas as a combination of any of multiple ferroptosis inhibitors with anti-PD-1 completely abrogated this effect. When we repeated these experiments with ferroptosis inducers instead of ferroptosis inhibitors, the combination of ferroptosis inducers and anti-PD-1 increased the rate of tumor clearance and survival to 100%. Overall, these findings support a key role for ferroptosis in antitumor immunity and underscore the therapeutic potential of ferroptosis inducers used in combination with existing immunotherapy regimens.

**Systematic literature review (SLR) on proportion and treatment outcomes of melanoma brain metastases (MBM) patients**

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Brain metastases (BM) are a major cause of mortality in advanced melanoma (Mel) patients. Treatment (tx) is challenging and clinical trials are limited. We conducted a SLR to evaluate the proportion and outcomes of MBM patients. Ovid MEDLINE, EMBASE, Cochrane Central Register of Controlled Trials, and Cochrane reviews were searched to capture relevant full text English publications within 5 years of Nov. 2020. MBM proportion and overall survival (OS) by tx, including immunotherapy (IO), MAPK inhibitors (MAPKi), and stereotactic radiosurgery (SRS) were summarized using weighted average estimates. Meta-analysis hazard ratio (mHR) summary estimates for OS and 95% confidence intervals (CI) were calculated using random-effects models. 123 studies were identified. For MBM proportion, 34 studies included predominantly cutaneous Mel; 21% (range: 8-42%) had BM at diagnosis (n=29) and 24% (18-44%) developed BM after diagnosis (n=5). 5 studies included non-cutaneous Mel and found only 5% (2-8%) had BM at diagnosis. 72 observational studies reported tx outcomes – most commonly for IO, MAPKi, SRS, and SRS + IO, with median OS from tx start of 7.2, 8.6, 7.3 and 14.1 months, respectively. Patient populations varied so no direct comparisons can be made. For OS from tx start, IO vs. no IO had HR of 0.42 (95%CI, 0.29-0.63) (n=5). SRS+IO vs. SRS had mHR 0.50 (0.37-0.69) (n=10). SRS+MAPKi vs. SRS showed mHR 0.70 (0.41-1.20) (n=7), while MAPKi vs. no MAPKi had mHR 0.82 (0.46-1.46) (n=3). 12 clinical trials were identified (7 with <100 subjects, 11 phase I/II, and 7 for targeted therapy or IO). This SLR found MBM proportion is lower for non-
cutaneous Mel. Overall, OS is poor, but evidence from observational studies indicates that IO or combination IO/MAPKi therapy with SRS may improve outcomes. More clinical trials would benefit this high unmet need population.

Real-world (RW) outcomes of second-line dabrafenib plus trametinib (D+T) in patients (pts) with unresectable stage III/IV cutaneous BRAF V600-mutant melanoma

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Data on the efficacy of treatments (tx) for melanoma in second-line are limited. This retrospective study reports RW effectiveness of D+T in adult pts with unresectable stage III/IV BRAF V600-mutant melanoma treated in second-line with the first progression event in the regimen directly preceding D+T tx (2L). Pts were selected from the US-based Flatiron Health de-identified database. Primary endpoints included RW progression-free survival (PFS) and overall survival (OS) from start date of 2L D+T tx. Among 68 eligible pts, median age was 66 y and 58.8% were male. ECOG status was ≤1 in 58.8% of pts, 2 in 11.8%, 3+ in 7.4%, and missing in 22.1%. Number of metastatic sites was 0, 1, 2, and ≥3 in 7.4%, 22.1%, 23.5%, and 47.1% of pts, respectively, with CNS metastases in 29.4% of pts. Lactate dehydrogenase (LDH) levels were low/normal in 29.4% of pts, elevated in 26.5%, and missing in 44.1%. Median PFS (mPFS) and median OS (mOS) (95% CI) were 4.8 mo (4.1-6.8) and 11.9 mo (8.3-15.2). mPFS and mOS were significantly longer in pts with normal LDH and <3 metastatic sites (n=12) vs pts with elevated LDH and ≥3 metastatic sites (n=11; mPFS: 8.2 mo [2.0-20.0] vs 2.9 mo [1.2-4.4], p=0.001; mOS: 17.1 mo [11.5-not reached] vs 6.0 mo [2.9-9.8], p=0.0001). mPFS and mOS did not significantly differ in pts treated with first-line (1L) anti–PD-1 + anti–CTLA-4 (n=23) vs pts treated with 1L anti–PD-1 alone (n=45; mPFS: 4.9 mo [4.1-6.8] vs 4.5 mo [3.3-8.1], p=0.503; mOS: 11.7 mo [5.4-32.2] vs 12.0 mo [8.3-15.6], p=0.935). In this study, pts receiving 2L D+T had a high burden of disease. Results suggest that D+T is effective in pts treated in 2L. Pts with normal LDH levels and <3 metastatic sites derive the greatest benefit from 2L D+T tx, indicating the continued prognostic value of LDH and number of metastatic sites beyond 1L tx.

Inhibition of FAK Attenuates Incidence of Melanoma Brain Metastasis

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Clinical trials utilizing combination therapies targeting mutant BRAF and MEK have shown that while many patients with brain metastases initially achieve intracranial responses, these responses are relatively short-lived due to resistance mechanisms. Interestingly, one mechanism of resistance to BRAF/MEK therapy is upregulation of the PI3K/AKT signaling pathway. We previously found that expression of activated AKT1 results in a highly aggressive melanoma with an increased propensity to metastasize to the brain and lungs due to increased phosphorylation of focal adhesion kinase (FAK). To address whether combined inhibition of mutant BRAF, MEK, and FAK is superior to standard of care targeted therapy directed against mutant BRAF and MEK in vivo, we utilized our autochthonous, RCAS/TVA metastatic melanoma mouse model driven by BRAFV600E and myristoylated AKT1 in combination with loss of CDKN2a and PTEN. Following tumor induction, mice were separated into cohorts treated with vehicle only, FAK inhibitor (FAKi) alone, combined mutant BRAF and MEK inhibitors (BRAFi/MEKi), or the triple combination of mutant BRAF, MEK, and FAK inhibitors (BRAFi/MEKi/FAKi). Each cohort was assessed for tumor onset, tumor growth, metastasis, and survival.
Tumor onset was significantly delayed in mice treated with BRAFi/MEKi and completely prevented within the treatment period for mice treated with BRAFi/MEKi/FAKi. All three treatment groups had significantly longer survival times when compared with vehicle alone. Importantly, cohorts that received FAKi alone or in combination with BRAFi/MEKi had significantly lower rates of metastasis to the brain. These data highlight the important role of FAK in driving BRAF-mutant melanoma brain metastasis and suggest that BRAF/MEK therapy resistance can be delayed or prevented through inhibition of FAK.

Splicing manipulation as a new strategy to enhance adoptive cell therapy

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Alternative splicing of immune receptors (IMRs) is a post-transcriptional process that can generate isoforms with a changed modulatory effect compared to the full-length receptor. Thus, splicing variants of IMRs are a treasure chest of therapeutic targets. To exemplify the impact of alternative splicing on the function of T lymphocytes, we chose the PD-1 receptor as a test case. RT-PCR of activated T cells showed two of the five documented PD-1 isoforms: full-length PD-1 (flPD-1) and soluble PD-1 (sPD-1). We hypothesized that these two variants could have opposing effects since flPD-1 is a well-documented inhibitor of T-cell activation, whereas the soluble form can block PD-L1, the ligand of PD-1.

To elucidate the role of the splicing isoforms of PD-1, we developed a system that interferes with splicing by targeting selected splice sites in Jurkat T cells. Using the CRISPR/Cas9 editing system, we introduced a double-strand break into a pre-defined splicing recognition sequence of the PD-1 gene. This manipulation increased exon 3 skipping and lowered the transcript level of flPD-1, although it did not abolish the gene entirely. As a result, sPD-1 was highly expressed by PCR and as a soluble protein in the T cell supernatant. Remarkably, when activated, the modified cells had significantly improved IL-2 secretion. In contrast, knocking down PD-1 did not affect IL-2 secretion, suggesting that the improved function was indeed due to the soluble PD-1 and not the loss of the PD-1 receptor.

In summary, our research shows that the preferential expression of sPD-1 was essential for the improved T cell function. However, at this point, the mechanism is still unclear. As PD-1 plays a critical role in the failure of cancer immunotherapies, the potential of splicing manipulation of PD-1 can be valuable and applicable for the success of advanced cell-based therapies.

A novel mouse model recapitulates all the phases of human melanoma

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Cutaneous melanoma, the most aggressive form of skin cancer, is caused by malignant transformation of epidermal melanocytes. At early stages, melanoma cells proliferate in the epidermis before entering the dermis, a key step towards the development of an aggressive and metastatic disease. The molecular and cellular mechanisms enabling dermal invasion of melanoma cells are largely unknown. This is due to a lack of an accurate modeling system, one that recapitulates the human anatomo-physiological features of early melanoma development. To fill this gap, we therefore decided to generate such a mouse model.

Herein we have developed a novel genetically engineered mouse model of melanoma on the Tabby background. These mice lack hair follicles in the tail. The model allows activation of a BrafV600E-driven melanomagenic program in epidermal melanocytes located in the tail of these mice and in vivo fate mapping of the melanoma cells by intravital microscopy. We leveraged single cell RNA-sequencing (10X Genomics) to portray the transcriptional heterogeneity of the melanoma cells and searched for evidence of transcriptional reprogramming during early melanoma development.

We validated that this model faithfully recapitulates the early phases of human melanoma development, including radial epidermal expansion and dermal invasion, and subsequent clonal expansion. Interestingly, we observed that individual melanoma cells enter the dermis simultaneously and found evidence that this process is accompanied by a partial de-differentiation/reprogramming event that is transient and reversible.

In conclusion, we have established a suitable in vivo model to study the cellular and molecular
mechanisms underlying dermal invasion. This model provides a unique platform for the discovery of novel biomarkers of dermal invasion and therapeutic interventions that intercept the disease before its lethal dissemination to vital organs.

Modulating p53 pathway activity in acral melanoma to kill drug resistant persister cells to prevent recurrent resistant disease

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Acral melanomas typically have wildtype p53 but the pathway is frequently dysregulated by MDM protein overexpression. This study investigates whether the p53 pathway can be activated pharmacologically to increase apoptosis of acral melanoma cells in order to inhibit tumor development. Current therapies directly targeting MDM proteins tend to be toxic or have to be combined with other approaches for improved efficacy, further increasing toxicity. Therefore, this study chose an alternative approach in which the AKT and WEE1 pathways were targeted simultaneously using AKT inhibitor AZD5363 [4-amino-N-{(1S)-1-(4-chlorophenyl)-3-hydroxypropyl]-1-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperidine-4-carboxamide] and WEE1 inhibitor AZD1775 [2-allyl-1-(6-(2-hydroxypropan-2-yl)pyridin-2-yl)-6-{4-(4-methylpiperazin-1-yl)phenyl}amino]-1H-pyrazolo[3,4-d]pyrimidin-3(2H)-one]. The approach induced p53 activation in cells with wildtype protein but not in those in which p53 expression had been removed, subsequently leading to inhibition of acral tumor development. When the approach was tested on persister cells which had acquired resistance to BRAF/MEK inhibitors, the approach could re-sensitize these cells to treatments and significantly delay tumor development. The significance of the study is that targeting AKT/WEE1 could activate p53 signaling in drug resistant persister cells, which truncates the drug resistance and tumor growth mediated by these cell subpopulations.

Transcriptional and immunohistochemistry analyses of acral lentiginous melanoma tumors from Mexican patients suggest an immunosuppressive microenvironment

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Acral lentiginous melanoma (ALM), although overall a rare type of melanoma, is the most common form of the disease in a number of countries in Latin America, Africa and Asia; it is associated with a poor prognosis and recurrence. As immune cells acting in the tumor microenvironment have been reported to correlate with clinical characteristics and treatment response, in this study we seek to define the immune landscape of ALM and its relationship to tumoral transcriptional programs and clinical variables. We have performed transcriptome sequencing through exome-capture RNA-seq on 113 tumors from 86 patients, and immunohistochemistry (intratumoral and invasive margin areas) on markers CD8, CD3, MHC-I, MHC-II and S100 on 150 tumor regions from 74 patients, which had sufficient material for such studies. Samples were collected at the National Cancer Institute of Mexico and have been annotated with vast clinical information. A comparison of differentially expressed genes between primaries and metastases identified cell-surface receptors and genes involved in cell-matrix interactions, and a comparison between ulcerated and non-ulcerated tumours identified genes such as CXCL8, MMP1 and TERT as overexpressed in ulcerated samples. Hierarchical non-supervised clustering based on gene expression identified three clusters that are currently being analysed. Deconvolution of immune cell fractions suggests that B cells, T-reg and M2 macrophages are found in larger proportions of ALM tumors, whereas CD8 and CD4 T cells were found to be scarce. Immunohistochemistry quantification is still ongoing but suggests differences in immune cell
density within tumor regions. So far, our analyses point to genes that could drive important prognostic characteristics of ALM, and that its immune profile may recapitulate an immunosuppressed TME.

**MAFG is a novel oncogene in melanoma that rewires the tumor immune microenvironment**

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The MAF bZIP Transcription Factor G (MAFG) is a member of the small MAF family of proteins. Previously, we have shown that miRNA-29 suppresses melanoma development, at least in part, by repressing MAFG expression. Moreover, analysis of the TCGA revealed frequent amplification and/or overexpression of MAFG in melanoma. However, despite the known roles of MAFG in regulating antioxidant responses, its cell-intrinsic and -extrinsic effects in melanoma are unknown. Here, we investigated the role of MAFG in melanomagenesis. By using siRNAs and overexpression vectors, we modulated the expression of MAFG and the other members of the small MAF family, MAFF and MAFK, to study the dependency of melanoma cells on this family of proteins. We found that silencing of MAFG, but not MAFF or MAFK, abrogates melanoma cell growth, while melanocyte cell lines are less reliant on MAFG. Notably, MAFG overexpression promoted proliferation and focus formation of immortalized melanocyte cell lines. Using a high-throughput melanoma mouse modeling (ESC-GEMM) platform developed by the Karreth lab, we found that in vivo overexpression of MAFG in BrafV600E, Pten+/- mice potently accelerated melanoma development compared to the control cohort. Interestingly, MAFG-overexpressing tumors from ESC-GEMM mice and MAFG-High melanomas from TCGA showed reduced tumor immune infiltration, indicating a potential crosstalk between MAFG and the inflammatory tumor milieu. Interestingly, RNA-sequencing revealed that MAFG regulates a transcriptional program that could alter the tumor immune microenvironment. Altogether, we have uncovered a new role of MAFG in melanomagenesis that may rely on rewiring of the tumor immune microenvironment.

**A graph-based approach unveils complex patterns of melanoma distant metastases**

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The administration of immune checkpoint blockade (ICB) therapies in patients with stage III and IV melanomas has revolutionised the field, resulting in durable responses. Recent work has shown that different metastatic sites have distinct response rates to ICB therapies, suggesting that the distribution of anatomical locations in each patient plays a role in treatment response and overall survival. In order to explore the association between anatomical patterns of distant disease, response and survival outcomes, we collated and manually curated the progression history of distant metastases in a cohort of 6,031 patients with melanoma. This cohort includes untreated patients as well as patients exposed to ICB therapies and BRAF/MEK inhibitors. For each patient, the temporal and spatial distribution of distant metastatic events occurring from primary disease was captured in a directed acyclic graph model. A distance metric between the progression histories of any two patients based on their graph representation was calculated. The resulting distances were used to pursue clustering of these 6,031 metastatic progression histories. This approach identifies subsets of patients with similar histories of metastatic disease progression. These subsets present significant survival differences among them and capture varying levels of complexity. Specifically, our preliminary results identify that (i) haematogenous spread is associated with worse survival compared to distant lymphatic spread, (ii) patients with combinations of liver, lung and bone are among those groups with worst overall survival, (iii) patients with parallel metastatic events have worse survival than patients with events occurring in series.
and involving the same sites, and (iv) some clusters are enriched with patients exposed with ICB therapies.

Lesion-Level Response to Single-Agent PV-10 in Stage III Cutaneous Melanoma
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PV-10 (10% rose bengal sodium) is a small molecule autolytic immunotherapy in development for solid tumors. In both the single-agent (SA) setting and in combination with immune checkpoint blockade, intralesional injection (inj) can induce immunogenic cell death and tumor-specific reactivity in circulating T cells.

To evaluate SA response in Stage III cutaneous melanoma, a meta-analysis utilized data from 774 lesions in 121 patients (pts) treated at 14 clinical sites between 2007 and 2019 under phase 2, phase 3, and expanded access (EA) protocols. Data from case report forms and site databases were analyzed for response, survival, and safety. Best response for each lesion was assessed using RECIST thresholds starting ≥8 wks after initial inj to avoid potential interference from local reactions. Time-to-response (TTR) was based on confirmed response starting ≥4 wks after initial inj; time-to-progression (TTP) was based on RECIST thresholds; and time-to-treatment-failure (TTF) was based on clinically-relevant progressive disease.

Overall, 56% of lesions achieved CR after a median of 1 inj (range 1-8); 7% PR (median 2 inj, range 1-10); and 14% SD (median 2 inj, range 1-4). CR and PR were achieved with ≤2 inj in 85% and 81% of responding lesions, respectively. Non-responding lesions exhibited a similar pattern, with progression evident after a median of 1 inj (range 1-9). Median TTR was 2.4 months, TTP and TTF were not reached; a sensitivity analysis assigning progression for 71 non-evaluable lesions to 1 day after inj yielded identical outcomes. Median overall survival for 184 pts (including 63 EA pts without lesion-level data) was 44.9 months; disease-specific survival was 53.8 months. Adverse events were predominantly transient, locoregional, and mild-to-moderate grade.

These data demonstrate rapid response kinetics, high lesion-response rate, and acceptable safety for minimally invasive SA PV-10.

EP300/SOX10 co-amplification as a biomarker for sensitivity to A-485 in acral and UV-induced melanomas
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Acral lentiginous melanoma (ALM), a rare form of melanoma on sun-protected skin, is often diagnosed late and accounts for about 2-3% of all melanomas. Unfortunately, traditional melanoma therapies (e.g. BRAF inhibitors) are not as applicable to ALM and new strategies are needed. A recent study demonstrated amplified expression of the EP300 gene in a subset of ALM (~16%). EP300 encodes the p300 lysine acetyltransferase (KAT) and is an emerging therapeutic target in cancer. We previously reported that p300 KAT inhibitor A-485 downregulates proliferation in melanoma cell lines that highly express microphthalmia-associated transcription factor (MITF), a known driver of proliferation. MITF expression is controlled by the activity of several transcriptional regulators, including CREB, ATF2, SOX10 and EP300. Interestingly, the EP300 and SOX10 genes are located in close proximity on chromosome 22 and we found their copy numbers are strongly correlated in UV-induced (R=0.83, n=83, p=0.00001) and acral melanoma cell lines. We also report EP300 and SOX10 genes are commonly co-amplified in patients in The Cancer Genome Atlas (TCGA) Melanoma (SKCM) dataset and this correlates with higher EP300 and SOX10 expression in these patients. Furthermore, bioinformatics analysis of the Cancer Dependency Map demonstrates melanoma cell lines with EP300/SOX10 co-amplifications have increased MITF expression versus those without co-amplifications (p < 0.05). Since EP300 and SOX10 regulate MITF, we hypothesize EP300/SOX10 co-amplifications in acral and UV-induced melanoma activate MITF expression and that A-485 represents a novel therapeutic in this subset of melanoma tumors. We are currently exploring whether acral and UV-
induced melanoma cell lines with EP300/SOX10 co-amplifications are more sensitive to A-485 than those lacking co-amplifications through fluorescence-based proliferation assays.

**MYBL2 is an oncogene in melanoma**  
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**BACKGROUND:** MYBL2, a MYB family transcription factor regulating the cell cycle, cell survival, and differentiation, is frequently deregulated in cancer and promotes tumor initiation and progression. We previously showed that MYBL2 is an important functional target of the tumor suppressive miR-29 in melanoma. Moreover, MYBL2 often undergoes copy number gains in melanoma. These findings suggest an oncogenic function of MYBL2 in melanoma. However, whether and how MYBL2 promotes melanoma initiation and progression remains unknown.

**METHODS:** MYBL2 expression in melanoma cell lines was determined by RT-PCR and Western blotting. Cell proliferation, colony formation assay, and anchorage-independent growth assays were used to determine the role of MYBL2 in vitro. Xenograft models were used to determine the regulation of melanoma tumor growth by MYBL2 in vivo.

**RESULTS:** We observed that MYBL2 expression is upregulated in melanoma cells compared to BRAF-wildtype and BRAF-mutant melanocytes. TCGA analysis revealed that higher MYBL2 expression is associated with poorer survival of melanoma patients. In vitro characterization showed that MYBL2 overexpression in melanoma cells modestly promotes proliferation and increases clonogenic and anchorage-independent growth. Conversely, knock down of MYBL2 in melanoma cell lines diminishes melanoma cell proliferation, clonogenic growth and anchorage-independent growth. In addition, silencing of MYBL2 in A375 melanoma cells xenografted into NSG mice significantly slows tumor growth and decreases tumor weight at end point, while overexpression of MYBL2 in WM115 melanoma cells significantly increased tumor growth and tumor weight at end point.

**CONCLUSIONS:** Our results suggests that MYBL2 promotes melanoma aggressiveness and MYBL2 could potentially represent a melanoma vulnerability. Our future studies are aimed at revealing the mechanism underlying the oncogenic role of MYBL2 in melanoma.

**Immune checkpoint inhibitor resistance mutations in the IFN-JAK-STAT pathway increase melanoma sensitivity to oncolytic virus treatment**  
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Next-generation sequencing studies and CRISPR-Cas9 screens have established mutations in the interferon (IFN)γ-JAK-STAT pathway as a ICI resistance mechanism in a significant proportion of melanoma patients. We hypothesized ICI resistance mutations in the IFNγ pathway would simultaneously render melanomas susceptible to oncolytic virus (OV) therapy. To address this, we studied matched melanoma cell lines generated from a baseline biopsy and a progressing lesion with complete JAK2 loss from the same patient that relapsed on anti-PD-1 therapy. To determine OV sensitivity, these lines were infected with modified vesicular stomatitis virus (VSV-Δ51) and herpes simplex virus 1 (HSV1-dICP0). We observed the melanoma line from an anti-PD-1 progressing lesion was 7- and 22-fold more sensitive to HSV1-dIPC0 and VSV-Δ51, respectively, compared to the line from the baseline biopsy. Next, we performed RNAi and JAK inhibitor studies that revealed a significant increase in OV sensitivity with JAK/STAT pathway inhibition. Finally, our in vivo studies of Jak2 KOs B16-F10 mouse melanomas revealed a significant increase in VSV-Δ51 sensitivity with JAK/STAT pathway inhibition. We analyzed the cutaneous melanoma TCGA data and estimated that ~11% of ICI-naïve cutaneous melanomas have alterations in IFNγ pathway genes that may confer OV susceptibility. In conclusion, we provide mechanistic support for the use of OVs as a precision-medicine strategy for both salvage therapy in ICI-resistant and first-line treatment in melanomas with IFNγ-JAK-STAT pathway mutations. Our study also supports use of JAK inhibitor-OV combination therapy for treatment-naïve melanomas without defects in IFN signaling.
The spatial immune landscape of cutaneous melanoma

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Melanoma is an immunogenic malignancy with one of the highest response rates to immune checkpoint inhibitors (ICIs). It harbors an elevated mutation burden compared to other cancers, and as a result, abundant neoantigens are presented to infiltrating lymphocytes within its microenvironment. Understanding the complex interplay between the stroma, tumor cells and distinct tumor infiltrating lymphocyte (TIL) subsets remains a significant challenge in immune oncology. A fundamental requirement to study this interplay is the ability to quantify spatial relationships of multiple cell types within the tumor microenvironment. To address this, we employed Cytometry Time of Flight (CyTOF) Imaging Mass Cytometry (IMC) to simultaneously quantify the expression of 35 protein markers, characterizing the microenvironment of 5 benign nevi and 67 melanomas. We profiled over 230,000 individual cells to identify melanoma, lymphocyte subsets, macrophage/monocyte and stromal cell populations, allowing for in-depth spatial quantification of the melanoma microenvironment with unprecedented detail. Here, we report that within pre-treatment melanoma TILs in our patient cohort, it is the abundance of proliferating antigen-experienced cytotoxic T cells (CD8+CD45RO+Ki67+) and their proximity to melanoma cells that best inform on ICI responses. Our study highlights the potential of multiplexed single cell technology with the capacity to quantify spatial cell-cell interactions for ICI biomarker studies.

ARTISTRY-6: Nemvaleukin Alfa Monotherapy in Patients With Advanced Mucosal and Cutaneous Melanoma

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Despite improved outcomes for melanoma patients with the introduction of checkpoint inhibitors (CPIs), ~50% of patients do not respond. A subset of responders ultimately progress and have limited treatment options, underscoring an unmet need for novel treatments with durable benefit. Patients with mucosal melanoma exhibit response rates and progression-free survival times ~2 times lower than those with cutaneous melanoma. Nemvaleukin alfa (nemvaleukin, ALKS 4230) is a novel, engineered cytokine that selectively binds the intermediate-affinity interleukin-2 receptor complex to preferentially activate CD8+ T and NK cells with minimal expansion of regulatory T cells. Nemvaleukin has been granted orphan drug designation and fast track designation for the treatment of mucosal melanoma by the FDA. In ARTISTRY-1, intravenous (IV) recommended phase 2 dose (RP2D) of 6 µg/kg nemvaleukin monotherapy demonstrated durable antitumor activity in patients with advanced melanoma, including mucosal melanoma, previously treated with a CPI. In ARTISTRY-2, subcutaneous (SC) RP2D of 3 mg q7d had pharmacodynamic effects consistent with IV. ARTISTRY-6 is a phase 2, global, multicenter, open-label study. Eligible patients have had prior treatment with an anti–PD-(L)1 therapy with or without anti–CTLA-4 therapy and have an ECOG performance status of 0 or 1, adequate hematologic reserve and hepatic and renal function. Patients with advanced cutaneous (cohort 1) and mucosal (cohort 2) melanoma will receive nemvaleukin at the SC and IV RP2D, respectively. Patients will receive nemvaleukin until progression or intolerable toxicity. The primary objective is to evaluate the antitumor activity of nemvaleukin monotherapy by cohort. Additional objectives include the evaluation of...
safety, health-related quality of life, predictive biomarkers, pharmacokinetics, immunogenicity, and pharmacodynamic effects.

Five-year outcomes with adjuvant nivolumab (NIVO) versus ipilimumab (IPI) in resected stage IIIB–C or IV melanoma (CheckMate 238)


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In the double-blind, randomized phase 3 CheckMate 238 study, NIVO showed improved recurrence-free survival (RFS) and distant metastasis-free survival (DMFS) vs IPI in patients (pts) with high-risk, resected stage IIIB–C or IV melanoma, sustained at 4 y. There were fewer than expected overall survival (OS) events; rates were similar between arms. Updated 5-y outcomes are presented. Pts aged >15 y, stratified by disease stage and tumor programmed death ligand 1 (PD-L1) status, were randomized 1:1 to NIVO 3 mg/kg Q2W (n=453) or IPI 10 mg/kg Q3W for 4 doses, then Q12W (n=453) for up to 1 y. The primary endpoint was RFS; other key endpoints were OS (secondary) and DMFS (exploratory). At a 5-y minimum follow-up, RFS remained superior for NIVO vs IPI (HR 0.72; 95% CI, 0.60–0.99), with benefit seen across most subgroups including stage and PD-L1 status. DMFS in stage III pts also continued to favor NIVO (HR 0.79; 95% CI, 0.63–0.99). The OS data continue to be immature (events=228/302 expected, 75%), with 5-y OS rates of 76% (NIVO) and 72% (IPI) and an HR of 0.86 (95% CI, 0.66–1.12), compared with 4-y rates of 78% and 77%, respectively. Of pts who received subsequent systemic therapy, fewer NIVO vs IPI pts (70% vs 81%) received immunotherapy, most notably anti–PD-1 regimens. No additional late-emergent treatment-related adverse events were voluntarily reported since the 4-y analysis. Updated analyses exploring the association of several single and composite immuno-oncology–related biomarkers with OS and RFS will be presented. In summary, at a minimum 5-y follow-up, adjuvant NIVO showed sustained long-term RFS and DMFS benefits vs IPI across clinically relevant subgroups in pts with stage IIIB–C or IV resected melanoma. With fewer than expected deaths at 5 y, median OS was not reached in either arm with no notable difference between NIVO and IPI.
All stressed out: Mechanisms of survival in metastatic melanoma cells
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Metastatic melanoma is an aggressive disease. Despite recent improvements in therapy, drug resistant populations emerge even in drug-sensitive tumors. The ability to adapt to multiple types of stress contributes to tumor progression and therapy resistance. We have found a subset of melanoma cells with high Wnt5A and wild type p53 expression can transition to a slow-cycling state, rendering them resistant to most targeted therapy. Wnt5A, a non-canonical Wnt ligand that drives a metastatic phenotype, stabilizes the half-life of p53 and uses p53 to initiate a slow-cycling state. Multiple types of stress, including DNA damage, targeted therapy and aging increase Wnt5A and p53 in these metastatic cells. WTp53 is typically thought of as a tumor suppressor due to its ability to regulate cell proliferation, stress response, and cell death. However, in metastatic melanoma cells, p53 promotes the expression of EDIL3, FAP, MMP3, and netrin G1, which are associated with poor prognosis, increased angiogenesis, and metastasis in multiple types of cancer. In melanoma, inhibiting p53 blocks the slow cycling phenotype and promotes sensitivity to BRAFi/MEKi targeted therapy. These data suggest that inhibiting p53 may sensitize resistant metastatic melanoma cells to therapy. Paradoxically, wild type p53, typically thought of as a tumor suppressor, may be promoting the survival of a subset of highly resistant metastatic cells.

MERTK induced secretion of PROS1 by melanoma cells in the aged lung regulates the stromal and immune microenvironment to drive efficient metastatic progression
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Our previous data have indicated that while tumors grow more slowly in aged mouse skin, they form more lung metastases. We queried whether the lung microenvironment undergoes age-related changes that provide a permissive niche for metastatic outgrowth. Our data showed that secreted factors by lung fibroblasts changed during aging that promoted proliferation of melanoma cells via expression of the tyrosine kinase receptor MER. Further investigation of this MER-high phenotype revealed that these melanoma cells induce dramatic changes to the lung microenvironment via secretion of its primary ligand PROS1, which creates a more permissive niche for metastasis. It first acts in a paracrine manner to induce proliferation of previously dormant melanoma cells in the lung. MER induced secretion of PROS1 by melanoma cells also regulates the production of extracellular matrix (ECM) from lung fibroblasts. Specifically, healthy human lung fibroblasts produce thick, unified cellular derived matrices (CDMs) in vitro that are growth-restrictive to melanoma cells. Treatment of lung fibroblasts with conditioned media from MER-High melanoma cells or with recombinant-PROS1 alters CDM density and orientation, which resulted in a CDM that dramatically increased melanoma proliferation. Finally, we found that MER induced metastatic outgrowth of melanoma cells within the lungs of mice decreased CD4 and CD8 T-cells whilst increasing infiltration of and immunosuppressive T-regulatory cells (Tregs) and Myeloid Derived Suppressor Cells (MDSCs). Overall, we find that age-induced activation of melanoma cells towards a MER-high state within the lung can induce a cancer phenotype which dramatically alters fibroblast ECM production and immune cell infiltration to promote aggressive metastatic outgrowth and a growth permissive microenvironment.
Real-world clinical outcomes of pembrolizumab for advanced melanoma in the German ADOREG skin cancer registry

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Pembrolizumab has demonstrated strong antitumor activity in advanced melanoma trials, but real-world evidence in large patient groups is needed. We aimed to evaluate real-world utilization and clinical outcomes for patients treated with pembrolizumab for advanced melanoma in ADOREG, a German skin cancer registry.

Adults who initiated pembrolizumab for advanced melanoma (index date) from 1-Aug-2014 to 18-Feb-2021 were eligible for inclusion. Participation in a clinical trial or receipt of other chemotherapy led to exclusion. The Kaplan-Meier method was used to estimate overall survival (OS), real-world progression-free survival (rwPFS), and real-world time to next treatment or death (rwTTNTD). Included patients were followed through first of end of data availability or 01-Mar-2021. OS and rwPFS were estimated in patients with ≥6 months of follow-up or experienced an event within ≥6 months of index. Overall, 1012 patients (median age 69; range 21–96) were included in the study; 59% of patients were male, 10% with non-missing values had ECOG PS of 2+, and 20% had brain metastasis present at baseline. Most (82%) primary tumors were cutaneous, and 38% were BRAF-mutant. Median follow-up for all patients was 34.4 months (IQR 15.2–50.6). Pembrolizumab was 1L therapy for 54% of patients. Across all lines of therapy, median OS was 24.7 months (95% CI 21.6–30.3), median rwPFS was 4.3 months (3.6–5.0), and median rwTTNTD was 9.9 months (95% CI 9.0–10.8). With 1L pembrolizumab, median OS was 32.4 months (25.7–41.2); median rwPFS was 5.0 months (3.7–6.2); and median rwTTNTD was 13.6 months (10.4–15.9).

The pembrolizumab outcomes observed in this large real-world advanced melanoma patient population support its real-world effectiveness. The time
between median rwPFS and median rwTTNTD for all and 1L patients suggest pembrolizumab benefits may extend beyond disease progression.

A Standardized Analysis of Tertiary Lymphoid Structures in Human Melanoma: Disease Progression- and Tumor Site-Associated Changes With Germinal Center Alteration
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There is increasing evidence that tertiary lymphoid structures (TLS) not only control local adaptive B cell responses at tumor sites but also the cellular composition and function of other immune cells. In human melanoma, however, a comprehensive analysis of TLS density, phenotypes and spatial distribution in different disease stages is lacking. Here we used seven color multiplex immunostaining of whole tissue sections from 103 human melanoma samples to characterize TLS phenotypes along the expression of established TLS-defining molecular and cellular components. TLS density and spatial distribution was determined by referring TLS counts to the tissue area within defined intra- and extratumoral perimeters around the invasive tumor front.

We show that only a subgroup of primary human melanomas contains TLS. These TLS rarely formed germinal centers and mostly located intratumorally within 1 mm distance to the invasive tumor front. In contrast, melanoma metastases had a significantly increased density of secondary follicular TLS. They appeared preferentially in stromal areas within an extratumoral 1 mm distance to the invasive tumor front and their density varied over time and site of metastasis. Interestingly, secondary follicular TLS in melanoma often lacked the presence of BCL6⁺ lymphatic cells as well as canonical germinal center polarity with formation of dark and light zone areas.

Our work provides a standardized qualitative, quantitative and spatial analysis of TLS in human melanoma and shows disease progression- and site-associated changes in TLS phenotype, density and spatial distribution. The frequent lack of canonical germinal center polarity in melanoma TLS highlights the induction of TLS maturation as a potential additive to future immunotherapy studies.

Spatiotemporal Analysis of B Cell and Antibody Secreting Cell-Subsets in Human Melanoma Reveals Metastasis-, Tumor Stage-, and Age-Associated Dynamics
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The role of tumor-associated B cells in human cancer is only starting to emerge. B cells typically undergo a series of developmental changes, however, data on the composition of the B cell population in human melanoma are largely absent including changes during tumor progression and their potential clinical significance.

In this study, we compared the number and distribution of six major B cell and antibody secreting cell subpopulations in whole tumor sections of 154 human cutaneous melanoma samples (53 primary tumors without subsequent metastasis, 44 primary tumors with metastasis, 57 metastatic samples) obtained by seven color multiplex immunohistochemistry and automated tissue imaging and analysis.

In primary melanomas, we observed the highest numbers for plasmablast-like, memory-like, and activated B cell subtypes. These cells showed a patchy, predominant paratumoral distribution at the invasive tumor-stroma margin. Plasma cell-like cells were hardly detected, germinal center- and transitional/regulatory-like B cells not at all. Of the major clinicopathologic prognostic factors for primary melanomas, metastasis was associated with decreased memory-like B cell numbers and a higher age associated with higher plasmablast-like cell numbers. When we compared the composition of B
cell subpopulations in primary melanomas and metastatic samples, we found a significantly higher proportion of plasma cell-like cells at distant metastatic sites and a higher proportion of memory-like B cells at locoregional than distant metastatic sites. These data provide a first comprehensive and comparative spatiotemporal analysis of major B cell and antibody secreting cell subpopulations in human melanoma and describe metastasis-, tumor stage-, and age-associated dynamics, an important premise for B cell-related biomarker and therapy studies.

**Neoadjuvant Canerpaturev (C-REV) Oncolytic Immunotherapy in Combination with Nivolumab (Nivo) in Resectable Advanced Melanoma**

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**Background:** In clinical trials involving neoadjuvant therapy for high-risk resectable melanoma, pathologic complete response (pCR) is associated with excellent recurrence-free survival (RFS). C-REV, a bioselected HSV-derived oncolytic virus, has demonstrated efficacy as monotherapy and in combination with immune checkpoint inhibitors in advanced solid tumors. We report clinical and correlative results from a single-arm, open-label, Phase II study (NCT03259425) evaluating neoadjuvant Nivo + C-REV in resectable advanced melanoma. **Methods:** Key entry criteria: age ≥18y, ECOG ≤ 1, Stage IIIB/C and IVM1a resectable melanoma with measurable/injectable subcutaneous/cutaneous or nodal metastasis. C-REV was injected into single or multiple tumors (1 x 10⁷ TCID50/mL/dose, up to 5mL) for a total of 9 weekly/biweekly doses. Seven Nivo IV infusions (240mg) were administered q2wk, followed by surgery at 12 wks. Adjuvant Nivo was then given at 480mg/dose x 1 year total. AEs were assessed per CTCAE 4.0. Primary endpoint was pathological response rate. Secondary endpoints included RFS/OS at 1-year post adjuvant Nivo, R0 resection rate, and RECIST 1.1 radiographic tumor response. **Results:** Of 7 patients enrolled, 6 underwent surgery after C-REV/Nivo and were evaluable. Pathological CR rate was 83.3% (5/6), and R0 rate was 83.3% (5/6). At a median follow-up of 27.3 mo, RFS was 50.0% (3/6). Radiographic complete or partial responses in both injected/non-injected target lesions were seen in 66.7% (4/6) pts prior to surgery. Exploratory blood and tumor biomarker analyses showed enriched tumor immune cell infiltration, enhanced serum levels of pro-inflammatory cytokines, and increased Natural Killer T cells in responders vs. non-responders. **Conclusions:** Neoadjuvant C-REV + Nivo in resectable advanced melanoma produces a high pCR rate, warranting further investigation.

**Inflammatory cytokine release from BRAF and MEK inhibitor-mediated pyroptosis restructures the tumor-immune microenvironment**

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BRAF and MEK inhibitor (BRAFi+MEKi) resistant melanomas are cross-resistant to immune checkpoint inhibitors; hence, understanding immune-based mechanisms of response and resistance to BRAFi+MEKi and immunotherapies are critical to improve patient responses and to develop novel therapies. Our lab has recently shown that melanoma cells treated with BRAFi+MEKi undergo pyroptosis through cleavage of the pore-forming protein gasdermin E (GSDME). Tumors lacking GSDME showed changes to immune population percentages when compared to parental tumors after treatment with BRAFi+MEKi. Additionally, BRAFi+MEKi resistant cells no longer showed GSDME cleavage when treated with drug. Thus, we are exploring how BRAFi+MEKi-mediated cell death alters immune recruitment and function in melanoma in the presence and absence of GSDME. We now show that inflammatory cytokines are released in a GSDME-dependent manner after cells are treated with BRAFi+MEKi. Tumor analysis also revealed changes in direct recruitment of immune cells over time in the GSDME WT and GSDME KO tumors with CD8+ T cells enriched in the GSDME WT tumors and macrophages enriched in the GSDME KO tumors. Analysis of these populations via flow cytometry showed that the CD8+ T cells express receptors for the inflammatory cytokines released by pyroptotic melanoma cells indicating that the CD8+ T cells are recruited in a cytokine-dependent manner. Thus, the mechanisms of cell death in the melanoma cells may be playing an underappreciated role in promoting an immune response to the tumor.
Control of Rac1 activity and cell invasion by GTP metabolism enzymes in melanoma

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The RHO-GTPase Rac1 promotes tumor progression and metastasis in several malignancies including melanoma. Our laboratory recently uncovered a fundamental connection between GTP metabolism enzymes and Rac1 activity. Specifically, we demonstrated that expression levels of GTP-producing enzymes inosine monophosphate dehydrogenase 2 (IMPDH2) and guanosine monophosphate synthase (GMPS) increase during melanoma progression, while expression of GTP-suppressing enzyme guanosine monophosphate reductase (GMPR) is reduced. Modest reduction of intracellular GTP pools via inhibition of IMPDH2 or GMPS results in reduced Rac1 activity and invasion in melanoma cells, while restoration of GMPR levels has the same effects. Here, we show that these GTP metabolism enzymes co-localize with Rac1 in the protrusions of invading cells, while GTP pools are higher in protrusions than cell bodies. We discovered that phosphorylation of GMPR at Tyr267 is critical for GMPR activity and reduces GTP pools in protrusions, ultimately suppressing Rac1 activity, invasion, and tumorigenicity in melanoma cells. This phosphorylation is largely driven by receptor tyrosine kinase EPHA4, controlling the net effect of EPHA4 signaling on Rac1 activity. Mechanistically, we found that Rac1 co-immunoprecipitates with GTP metabolism enzymes in an IMPDH2-dependent manner, while Rac1 activity is sensitive to loss of local GTP production by IMPDH2. Importantly, the oncogenic Rac1P29S mutant protein present in 5-10% of sun-exposed melanomas is also dependent on IMPDH2 activity. Therefore, targeting GTP production may be a strategy for inhibiting Rac1 activity in melanoma, particularly in patients presenting with aggressive Rac1P29S-positive disease.

Targeting natural miRNA sponge as a novel strategy for melanoma therapy

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Despite metastatic melanoma being the leading cause of skin cancer-related deaths worldwide, the molecular mechanisms of melanoma metastasis have remained largely unknown. Genomic alterations such as deletions and amplifications occur frequently in human melanoma and are thought to be drivers of malignant progression. Aberrant expression of driver genes that are localized within genomic alterations may represent actionable cancer vulnerabilities; however, drivers are not easily distinguished from passengers based solely on the genomic information or the function of the encoded proteins. The previous work of our lab demonstrated that messenger RNAs engage in protein coding-independent posttranscriptional regulation by sequestering microRNAs (miRNAs) from other transcripts, and we termed such natural miRNA sponges competitive endogenous RNAs (ceRNAs). Deregulated ceRNA expression has been causally linked to cancer development, and thus amplification of ceRNA genes may contribute to malignant progression. Here we identified a highly interconnected ceRNA network that is largely controlled by three ceRNAs (CEP170, NUCKS1, and ZC3H11A) localized on chromosome 1q. Amplification of 1q occurs in up to 25% of human melanoma cases and is associated with melanoma progression. We validated that the 3' UTRs, but not the proteins encoded by CEP170, NUCKS1, and ZC3H11A promote melanoma cell invasiveness in vitro and metastasis in vivo. Notably, the combination of these three ceRNA 3' UTRs induced a potent oncogenic effect. Importantly, we found that the CEP170, NUCKS1, and ZC3H11A 3' UTRs are able to sequester 8 tumor suppressive miRNAs, and thus de-repress their oncogenic targets to promotes melanoma metastasis. Moreover, targeting these 3 ceRNA transcripts significantly inhibit melanoma cell metastasis, indicating the potential of novel therapeutic targets and treatment strategies.
VALIDATION OF AN ACCURATE IMMUNOPROFILING MELANOMA METHOD USING AUTOMATED MULTIPLEX IMMUNOFLUORESCENCE

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Multiplex immunofluorescence is a useful tool for the identification of multiple cell sub-populations within the tumour microenvironment in a single tissue section. However, this staining technique needs to be validated against clinical immunohistology before implementation into clinical practice. This study sought to investigate the reproducibility of a multiplex immunofluorescence workflow in characterising the expression of immune and melanoma markers in the tumour microenvironment.

Chromogenic immunohistochemistry (IHC), single-plex immunofluorescence and multiplex immunofluorescence were performed on serial tissue sections of a formalin-fixed paraffin-embedded (FFPE) tissue microarray containing metastatic melanoma specimens from 67 patients. The panel included the following markers: CD8, CD68, CD16, and PD-L1, and SOX10. Slides were stained with the Opal™ 7 colour Kit (PerkinElmer) on the Dako autostainer and imaged using the Vectra 3.0.5 microscope. Marker expression was quantified using Halo v.3.2.181 (Indica Labs). Significant positive correlations between the percentages of CD8, CD68, CD16, PD-L1, and SOX10 markers stained with IHC and single-plex immunofluorescence was observed (Spearman $r = 0.965$ to $r = 0.657$, $P < 0.0001$). Significant correlations were also observed for all markers following comparison between single-plex immunofluorescence and multiplex immunofluorescence staining (Spearman $r > 0.9$, $P < 0.0001$). Finally, correlation analysis of the three multiplex replicates revealed a high degree of reproducibility (Spearman $r > 0.9$, $P < 0.0001$). This points to the reliability and validity of this multiplex immunofluorescence workflow in the accurate profiling of multiple cell sub-populations in FFPE metastatic melanoma specimens and is useful for predictive clinical research evaluating melanoma and its microenvironment.

Personalized strategies with BCL2 family inhibitors to overcome acquired resistance to BRAF (BRAFi) and MEK (MEKi) in BRAF-mutant melanomas

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There is a critical need to identify new strategies for BRAF-mutant melanomas with acquired resistance to BRAFi + MEKi. Increased mitochondrial activity plays important roles in tumor survival and therapeutic resistance. These are promoted at least in part by antiapoptotic BCL2 family member proteins which demonstrate heterogeneous expression in this disease. We tested the efficacy of targeting BCL2 family members in combination with BRAFi/MEKi in molecularly characterized melanoma patient derived xenografts (PDX) established from BRAFi/MEKi-refractory patients to evaluate therapeutic potential of this approach and identify biomarkers to personalize such treatments.

The effects of navitoclax (NAV; inhibits BCL2 and BCLxL), alone and in combination with BRAFi+MEKi, were tested in vivo in a total of 18 PDX models. NAV had minimal single-agent activity, but in combination induced tumor regression or complete growth arrest in 6 of 10 (60%) BRAFi/MEKi-refractory PDX. Analysis of baseline features showed that sensitivity to NAV + BRAFi+MEKi was associated with increased BCL2 expression and low MCL1 expression. NAV + BRAFi+MEKi potently inhibited PI3K/mTOR activity and cell cycle regulatory proteins compared to single agents. Further testing with venetoclax (VEN; inhibits BCL2, not BCLxL) confirmed in vivo efficacy unless PDX had high expression of BCLxL or MCL1. Enforced expression of MCL1 functionally confirmed its role in resistance to NAV and VEN combinations with BRAFi+MEKi. AZD5991 (MCL1 inhibitor) demonstrated significant single agent anti-tumor activity, and additional
benefit when combined with BRAFi + MEKi, in MCL1-overexpressing melanomas. Together these results identify candidate personalized strategies targeting BCL2 family members for further testing in BRAF-mutant patients with acquired resistance to BRAFi + MEKi therapy.

Baseline autoantibody profiles may predict response to immune-checkpoint inhibitor therapy in patients with metastatic melanoma – a comparison of two studies

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Immunotherapies have revolutionised the melanoma treatment landscape. However, only 20-60% of patients respond to the therapy and biomarkers predictive of response are needed. Autoantibodies (AAbs) reflect a biologically amplified, stable signature of the anti-tumour immune response that is secreted in relatively large quantities in serum and may serve as valuable predictive biomarkers of therapy response. Blood from stage IV melanoma patients (n=25, study 1) was screened against the Immunome™ microarray containing 1627 functional antigens. In study 2, a total of 100 stage IV melanoma blood samples were collected and screened against the HuProt™ array containing >21000 antigens. IgG responses were measured in relative fluorescence units (RFU) from patient serum collected prior to the commencement of either CTLA-4, PD-1 or combination immunotherapy and the intra- and inter-array normalised RFU median was reported for each antigen for each patient. The serum score, the sum of all signal intensities above cutoff were calculated to quantify individual patient seroreactivity for multiple antibodies. Response to treatment was evaluated using the RECIST guidelines and was assessed via routine PET/CT scan at 12 weeks post therapy commencement. We observed distinct pre-treatment antibody profiles between patients on CTLA-4 and PD-1 therapy. In both studies, a shortlist of identified AAbs at baseline was predictive of response to immunotherapy. A high serum score prior to treatment commencement was associated with an increased overall survival and progression free survival. The identified autoantibody profiles may serve as valuable predictive blood-based biomarkers of response to current immune checkpoint inhibitors. However, studies involving larger cohorts of patients must be performed to validate these findings.

PV-10 and anti-PD-1 in cutaneous melanoma refractory to checkpoint blockade

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PV-10 (10% rose bengal sodium for injection) is a small molecule autolytic immunotherapy in development for solid tumors; intralesional injection can induce immunogenic cell death and tumor-specific reactivity in circulating T cells. A phase 1b/2 study is evaluating PV-10 in combination with systemic anti-PD-1 (pembrolizumab, “pembro”) in patients (pts) with at least 1 injectable lesion. The combination is administered q3w for 5 cycles followed by pembro alone q3w for up to 24 months. The primary endpoint is safety and tolerability; objective response rate (ORR) is a key secondary endpoint; exploratory immune correlative assessments are being performed on a subgroup of pts.

Between mid-2017 and the abstract deadline, 22 pts (4 IIIB-D, 8 M1a, 3 M1b, 4 M1c, 3 M1d; median age 72 yrs, range 28-90) refractory to at least one prior line of checkpoint blockade (CB) were enrolled in phase 1b (13 were refractory to CTLA-4 and PD-1). Adverse events were consistent with established patterns for each drug, principally Grade 1-2 injection site reactions attributed to PV-10 and Grade 1-3 immune-mediated reactions attributed to pembro, with no significant overlap or unexpected toxicities. Seventeen pts were evaluable for overall response by RECIST: 1 pt achieved CR (M1a), 3 PR (IIID, M1a, ...)
and M1d) (24% ORR), and 5 SD (IIIC, M1a, M1b, and 2 M1c) (53% disease control rate). Initial correlative assessments demonstrated increased HMGB1, a Damage Associated Molecular Pattern molecule associated with activation of dendritic cells, in post-PV-10 serum from 2 of 4 pts; additionally, 2 pts refractory to CTLA-1 and PD-1 exhibited enhanced T cell reactivity to HLA-matched melanoma cell lines that preceded a durable CR (M1a) and a durable SD (M1c).

Encouraging response and acceptable safety support expanded enrollment. Pharmacodynamic assessments substantiate the immune-mediated mechanism of PV-10 in CB-refractory pts.

KDM5B Promotes Immune Evasion by Recruiting SETDB1 to Silence Retroelements

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Tumors utilize various strategies to evade immune surveillance. Immunotherapies targeting tumor immune evasion such as immune checkpoint blockade have shown unprecedented efficacy on multiple cancers, but are ineffective for most patients due to primary or acquired resistance. Recent studies showed that some epigenetic regulators suppress anti-tumor immunity, suggesting epigenetic therapies could boost anti-tumor immune responses and overcome resistance to current immunotherapies. Here we show that depletion of KDM5B, an H3K4 demethylase critical for melanoma maintenance and drug resistance, induces robust adaptive immune responses and enhances responses to immune checkpoint blockade. Mechanistically, KDM5B recruits the H3K9 methyltransferase SETDB1 to repress endogenous retroelements such as MMVL30 in a demethylase-independent manner. De-repression of these retroelements activates cytosolic RNA and DNA sensing pathways and subsequent type I interferon response, leading to tumor rejection and induction of immune memory. Our results demonstrate that KDM5B suppresses anti-tumor immunity by epigenetic silencing of retroelements. Thus, we reveal the novel roles of KDM5B in heterochromatin regulation and immune evasion in melanoma, opening a new venue for the development of KDM5B and SETDB1 targeting therapies to enhance tumor immunogenicity and overcome immunotherapy resistance.

Malignant melanoma of the gallbladder in the era of modern therapies: outcomes of cholecystectomy.

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Historically, melanoma metastases to the gallbladder were considered a sign of advanced disease. The introduction of targeted and immunotherapy has reshaped the management of metastatic melanoma. Thus, we occasionally encounter gallbladder disease as an isolated site of treatment resistance or breakthrough. There is sparse literature on cholecystectomy for malignant melanoma, all of
We reviewed all patients at a single tertiary center that had a biopsy proven gallbladder melanoma after cholecystectomy between 2010-2021. We reviewed their clinical, pathological, perioperative variables, as well as short- and long-term outcomes.

Eight patients underwent cholecystectomy for gallbladder melanoma metastases. Six were treated with preoperative immunotherapy and 2 with targeted therapy. Six were asymptomatic and 2 presented with cholecystitis. Indication for cholecystectomy was control of limited resistant or progressive disease or cholecystitis. The mean age was 65 years. The location of the primary lesion was trunk (n=3), extremities (n=3), neck (n=1), and esophagus (n=1). Seven underwent laparoscopic resection and one required an open approach. The mean duration of surgery was 30.2 minutes. Mean length of stay was 2.5 days. There were no procedure related complications.

The median survival from the time the primary lesion was diagnosed was 150.1 months, and from gallbladder diagnosis was 17.1 months. The estimated DFS was 12.2 months. Both symptomatic patients were symptom free after cholecystectomy. Four patients died from metastatic disease, 3 alive with disease and 1 without evidence of disease. In the era of modern therapeutics, cholecystectomy for symptomatic disease or resistant/ breakthrough gallbladder metastases may be considered to achieve systemic control of the disease and symptom relief.

after Initial Progression of BRAF Mutant Metastatic Melanoma on Checkpoint Inhibitor Therapy

In order to test this hypothesis, we optimized a DTP cell colony formation assay in 1205Lu and 451Lu melanoma cells. Cells were treated with 1 µM vemurafenib (PLX4032) for 10 days, which resulted in distinct colonies of drug tolerant persister cells. While control DMSO-treated 451Lu cells reached confluence after 10 days, vemurafenib-treated 451Lu cells reached 1.48% colony area confluency after 10 days. These remaining vemurafenib-treated cells represent DTPs. We evaluated the ability of novel epigenetic therapies to influence the development of melanoma DTP cells after 10 days of treatment with vemurafenib or a vemurafenib/cobimetinib combination, including an inhibitor of the CoREST complex, corin, and the p300 HAT inhibitor, A-485 in addition to the LSD1 inhibitor GSKLSD1 and HDAC inhibitors currently in clinical trials. We expect epigenetic agents that prevent the development of DTP cells will set the stage for the use of such therapies to delay or prevent the development of MAPKi resistance, ultimately preventing clinical relapse and improving patient outcomes.