**Specific Aims**

Immune checkpoint inhibitors (ICIs) prolong recurrence-free survival and distant metastasis-free survival for patients with resected stage IIB-III melanoma in the adjuvant setting1–3. ICI therapy overcomes tumor immune suppression mechanisms that impact T cell effector function. Several potential predictive biomarkers for response to ICI therapy have been studied in melanoma, including factors intrinsic to the tumor as well as the tumor microenvironment (TME)4. Specifically, overall tumor mutational burden (TMB)5–7, the tumor neoantigen profile6–8, and the relative abundance of different subsets of tumor infiltrating lymphocytes (TILs)9–12 have been associated with response to ICI therapy.

The impact of host factors, such biologic sex, on the immune and clinical response to ICI therapy has not been well established. Biologic sex has long been known as a prognostic factor for melanoma13–17, with females experiencing a survival advantage across all stages of melanoma18,19. Differences in sex-linked gene expression and sex steroid signaling modulate innate and adaptive immune responses involved in disease pathogenesis and response to immunotherapies20,21. In our group, we have observed sex differences in clinical outcomes after shared multipeptide vaccination in patients with resected high-risk melanoma22. We found durable clinical benefit confined to males after vaccination with melanoma antigens for both CD8+ and CD4+ T cells22, while only females experienced durable clinical benefit after vaccination with antigens restricted to CD8+ T cells (unpublished results). Similarly, the impact of sex on the efficacy of ICI therapy in patients with melanoma is variable and inconclusive, with some studies suggesting a greater survival benefit for males compared to females23 and other studies suggesting no sex differences in survival benefit24.

While ICI therapy has revolutionized the treatment of patients with melanoma, the ability to successfully predict the patients who will benefit most from this treatment remains elusive. Understanding how host factors, such as biologic sex, modulate the response to ICI therapy and associated immune-related toxicities may better inform patients and clinicians on the treatment risk-benefit ratio. Further investigation is needed to better characterize the interactions among immune cells and signaling in the melanoma TME and if sex-based heterogeneity in the antitumor response translates into sex differences in response to ICI therapy. The objective of this study is to characterize the impact of biologic sex on the immune response within the TME and clinical outcomes after ICI therapy in patients with melanoma. We hypothesize that sex-based heterogeneity in the TME and antitumor response exists and contributes to more favorable outcomes in males after ICI therapy.

**Aim 1. Characterize the immune cell infiltrate in the melanoma tumor microenvironment.**

*1.1. Evaluate sex differences in immune cell infiltrate composition.*

*1.2. Evaluate sex differences in phenotypes of T cell populations*.

**Aim 2. Characterize key features of the antitumor response in the melanoma tumor microenvironment.**

*2.1. Evaluate sex differences in immune signaling pathways*.

*2.2. Evaluate sex differences in immune evasion mechanisms*.

*2.3 Evaluate sex differences in the tumor mutational burden.*

**Aim 3**. **Investigate sex differences in clinical outcomes after immune checkpoint inhibitor therapy.**

*3.1. Evaluate sex as an independent predictor for clinical outcome after immune checkpoint inhibitor therapy.*

**Research Strategy**

**Significance**

Identification of factors that influence response to immune checkpoint inhibitor (ICI) therapy is necessary for better characterizing the risk-benefit ratio of treatment for patients with melanoma. Several biomarkers have been proposed to predict response to ICI therapy4; however, the influence of host factors, such as biologic sex, on ICI therapy outcomes has not been well established, despite evidence supporting how biologic sex broadly influences immune function20,21. The goal of this study is to better characterize sex-based heterogeneity in the antitumor response and its influence on response to ICI therapy in patients with melanoma. These results may inform future clinical trial design to improve reporting of sex comparisons in immunologic and clinical outcomes25.

**Innovation**

Prior work to assess sex differences in ICI therapy response has primarily involved meta-analyses of published clinical trials outcomes23,24. Few studies26 have evaluated such differences at a molecular level for patients. To our knowledge, the ORIEN database has not been used for this type of analysis. The ORIEN database provides a unique opportunity to use clinical and immunologic data from a large real-world experience to analyze the impact of biologic sex on immune function and clinical outcomes in patients treated with ICI for melanoma.

**Approach**

We propose to analyze genomic and transcriptome data from tumor samples of patients age ≥ 18 years with high-risk (stage IIB-IV) melanoma. We will implement a comprehensive analysis pipeline to characterize sex-based differences in the tumor immune microenvironment. Clinical data associated with patient samples will be used to assess sex-specific differences in ICI therapy outcomes.

**Aim 1. Characterize the immune cell infiltrate in the melanoma tumor microenvironment.**

*1.1. Evaluate sex differences in immune cell infiltrate composition.* The female survival advantage in melanoma, particularly at earlier stages18,19, may reflect a more effective spontaneous antitumor response. However, prior analysis of a subset of TCGA samples suggested no sex differences in the abundance of six immune cell subsets comprised of T cells and myeloid-derived suppressor cells (MDSC)26. Detailed selection criteria of the TCGA sample subset was not reported in that study, though previously published data show that TCGA samples primarily represent metastatic tumors27. We hypothesize that females will have a greater abundance of immune cell infiltrates in the TME compared to males and that this difference may be abrogated, at least partially, in metastatic tumors able to evade the antitumor immune response. The xCell algorithm28 will be applied to bulk RNA-seq data to estimate enrichment scores for 64 distinct cell types, including immune and stromal populations. For each cell type, we will fit linear mixed models with enrichment scores as the dependent variable and sex as the primary predictor, adjusting for key clinical covariates (age, stage, prior treatments) and technical factors (batch, sequencing depth). Random effects will account for multiple samples per patient where available. The Benjamini-Hochberg procedure will be used to control the false discovery rate at 10% across all cell types tested.

*1.2. Evaluate sex differences in phenotypes of T cell populations*. Evidence of improved clinical response to ICI therapy in males compared to females with melanoma23,29 is supported by studies demonstrating greater abundance of partially exhausted cytotoxic T cells, a subset expressing high levels of PD-1 and CTLA-4 targeted by ICI therapy, in male tumors compared to females30. We hypothesize a greater abundance of T cell subtypes susceptible to rescue by ICI therapy in male tumors compared to females. Gene signature enrichment analysis (GSEA)31 will be performed using the fgsea R package to assess enrichment of previously validated signatures of melanoma tumor infiltrating lymphocytes (TILs)32,33 derived from single-cell RNA-seq studies. We will focus particularly on signatures of partially exhausted cytotoxic T cells characterized by high PD-1 and CTLA-4 expression. Genes will be pre-ranked by their correlation with sex (point-biserial correlation coefficient), and enrichment significance will be assessed through 10,000 permutations. Normalized enrichment scores and FDR-adjusted p-values will be calculated for each signature.

**Aim 2. Characterize key features of the antitumor response in the melanoma tumor microenvironment.**

*2.1. Evaluate sex differences in immune signaling pathways*. The complexity of the antitumor response may be better characterized by evaluating immune response pathways that capture specific gene interactions instead of assessing differentially expressed gene sets for immune cells alone. Thus, even if we do not find significant differences in the immune cell infiltrate in the melanoma TME as outlined in Aim 1, sex differences in immune signaling pathways may still exist that impact antitumor immunity and the response to ICI therapy. Consistent with Aim 1.1, we expect that female tumors will have gene signatures indicative of greater immune activation compared to males, particularly in earlier stage tumors, and that more immunosuppressive signatures will be present in metastatic tumors. GSEA will be conducted using both MSigDB Hallmark34 (n=50) and BioCarta pathway collections to examine immune-related pathways including inflammatory response, interferon signaling, T cell receptor signaling, and cytokine networks. The previously validated T cell-inflamed gene expression profile35 will be analyzed as a custom gene set. Pathway scores will be calculated using single-sample GSEA (ssGSEA) to enable correlation with clinical outcomes..

*2.2. Evaluate sex differences in immune evasion mechanisms*. While T cell subsets have been well-studied to identify potential biomarkers predictive of response to ICI therapy7,30, including identification of a T cell-inflamed gene expression profile35, another important mechanism of immune evasion involves T cell exclusion36,37. The Tumor Immune Dysfunction and Exclusion (TIDE) model38 addresses both T cell dysfunction and exclusion, with superior ability in predicting response to ICI therapy in ICI-naïve melanoma tumors compared to other biomarkers. Even if we do not find significant differences in abundances of T cell phenotypes outlined in Aim 1.2, sex differences in immune evasion may still exist due to differences in interactions between TILs and the other cells in the TME. We hypothesize that female and male tumors will exhibit greater expression of gene signatures associated with T cell exclusion and T cell dysfunction, respectively, particularly among those with poor response to ICI therapy. The TIDE computational framework will be applied to quantify two distinct immune evasion mechanisms: T cell dysfunction in T cell-inflamed tumors and T cell exclusion in non-T cell-inflamed tumors. TIDE scores will be integrated with tumor mutational burden (TMB) data (described in Aim 2.3). Sex-specific differences in these metrics will be assessed using multivariate regression models adjusting for tumor purity and mutation calling parameters.

*2.3 Evaluate sex differences in the tumor mutational burden.* A high TMB has been associated with clinical benefit from ICI therapy, leading to FDA approval of pembrolizumab (PD-1 inhibitor) for the treatment of unresectable solid tumors with TMB ≥ 10 mutations/megabase39. Sex differences in TMB have been attributed to more efficient immune escape mechanisms in females40. While we expect TMB to be associated with improved response to ICI therapy in our analyses, we hypothesize that male tumors will have a greater TMB compared to females consistent with previous work26,41. TMB will be calculated from matched tumor-normal whole-exome sequencing (WES) pairs using established bioinformatic pipelines (minimum coverage threshold of 100x, variant allele frequency ≥ 5%). Sex-specific differences in TMB will be assessed as described in Aim 2.2.

**Aim 3**. **Investigate sex differences in clinical outcomes after immune checkpoint inhibitor therapy.**

*3.1. Evaluate sex as an independent predictor for clinical outcome after ICI therapy.* We will obtain data on clinical outcomes from ORIEN, including best response to ICI therapy, date of disease progression or recurrence, and date of death. We hypothesize that males will have improved outcomes after ICI therapy compared to females. We will first establish the relationship between sex and immune features (TIDE scores, TMB, immune cell proportions) through our earlier analyses, then employ a mediation analysis framework to understand how these sex-dependent immune characteristics influence clinical outcomes. For advanced melanoma patients (unresectable stage IIIB-IV), we will construct hierarchical models that account for the causal pathway from sex through immune features to best clinical response. For survival outcomes, we will implement time-to-event analyses using Cox proportional hazard models, stratifying by ICI treatment modality (no ICI, neoadjuvant, or adjuvant) and carefully adjusting for clinical features not in the causal pathway between sex and outcomes. The primary endpoints will be overall survival (OS), progression-free survival (PFS), and recurrence-free survival (RFS), with sex-stratified Kaplan-Meier estimates and hazard ratios calculated to quantify male versus female outcome differences. This analysis will appropriately account for immune features as mediators rather than confounders.

**Statistical Considerations**

From the ORIEN database, we anticipate approximately 400 melanoma patients (stages IIB-IV) with an expected sex distribution of 60% male and 40% female. Of these, approximately 200 patients will have received ICI therapy, with matched molecular data (RNA-seq and WES) available for approximately 100 patients. Power calculations were performed using G\*Power software, assuming two-sided tests with α = 0.05. For the primary comparison of molecular features between sexes, our sample size will provide 80% power to detect moderate to large effect sizes (Cohen's d ≥ 0.5). For survival analyses, assuming a median follow-up of 24 months and a hazard ratio of 1.5, we will have 80% power to detect sex differences in survival outcomes.

**Budget Justification**

This proposal for this Computational Genomics and Data Science Pilot Awards FY2025 is for analysis of national ORIEN data (through Aster Insights) to understand differences, as a function of biologic sex, in the tumor immune microenvironment and clinical responses to immune checkpoint blockade. Given the scope of the analysis required (RNA-seq, WES data, cell type deconvolution, GSEA, TIDE modeling) we believe this project will be supported best by full-time work of a graduate student in the School of Data Science. Thus, we request funding for a graduate research assistantship for one semester. The cost for this graduate research assistant is $25,000.

No funding is requested for faculty effort (for Dr. Slingluff and Dr. Shakeri). Dr. Emily Ninmer is a surgical research fellow who works with Dr. Slingluff and is funded on the Rebecca Clary Harris Fellowship; funding for her is not requested in this pilot award.

Any other costs (publication costs, travel for presentation, administrative costs) will be covered by Dr. Slingluff and his team. The request to Aster Insights for the ORIEN data has been written by Dr. Ninmer and is being finalized with Drs. Slingluff and Shakeri with the expectation of submission in early December 2024. We fully expect that request for the data to be approved and to result in the data becoming available before the funding period for this pilot award.

**References**

1. Eggermont AMM, Blank CU, Mandalà M, et al. Adjuvant pembrolizumab versus placebo in resected stage III melanoma (EORTC 1325-MG/KEYNOTE-054): distant metastasis-free survival results from a double-blind, randomised, controlled, phase 3 trial. *Lancet Oncol*. 2021;22(5):643-654. doi:10.1016/S1470-2045(21)00065-6

2. Luke JJ, Rutkowski P, Queirolo P, et al. Pembrolizumab versus placebo as adjuvant therapy in completely resected stage IIB or IIC melanoma (KEYNOTE-716): a randomised, double-blind, phase 3 trial. *Lancet*. 2022;399(10336):1718-1729. doi:10.1016/S0140-6736(22)00562-1

3. Long GV, Luke JJ, Khattak MA, et al. Pembrolizumab versus placebo as adjuvant therapy in resected stage IIB or IIC melanoma (KEYNOTE-716): distant metastasis-free survival results of a multicentre, double-blind, randomised, phase 3 trial. *Lancet Oncol*. 2022;23(11):1378-1388. doi:10.1016/S1470-2045(22)00559-9

4. Gibney GT, Weiner LM, Atkins MB. Predictive biomarkers for checkpoint inhibitor-based immunotherapy. *Lancet Oncol*. 2016;17(12):e542-e551. doi:10.1016/S1470-2045(16)30406-5

5. Hugo W, Zaretsky JM, Sun L, et al. Genomic and Transcriptomic Features of Response to Anti-PD-1 Therapy in Metastatic Melanoma. *Cell*. 2016;165(1):35-44. doi:10.1016/j.cell.2016.02.065

6. Snyder A, Makarov V, Merghoub T, et al. Genetic basis for clinical response to CTLA-4 blockade in melanoma. *N Engl J Med*. 2014;371(23):2189-2199. doi:10.1056/NEJMoa1406498

7. Van Allen EM, Miao D, Schilling B, et al. Genomic correlates of response to CTLA-4 blockade in metastatic melanoma. *Science*. 2015;350(6257):207-211. doi:10.1126/science.aad0095

8. McGranahan N, Furness AJS, Rosenthal R, et al. Clonal neoantigens elicit T cell immunoreactivity and sensitivity to immune checkpoint blockade. *Science*. 2016;351(6280):1463-1469. doi:10.1126/science.aaf1490

9. Chen PL, Roh W, Reuben A, et al. Analysis of Immune Signatures in Longitudinal Tumor Samples Yields Insight into Biomarkers of Response and Mechanisms of Resistance to Immune Checkpoint Blockade. *Cancer Discov*. 2016;6(8):827-837. doi:10.1158/2159-8290.CD-15-1545

10. Daud AI, Loo K, Pauli ML, et al. Tumor immune profiling predicts response to anti-PD-1 therapy in human melanoma. *J Clin Invest*. 2016;126(9):3447-3452. doi:10.1172/JCI87324

11. Hamid O, Schmidt H, Nissan A, et al. A prospective phase II trial exploring the association between tumor microenvironment biomarkers and clinical activity of ipilimumab in advanced melanoma. *J Transl Med*. 2011;9:204. doi:10.1186/1479-5876-9-204

12. Tumeh PC, Harview CL, Yearley JH, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature*. 2014;515(7528):568-571. doi:10.1038/nature13954

13. Lasithiotakis K, Leiter U, Meier F, et al. Age and gender are significant independent predictors of survival in primary cutaneous melanoma. *Cancer*. 2008;112(8):1795-1804. doi:10.1002/cncr.23359

14. Thompson JF, Soong SJ, Balch CM, et al. Prognostic significance of mitotic rate in localized primary cutaneous melanoma: an analysis of patients in the multi-institutional American Joint Committee on Cancer melanoma staging database. *J Clin Oncol*. 2011;29(16):2199-2205. doi:10.1200/JCO.2010.31.5812

15. Scoggins CR, Ross MI, Reintgen DS, et al. Gender-related differences in outcome for melanoma patients. *Ann Surg*. 2006;243(5):693-698; discussion 698-700. doi:10.1097/01.sla.0000216771.81362.6b

16. Radkiewicz C, Johansson ALV, Dickman PW, Lambe M, Edgren G. Sex differences in cancer risk and survival: A Swedish cohort study. *Eur J Cancer*. 2017;84:130-140. doi:10.1016/j.ejca.2017.07.013

17. de Vries E, Nijsten TEC, Visser O, et al. Superior survival of females among 10,538 Dutch melanoma patients is independent of Breslow thickness, histologic type and tumor site. *Ann Oncol*. 2008;19(3):583-589. doi:10.1093/annonc/mdm498

18. Joosse A, Collette S, Suciu S, et al. Superior outcome of women with stage I/II cutaneous melanoma: pooled analysis of four European Organisation for Research and Treatment of Cancer phase III trials. *J Clin Oncol*. 2012;30(18):2240-2247. doi:10.1200/JCO.2011.38.0584

19. Joosse A, Collette S, Suciu S, et al. Sex is an independent prognostic indicator for survival and relapse/progression-free survival in metastasized stage III to IV melanoma: a pooled analysis of five European organisation for research and treatment of cancer randomized controlled trials. *J Clin Oncol*. 2013;31(18):2337-2346. doi:10.1200/JCO.2012.44.5031

20. Klein SL, Flanagan KL. Sex differences in immune responses. *Nat Rev Immunol*. 2016;16(10):626-638. doi:10.1038/nri.2016.90

21. Klein SL, Morgan R. The impact of sex and gender on immunotherapy outcomes. *Biol Sex Differ*. 2020;11(1):24. doi:10.1186/s13293-020-00301-y

22. Ninmer EK, Zhu H, Chianese-Bullock KA, et al. Multipeptide vaccines for melanoma in the adjuvant setting: long-term survival outcomes and post-hoc analysis of a randomized phase II trial. *Nat Commun*. 2024;15(1):2570. doi:10.1038/s41467-024-46877-6

23. Conforti F, Pala L, Bagnardi V, et al. Cancer immunotherapy efficacy and patients’ sex: a systematic review and meta-analysis. *Lancet Oncol*. 2018;19(6):737-746. doi:10.1016/S1470-2045(18)30261-4

24. Wallis CJD, Butaney M, Satkunasivam R, et al. Association of Patient Sex With Efficacy of Immune Checkpoint Inhibitors and Overall Survival in Advanced Cancers: A Systematic Review and Meta-analysis. *JAMA Oncol*. 2019;5(4):529-536. doi:10.1001/jamaoncol.2018.5904

25. Kammula AV, Schäffer AA, Rajagopal PS, Kurzrock R, Ruppin E. Outcome differences by sex in oncology clinical trials. *Nat Commun*. 2024;15(1):2608. doi:10.1038/s41467-024-46945-x

26. Ye Y, Jing Y, Li L, et al. Sex-associated molecular differences for cancer immunotherapy. *Nat Commun*. 2020;11(1):1779. doi:10.1038/s41467-020-15679-x

27. Cancer Genome Atlas Network. Genomic Classification of Cutaneous Melanoma. *Cell*. 2015;161(7):1681-1696. doi:10.1016/j.cell.2015.05.044

28. Aran D, Hu Z, Butte AJ. xCell: digitally portraying the tissue cellular heterogeneity landscape. *Genome Biol*. 2017;18(1):220. doi:10.1186/s13059-017-1349-1

29. Jang SR, Nikita N, Banks J, et al. Association Between Sex and Immune Checkpoint Inhibitor Outcomes for Patients With Melanoma. *JAMA Netw Open*. 2021;4(12):e2136823. doi:10.1001/jamanetworkopen.2021.36823

30. Loo K, Tsai KK, Mahuron K, et al. Partially exhausted tumor-infiltrating lymphocytes predict response to combination immunotherapy. *JCI Insight*. 2017;2(14):e93433, 93433. doi:10.1172/jci.insight.93433

31. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A*. 2005;102(43):15545-15550. doi:10.1073/pnas.0506580102

32. Oliveira G, Stromhaug K, Klaeger S, et al. Phenotype, specificity and avidity of antitumour CD8+ T cells in melanoma. *Nature*. 2021;596(7870):119-125. doi:10.1038/s41586-021-03704-y

33. Sade-Feldman M, Yizhak K, Bjorgaard SL, et al. Defining T Cell States Associated with Response to Checkpoint Immunotherapy in Melanoma. *Cell*. 2018;175(4):998-1013.e20. doi:10.1016/j.cell.2018.10.038

34. Liberzon A, Birger C, Thorvaldsdóttir H, Ghandi M, Mesirov JP, Tamayo P. The Molecular Signatures Database (MSigDB) hallmark gene set collection. *Cell Syst*. 2015;1(6):417-425. doi:10.1016/j.cels.2015.12.004

35. Ayers M, Lunceford J, Nebozhyn M, et al. IFN-γ-related mRNA profile predicts clinical response to PD-1 blockade. *J Clin Invest*. 2017;127(8):2930-2940. doi:10.1172/JCI91190

36. Gajewski TF, Schreiber H, Fu YX. Innate and adaptive immune cells in the tumor microenvironment. *Nat Immunol*. 2013;14(10):1014-1022. doi:10.1038/ni.2703

37. Joyce JA, Fearon DT. T cell exclusion, immune privilege, and the tumor microenvironment. *Science*. 2015;348(6230):74-80. doi:10.1126/science.aaa6204

38. Jiang P, Gu S, Pan D, et al. Signatures of T cell dysfunction and exclusion predict cancer immunotherapy response. *Nat Med*. 2018;24(10):1550-1558. doi:10.1038/s41591-018-0136-1

39. Marabelle A, Fakih M, Lopez J, et al. Association of tumour mutational burden with outcomes in patients with advanced solid tumours treated with pembrolizumab: prospective biomarker analysis of the multicohort, open-label, phase 2 KEYNOTE-158 study. *Lancet Oncol*. 2020;21(10):1353-1365. doi:10.1016/S1470-2045(20)30445-9

40. Castro A, Pyke RM, Zhang X, et al. Strength of immune selection in tumors varies with sex and age. *Nat Commun*. 2020;11(1):4128. doi:10.1038/s41467-020-17981-0

41. Conforti F, Pala L, Bagnardi V, et al. Sex-based differences of the tumor mutational burden and T-cell inflammation of the tumor microenvironment. *Ann Oncol*. 2019;30(4):653-655. doi:10.1093/annonc/mdz034