Immunopeptidomics Profiling of Healthy Tissue Matched to Primary and Metastatic Tumor Samples

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INTRODUCTION

Therapeutic peptide-based vaccination is a promising strategy for cancer immunotherapies. The identification of tumor-specific antigens is key to developing therapies. Mass spectrometry has emerged as the primary technique for the identification of peptides presented on human leukocyte antigen (HLA), i.e., immunopeptides. While tremendous progress has been made, the analysis of immunopeptides remains challenging. This is especially true in a clinical context where tissue material is scarce, often limited to a handful of biopsies, with amounts below 20mg. Here, we introduce a workflow that allows deep immunopeptidomics profiling of clinically relevant samples.

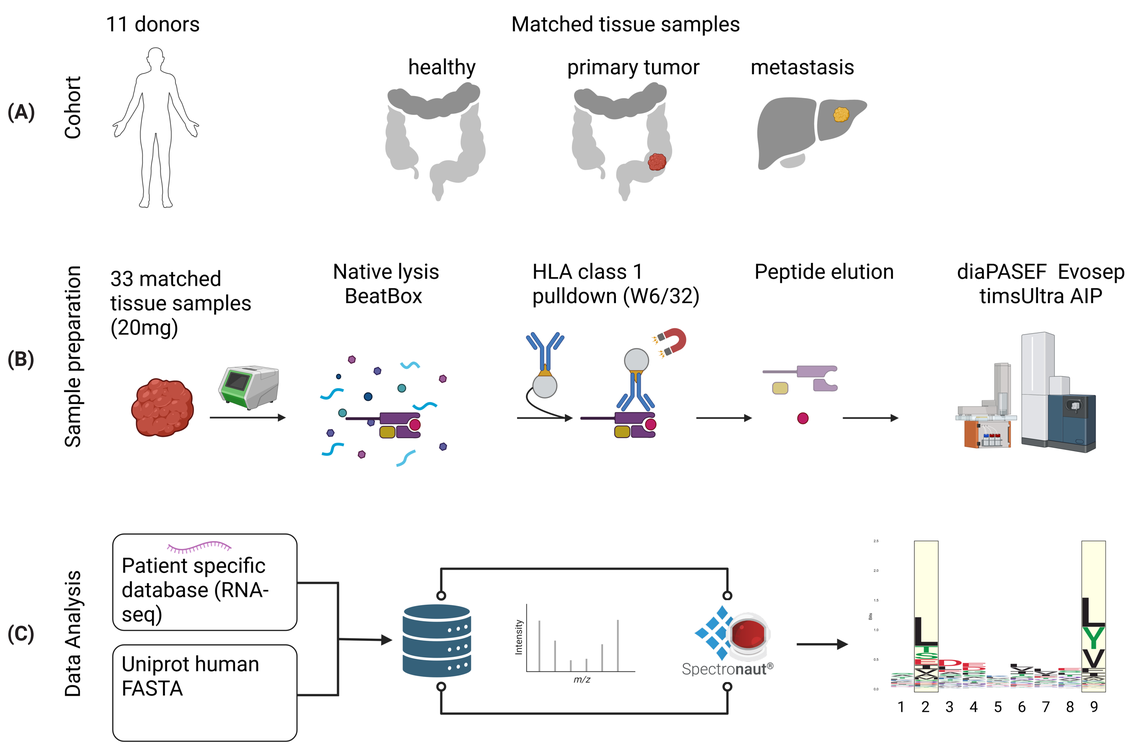

Figure 1 Overview of the cohort (A), sample preparation workflow (B), and the data analysis strategy (C). (**A**) The cohort comprises 33 tissue samples collected from 11 donors. For each donor, tumor tissue, adjacent healthy tissue, and metastatic tissue were collected. (**B**) 20mg of tissue was homogenized using the BeatBox Tissue Kit 24x under native conditions. Immunopeptides were isolated using anti-HLA class I antibodies (W6/32). Immunopeptides were then loaded onto Evotips (Evosep) and analyzed using the Whisper Zoom method from Evosep coupled to a timsUltra AIP (Bruker) using diaPASEF. (**C**) Data analysis was performed using Spectronaut® 20 (SN20) in directDIA®, either with the canonical human FASTA database or a patient-specific database.

METHODS

Tissue samples were lysed under native conditions using the BeatBox (PreOmics) Tissue Kit 24x (2x 10min standard settings). Immunopeptides were isolated using anti-HLA class I antibodies coupled to magnetic protein A beads in a semi-automated manner using the KingFisher. The peptides were eluted under acidic conditions and filtered using a 10kDa MWCO plate. The immunopeptides were then loaded onto Evotips and analyzed using the Whisper Zoom method from Evosep coupled to a timsUltra AIP (Bruker) mass spectrometer. Data analysis was performed using Spectronaut 20 (Biognosys). For the RNA sequencing, total RNA was extracted from fresh frozen tissue samples and sequenced using Illumina 2×150 bp paired-end reads (~100M reads per sample).

RESULTS

PEPTIDE IDENTIFIED PER DONOR AND TISSUE TYPE

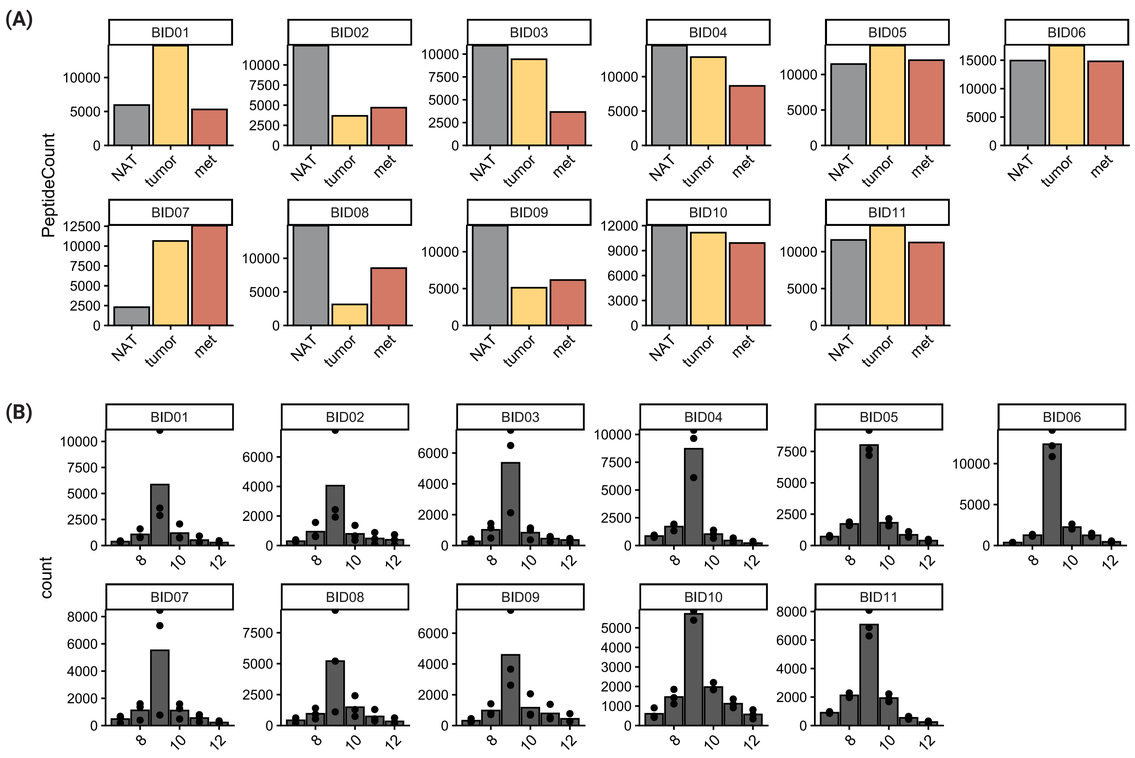

Figure 2 The MS data was analyzed in directDIA using Spectronaut 20. (**A**) Number of immunopeptides identified per donor and tissue type: healthy tissue (grey), tumor tissue (yellow), and metastasis (red). The peptide count is based on stripped sequences. On average, 10,000 peptides were identified per sample. (**B**) To investigate the data quality, peptide length distribution was plotted per donor, highlighting a pronounced enrichment of 9-mers.

PROPORTION OF PEPTIDE PREDICTED TO BE BINDERS PER SAMPLE

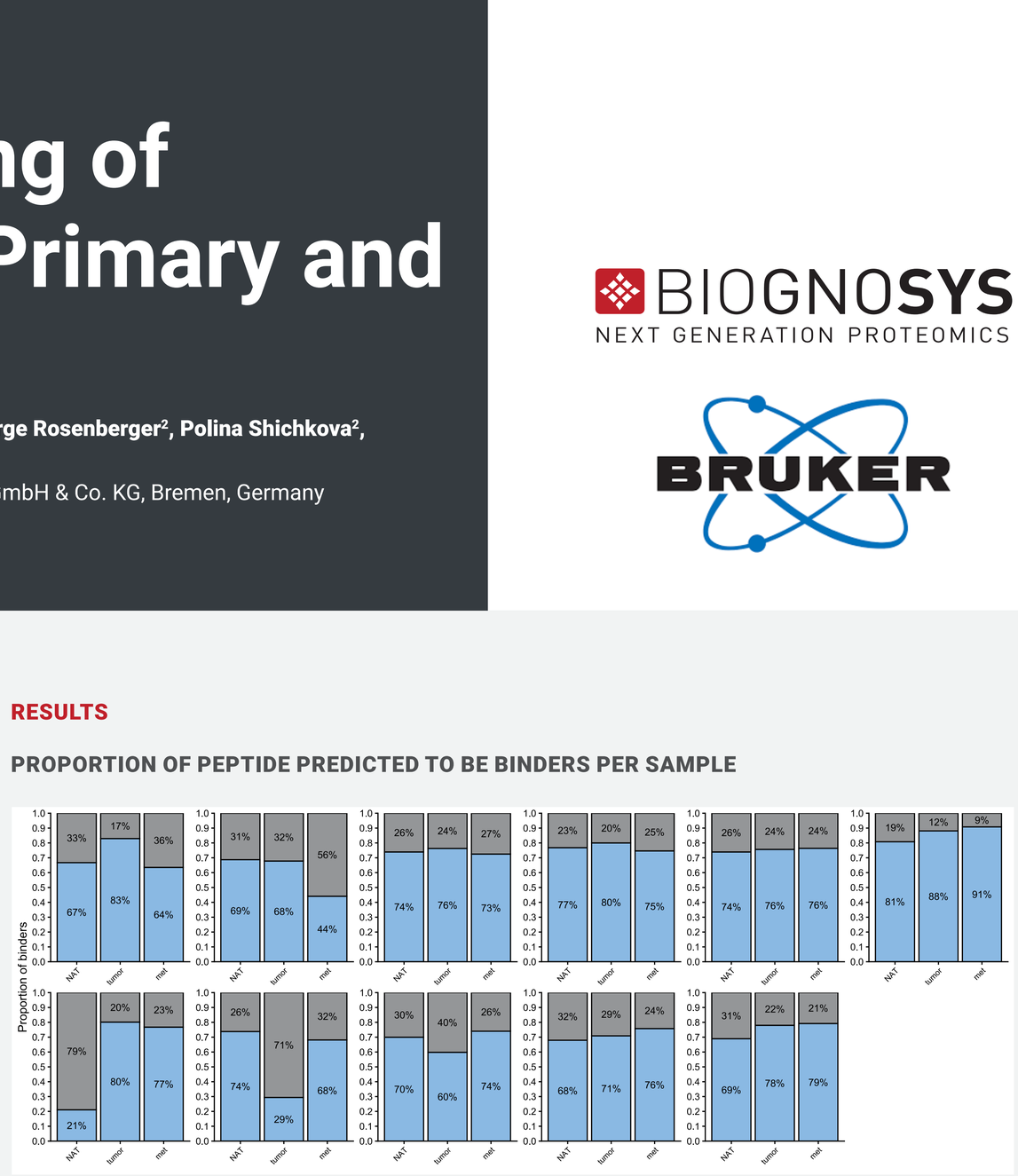

Figure 3 To further investigate data quality, peptide binding predictions were performed using the MHCflurry 2.0 tool (PMID: 32711842) for each sample. Overall, 75% of the peptides were predicted as strong binders (presentation score >0.5). The large proportion of binders supports the robustness of the immunopeptidomics workflow.

INTEGRATION OF RNA-SEQ DATA REVEALS NON-CANONICAL PEPTIDES FROM TISSUE BIOPSIES WITH IMMUNOTHERAPY POTENTIAL

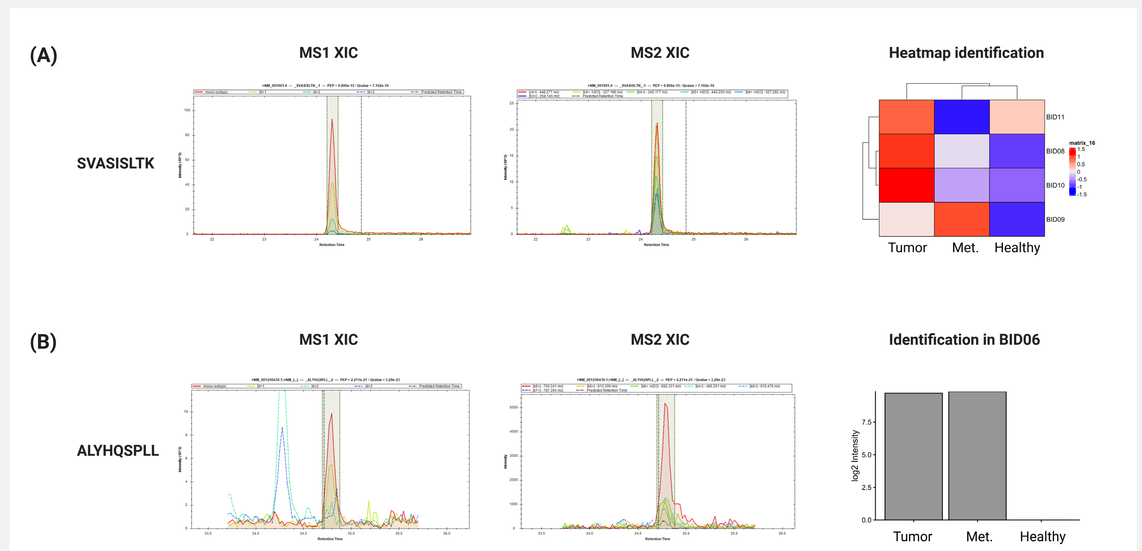

Figure 4 Integrating RNA-seq data reveals over 200 non-canonical peptides with immunotherapy potential. Two examples are highlighted:

(**A**) SVASISLTK , a non-canonical immunopeptide previously reported in Waleron et al. (BiorXiv, 2025). This peptide was identified in 12 samples and 4 donors. The heatmap shows Z-scored peptide intensities across the tissue type (tumor, metastasis, and healthy) , with enrichment observed in tumor samples compared to metastasis and healthy tissue.

(**B**) ALYHQSPLL, described in US patent US20200093862A1 as a candidate for cancer immunotherapy, was identified in one sample (BID06) and exclusively in tumor and metastasis tissues, highlighting its potential as an immunotherapy target.

CONCLUSIONS

- **We developed and optimized sample preparation to process clinical tissue samples (20mg). This workflow will be further evaluated for lower input, down to 5mg.**
- **A cohort of 11 donors was analyzed. Using directDIA we identified on average 10'000 peptides per sample, including 75% of predicted strong MHC binders.**
- **By integrating RNA-sequencing and mass spectrometry data we identified over 200 non-canonical 9-mer peptides predicted to be strong binders. Notably, several of these peptides have been reported in publications or listed in patents. Further validation is needed to confirm these findings.**

CONTACT

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Conflict of interest Disclosure: Arthur Viodé, Monika Puchalska, Anamarija Pfeiffer, Lucy Yang, Monika Peplnjak, George Rosenberger, Polina Shichkova, Christopher Below and Roland Bruderer are current employees of Biognosys AG, Schlieren (Switzerland). Kristina Marx is a current employee of Bruker Daltonics GmbH & Co. KG, Bremen (Germany). Daniel Hornburg is a current employee of Bruker Scientific LLC, Billerica (MA, USA)