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Tissue biomarkers for routine diagnostics and proteomic research in cutaneous melanoma malignum

Ph.D. Thesis

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List of publications

Scientific papers included in this thesis:

I. Leticia Sz., Erika V., Beáta Sz., Natália P. de A., Gilberto D., Lazaro H. B., Jeovanis G., Matilda M.-V., Henriett O., Ágnes Judit J., Maria Del Carmen B.-A., Lajos K., Bo B., Johan M., Peter H., A Marcell Sz., István Balázs N., György M.-V. Deep Proteomic Analysis on Biobanked Paraffine-Archived Melanoma with Prognostic/Predictive Biomarker Read-Out. Cancers (Basel) (2021) Dec 3;13(23):6105. doi: 10.3390/cancers13236105. **IF:6.575** (Journal specialization: Scopus – Oncology, SJR indicator: Q1)

II. Leticia Sz., Jéssica de S. G., Nicole W., Natalia P. de A., Ágnes J., Ahmed R., Ferenc K, András K., Ede M., Guihong W., Nga N., Henriett O., Roger A., Fábio N., Gilberto D., Kun-Hsing Y., Yevgeniy R. S., Johan M., Melinda R., Elisabet W., David F., Lajos K., Peter H., István B. N., György M.-V., Jeovanis G., Mitochondrial and Immune Response Dysregulation in Melanoma Recurrence. Clinical and Translational Medicine. Clin Transl Med. 2023 Nov;13(11): e1495. doi: 10.1002/ctm2.1495. **IF: 7.9** (Journal specialization: Scopus – Medicine (miscellaneous), SJR indicator: D1)

Publications not directly related to the thesis:

III. István N.B., **Leticia Sz.,** Ágnes J.J., Zsuzsanna Ú., Tibor P., György M-V., Lajos K., Erika V., A molekuláris biológiai módszerek dermatopatológiai vonatkozásai. BŐRGYÓGYÁSZATI ÉS VENEROLÓGIAI SZEMLE, 2022, 98. ÉVF.3.152–158.DOI 10.7188/bvsz.2022.98.3.7.

IV. Erika V., **Leticia Sz.,** Qimin Z., Yonghyo K., Indira P., Aniel S., Roger A., Henriett O., Matilda M.-V., Boram L., Ho J. K., Johan M., Attila M. Sz., Jeovanis G., Lazaro H. B., István B. N., György M.-V. A biobanking turning-point in the use of formalin-fixed, paraffin tumor blocks to unveil kinase signaling in melanoma. Clin Transl Med. 2021 Aug;11(8):e466. doi: 10.1002/ctm2.466.

V. Lazaro H. B., Jeovanis G., Aniel S., Viktória D., Magdalena K., Jimmy R. M., Erika V., Uğur Ç., Yonghyo K., Yutaka S., Indira P. P., Beáta Sz., Roger A., Elisabet W., Charlotte W., Natália P. de A., Nicole W., Matilda M.-V., Jonatan E., Krzysztof P. , Bo B., Christian I., Håkan O., Lotta L., Henrik L., Henriett O., Boram L., Ethan B., Marie S., Carina E., Dasol K., Ho J. K., Beatrice K., Melinda R., Johan M., Runyu H., Peter H., A Marcell Sz., József T., Sarolta K., Peter H., Tasso

M., Toshihide N., Harubumi K., Erik S., Madalina O., Ken M., Francesco F., Quimin Z., Gilberto B D., Luciana P., Fábio C S N., **Leticia Sz.,** István B. N., Henrik E., David F., György M.-V. The Human Melanoma Proteome Atlas-Complementing the melanoma transcriptome. Clin Transl Med. 2021 Jul;11(7):e451. doi: 10.1002/ctm2.451. **IF:8.554** (Journal specialization: Scopus – Medicine (miscellaneous), SJR indicator: Q4)

VI. Lazaro H. B., Jeovanis G., Yonghyo K., Viktória D., Uğur Ç., Aniel S., Jimmy R. M., Magdalena K., Indira P. P., Yutaka S., Roger A., Elisabet W., Charlotte W., Erika V., Natália P. de A., Nicole W., Matilda M.-V., Krzysztof P., Jonatan E., Beáta Sz., Bo B., Christian I., Håkan O., Lotta L., Henrik L., Henriett O., Boram L., Ethan B., Marie S., Carina E., Dasol K., Ho J. K., Beatrice K., Melinda R., Runyu H., Peter H., Tasso M., Toshihide N., Harubumi K., Erik S., Madalina O., Ken M., Francesco F., Qimin Z., Gilberto B. D., Luciana P., Fábio C.S. N., Peter H., **Leticia Sz.,** József T., Sarolta K., Marcell A. Sz., Johan M., Dávid F., Henrik E., István N. B., György M-V., The human melanoma proteome atlas-Defining the molecular pathology. Clin Transl Med. 2021 Jul;11(7):e473. doi: 10.1002/ctm2.473. **IF:8.554** (Journal specialization: Scopus – Medicine (miscellaneous), SJR indicator: Q4)

VII. Leticia Sz., Aron B., Indira P. P., Alexandra L., Dorottya P., Anna S. L., Natália P de A., Ágnes J. J., Fábio N., Beata Sz., Viktória D., Nicole W., Jéssica G., Zsuzsanna U., Zoltán G. P., Tibor P., Yonghyo K., Balázs Gy., Bo B., Charlotte W., Marcell A. Sz., Lazaro B., Jeovanis G., Roger A., Ho J. K., Sarolta K., Magdalena K., Jimmy R. M., István B. N., Johan M., David F., Krzysztof P., Peter H., Elisabet W., Lajos V. K., Gilberto D., György M-V., Aniel S. Predicting immune checkpoint therapy response in three independent metastatic melanoma cohorts. Front. Oncol. 2024, 14:1428182. doi: 10.3389/fonc.2024.1428182. **IF:3.5** (Journal specialization: Scopus – Oncology, SJR indicator: Q2)

1. Introduction

1.1.   Management of melanoma malignum in patient care

Melanoma malignum (MM) is a highly lethal skin cancer responsible for 80% of skin cancer-related deaths, posing challenges to healthcare systems with high treatment costs. Early diagnosis of the disease via clinical detection and histological examination is crucial. Detection of metastases and clinical staging according to the American Joint Committee on Cancer eighth edition (AJCC8) guidelines are essential before initiating therapy. Regarding the therapy options, kinase and immune checkpoint inhibitors have revolutionized the management of melanoma in the last 10 years by inhibiting cell proliferation and activating lymphocytes, yet therapy resistance and toxicity, such as immune-related adverse effects (irAE), remain a challenging issue.

1.2.   Role of prognostic and predictive biomarkers

Proteins are increasingly significant as biomarkers in predicting disease outcomes. They play a central role in diagnosing and monitoring various conditions, such as autoimmune diseases, inflammation, and cancers. For example, prostate-specific antigen help detecting localized prostate cancers, and high thyroglobulin levels can predict thyroid tumor recurrence. In the past decade, biomarkers such as Breslow level, blood lactate dehydrogenase levels, gene mutations (e.g., MITF, CDKN2A), and immune cell density have shown promise in predicting melanoma progression, but they lack standardization and accuracy.

In melanoma histopathology, biomarkers can be categorized by their functions: prognostic biomarkers for predicting disease progression, and predictive biomarkers for anticipating therapy response. The BRAF mutation serves as a key target in melanoma. It mainly occurs in the V600. position of the BRAF gene (e.g., V600E, V600K, V600R, V600D), and plays a pivotal role in treatment selection as well. DNA-based PCR analysis is the gold standard for mutation detection, nonetheless it is a time-consuming and expensive technique. There are alternative methods, like protein-based immunohistochemistry (IHC) staining with the VE1 clone antibody, which is cost-effective and accessible for detecting mutated BRAF proteins, requiring minimal tumor content and offering preserved protein integrity. IHC staining provides information about the tumor, complementing PCR analysis in diagnostic evaluation. This thesis will outline the comparative analysis of these two methods.

Regarding therapy response, it is known that due to the tumor mutation burden and the immunogenic nature of melanoma, there is an increased likelihood of producing mutant proteins that can serve as neoantigens, thereby enhancing immunogenicity. These mutant proteins can also contribute to the prediction of therapy response. Nowadays, artificial intelligence-based digital pathology plays a vital role in enabling the prediction of disease recurrence risk and survival outcomes, with the help of machine learning and deep learning algorithms. In addition, proteomics studies using formalin-fixed paraffin-embedded (FFPE) samples can provide insights into the tumor microenvironment and the underlying mechanisms of progression. In summary, our biomarker research seeks to deepen our understanding of the molecular features of melanoma through novel methodologies such as quantitative proteomics and digital pathology with AI-driven imaging, promising more personalized treatment approaches and ultimately improving patient outcomes.

2. Aims

Our research group is dedicated to uncover proteins from paraffin-archived melanoma samples for predictive and prognostic purposes with novel methodologies. In the scope of this discovery project, our goals are the following: (i) To compare the use of PCR technique and IHC staining on BRAF mutation detection in routine diagnostics. (ii) For predictive purposes, we conduct a comprehensive proteomic analysis on FFPE melanoma samples to unveil potential proteins predicting therapy outcomes. (iii) For prognostic purposes, we introduce an AI-powered digital pathology approach alongside an in-depth quantitative proteomics analysis of 12 early-stage primary melanomas to detect potential proteins predicting progression.

3. Materials and Methods

I will summarize the materials and methods of the BRAF detection study, predictive biomarker study **(paper I)** and prognostic biomarker study **(paper II)**.

3.1.　Workflow of the studies involved in the thesis

In our BRAF detection study, we collected retrospectively 94 FFPE melanoma samples along with their clinical data, including PCR data of BRAF mutations. These samples were stained with VE1 antibody to detect BRAF mutation. Samples that were PCR-negative for BRAF mutations but showed diffuse positive staining with intratumoral heterogeneity were sent for

quantitative PCR analysis. Samples that were PCR-negative for BRAF mutations but showed focal positive staining were submitted for next-generation sequencing. The results were then summarized. In the predictive biomarker study (**paper I**) 90 FFPE melanoma samples were collected retrospectively with detailed clinical information for each sample during the oncology care. The samples underwent histopathology analysis and were also sent for proteomic analysis using high-resolution mass spectrometry. The results were eventually analyzed. In the prognostic biomarker study (**paper II**), six early-stage FFPE melanoma samples with recurrence and six without recurrence were involved. The samples were sectioned, H&E stained, and analyzed using AI-based topographic image analysis to create a digital pathology profile for automatically identifying and annotating tumor and stromal areas. Annotated sections underwent laser capture microdissection to isolate tumor and stromal cells for quantitative proteomics. Finally, bioinformatics analysis and biological interpretation of the proteomic data were conducted.

3.2. Patient cohorts

In the BRAF detection study, 94 melanoma samples were obtained from 94 patients. Inclusion criteria encompassed archived FFPE tissue blocks with data about BRAF immunohistochemistry staining and the availability of DNA-based BRAF mutational status. The predictive biomarker study (**paper I**) involved 53 primary and 37 metastatic FFPE melanoma samples. **Paper II** included twelve patients with primary melanoma. All samples were from early-stage patients classified as AJCC8 IA-IIA at diagnosis. All the samples from the three studies were collected retrospectively from the Department of Dermatology and Allergology in the University of Szeged with clinical data including gender, age at primary melanoma diagnosis, histological parameters, therapy response and survival outcomes.

3.3. Molecular analysis in the BRAF detection study (PCR with Sanger sequencing, NGS, qPCR)

PCR analysis for BRAF V600 mutations was performed on FFPE samples via Sanger Sequencing technique. DNA isolation and PCR amplification were conducted, and mutations in the BRAF gene (V600E, V600K, V600R, K601E) were identified. For detailed mutation analysis of focal positive cases identified by IHC, next-generation sequencing (NGS) was employed including the following steps: extraction, library construction, and variant calling from FFPE tissue samples. For further mutation analysis of cases with diffuse positive intratumoral heterogeneous

staining, qPCR was applied involving the steps of DNA isolation, the detection of BRAF mutated allele and the calculation of mutation status.

3.4. Immunohistochemistry validation

Archived FFPE tissue samples underwent stepwise sectioning and staining for hematoxylin and eosin (H&E) in **paper I and II**. In addition, immunohistochemistry (IHC) with BRAF VE1 monoclonal antibody was applied in the BRAF detection study, and it resulted in various staining patterns including diffuse negative, diffuse positive with or without intratumoral heterogeneity and focal positive cases. For visualization, a high-affinity polymer-based, alkaline-phosphatase-linked secondary antibody with either fast red chromogen or specific horseradish peroxidase based detection with DAB chromogen was utilized. Finally, for scanning, the slides were placed in an automated slide scanner system (3D Histech Ltd., Budapest, Hungary) in the BRAF detection, predictive **(paper I)** and prognostic biomarker studies **(paper II)**.

3.5. Digital pathology and laser capture microdissection in paper II

Deep learning and machine learning algorithms were utilized for image analysis of H&E-stained FFPE tissue scans. Integrative image analysis was performed using Biological Image Analysis software (BIAS, v. 1.1.1, Single-Cell Technologies), encompassing image pre-processing, deep-learning-based image segmentation, feature extraction, and machine-learning approaches for tissue part categorization. Tumor and stromal content were differentiated using annotation and segmentation methods. The previously annotated tumor and surrounding stromal cells were isolated by automated laser capture microdissection, and then they were submitted for quantitative proteomics.

3.6. Proteomic analysis covering sample preparation, mass-spectrometry-based analysis, data analysis in paper I and II

In **paper I,** sample preparation protocol encompassed deparaffinization, protein extraction, digestion, LC/MS-MS analysis, and database searching using data-dependent acquisition (DDA) mode. Proteomic data was searched against the UniProt human database and spectral libraries, with application of batch correction. In **paper II**, microdissected tissue samples underwent proteomic analysis using a variable window data-independent acquisition (DIA) method. DIA neural network software was conducted to protein database search. The data

underwent processing using the Perseus platform. The abundance values were log2 transformed and then normalized by subtracting the median of all identified proteins in the sample as well as in **paper I**.

3.7.   Code availability and statistical analysis of proteomic data

The scripts for proteomic data normalization and batch effect correction from **paper I,** are available online, at the following link: https://github.com/bszeitz/MM_pilot. Various statistical methods, including Kaplan-Meier survival analysis, Pearson Chi^2 test with cross tabulation, Cox regression and GSEA, were employed for survival and proteomic data analysis. Alpha was set to 0.05 and nominal p-values less than 0.05 were considered as significant. GraphPad Prism, SPSS, STRING, Cytoscape and RStudio were utilized for statistical analyses and data visualization.

3.8.   Ethical Approval

The three studies were conducted according to the guidelines and regulations from the Swedish biobanking laws and from the Declarations of Helsinki, and approved by the Hungarian Ministry of Human Resources, Deputy State Secretary for National Chief Medical Officer, Department of Health Administration. The protocol code is MEL-PROTEO-001, the approval number is 4463-6/2018/EÜIG and the date of approval is 12 March 2018. The approval numbers for the most recent modifications are 2852-5/2023/EÜIG (10th February, 2023) and 2852-10/2023/EÜIG (12th July, 2023).

4. Results

I will summarize the results of the BRAF detection study, predictive biomarker study (**paper I**) and prognostic biomarker study (**paper II**).

4.1.   Results of the BRAF detection study

In managing advanced melanomas, the identification of detailed characteristics of melanoma, such as BRAF mutation status, is pivotal for targeted therapy. Our study aimed to compare two diagnostic methods, DNA-based polymerase chain reaction (PCR) and protein-based immunohistochemistry (IHC) for BRAF mutation detection. In our cohort, Sanger sequencing detected BRAFV600 mutations in 43 samples out of 94 samples, predominantly BRAFV600E mutations. In parallel, all the 94 slides from FFPE samples underwent staining using the VE1 antibody. PCR analysis detected additional mutations such as V600K, V600R, and K601E, which

were not identified by the VE1 antibody clone designed specifically for the BRAFV600E mutated protein. Therefore, these cases were excluded from the immunohistochemistry results as well as from the specificity, sensitivity values. We classified the samples into four groups based on VE1 antibody staining patterns. Roughly one-third displaying diffuse negative or very weak staining, while others exhibited varying degrees of positive staining including 32 cases with diffuse positive staining, 8 cases with diffuse positive staining with intratumoral heterogeneity, and 11 cases with focal positive staining. A significant discordance between PCR and IHC methods was noted (Pearson Chi^2 test, $p < 0.05$). The comparative analysis showed 100% sensitivity of BRAFV600E detection via IHC alongside 63% specificity, with positive and negative predictive values of 63% and 100%, respectively. In order to validate our results, further molecular analyses were performed. Next generation sequencing of PCR-negative cases exhibiting focal expression of the V600E mutated protein, revealed BRAF D594N aberration in one sample out of nine focal positive cases. Real-time polymerase chain reaction (qPCR) was performed on PCR-negative cases showing diffuse positivity with intratumoral heterogenous staining of the V600E mutated protein. qPCR confirmed the presence of BRAFV600E mutation in only one case among eight samples, with three cases remaining in the borderline range.

4.2. Results of the predictive biomarker study (paper I)

In our predictive biomarker study (**paper I**), we aimed to uncover potential proteins predictive of immunotherapy response. Using clinical and global proteomic expression data, we analyzed 90 samples from 77 patients to identify potential predictors for immunotherapy response based on progression-free survival (PFS). Multiple Cox regression models were constructed, revealing significant correlations between protein expression and survival in the immunotherapy patient group (Multiple Cox regression p-value < 0.05). Functional analysis of the proteins highlighted distinct pathways associated with different PFS durations. Upregulated proteins were linked to cellular and metabolic processes, including the VEGFA-VEGFR2 pathway (KEGG pathway database: FDR < 0.05), while downregulation of NOS3 was observed in patients with longer PFS after immunotherapy treatment (Cox regression test $p < 0.05$). Additionally, patients with a lack of tumor response to immunotherapy (i.e., started to progress after a few months) showed upregulation of proteins involved in RNA splicing mechanisms, such as SNRPB2, SNRNP70, and SNRPA1 (GO Biological Process, FDR < 0.05). We also identified proteins potentially predictive of improved response to immunotherapy, including those associated with

mechanisms linked to extracellular matrix and the immune system (KEGG pathways, GO biological processes, FDR < 0.05). Moreover, proteins involved in neutrophil degranulation, (e.g., PNP (Purine nucleoside phosphorylase), FTH1 (Ferritin heavy chain 1)), immunoregulation (e.g., ADAM17 (ADAM metallopeptidase domain 17), ECM-receptor interaction (e.g., AGRN (Agrin) protein), integrin cell surface interactions and cell adhesion (e.g., ICAM2 protein, COL4A2 and COL6A2 proteins) were significantly upregulated in patients with longer progression-free survival after immunotherapy.

4.3. Results of the prognostic biomarker study (paper II)

In this study, we conducted a detailed analysis of 12 early-stage primary melanoma samples (AJCC8 IA-IIA at diagnosis) to explore molecular and histopathological markers linked to disease recurrence. The samples were divided into two groups: those with recurrence in 5 years (n = 6) and those without recurrence (n = 6) in 5 years. Our histopathological assessment revealed a notable correlation between tumor thickness (Breslow and Clark indexes, Mann–Whitney U test: p = 0.0022) and recurrence. Moreover, analysis of clinical data indicated the influence of clinical stages (IA–IIA) (Fisher exact test: p = 0.0606) on recurrence. Prior to the quantitative proteomics, the isolation of the tumor and stromal cells of the FFPE melanoma samples were carried out with laser capture microdissection using digital pathology. AI-based digital pathology (AI-DP) was utilized, integrating deep learning and machine learning algorithms for precise identification of tumor and stroma cells in early-stage primary melanomas from histological images. After training and optimization, the algorithm accurately identified tumor and stroma regions, as well as normal epidermis, dermis, glands, and connective tissues, achieving an overall segmentation accuracy of approximately 80%. Subsequently, laser microdissection was performed to isolate tumor and stroma for quantitative proteomics. A comprehensive proteome profiling was conducted using HR-DIA-MS, identifying over 7000 proteins across all samples. Hierarchical clustering and PLS-DA analysis revealed clear proteomic differences between tumor and stromal compartments across different recurrence status groups. Stromal components exhibited better differentiation between recurrence status groups compared to tumor regions. Proteomic disparities between recurrent and non-recurrent melanoma cells were further explored using Gene Set Enrichment Analysis (GSEA). Recurrent melanoma cells showed increased expression of mitochondrial translation machinery (e.g., ADP/ATP translocases (ANT1, ANT2, and ANT3 proteins), MCAM and HNRNPA1 proteins) and metabolic pathways like oxidative phosphorylation and TCA cycle, while non-

recurrent melanoma cells demonstrated enrichment in pathways associated with immune response (FDR, q-value < 0.05). Moreover, the downregulated proteins in recurrent melanoma cells were primarily involved in immune responses. Additionally, non-recurrent melanomas displayed higher enrichment in pathways related to extracellular matrix, mitophagy, immune response pathways linked to both adaptive immunity and initial immune system responses (FDR, q-value < 0.05). Functional pathway enrichment analysis revealed distinct patterns between the stromal cells of recurrent and non-recurrent melanomas. The mitochondrial translation was significantly enriched in both tumor cells and stromal regions of recurrent melanomas (FDR, q-value < 0.05). In recurrent melanomas, the stromal cells showed enrichment in pathways like epithelial-mesenchymal transition and PD-1 signaling, compared to the stromal cells in the non-recurrent group (FDR, q-value < 0.05). Conversely, the stromal cells in non-recurrent melanomas displayed more abundant features such as interleukin-related signaling pathways, collagen degradation, and complement mechanisms, compared to recurrent cases (FDR, q-value < 0.05).

5. Discussion

In this thesis, we summarized three studies aimed to identify biomarkers for melanoma using prognostic, and predictive approaches via immunohistochemistry (IHC), quantitative proteomics, and digital pathology with AI-driven imaging.

Our BRAF detection study compared PCR and IHC methods for BRAF mutation detection in routine diagnostics. IHC staining of BRAFV600E on FFPE slides provides valuable and additional information about the characteristics of the tumor and protein expression patterns. Our IHC validation revealed four staining patterns, highlighting intratumoral and focal heterogeneity. Significant discrepancies between PCR and IHC results were noted.

Despite the high sensitivity of IHC in detecting BRAF V600E mutations, it demonstrated limited specificity, potentially due to false-positive results. According to the literature, false positive results could be caused by various BRAF point mutations or antibody cross-reactions. Significant mismatched results were further validated by NGS and qPCR. It also revealed a PCR-negative case with diffuse positive intratumoral heterogeneity staining detected by IHC that was positive on qPCR. This result indicated a potential dilution artifact in the mutated DNA and the limitations of PCR. Thorough NGS identified a BRAF D594N aberration in one PCR–negative sample with focal positivity staining detected by IHC, emphasizing the need for detailed DNA-based PCR analysis, particularly in equivocal or focal BRAF IHC staining cases. Both NGS and qPCR results

emphasize that relying solely on a single technique for analyzing a tumor with a high mutation rate might be insufficient, and suggest the combined usage of the PCR and IHC techniques.

Moving forward to proteomics studies in this thesis, in the predictive biomarker study (**paper I**), we were able to reveal distinct proteins and pathways linked to immunotherapy response from FFPE melanoma samples. For patients in immunotherapy subgroup with worse response (low progression free survival), VEGFA-VEGFR2 proteins were upregulated, which is linked to vasculogenesis. Several studies suggest that VEGFA-VEGFR2 pathway contributes to progression through locoregional lymphatic metastasis. Recently, VEGFA blockers like bevacizumab (Avastin) have been used in anticancer therapies, such as in the case of lung and metastatic colorectal cancer, suggesting their potential role in melanoma treatment. Moreover, the upregulation of abnormal RNA splicing pathways was also associated with worse immunotherapy response. Based on studies, RNA splicing could facilitate the loss of cell surface antigens leading to immunotherapy resistance and progression. Numerous studies have investigated small molecules that disrupt splicing mechanisms, and while their efficacy and safety are still being evaluated, anti-spliceosome drugs may offer potential strategies to overcome immunotherapy resistance. In the subgroup with better immunotherapy response, proteins related to the ECM and immune system were notably increased. Particularly, neutrophil degranulation and immunoregulation exhibited a pattern of overexpression with potential to treatment targets, aligning with previous research.

In **paper II**, we identified prognostic pathways in tumor and stroma in early-stage melanoma patients, emphasizing tumor-microenvironment interactions. Regarding histopathology assessment between the melanoma samples in different recurrence status subgroups, consistent with previous research, Breslow and Clark levels were predictive of a high risk of recurrence. Our application of AI-based digital pathology with BIAS software and deep learning models has revolutionized clinical pathology by achieving approximately 80% accuracy in distinguishing tumor and stroma regions, enabling automated selection and retrieval of tumor-relevant areas. Although the results are promising, the small sample size of the study restricts the generalizability of the findings. To validate the outcomes, larger cohorts are required. Comparing proteomic results, we found that mitochondrial function and immune response pathways played key roles in melanoma recurrence. The overexpression of mitochondrial metabolic pathways, including oxidative phosphorylation and TCA cycle, in recurrent melanoma tumor cells suggests a high energy demand essential for tumor cell proliferation. Specific proteins such as mitochondrial ADP/ATP translocases (ANT1, ANT2,

and ANT3) indicate increased ATP flux, while MCAM promotes angiogenesis and metastasis, and HNRNPA1 contributes to metabolic reprogramming in melanoma cells. These findings highlight a more aggressive phenotype and increased risk of disease recurrence in the recurrence group. Regarding mitochondrial targeting therapies, studies have shown that antibiotics like Doxycycline, Tigecycline, and Azithromycin can inhibit *in vivo* melanoma cell proliferation by targeting mitochondrial subunits. Moreover, it was shown that metformin inhibits mitochondrial function and promotes immune-mediated tumor cell death, suggesting it as a promising therapeutic option. Additionally, non-recurrent melanomas exhibited pathways associated with immune reactions and mitophagy, suggesting protective mechanisms against recurrence. Stromal components of recurrent melanomas displayed heightened proliferation pathways, particularly PD-1 signaling. The increased expression of PD-L1 in stromal cells, along with its binding to the PD-1 receptor on adaptive immune cells, triggers a signaling cascade that inhibits immune surveillance. This mechanism has significant therapeutic applications, including the potential use of PD-L1 and PD-1 inhibitors in early-stage melanomas. Furthermore, in line with our results, previous research has highlighted that the dysregulation of immune system-related pathways and immune cell infiltration in the tumor microenvironment correlates with cancer advancement and unfavorable prognosis. Moreover, the increased mitochondrial translation in the stromal area of recurring melanomas is noteworthy, hinting at a possible transmission of the cancer cell characteristics to the microenvironment. Our research identifies distinct molecular signatures from both tumor and stromal cells in early-stage primary melanomas, which may help predict high risk of melanoma recurrence within five years.

6. Summary, novel findings of the PhD work

In our discovery studies, we compared DNA-based PCR and protein-based IHC techniques to assess BRAF expression. Additionally, we applied new methodologies, including quantitative proteomics and AI-powered digital pathology, to identify predictive and prognostic proteins from formalin-fixed paraffin-embedded melanoma samples.

- Despite the rapid and cost-effective nature of IHC, significant discrepancies between BRAFV600E mutation detection by PCR and via IHC techniques highlighted the importance of the combined use of PCR and IHC, especially in cases of inhomogeneous and focal positive BRAF cases.

- At the first time, we were able to identify predictive proteins with deep proteomic sequencing from formalin-fixed paraffin-archived melanoma samples. The VEGFA-VEGFR2 pathway and RNA splicing pathways were connected to short progression-free survival, while increased activity of proteins and pathways from immune cells and extracellular matrix were associated with long progression-free survival in melanoma patients after the application of immunotherapy.

- For the first time, our findings revealed prognostic mechanisms from laser capture microdissected, formalin-fixed paraffin-embedded early-stage melanoma samples using quantitative proteomics and digital pathology with AI-driven imaging.

- We observed that upregulation of mitochondrial translation and cellular proliferation pathways, coupled with downregulation of immune response pathways, play pivotal roles in early-stage melanoma progression both in tumor and stromal cells.

In conclusion, diverse protein expression patterns observed across patient subgroups in our results suggest that immunohistochemistry, quantitative proteomics and digital pathology with AI-powered imaging, as aspects of spatial proteomics, are emerging, crucial technical components supporting therapy selection and precision medicine.

7. Acknowledgement