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FACULTY OF INFORMATION TECHNOLOGY AND BIONICS

ROSKA TAMÁS DOCTORAL SCHOOL OF SCIENCES AND TECHNOLOGY

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Role of extracellular vesicles in cancer progression and diagnosis

Theses of PhD Dissertation

Supervisor:

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Introduction:

During my PhD, my primary focus was to develop expertise in the field of extracellular vesicles (EVs) within the context of cancer biology. EVs are nanoscale, membrane-bound particles that carry diverse macromolecular cargo, including proteins, lipids, nucleic acids and other signal molecules [1]. They are increasingly recognized as pivotal mediators of intercellular communication due to their ability to transfer and protect bioactive molecules [2]. As a relatively young and rapidly evolving field, EV research holds substantial promise for advancing our understanding of cancer progression.

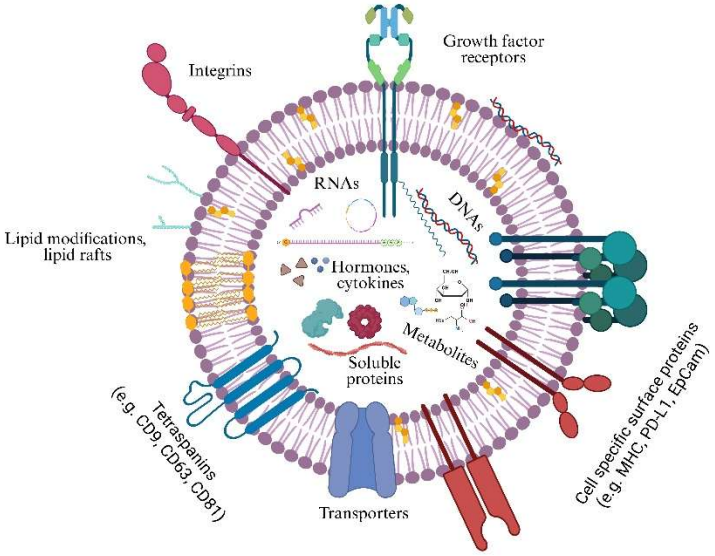

Figure 1. Representative image of an extracellular vesicle. EVs are nanosized membrane-bound particles releases by all cells and present in all body fluids. Their cargo includes diverse macromolecules such as lipids, proteins, multiple RNA types, DNA fragments, and various signaling molecules. Created with Biorender.com.

In oncology, EVs have been implicated in nearly every stage of **tumor progression** [3]. Tumor-derived EVs can transfer oncogenic proteins and RNAs that influance migratory behavior, morphological plasticity, and invasive capacity [4] - [6]. In addition to enhancing cell-autonomous traits, they reprogram the tumor microenvironment – activating fibroblasts [7], modulating immune cells [8], remodeling the extracellular matrix (ECM) [9], and promoting angiogenesis [10]. Moreover, EVs can influence distant tissues through integrin-guided organotropism and supporting pre-metastatic niche formation [11].

Despite their diverse roles, it is still not fully understood during which stages of cancer progression EVs exert their most critical effects. Do they primarily support early tumor expansion or do they play a more central role in systemic dissemination and metastasis formation? This question served as a key motivation for my PhD work.

To address it, I focused on melanoma – a clinically aggressive skin cancer with high metastatic potential and strong dependence on specific oncogenic drivers [12]. Notably, the poor prognosis associated with advanced melanoma is compounded by the emergence of **resistance to targeted therapies** in almost all patients [13]. As the majority of melanoma patients harbor activating BRAF mutations [14], therapeutic efforts have predominantly centered on BRAF inhibitors such as vemurafenib, dabrafenib, and encorafenib [15] - [17]. Although MEK inhibitors (MEKi) are also employed in clinical settings, resistance remains a frequent and challenging obstacle [18]. Emerging evidence suggests that EVs may contribute to therapy resistance by disseminating resistance-associated molecules [19], [20], sequestering therapeutic agents [21], or modulating the tumor microenvironment to enhance cellular survival under pharmacologic stress [21]. Elucidating the role of EVs in these adaptive processes may uncover novel vulnerabilities and therapeutic targets to improve treatment outcomes.

Another notable feature of EVs is their presence in body fluids and ability to reflect the molecular characteristics of their cells of origin and possibly represent tumor heterogeneity. These properties render them highly promising as less invasive **biomarkers for cancer diagnostics** and disease monitoring [22]. In this context, I examined EVs isolated from pleural effusions – fluids that accumulate in the pleural cavity and represent the tumor microencironment and often aspirated to ease symtopms in thoracic malignancies [23]. Notably, non-small cell lung cancer (NSCLC) and pleural mesothelioma (PM) patients – frequently present with malignant pleural effusions – pose considerable diagnostic challenges. To provide a comparative framework and capture benign inflammatory conditions, pleuritis was also investigated [24]. EVs could represent the tumor and the microenvironment present in the pleural cavity, therefore analysing these particles surface-marker expression across the patient groups could hold diagnostic potential [25]. Given the complexity and subtlety of EV-associated molecular signatures, advanced computational approaches such as machine learning offer powerful tools for identifying patterns that may not be readily discernible through conventional analysis

Objectives:

The overarching aim of this dissertation is to investigate the role of EVs in cancer progression, resistance to targeted therapies, and their potential application in liquid biopsy-based diagnostics. The specific objectives of the research are as follows:

- **To assess the role of EVs in tumor progression,** including their impact on primary tumor growth and metastatic potential, through proliferation assays, sphere formation assays, and migration analyses. For these experiments, cell lines representing varying stages of cancer progression, but sharing similar genetic backgrounds were employed.
- **To elucidate the contribution of EVs to resistance against BRAF and MEK inhibitors,** primarily through single-cell migration analyses using EVs derived from cells with distinct drug sensitivities:
    - To investigate the role of EVs in resistance to vemurafenib, the first-generation BRAF inhibitor used in the treatment of patients from whom some of the cell lines were derived.
    - To evaluate EV-mediated support of dual resistance to dabrafenib (BRAFi) and trametinib (MEKi) in cell lines that had been previously exposed to BRAF inhibition.
    - To establish melanoma cell line clones resistant to long-term combined encorafenib (BRAFi) and binimetinib (MEKi) exposure, characterize the resulting phenotypic alterations, and assess the potential of EVs from these clones to promote dual therapy resistance, also in the context of EV preconditioning.
- **To investigate the diagnostic utility of EVs in thoracic malignancies,** by analyzing surface marker expression profiles of EVs isolated from pleural effusion samples of patients with NSCLC, PM, and benign pleuritis using a multiplex bead-based assay:
    - To assess the diagnostic relevance of individual surface markers in distinguishing between disease groups.
    - To explore the potential of machine learning algorithms to improve patient classification based on EV surface marker signatures.
    - To evaluate alternative patient classification strategies by comparing diagnostic performance when (i) all patients are included based on clinical cancer history and (ii) patients with confounding clinical backgrounds or additional tumors are excluded. To help determine whether stricter patient selection improves the accuracy of EV-based diagnostic classification.

Methods:

EVs were **isolated** from the conditioned media of five syngeneic cell line pairs, each representing distinct stages of cancer progression, using differential ultracentrifugation. The isolated EV populations were comprehensively characterized by nanoparticle tracking analysis (NTA), Qubit protein quantification, sulfophosphovanillin (SPV) lipid assays and bead-based flow cytometry to verify the presence of commonly used EV markers. Each EV isolate was subsequently applied to its corresponding or paired cell line to assess **functional effects** on cell viability, spheroid formation, and single-cell migratory behavior. Cell viability was evaluated at 72 hours post-treatment using SRB assay, while spheroid growth was monitored longitudinally over a 7-day period. Single-cell migration was tracked for 24 hours using semi-automated CellTracker software [26], enabling calculation of total traveled distance (TTD) and mean squared displacement (MSD) from xy-coordinate data. To facilitate quantitative comparison of time-dependent responses, differential area under the curve (ΔAUC) values were derived from the relevant kinetic curves.

Drug sensitivity profiles of the cell lines were established using the SRB assay, and the synergistic potential of combined BRAF and MEK inhibition was quantified by calculating combination indices. The capacity of **EVs to mediate drug resistance** was interrogated through single-cell migration assays. Moreover, drug-resistant clones were generated by prolonged exposure to sub-lethal doses of BRAF and MEK inhibitors. Phenotypic alterations in these resistant clones – including proliferation rates, morphological changes, and migratory behavior – were evaluated from videomicroscopy recordings. Comparative analysis of EV production from resistant versus parental cells was performed as described above. Finally, the contribution of EVs to drug resistance was assessed through migration assays involving co-treatment with inhibitors and EVs, as well as EV preconditioning prior to drug exposure.

To evaluate **EVs as potential biomarkers**, EVs were enriched from pleural effusions of NSCLC, PM, and pleuritis patients using size exclusion chromatography (SEC) combined with ultrafiltration. Particle size distribution was assessed by NTA, protein content quantified via Qubit assay, and tetraspanin expression at the single-EV level confirmed by nano flow cytometry (nFCM). Surface marker profiling was performed using a multiplex bead-based assay (MACSPlex), targeting 37 markers plus two isotype controls according to manufacturer protocols. Patients were classified using four alternative schemes based on clinical history. Surface marker expression patterns were further analyzed with a machine learning algorithm to classify patients into their respective groups.

New scientific results:

I. **Melanoma cell–derived extracellular vesicles (EVs) promote cell migration more effectively than proliferation or sphere formation.**

Syngeneic melanoma cell line pairs were used in the study, and EVs were successfully produced and isolated from all cell lines. The protein and lipid content of the EV isolates did not show major differences across cell lines with varying malignancy. EV treatment altered proliferation in some cases, they maintained sphere formation at baseline level, while their overall effect on migration was more pronounced, with a broader trend of enhanced motility observed across cell lines. A notable strength of this work is the analysis of EV-mediated migration at the single-cell level, which has not been previously addressed.

II. **EVs originating from drug-resistant melanoma cell lines contribute to resistance transmission.**

a. **EV-mediated promotion of migration persists under BRAF inhibitor treatment**

The baseline sensitivity of cell lines to vemurafenib and dabrafenib was assessed, and drug concentrations resulting in ~50% inhibition were selected for further analysis. EVs derived from more resistant cell lines were able to counteract the migration-inhibitory effects of BRAF inhibitors, indicating that EVs can maintain pro-migratory signals under targeted therapy. Furthermore, resistant cells-derived EVs carry distinct infomration compared to the ones from the sensitive cells and this leads to resistance transfer.

b. **Dual BRAF-MEK inhibition remains largely unaffected by EVs from resistant cells**

Sensitivity to dual BRAF-MEK inhibition was evaluated, and combination index calculations confirmed a synergistic interaction between the inhibitors. The inhibitory effects of either MEK inhibition alone or combined BRAF-MEK inhibition remained effective when EVs were administered simultaneously. However, slight modulation of inhibition was observed when cells were preconditioned with EVs, suggesting a time-dependent and subtle influence in this setting.

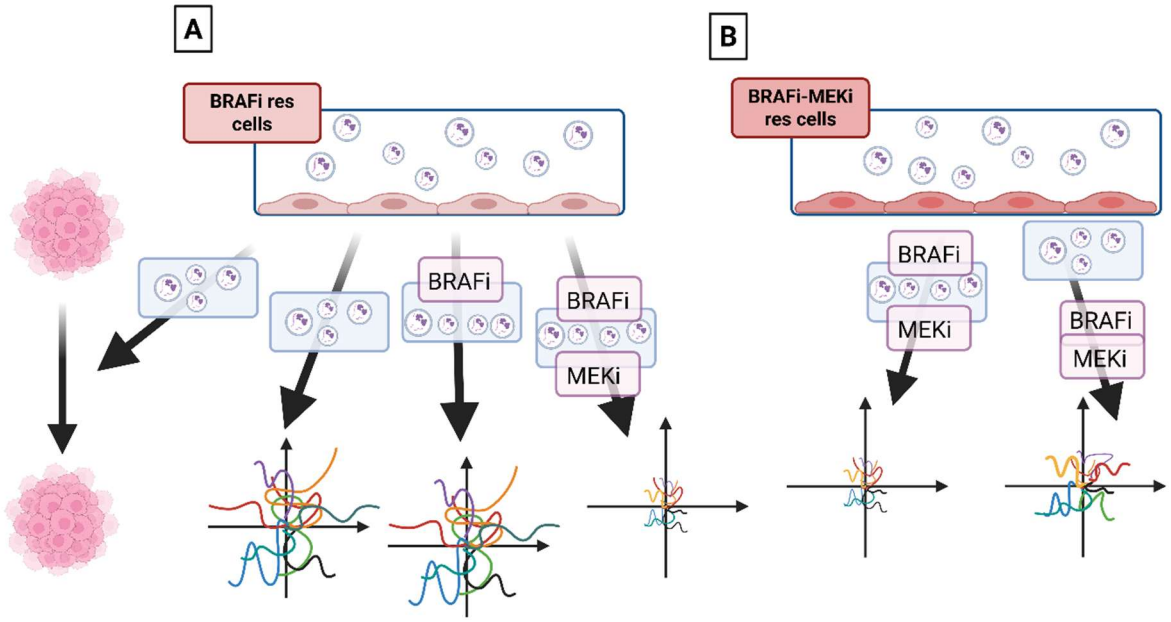

Figure 2. Overview of EV-mediated effects in the context of melanoma progression and targeted therapy resistance. *A) EVs promote cell migration rather than proliferation and sphere growth. Resistant cells-derived EVs retain their ability to promote cell migration under BRAF inhibitor treatment (vemurafenib, dabrafenib), but show limited effect when BRAF and MEK inhibitors are combined (dabrafenib + trametinib). B) In the established BRAFi-MEKi resistant cell lines, only EV preconditioning partially counteracted the migration-inhibitory effects of combined encorafenib-binimetinib therapy.*

III. Pleural effusion-derived EVs from NSCLC, pleural mesothelioma (PM), and pleuritis patients exhibit distinct surface marker profiles.

a. CD4, CD8, CD44, CD326, and MCSp can differentiate between disease groups.

EVs were successfully isolated from pleural effusion samples, with no significant differences observed in particle size distribution or protein content across the patient groups. Surface marker expression was assessed using a multiplex bead-based assay, revealing that the EV marker profile in pleural effusions differs markedly from plasma-derived EVs, and reflect active EV-based communication in the tumor microenvironment. CD44 and MCSp emerged as markers associated with PM, CD8 was linked to pleuritis, and CD326 was indicative of NSCLC and malignancy. Additionally, CD4 and CD44 were capable of distinguishing between PM subtypes.

b. Machine learning analysis of the EV surface markers able to highlight classification inconsistencies primarily linked to patients with secondary malignancies; excluding these cases led to the highest accuracy.

Machine-learning algorithm was used to classify patients based on their surface maker expression pattern and most of the patients were classified correctly. Furthermore, the

algorithm was able to point out some inaccuracies in our classification scheme. Therefore, several alternative classifications were created based on the patients clinical history: 'initial' (based on cytological diagnosis), 'tumor-focused' (based on the presence of any tumor in the body), 'pleural involvement' (based on whether the pleura was affected), 'cancer type' (categorizing NSCLC, PM, and other cancers separately), and 'strict criteria' (excluding patients with secondary malignancies). Among these, the strict criteria approach yielded the most accurate classification performance for distinguishing between NSCLC and PM patients.

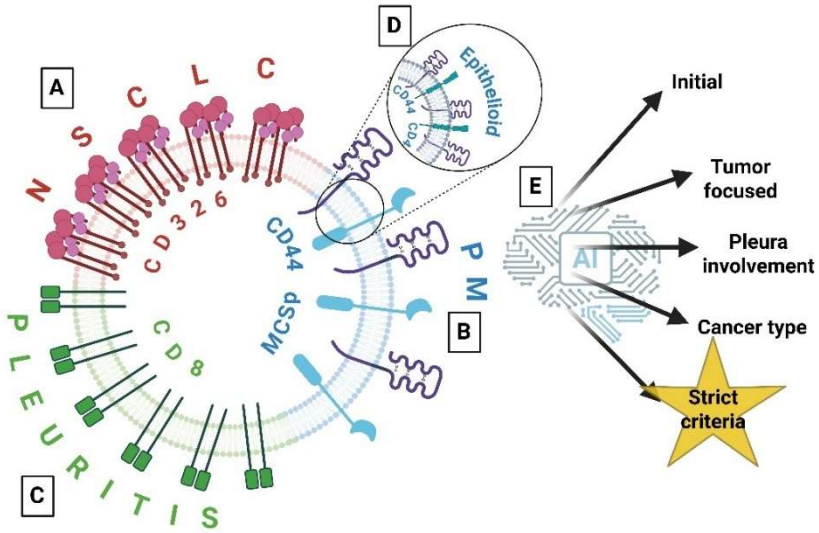

Figure 3. Summary of pleural effusion-derived EV surface markers and their diagnostic potential*. A) CD326 (EpCAM) is highly expressed in EVs from NSCLC patients; B) CD44 and MCSp is elevated in PM; C) CD8 a pleuritis-associated marker; D) CD4 and CD44 is overexpressed in epithelioid PM compared to non-epithelioid; E) Among several classification strategies, the 'strict criteria' approach yielded the most accurate group separation in machine learning analysis*

Potential applications and benefits

My PhD research advances our understanding of how EVs contribute to cancer progression and treatment resistance, providing promising avenues for improving patient management in melanoma. By demonstrating that melanoma cell–derived EVs predominantly enhance cell migration rather than proliferation or sphere formation, this work elucidates key mechanisms underlying tumor invasiveness and metastatic potential. The application of single-cell migration analyses further reveals novel layers of EV-associated functions. In the context of therapy resistance, EVs from drug-resistant melanoma cells were shown to sustain migratory activity even under BRAF inhibitor treatment, highlighting the complex role of EVs in modulating tumor behavior beyond survival signals. Although dual BRAF-MEK inhibition largely retains efficacy despite EV presence, subtle modulatory effects suggest that EVs could influence therapeutic responses in nuanced ways, warranting further study. These insights contribute to the growing recognition of EVs as active participants in cancer biology, emphasizing their potential as **therapeutic targets**.

A significant societal contribution of this work lies in the advanced biomarker profiling of EVs isolated from pleural effusions – a readily accessible, minimally invasive liquid biopsy source that directly reflects the tumor microenvironment. Despite their clinical relevance, pleural effusions have been relatively underutilized for EV analysis, especially in the context of pleural mesothelioma and differential diagnosis among NSCLC, PM, and pleuritis. This study identified distinct EV surface marker patterns that reflect disease-specific microenvironments, confirming markers with established potential, such as CD326 (EpCAM) and CD44, while also suggesting novel candidates for further investigation. The integration of machine learning algorithms enhanced classification accuracy, demonstrating that simple, well-defined patient groupings yield the most reliable diagnostic distinctions.

Overall, these findings support the development of more sensitive and **specific EV-based liquid biopsy diagnostics**, which are less invasive and more convenient than traditional tissue biopsies. By leveraging pleural effusions – fluids that accumulate naturally and can be readily collected – this research lays the groundwork for improved diagnostic tools that can facilitate earlier detection and more precise disease characterization. Such advances have the potential to enhance patient care while promoting more efficient and cost-effective healthcare delivery.

List of publications

Journal publications:

Ekström K, Riaz N, Larsson K, Németh A, Crescitelli R, Linderholm B, Olofsson Bagge R. Plasma extracellular vesicles reflect response and prognosis in patients with breast cancer undergoing neoadjuvant treatment. Breast Cancer Res (2026). doi:10.1186/s13058-025-02209-0

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Submitted journal publications:

Németh A, Ekström K, Crescitelli R, Bagge RO, Bányai GL, Rák Á, Cserey Gy, Bölükbas S, Visnovitz T, Hegedűs B, Garay, T Machine learning-enhanced analysis of extracellular vesicle profiles in pleural effusions for cancer diagnosis – submitted to MedComm journal

Németh A., Simon A, Bányai GL , Lenzinger D, Garay T Acquired resistance to MAPK inhibition and the contribution of extracellular vesicles – submitted to Cancer Reports

Conference talk:

Németh A, Bányai GL, Dobos NK, Kós T, Gaál A, Varga Z, Buzás EI, Khamari D, Dank M, Takács I, Szász AM, Garay T; Extracellular vesicles promote migration despite BRAF inhibitor treatment; *2nd MOVE symposium 2024 Belgrade*

Conference publications:

Németh A, Ekström K, Crescitelli R, Bagge RO, Bányai GL, Rák Á, Cserey Gy, Bölükbas S, Visnovitz T, Hegedűs B, Garay, T; Diagnostic potential of pleural fluid-derived extracellular vesicles in differentiating pleural-associated diseases using machine learning; *ISEV2025 Vienna*

Bányai GL, Merényi A, Szabó A, Németh A, Gaál A, Pongor Cs, Garay T; Utilizing microfluidic techniques to differentiate cancer patient samples based on extracellular vesicle profiles; *ISEV2025 Vienna*

Németh A; Extracellular vesicles promote migration despite BRAF inhibitor treatment; *Phd Proceedings Annual Issues of the Doctoral School Faculty of Information Technology and Bionics 2024*

Németh A; The role of extracellular vesicles in melanoma progression; *Phd Proceedings Annual Issues of the Doctoral School Faculty of Information Technology and Bionics 2023*

Németh A, Kós T, Bányai GL, Gaál A, Varga Z, Dank M, Takács I, Szász AM, Garay T; Effects of extracellular vesicles on vemurafenib sensitivity in syngeneic melanoma cell lines; *ISEV22 Lyon*

Németh A, Kós T, Bányai GL, Gaál A, Varga Z, Dank M, Takács I, Szász AM, Garay T; Can extracellular vesicles transmit vemurafenib resistance in melanoma cells in vitro?; *EACR 2022 Sevilla*

Németh A; Isolation methods and storage stability of extracellular vesicles; *Phd Proceedings Annual Issues of the Doctoral School Faculty of Information Technology and Bionics 2022*

Conference posters:

Németh A, Simon A, Bányai GL, Garay T; Extracellular vesicles pretreatment enhances melanoma cell migration despite dual MAPK pathway inhibition; *PhD Scientific Days 2025 Budapest*

Németh A, Lässer C, Bagge RO, Ekström K; Evaluating Nano-Flow Cytometry for Detecting HER2-Positive Extracellular Vesicles in Breast Cancer Patients; *1st Swedish Extracellular Vesicles Network Meeting Stockholm*

Kvasznicza J, Németh A, Bányai GL, Szőcs L, Garay T Cyclodextrin's Role in Modifying Cancer Cell Extracellular Vesicles; *UKEV2024 Newcastle*

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Németh A, Bányai GL, Dobos NK, Kós T, Gaál A, Varga Z, Buzás EI, Khamari D, Dank M, Takács I, Szász AM, Garay T; Extracellular vesicles promote migration despite BRAF inhibitor treatment; *HSEV2024 Budapest*

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Choi J, Görög D, Gaál A, Khamari D, Buzás EI, Németh A, Garay T, Bányai GL; Investigating the effect of extracellular vesicles on proliferation and migration on syngeneic colorectal cancer cell lines; *Small New word 2.0 2023 Graz*

Németh A, Linderholm B, Bagge RO, Ekström K; Is it possible to detect HER2-positive extracellular vesicles in human plasma?; *Small New word 2.0 2023 Graz*

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Dobos NK, Németh A, Kvasznicza J, Magyar R, Görög D, Dank M, Takács I, Szász AM, Garay T; Are the observed effects on cell proliferation EV-specific or are other cell culture supernatants important players too? *Small New word 2.0 2023 Graz*

Kvasznicza J, Magyar R, Bányai GL, Rohan Zs, Szőcs L, Németh A; Cyclodextrins affect extracellular vesicle production in melanoma in vitro, *Small New word 2.0 2023 Graz*

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