Ph.D. Thesis

Molecular background examinations to identify differential diagnostic and predictive biomarkers in NSCLC

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1. Introduction

1.1 Epidemiology and diagnosis of lung cancer (LC)

On a global scale, LC remains the foremost cause of cancer-related fatalities among both men and women.[1] A disaggregated analysis based on economic development levels reveals no significant disparities in cancer mortality rates among men. However, a higher incidence of LC mortality is observed among women in industrialized countries compared to developing nations.[2] The strong correlation between LC incidence and mortality and cigarette smoking patterns is well-documented. As smoking rates reach their peak, first among men and then among women, there is an observed rise in lung cancer incidence and mortality in subsequent decades. In recent decades, Hungary has been recognized as having one of the highest incidence and mortality rates of LC worldwide. According to the findings of the HeLP3 study conducted in 2016, the number of new LC cases clinically diagnosed in Hungary amounted to 6,996 cases (with 59.7% of the cases affecting males), with an average age of 65.88 years (SD ± 9.84).[8] Concurrently, 6,465 patients passed away (63.2% of whom were male), with an average age at death of 67.95 years (SD ± 9.68).[8]

Accurate radiological staging of cancer necessitates the use of various imaging modalities. For patients with LC undergoing staging, various imaging modalities such as CT scanning, magnetic resonance imaging, positron emission tomography (PET) CT, bone scans, and ultrasound can all be utilized. FDG-PET is increasingly used alongside CT for the staging of LC. FDG-PET effectively highlights metabolic activity in lesions measuring 1 cm or larger. The combination of FDG-PET and CT is superior to either modality alone for detecting affected mediastinal lymph nodes, boasting a sensitivity of 58–94% and a specificity of 76–96%.[10] The pathomorphologic verification of tumors is required for establishing the diagnosis of LC. Biopsy is most often obtained using bronchoscopy, and it is supplemented with conventional or endobronchial ultrasound-guided transbronchial needle aspiration for the assessment and staging of mediastinal lymph nodes. CT-guided transthoracic needle biopsy, mediastinoscopy, and video-assisted thoracic surgery (VATS) are other possible but less frequently used invasive diagnostic procedures. These are performed when less invasive methods fail to provide sufficient tissue. LC staging is an integral component of determining the patient's treatment options. The Tumor, Node, Metastasis (TNM) staging system for LC is an internationally accepted system used to characterize the extent of disease.[11] The TNM system integrates tumor-

related features to create stage groups that correspond to patient prognoses and are associated with treatment recommendations.

1.2 Histological features and molecular genetics of LC

LC is a diverse disease characterized by a variety of clinical and pathological features. It is primarily categorized into two types: non-small cell lung cancer (NSCLC), accounting for 85% of diagnoses, and small cell lung cancer (SCLC), which represents 15%. Among the NSCLC classifications, adenocarcinomas (LUAD) are the most prevalent subtype, followed by squamous cell carcinomas (LUSC). The incidence of LUSC, once the most prevalent histological type, has significantly decreased, primarily due to declining smoking rates in high-income countries and changes in cigarette formulations.[10] The molecular basis of LC is complex and heterogeneous. Advances in our understanding of molecular changes at genetic, epigenetic, and protein expression levels have the potential to impact diagnosis, prognosis, and treatment of LC. The development of LCs is a multistep process that activate growth-promoting pathways while inhibiting tumor suppressor pathways, characterized by multiple genetic and epigenetic alterations. A comprehensive understanding of the multifaceted biochemical pathways involved in the molecular pathogenesis of LC is imperative for the development of treatment strategies that can target molecular aberrations and their downstream activated pathways.[5] The molecular alterations that drive tumor growth and provide targets for therapy have been most thoroughly delineated in LUADs.[5] However, there is an increasing interest in the molecular landscape of LUSC, which is more strongly associated with cigarette smoking, resulting in a highly variable mutational background, often in combinations of gene amplifications, gene fusions, tumor suppressor mutations and point mutations in a variety of genes.[15] Therefore, to date, mutation-based targeted therapy is not available for LUSC patients. In the context of LC, as with other malignancies, the process of tumorigenesis is associated with the activation of growth-promoting proteins (e.g. KRAS, EGFR, BRAF, MEK-1, HER2, MET, ALK, RET), as well as inactivation of tumor suppressor genes.[5] The process of activation of growth-promoting oncogenes can occur through gene amplification or other genetic alterations, including point mutations and structural rearrangements, which lead to uncontrolled signaling through oncogenic pathways. The phenomenon of "oncogene addiction" arises when cell survival is contingent upon the persistent activation of the aberrant signaling pathways. These aberrant signaling pathways are considered ideal candidates for targeted therapeutic interventions. Oncogenic driver mutations have been identified in over 50% of LUAD cases and are typically exclusive of other driver mutations.[5] The advent of high-throughput sequencing techniques has

led to the identification of comprehensive molecular profiles of LC. The Cancer Genome Atlas (TCGA) research network has identified genomic and other molecular alterations among various types of cancer, including LC.

1.2.1 WNT Signaling in lung cancer

An increasing amount of evidence has demonstrated that the WNT pathway, a pivotal signaling pathway crucial for maintaining lung homeostasis, is implicated in the pathogenesis of numerous debilitating lung diseases.[23] In a similar manner to other human cancers, WNT signaling plays an important part in lung carcinogenesis. WNT proteins are secreted glyco-lipoprotein morphogens that are required during lung development for cell-fate specification, cell proliferation, and the control of asymmetric cell division. The human lung has been found to contain 19 WNT ligands and the 10 main receptors, Frizzleds (FZD), that have been identified in mammalian cells. Two major WNT pathways have been identified: i) the β-catenin-dependent or canonical pathway, and ii) the β-catenin-independent or non-canonical pathways, which include the planar cell polarity (PCP) and the WNT/Ca2+ pathways. The most well-known activator of Ca2+ -dependent WNT signaling is WNT5A. Despite the apparent distinctiveness of WNT signaling pathways, WNT proteins exhibit promiscuity, capable of sharing receptors and regulating the expression of WNT signaling molecules, in addition to modulating WNT signaling pathway activity.[23,24] Alterations in the WNT/β-catenin pathway are a hallmark of human cancers. In NSCLC, mutations in β-catenin and APC are uncommon, yet WNT signaling maintains its significance for NSCLC cell lines, as its inhibition leads to a reduction in cell proliferation.[32] The expression of WNT-1, -2, -3, and -5a, in conjunction with other WNT pathway components such as Frizzled-8, DVL, PORCN and TCF-4, is prevalent in resected NSCLC and is associated with a poorer prognosis.[32,33] In contrast, WNT-7a has been observed to exhibit a suppressive effect on NSCLC development and is frequently found to be downregulated in the resected tumor tissues. Although β-catenin is frequently expressed in NSCLC, some studies have associated its presence with improved prognosis, possibly due to interactions with E-cadherin.[23] Downregulation of WNT inhibitors, frequently attributable to hypermethylation, has been observed in a variety of NSCLC tumor cell lines and resected tissues.[32] This loss has been associated with advanced stage, dedifferentiation, and unfavorable outcomes. Preliminary investigations indicate that the restoration of WNT inhibitor function results in a reduction of WNT signaling, an inhibition of proliferation, and the promotion of apoptosis.[32] WNT signaling may also contribute to resistance against chemotherapeutic agents such as cisplatin, docetaxel, and radiotherapy, with the potential for WNT inhibitors to enhance

sensitivity.[34] The clinical targeting of WNT pathway antagonists, encompassing agents that impede specific β-catenin interactions or augment WNT inhibitor expression, merits further investigation.

1.2.2 The role of ABC drug transporter expression in LC

Drug transporters are integral membrane proteins that facilitate the transport of drugs and xenobiotics into and out of cells in all organs, including the lungs. Since LC treatment involves chemotherapy, it is not surprising that tumor cells increase transporter expression to actively remove the drugs.[35] This process contributes to drug resistance and increases cellular survival. The best-studied drug transporters belong to the ATP-Binding Cassette (ABC) superfamily.[36] The ABC transporter family is classified into seven subfamilies based on genomic sequences and the structure of their transmembrane domains (TMDs). They play a crucial role in the efflux of various chemotherapeutic agents, which often leads to reduced effectiveness of these drugs. ABC transporters can significantly affect therapy, as variations in expression levels can influence the extent and duration of drug outflow. This, in turn, impacts patient outcomes.[37]

1.2.3 Regulation of protein translation - miRNAs in LC

MicroRNAs (miRNAs) are a family of small noncoding RNAs (21–25 nucleotides) that have the capacity to inhibit messenger RNA (mRNA) translation into protein due to promoting mRNA degradation by base pairing to complementary sites of target mRNAs. Through this mechanism, miRNAs alter gene expression post-transcriptionally. The first noncoding RNA to be identified as a member of the miRNA family was lin-4, which was discovered in Caenorhabditis elegans in 1993.[44] However, it was not until the early 2000s that miRNAs were first defined as a distinct group of regulatory molecules. Since then, there has been a rapid increase in the number of studies about miRNAs, and the miRBase database now contains over 2500 mature miRNAs (from 1188 precursor molecules).[44] miRNAs play a crucial role in carcinogenesis, as mutations in genes related to LC can lead to changes in protein expression. The regulation of these proteins during translation can significantly influence disease progression, response to therapy, and overall survival.

1.2.4 Extracellular Vesicles (EVs)

Exosomes are a type of EV characterized by a double lipid layer and have recently emerged as crucial players in intracellular communication. These vesicles have the capacity to facilitate the

transfer of bioactive molecules, including proteins, DNA, mRNA, and non-coding RNAs, from one cell to another. This process can result in the exchange of genetic information and the reprogramming of the recipient cell.[49] Research findings have demonstrated that tumor cells release substantial quantities of exosomes, which can influence tumor growth, progression, dissemination, and drug resistance.[50] These particles have been shown to enable communication between tumor cells and various other cell types, including immune and stromal cells. This interaction contributes to tumor development, progression, and immune evasion.[50]

1.3 The systemic therapy of lung cancer

The systemic treatment of advanced stage LC encompasses a range of therapeutic modalities, including chemotherapy, targeted molecular therapies, and immunotherapy.[54] The selection of a systemic treatment is contingent upon the patient's characteristics and the characteristics of the tumor, as well as the stage of the disease. The utilization of conventional chemotherapeutic agents, including cisplatin, carboplatin, and third-generation cytotoxic agents such as taxanes (paclitaxel, docetaxel) or gemcitabine, constitutes a standard component of clinical practice for NSCLC. In addition, pemetrexed and bevacizumab are commonly used in the treatment of advanced stage NSCLC. In recent years, therapeutic options have expanded significantly, with the advent of targeted therapy and immunotherapy.

1.3.1 Targeted Therapy in NSCLC

The molecular targeted therapy is tailored to the molecular profile of the tumor.[55] LUAD is classified into distinct molecular subtypes, characterized by specific genetic alterations in oncogenic drivers.[5] Molecular drivers in NSCLC include a broad spectrum of genomic alterations across genes such as EGFR (both common and rare mutations), ALK, ROS1, BRAF, KRAS, MET, RET, NTRK, and HER2. However, many of these alterations are relatively rare—occurring in less than 2%–5% of patients—and are often not covered by standard diagnostic methods like polymerase chain reaction (PCR) or immunohistochemistry (IHC) due to limitations in cost, turnaround time, and the small size of available tumor tissue samples.[5] The advent of next-generation sequencing (NGS) technologies has addressed these challenges by enabling the simultaneous detection of multiple genetic alterations with greater sensitivity, accuracy, and cost-efficiency. NGS can be applied to formalin-fixed, paraffin-embedded (FFPE) tumor tissue as well as to circulating free DNA or messenger RNA obtained through liquid biopsy from peripheral blood.[56]

1.3.2 Immunotherapy in NSCLC

LC immunotherapy—aimed at enhancing the immune system's ability to recognize and eliminate cancer cells—has gained significant attention in recent years. The effectiveness of the immune response against tumors is largely determined by the interaction between cancer cells and immune cells within the TME.[59] Emerging evidence highlights the dual role of immune cells in LC progression: they can support anti-tumor immunity or foster an immunosuppressive, tumor-promoting TME.[60] The TME is a dynamic and complex network composed of endothelial cells, fibroblasts, mesenchymal cells, adipocytes, immune cells, and the extracellular matrix. Over the past decade, there has been intense focus on understanding the composition and function of the tumor immune microenvironment (TIME), which plays a crucial role in shaping the immune response and determining the efficacy of immunotherapies. A breakthrough in immunotherapy was the discovery of immune checkpoints (ICPs)—regulatory proteins expressed on immune and cancer cells. Under normal physiological conditions, these checkpoints maintain immune homeostasis and prevent autoimmunity by transmitting inhibitory signals that downregulate T cell activity upon ligand binding. However, tumor cells exploit these pathways to avoid immune-mediated destruction by overexpressing ICP ligands and suppressing T cell function.[59]

Checkpoint inhibitors (CKIs), which block these immune checkpoints, have revolutionized cancer treatment by restoring anti-tumor T cell activity. The first discovered and most widely studied ICPs are cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and programmed death-1 (PD-1). Checkpoint inhibitors have emerged as a cornerstone of treatment for advanced NSCLC. In 2015, the U.S. Food and Drug Administration (FDA) approved nivolumab (an anti–PD–1 antibody) for LUSC that had progressed following platinum-based chemotherapy, with subsequent approval for all NSCLC subtypes. PD-1, a receptor, which is normally expressed on activated T cells, and interacts with its ligand, PD-L1, to prevent overstimulation of immune responses. Tumors upregulate PD-L1 to evade immune detection, but the blockade of the PD-1/PD-L1 pathway using monoclonal antibody inhibitors restores T cell function and consistently shows remarkable anti-tumor effects in patients with advanced cancers. Currenty, the CKI is recognized as the gold standard for developing new immune checkpoint blockade (ICB) and combination therapies.[60] Pembrolizumab was similarly approved in 2015 for PD-L1-positive NSCLC after chemotherapy failure, and in 2016, it received first-line approval for patients with high PD-L1 expression (≥50%). Atezolizumab, another anti–PD-L1 antibody, was approved for advanced NSCLC following progression after chemotherapy.[61] As research into

cancer immunology advances, immunotherapy is evolving beyond checkpoint inhibitors. Promising alternatives include cancer vaccines, other monoclonal antibodies (mAbs), and adoptive cell therapies, which will offer additional treatment options for LC therapy in the future.

2. Aims of the study

In the context of NSCLC, which consists of two main primary subtypes – LUSC and LUAD – research suggests that these tumors exhibit differential responses to therapy.[66] Upon review of available information on molecular differences and analysis of patient statistics, several areas emerged for further studies. For example, we questioned the current trends, which mainly investigated the tumor tissues for markers leaving out the association with patient characteristics and therapy response in the circulation, where several lipid-vesicles including heparan sulfate proteoglycans (HSPGs), cytonemes, and the most extensively studied members of the EVs, exosomes, carry a vast array of molecules including nucleic acids, proteins, etc. Additionally, some disturbing trends emerged towards certain therapies. Patients diagnosed with LUSC were presented with a significantly increased incidence of bleeding as a side effect associated with inhibition of vascularization.[66,67] This alarming discovery led to a major change in clinical practice; as a result, patients diagnosed with LUSC were subsequently excluded from receiving anti-angiogenic therapies, including bevacizumab. However, it is important to emphasize that this exclusion does not mean that patients with LUAD are completely free from the risk of similar side effects, including gastrointestinal bleeding or perforation, if their treatment involves inhibition of vascularization. Indeed, episodes of bleeding side effects may still occur sporadically in some adenocarcinoma patients, raising questions about the underlying mechanisms contributing to these reactions. While targeted therapies have improved survival rates in LUAD, effective treatment options for LUSC are still limited. In light of the above therapy responses, we hypothesized that there might be specific molecular microenvironmental elements that facilitate the differential response to specific treatment regimens. The differences in therapy response could be associated with molecules carried in extracellular vesicles, or differential expression in drug transporters. By identifying and measuring these potentially influential factors, we aimed to gain a deeper insight into the biological basis of therapy response and overall survival.

The objectives of this study were as follows:

1. Identification of differential molecular characteristics of LUAD and LUSC in patient sera and serum-derived exosomes with a focus on specific proteins and a broad range of miRNAs.
2. Monitoring patients during and after clinical therapy and characterizing patient response and overall survival in light of the identified pre-therapy molecular characteristics.
3. Investigation of drug transporters and their association with KRAS and EGFR mutations in one of the NSCLC subtypes using LUAD cell lines.

3. Materials and Methods

3.1 Patients and Sample Collection for circulating EV studies

From February to December of 2018, serum samples were collected from 60 patients, the majority of whom were diagnosed with stage III/b-IV NSCLC, at the Department of Pulmonology and the Ist. Department of Internal Medicine at PTE. This study was conducted with ethics committee approval (PTE_KK_RIKEB_6444/2016) and was conducted in compliance with the Declaration of Helsinki. All patients provided written informed consent before participating. The patient data were stored in an Excel spreadsheet, in which the following information was recorded for each patient: gender, age, histological diagnosis, TNM stage of the tumor, molecular profile (KRAS, EGFR, ALK), diagnostic procedure used for histological sampling, tumor location (central or peripheral), date of sampling, clinical treatment, and type of treatment (curative or palliative), start and end of treatment, number of treatment cycles, response to therapy RECIST 1. 1 (Response Evaluation Criteria in Solid Tumors), the patient's further oncological treatment, fate, whether the patient developed bleeding complications or hypertension during treatment, the patient's smoking history, and whether the patient had known chronic obstructive pulmonary disease (COPD).

On the day of the initial cycle of chemotherapy treatment, 5 ml of blood was extracted from a peripheral vein of each patient into a native tube. The tubes were then subjected to centrifugation at 3000 g for 10 min at 4°C within two hours of sample collection. After centrifugation, the serum was transferred into Eppendorf tubes and stored at -80°C until further use. Patients were observed during their respective therapeutic regimens, and details regarding treatment response and comorbidities were meticulously recorded during clinical visits.

The study's inclusion criteria were as follows: Firstly, patients with histologically confirmed NSCLC stage I-IV (LUAD or LUSC) based on the American Joint Committee on Cancer

(AJCC, version 8) staging were included. Secondly, patients with no history of radiotherapy, immunotherapy, chemotherapy, or other treatments before diagnosis were included. Thirdly, patients with the availability of complete follow-up data, including best overall response (BOR) and overall survival (OS), were included. Lastly, patients with the availability of sufficient quantity and quality of serum samples were included. The exclusion criteria were as follows: (i) pathologically different diagnoses of NSCLC; (ii) patients with a history of a second primary malignancy; and (iii) serum samples or any other reason for the failure of quality control at any stage of the study. Best overall response (BOR) was determined according to RECIST version 1.1. The time from the start of treatment to the end of any cause or the last follow-up date was defined as overall survival (OS). Serum from healthy blood donors (HC) selected by a comparable age distribution to the study population was used as controls. The study was declared complete three years later, in 2021.

3.2 Cell Cultures

Human NSCLC AC cell lines A549 (KRAS mutant) and PC9 (EGFR mutant: DelE746A750) (American Type Cell Culture Collection, Rockville, MD, USA) were used in the experiments. A549 cells were cultured in complete Dulbecco's Modified Eagle's Medium (DMEM) (Lonza, Walkersville, MD, USA), supplemented with 10% foetal calf serum (FCS), 3% Penicillin/Streptomycin, 2% L-Glutamine, 1% non-essential amino acid, 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer and 1% β-mercaptoethanol. PC9 cells were cultured in Rosewall Park Memorial Institute 1640 Medium (RPMI) (Thermo Fisher Scientific, Waltham, USA) supplemented with 10% FCS, 3% Penicillin/Streptomycin, 2% L-Glutamine. Primary small airway epithelial cells (SAEC) were purchased from Lonza (Lonza, Walkersville, MD, USA) and cultured in Small Airway Growth Medium (Lonza, Walkersville, MD, USA). Normal human lung fibroblasts cells (NHLF) were cultured in Fibroblast Growth Medium (FGM-2) and Primary Umbilical Vein Endothelial Cells; Normal, Human (HUVEC) (American Type Cell Culture Collection, Rockville, MD, USA, ATCC® PCS-100-010™), were maintained in F-12K Medium (Kaighn's Modification of Ham's F-12 Medium) (ATCC® 30-2004™), respectively. All types of cells were cultured at 37 °C in humidified atmosphere containing 5% CO_2. Trypan blue dye exclusion test was used to assess cell viability.[68]

3.3 Three Dimensional (3D) Aggregate Cultures

3D aggregates consisted of NHLF, HUVEC and A549 or PC9 cells at 4:3:3 ratio. The aggregates were prepared by a commercially available magnetic cell levitation technique using the 96 Well Bioprinting Kit and the 96 Well BiOAssay™ Kit provided by Greiner Bio-One Ltd., (Kremsmünster, Austria). The cells were incubated with NanoShuttle^{TM}-PL at 4°C for 4 h, respectively (A549, A549-CR, PC9, PC9-CR, NHLF, HUVEC). The magnetised cells were detached, counted, and dispensed in a cell-repellent plate. Cell-repellent plate was placed on the holding drive to aggregate the cells and incubated at 37 °C for 15 min. The aggregates were suitable for gene expression testing and immunohistochemical staining of drug transporters.[68]

3.4 Immunohistochemistry

3.4.1 Detection of WNT5A

Immunohistochemistry was performed on 4 μm-thick tissue sections of FFPE tissue blocks. Tris/EDTA buffer, pH 9.0, was used for 20 min for antigen retrieval. Reactions were visualised via BOND polymer refine detection (Leica DS9800) (Leica Biosystems, Deer Park, IL, United States). A monoclonal antibody WNT5A (Clone 3D10) (MA5-15511, Thermo Fisher Scientific, Waltham, MA, United States) was used.

3.4.2 Detection of drug transporters

The 3D aggregates were sectioned using a Leica CM1950 cryostat (Leica, Wetzlar, Germany), then fixed and stained using a routine IHC staining procedure in a Vision Biosystems bond™ automated immune-stainer (Leica, Wetzlar, Germany). Anti-human ABCG2 mouse monoclonal antibody (CD338, clone 5D3, BD Biosciences, San Jose, CA, USA) and anti-human ABCB1 rabbit monoclonal antibody (clone D3H1Q, Cell Signaling Technology, Danvers, MA, USA) were used as primary antibodies, both in 1:50 dilution. EpCAM (CD326, 323/A3, MA5-12436, ThermoFisher Scientific, MA, USA), an unlabeled mouse monoclonal antibody in 1:50 dilution, and Cytokeratin 5 polyclonal rabbit (SC-66856, Santa Cruz Biotechnology, Dallas, TX, USA) in 1:100 dilution were used. The secondary antibody was a goat anti-rabbit IgG antibody (Alexa Fluor® 647) (ab150087) (1:2000) (Abcam Plc, Cambridge, United Kingdom) and the anti-mouse antibody was an Alexa Fluor® 488 conjugated IgG (Thermo Fisher Scientific, Waltham, USA) (dilution 1:200). Nuclei were counterstained with Dapiprazole hydrochloride (DAPI) (ab142859) (1:1000) (Abcam Plc, Cambridge, United

Kingdom). Images were acquired applying Nikon Eclipse Ti-U microscope (Nikon GmbH CEE, Vienna, Austria) equipped with CCD camera (AndorZyla 5.5), ImageJ (Java) applied for densitometry. Drug transporter protein intensity quantification was normalized to the respective epithelial marker intensity.[68]

3.5 WNT Signaling Arrays in drug transporter studies

Relative mRNA expression (2^{-ddCt}) of WNT signaling pathway genes in A549, A549-CR and PC9, PC9-CR in 3D aggregates was assessed using Applied Biosystems™ TaqMan™ Array, Human WNT Pathway, Fast 96-well (Thermo Fisher Scientific Inc. TMO, Waltham, MA, USA).

3.6 Extraction of exosomes from serum

In general, blood samples were collected, allowed to clot at 37°C for 20 min, and then centrifuged at 1500×g for 10 min at room temperature (RT). The serum was then stored at -80°C until further processing. Following the manufacturer's protocol, exosomes were isolated from 400 µl of serum samples using Total Exosome Isolation Reagent (TEI) (from serum) (4478360, Invitrogen, Thermo Fisher Scientific, Waltham, MA, United States). Briefly, serum samples were spun at 2000× g for 30 min to remove cells and debris. Next, 0.2 volumes of TEI reagent were added to each supernatant, and the samples were incubated at 4°C for 30 min. The precipitated exosomes were recovered by centrifugation at 10,000×g for 10 min at RT. The exosome pellets were subsequently resuspended in PBS, pH 7.4, at RT.3.7

3.7 Nanoparticle tracking analysis

A NanoSight NS300 instrument (Malvern Panalytical Ltd., Malvern, United Kingdom) equipped with a 488-nm blue laser was used for real-time tracking and analysis. All analyzed samples were diluted in Ca- and Mg-free PBS to a final volume of 1 ml. Exosome isolates were subsequently diluted to the optimum NTA detection range before measurements (10–50 particles/frame). For each measurement, five 1-minute videos were captured under the following conditions: cell temperature: 25°C; syringe speed: 50 µl/s. The videos were analysed via in-built NTA v3.2 software.

3.8 Transmission electron microscopy (TEM)

TEM was used to visualize the exosomes. A 2.5 µl sample volume was placed individually on a 300-mesh grid for each sample. The grid was dried overnight at RT, and then 5% uranyl acetate and 3% sodium citrate were added to the grid. After 5 min of incubation, the grid was air-dried. Twenty-four hours later, the grid was analyzed using JEOL TEM (JEOL Ltd., Tokyo, Japan) 1,200 EX.

3.9 EV Antibody array

EV-specific marker analysis was performed using the Exo-Check antibody array (EXORAY210B-8, System Biosciences, Palo Alto, CA, USA). 60 µg of EV preparation was added to the membrane-based blot array, and the manufacturer's instructions were followed. The intensity of chemiluminescence was detected with a G:BOX Chemi XRQ (Syngene, Cambridge, UK).

3.10 WNT5A ELISA

A human WNT5A (protein Wnt-5a) ELISA Kit (EH1164, FineTest, Wuhan, China) was used to quantify the serum, exosome-free serum, and exosome WNT5A contents. The starting volume was 400 µl of serum in all cases. Furthermore, to study the association and distribution of WNT5A with exosomes, the isolated pellet was resuspended in 395 µl of PBS to quantify the WNT5A content on the surface of exosomes. To determine the total WNT5A content of exosomes the samples were treated with 95 µl of ice-cold RIPA buffer (89900, Pierce RIPA buffer, Thermo Fisher Scientific, Waltham, MA, United States) to disrupt the exosome membranes. All samples were incubated with 5 µl of 100x Halt-Protease inhibitor cocktail (87786, Thermo Fisher Scientific, Waltham, MA, United States). The samples were mixed with their respective buffers and incubated on ice for 15 min. The protein concentration was quantified by a BCA assay using the Pierce™ BCA Protein Assay Kit (23225, Thermo Fisher Scientific, Waltham, MA, United States) according to the manufacturer's instructions. All reagents, standards, and samples were prepared according to the manufacturer's instructions. The absorbance of the samples was recorded at 450 nm via a Perkin Elmer Enspire Multiplate Reader (Perkin Elmer, Waltham, MA, USA). The target concentrations were interpolated from a standard curve created from the standards with predefined concentrations. Each sample was measured in duplicate.

WNT5A protein per particle

The amount of WNT5A protein per particle was calculated by dividing the WNT5A concentration determined by the ELISA by particle concentration detected by NTA.

The amount of WNT5A protein per particle $\frac{\text{WNT5A concentration measured by ELISA } \left(\frac{\text{pg}}{\text{ml}}\right)}{\text{Particle concentration detected by NTA } \left(\frac{\text{particles}}{\text{ml}}\right)}$

3.11 RNA isolation and further studies in EV and drug transporter studies

3.11.1 Total RNA isolation from EV and Nanostring analysis

Total RNA was extracted from exosomes using the Total Exosome RNA and Protein Isolation Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's protocol. The extracted RNA was eluted with 50 µl of RNase-free water. The eluent (50 µl) was concentrated to 5 µl each using a vacuum concentrator. For miRNA profiling, the NanoString nCounter® microRNA platform (version 3) (NanoString Technologies, Seattle, WA, USA) was used according to the manufacturer's instructions. Quality checks confirmed the reliability of the run and the validity and reproducibility of the miRNA screening protocol. nSolver software was used for data analysis and normalization. Normalization was performed using the "Housekeeping" method according to the nCounter miRNA expression analysis in plasma and serum sample technote instructions.

3.11.2 RNA Isolation, cDNA Synthesis and qRT-PCR in drug transporter studies

Cell cultures were harvested in RA1 Buffer Solution then RNA isolation was performed using NucleospinII RNA isolation kit according to the manufacturers' protocol (Macherey-Nagel, Düren, Germany). RNA concentration was measured using Nanodrop technology (Thermo Fisher Scientific, Waltham, MA, USA). cDNA synthesis was performed using random hexamer primers of the high-capacity RNA to cDNA kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturers' protocol. SYBRGreen (Roche, Basel, Switzerland) real-time qRT-PCR reaction using sequence specific primers was set up using Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The relative quantities of different drug-transporters were calculated using the 2^{-ddCt} method. The inner control was β-actin for each sample.

3.12 Functionality Test of ABC Drug Transporters

Wild-type and cisplatin-resistant (CR) A549 and PC9 cells were washed with PBS and then trypsinized. Cells were counted, and sorted into FACS tubes, 100000 cells into each, and in triplicate. MultiDrugQuantTM Kit (SOLVO Biotechnology, Szeged, Hungary) and flow-cytometry were used to measure the functional activity of ABCB1 and ABCG2 transporters. BD FACScanto II (BD Biosciences, San Jose, CA, USA) was used for flow-cytometry with BD FACSDiva V5.1 software (BD Biosciences, San Jose, CA, USA). The results were calculated as multidrug resistance (MDR) activity factor values (MAF) according to the instructions of the manufacturer. The calculations were performed using the median of triplicate. MAF values at 20 and above are regarded as representing functionally active drug transporter proteins.[68]

3.13 Drugs and Reagents

Drugs including cisplatin, carboplatin, and paclitaxel were purchased from Selleckchem (Houston, TX, USA). For the generation of CR NSCLC cell lines, the starting concentration of cisplatin in A549 cell cultures was 1 μM, while the final concentration reached 12 μM within 3 weeks. In the PC9 cell cultures starting concentration of cisplatin was 0.67 μM which was increased to 4.67 μM within 3 weeks. Carboplatin and paclitaxel treatment were applied for 24 h in 100 μM and in 0.002 μM concentrations, respectively.

3.14 Statistical analysis

3.14.1 Statistical analysis of exosome studies

Data are expressed as the means ± SDs. The normal distribution was tested by Shapiro-Wilk test. The Mann–Whitney U test or unpaired t-test was used for comparison of two specifications; the Kruskal-Wallis or one-way ANOVA was used for comparison of three or more specifications. Cut-off values for serum, exosome-free serum, and exosome WNT5A were determined by receiver operating characteristics (ROC) curve analysis or the best cuff-off method, a web-based survival analysis tool tailored for medical research. Briefly, all possible cut-off values between the lower and upper quartiles are computed, and the best-performing threshold is used as a cut-off. The Kaplan–Meier method was used to generate survival curves based on the length of time between primary treatment and exit. The log-rank test (Mantel–Cox) was used to compare the survival distributions. For all analyses, a two-sided $p<0.05$ was

considered statistically significant. Statistical analysis was performed using GraphPad Prism 9.0.0 software (GraphPad, Palo Alto, USA).

Differential expression analysis was performed using t-tests, and fold changes were calculated. Benjamini- and Hochberg-adjusted p-values were also calculated. Data preprocessing and differential expression analysis were performed using R statistical package [69]. The heatmap package, R, was used for heatmap visualization [70].

3.14.2 Statistical analysis of drug transporter studies

Statistical analysis was performed using the SPSS 26.0 package for Windows (IBM SPSS Statistics, Chicago, IL, USA). Normal distribution was tested using the Kolmogorov–Smirnov test. The differences between 2 independent groups were calculated using the Mann–Whitney-U test as non-parametric calculation. For normal distribution, the independent-samples t-test and Kruskal–Wallis test were performed. Results were considered statistically significant if $p < 0.05$.

3.15 IPA analysis

The Ingenuity program (Ingenuity Systems, USA; http://analysis.ingenuity.com) was used to conduct functional analysis. Differentially expressed miRNAs were subjected to miRNA target filters, and mRNA target lists were created by applying cancer disease, VEGF, VEGF family ligand-receptor interactions, epithelial adherens junction, tight junction signaling and NSCLC signaling filters. Then, Molecule Activity Predictor (MAP) analysis was applied to predict molecular activation or inhibition.

4. Results and Discussion

4.1 WNT5A as a biomarker

WNT5A has a consistent association with various cancer types,[33,76] yet its potential as a biomarker remains largely unverified. In our study, we hypothesized that separating vesicle-free and vesicle-bound fractions of the serum might have reliably different WNT5A levels and yield predictive, prognostic, or diagnostic value for NSCLC patients. We initially observed that elevated levels of WNT5A in resected tumor tissue were not associated with diminished OS and did not differentiate NSCLC subtypes, LUAD and LUSC, confirming data in the literature. However, significant differences were detected in WNT5A levels between the LUAD and LUSC subtypes by separating the exosome-free serum and exosome fractions. LUSC patients had lower levels of circulating exosomes compared to LUAD patients or HCs. Moreover, LUSC exosomes contained significantly more WNT5A both on their surface and inside as cargo, in comparison to LUAD patients. Additionally, the exosome-free serum of LUAD patients had notably higher levels of WNT5A than LUSC patients, indicating that the two subtypes of NSCLC have distinct extracellular WNT5A profiles. The difference in WNT5A contents in exosomes and exosome-free serum suggests that the location of WNT5A may play a significant role in the clinical outcomes of LUAD and LUSC. Previous research into the role of vesicle-free and vesicle-bound forms of WNT5A has shown that the ratio of secreted versus vesicle-bound WNT5A depends on the cell type and the cellular context. While secreted WNT5A appears to promote a more aggressive cancer phenotype, WNT5A in extracellular vesicles may exhibit context-dependent effects, potentially inhibiting or promoting cancer progression.[77,78] In our study, high levels of WNT5A bound to exosomes were associated with LUSC patients and shorter OS, while higher WNT5A in exosome-free serum predicted better survival associated with LUAD, suggesting that the context of WNT5A presentation and mode of secretion is crucial for its function in cancer. Further studies are needed to elucidate the molecular mechanisms underlying the selection and sorting process of WNT5A into the extracellular space and vesicles in NSCLC subtypes to elucidate the distinctively different effects on survival. Understanding such differences might also lead to the identification of novel therapeutic targets, which are especially important since exosome-associated high levels of WNT5A are an indicator of far more aggressive disease with higher metastatic potential and reduced OS.[77,78] It is also significant, that a simple blood sample and measuring WNT5A in serum and exosome fractions at the time of diagnosis can identify advanced disease stage, lymph node involvement, distant metastasis, and predict lack of therapy response. Perhaps less

surprising is that all such characteristics are associated with LUSC patients and their reduced OS. In LUAD, the exosome-associated WNT5A levels were significantly lower and associated with significantly higher OS time. The difference in the location of serum WNT5A can also serve as a novel diagnostic biomarker to differentiate LUSC from LUAD. While other studies have also found that high WNT5A expression is somehow associated with poor prognosis in NSCLC patients, especially in the LUSC subgroup,[79] our study is the first one to identify the precise location and diagnostic and prognostic value of serum and serum-derived exosome-associated WNT5A with specified cut-off values. In summary, measuring specific extracellular forms of WNT5A in liquid biopsies can provide important clinical information that complements tissue-based biomarkers and supports treatment decisions in NSCLC.

4.2 Serum exosome-derived miRNAs as predictive markers in NSCLC

Circulating miRNAs have been suggested as NSCLC biomarkers in the past couple of years.[48, 47] The primary aims of such studies were to aid early disease detection and increase chances of long-term survival due to fast and effective therapy selection or even identification of novel therapeutic targets.[47] In contrast to previous studies, our research is the very first one that was able to correlate serum exosome-derived miRNA profiles with differential diagnosis and further studies are on the way to identify serum exosome-derived miRNAs as potential predictive biomarkers for therapy response in NSCLC.

4.3 Drug transporter analysis of LUAD cell lines to investigate therapy resistance

In this study, we investigated the effects of cisplatin in the context of different oncogenic mutation backgrounds, specifically KRAS and EGFR mutations. We analyzed the molecular microenvironment of two LUAD cell lines and compared them to normal lung epithelial cells. Both LUAD cell lines exhibited elevated expression of ABC transporters ABCB1 and ABCG2 relative to the normal control. Notably, the KRAS-mutant A549 cells demonstrated higher levels of both ABCB1 and ABCG2 compared to the EGFR-mutant PC9 cells, suggesting that the mutational context influences the expression of drug transporters. Furthermore, our data indicate that chronic cisplatin exposure modulates the expression of these transporters. The expression and functional activity of ABCB1 and ABCG2 were differentially altered depending on whether the cells harbored a KRAS or EGFR mutation, highlighting the mutation-dependent nature of MDR mechanisms in LC. To better mimic the human lung tumor microenvironment, KRAS-mutant (A549), EGFR-mutant (PC9), and their cisplatin-resistant counterparts (A549-

CR, PC9-CR) were grown in 3D aggregate co-cultures for more clinically relevant experiments. Although cisplatin remains one of the oldest chemotherapeutic agents, it is still widely used in combination regimens. Due to its toxicity and often limited tolerability, cisplatin can be replaced or supplemented with other platinum-based agents like carboplatin. Carboplatin is typically paired with drugs such as paclitaxel, vinorelbine, or gemcitabine, with the choice influenced by the patient's mutational background. Using this 3D co-culture model, we found that cisplatin treatment, especially due to its high efficacy, before transitioning to carboplatin or combination therapies, significantly elevated ABCB1 (P-gp) mRNA levels, a key mediator of multidrug resistance.[83] ABCB1 is known to efflux various chemotherapeutic agents, including vinca alkaloids, anthracyclines, taxanes, and notably paclitaxel. Since paclitaxel is a staple drug in advanced LUAD treatment,[84,85] ABCB1 expression may serve as a predictive marker of therapeutic response.[86] In cisplatin-resistant A549-CR cells, ABCB1 levels further increased following treatment with carboplatin and paclitaxel, alone or in combination. Conversely, ABCG2 expression was reduced, highlighting the sensitivity of transporter expression to both mutational background and treatment. These findings support the utility of 3D co-cultures in modeling drug responses. In principle, inhibiting ABCB1 could improve treatment efficacy. Accordingly, pharmaceutical efforts have explored ABC transporter inhibition. For example, Tariquidar, a potent ABCB1 inhibitor, was tested in combination with carboplatin/paclitaxel in phase III trials for NSCLC, based on its expected enhancement of chemotherapeutic efficacy. However, these trials were discontinued due to increased chemotherapy-related toxicity in the Tariquidar arm.[86] Despite this, ongoing and completed trials involving Tariquidar, paclitaxel, docetaxel, carboplatin, and cisplatin suggest that selective modulation of ABC transporter activity may hold promise, provided cancer cell specificity can be improved.[8786] Although cisplatin itself is not a substrate for ABCB1 or ABCG2, resistance to cisplatin alters the expression of these transporters. Consequently, if a patient can no longer tolerate cisplatin, prior exposure may reduce the efficacy of second-line therapies due to transporter upregulation. The WNT signaling pathway plays a pivotal role in regulating ABC transporter expression. Specifically, TCF/LEF-mediated β-catenin-dependent WNT signaling activates the ABCB1 promoter, increasing its transcription.[88,89] Thus, WNT pathway modulation directly impacts chemoresistance.[89,90] This complex, evolutionarily conserved pathway controls numerous developmental and homeostatic processes,[24,33] and though rarely mutated in LC, its regulation differs among LC subtypes. Our study revealed mutation-specific differences in WNT pathway gene expression that modulate cisplatin response. RHOU, a Rho GTPase family member involved in cytoskeletal organization and

metastasis regulation,[91] was ~10,000-fold more expressed in KRAS-mutant A549 cells than in EGFR-mutant PC9 cells. In contrast, PC9 cells overexpressed multiple WNT ligands (WNT1, WNT7A, WNT7B, WNT9A), which have been implicated in LUAD.[24] WNT9A is especially notable for activating canonical WNT/β-catenin signaling via FZD receptors. Prolonged cisplatin treatment in PC9 cells upregulated several β-catenin pathway regulators, including DACT1, a Disheveled-binding antagonist of JNK and WNT signaling.[92,93] DACT1 expression is typically low in NSCLC but can be elevated in other cancers,[93] and its dysregulation correlates with poor prognosis. DACT1 is currently under investigation as a therapeutic target.[93] Additionally, WNT16, which supports malignancy by preventing apoptosis, was also upregulated. Recent studies suggest that WNT16-neutralizing antibodies combined with chemotherapy may enhance survival.[94] Other WNT-associated genes were similarly modulated. RHOU expression increased in PC9-CR cells,[91] and TLE4, an established oncogene in lung and colorectal cancers, was induced by cisplatin.[95–97] WISP1, previously linked to platinum-induced toxicity in LC, was also upregulated.[98] These findings indicate that cisplatin can reshape the WNT microenvironment in EGFR-mutant cells, potentially influencing treatment outcomes. In A549-CR cells, cisplatin dramatically upregulated LEF1, a transcription factor in the β-catenin pathway, along with NKD1, a negative feedback regulator of WNT signaling.[99] While A549-CR cells expressed more soluble WNT inhibitors (e.g., frizzled-related proteins), PC9-CR cells showed higher levels of WNT ligands, DACT1, WISP1, and the co-receptor KREMEN2. Despite these molecular differences, the ultimate response—namely, upregulation of ABCB1—was common to both cell types. Importantly, the WNT pathway activation seen in EGFR-mutant PC9 cells involves molecules such as DACT1 and WNT16, which are already being investigated as therapeutic targets. These findings suggest that patients with EGFR-mutant LUAD may benefit from therapies targeting WNT signaling components rather than direct ABCB1 inhibition, potentially offering a more precise and less toxic treatment strategy. In summary, the impact of these pharmaceutical agents on the tissue environment, as evidenced by the three-dimensional imaging, remains to be elucidated. The pharmaceuticals utilized in this study are identical to those administered to patients, both in 2D and 3D. A subsequent comparison was made to ascertain which transporters were expressed. An examination of the treatment of LUAD KRAS or LUAD EGFR reveals that they exhibit divergent responses to the same chemotherapy regimen. Moreover, increased expression of drug transporters leads to the efflux of drugs into the tumor, which hinders the death of tumor cells. Our findings demonstrate that cisplatin resistance in LUAD is closely associated with mutation-specific alterations in WNT signaling and ABC transporter expression. Notably, EGFR-mutant cells exhibited

enhanced expression of WNT pathway components, including DACT1 and WNT16, alongside increased ABCB1 levels. These alterations may contribute to multidrug resistance and influence treatment efficacy. Importantly, the WNT-related changes observed in EGFR-mutant cells point to additional, potentially targetable pathways that could be leveraged to enhance treatment response and reduce toxicity. Thus, integrating WNT pathway inhibitors with conventional chemotherapy may offer a mutation-specific, more effective therapeutic strategy for patients with EGFR-mutant LUAD.

5. Summary of novel findings

1. Our study identifies WNT5A as a promising non-invasive biomarker for both diagnosis and prognosis in NSCLC, with its clinical significance strongly influenced by its mode of secretion.
2. By analyzing both exosome-free serum and exosome-bound WNT5A, we found distinct extracellular profiles between LUAD and LUSC patients. High levels of exosome-associated WNT5A were linked to LUSC, advanced disease features, and poorer overall survival, while elevated WNT5A in exosome-free serum predicted better outcomes in LUAD patients. These findings suggest that the compartmentalization of WNT5A, rather than its total expression alone, may serve as a valuable tool for subtype differentiation, prognostic stratification, and potentially guiding therapy selection in NSCLC.
3. Measuring specific extracellular forms of WNT5A in liquid biopsies can provide important clinical information that complements tissue-based biomarkers and supports treatment decisions in NSCLC.
4. Our research is the first to demonstrate that serum exosome-derived miRNAs can distinguish between healthy and NSCLC miRNA profiles.
5. This study underscores the mutation-specific mechanisms of multidrug resistance (MDR) in lung adenocarcinoma, particularly highlighting how EGFR and KRAS mutations differentially influence ABC transporter expression and WNT signaling in response to cisplatin treatment. Cisplatin-resistant cells, especially those with EGFR mutations, showed significant upregulation of ABCB1 and key WNT pathway components such as DACT1 and WNT16, factors associated with chemoresistance and

poor prognosis. These findings suggest that targeting WNT signaling, rather than ABCB1 directly, may offer a more effective and less toxic strategy to overcome MDR in EGFR-mutant lung adenocarcinoma, enabling more personalized and mutation-adapted treatment approaches.

6. References

1. Chaitanya Thandra, K., Barsouk, A., Saginala, K., Sukumar Aluru, J. & Barsouk, A. Epidemiology of lung cancer. *Współczesna Onkologia* **25**, 45–52 (2021).

2. de Groot, P. M., Wu, C. C., Carter, B. W. & Munden, R. F. The epidemiology of lung cancer. *Transl Lung Cancer Res* **7**, 220–233 (2018).

3. Barta, J. A., Powell, C. A. & Wisnivesky, J. P. Global Epidemiology of Lung Cancer. *Ann Glob Health* **85**, (2019).

4. SEER Cancer Statistic Review, 1975-2014 .

5. Cooper, W. A., Lam, D. C. L., O'Toole, S. A. & Minna, J. D. Molecular biology of lung cancer. *J Thorac Dis* **5 Suppl 5**, S479-90 (2013).

6. Francisci, S. *et al.* Survival patterns in lung and pleural cancer in Europe 1999–2007: Results from the EUROCARE-5 study. *Eur J Cancer* **51**, 2242–2253 (2015).

7. De Angelis, R. *et al.* Cancer survival in Europe 1999–2007 by country and age: results of EUROCARE-5—a population-based study. *Lancet Oncol* **15**, 23–34 (2014).

8. Lung Cancer in Hungary.

9. Bray, F. *et al.* Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* **68**, 394–424 (2018).

10. Thai, A. A., Solomon, B. J., Sequist, L. V, Gainor, J. F. & Heist, R. S. Lung cancer. *The Lancet* **398**, 535–554 (2021).

11. Detterbeck, F. C. *et al.* The IASLC Lung Cancer Staging Project: Summary of Proposals for Revisions of the Classification of Lung Cancers with Multiple Pulmonary Sites of Involvement in the Forthcoming Eighth Edition of the TNM Classification. *Journal of Thoracic Oncology* **11**, 639–650 (2016).

12. Raso, M. G., Bota-Rabassedas, N. & Wistuba, I. I. Pathology and Classification of SCLC. *Cancers (Basel)* **13**, 820 (2021).

13. J. Saller, J. & Boyle, T. A. Molecular Pathology of Lung Cancer. *Cold Spring Harb Perspect Med* **12**, a037812 (2022).

14. Gurda, G. T. *et al.* Utility of five commonly used immunohistochemical markers TTF-1, Napsin A, CK7, CK5/6 and P63 in primary and metastatic adenocarcinoma and squamous cell carcinoma of the lung: a retrospective study of 246 fine needle aspiration cases. *Clin Transl Med* **4**, (2015).

15. Comprehensive genomic characterization of squamous cell lung cancers. *Nature* **489**, 519–525 (2012).

16. Comprehensive molecular profiling of lung adenocarcinoma. *Nature* **511**, 543–550 (2014).

17. Altorki, N. K. *et al.* The lung microenvironment: an important regulator of tumour growth and metastasis. *Nat Rev Cancer* **19**, 9–31 (2019).

18. López-Camarillo, C., Ruiz-García, E., Starling, N. & Marchat, L. A. Editorial: Neovascularization, Angiogenesis and Vasculogenic Mimicry in Cancer. *Front Oncol* **10**, (2020).

19. Zhao, Y. *et al.* VEGF/VEGFR-Targeted Therapy and Immunotherapy in Non-small Cell Lung Cancer: Targeting the Tumor Microenvironment. *Int J Biol Sci* **18**, 3845–3858 (2022).

20. Frezzetti, D. *et al.* VEGF as a potential target in lung cancer. *Expert Opin Ther Targets* **21**, 959–966 (2017).

21. Assoun, S., Brosseau, S., Steinmetz, C., Gounant, V. & Zalcman, G. Bevacizumab in advanced lung cancer: state of the art. *Future Oncology* **13**, 2515–2535 (2017).

22. Hendrixson, M., Gladkiy, Y., Thyagarajan, A. & Sahu, R. P. Efficacy of Sorafenib-Based Therapies for Non-Small Cell Lung Cancer. *Medical Sciences* **12**, 20 (2024).

23. Stewart, D. J. Wnt Signaling Pathway in Non-Small Cell Lung Cancer. *JNCI Journal of the National Cancer Institute* **106**, djt356–djt356 (2014).

24. Rapp, J., Jaromi, L., Kvell, K., Miskei, G. & Pongracz, J. E. WNT signaling – lung cancer is no exception. *Respir Res* **18**, 167 (2017).

25. De Langhe, S. P. & Reynolds, S. D. Wnt signaling in lung organogenesis. *Organogenesis* **4**, 100–108 (2008).

26. Mucenski, M. L. *et al.* β-Catenin Is Required for Specification of Proximal/Distal Cell Fate during Lung Morphogenesis. *Journal of Biological Chemistry* **278**, 40231–40238 (2003).

27. Li, C., Xiao, J., Hormi, K., Borok, Z. & Minoo, P. Wnt5a Participates in Distal Lung Morphogenesis. *Dev Biol* **248**, 68–81 (2002).

28. Li, C. *et al.* Wnt5a regulates Shh and Fgf10 signaling during lung development. *Dev Biol* **287**, 86–97 (2005).

29. Qin, L. *et al.* WNT5A promotes stemness characteristics in nasopharyngeal carcinoma cells leading to metastasis and tumorigenesis. *Oncotarget* **6**, 10239–10252 (2015).

30. Okubo, T. & Hogan, B. L. Hyperactive Wnt signaling changes the developmental potential of embryonic lung endoderm. *J Biol* **3**, 11 (2004).

31. Sunaga, N. *et al.* Constitutive activation of the Wnt signaling pathway by CTNNB1 (beta-catenin) mutations in a subset of human lung adenocarcinoma. *Genes Chromosomes Cancer* **30**, 316–21 (2001).

32. Stewart, D. J. Wnt Signaling Pathway in Non-Small Cell Lung Cancer. *JNCI Journal of the National Cancer Institute* **106**, djt356–djt356 (2014).

33. Pongracz, J. E. & Stockley, R. A. Wnt signalling in lung development and diseases. *Respir Res* **7**, 15 (2006).

34. Yang, J. *et al.* Wnt signaling as potential therapeutic target in lung cancer. *Expert Opin Ther Targets* **20**, 999–1015 (2016).

35. Linton, K. J. Structure and Function of ABC Transporters. *Physiology* **22**, 122–130 (2007).

36. Sharma, P., Singh, N. & Sharma, S. Impact of *ABCB1* , *ABCC1* , *ABCC2* , and *ABCG2* variants in predicting prognosis and clinical outcomes of north Indian lung cancer patients undergoing platinum-based doublet chemotherapy. *J Gene Med* **25**, (2023).

37. Li, C. *et al.* The role of Exosomal miRNAs in cancer. *J Transl Med* **20**, 6 (2022).

38. Iwakawa, H. & Tomari, Y. Life of RISC: Formation, action, and degradation of RNA-induced silencing complex. *Mol Cell* **82**, 30–43 (2022).

39. Fang, H. & DeClerck, Y. A. Targeting the Tumor Microenvironment: From Understanding Pathways to Effective Clinical Trials. *Cancer Res* **73**, 4965–4977 (2013).

40. Duan, X. *et al.* Circulating miRNAs in Serum as Biomarkers for Early Diagnosis of Non-small Cell Lung Cancer. *Front Genet* **12**, (2021).

41. Florczuk, M., Szpechcinski, A. & Chorostowska-Wynimko, J. miRNAs as Biomarkers and Therapeutic Targets in Non-Small Cell Lung Cancer: Current Perspectives. *Target Oncol* **12**, 179–200 (2017).

42. Tenchov, R. *et al.* Exosomes─Nature's Lipid Nanoparticles, a Rising Star in Drug Delivery and Diagnostics. *ACS Nano* **16**, 17802–17846 (2022).

43. Zhu, L. *et al.* Isolation and characterization of exosomes for cancer research. *J Hematol Oncol* **13**, 152 (2020).

44. Brunelli, A. *et al.* Morbidity and mortality of lobectomy or pneumonectomy after neoadjuvant treatment: an analysis from the ESTS database. *European Journal of Cardio-Thoracic Surgery* **57**, 740–746 (2020).

45. Zhu, Z. *et al.* International consensus on radiotherapy in metastatic non-small cell lung cancer. *Transl Lung Cancer Res* **11**, 1763–1795 (2022).

46. Levy, A. *et al.* Stereotactic Body Radiotherapy for Centrally Located Inoperable Early-Stage NSCLC: EORTC 22113–08113 LungTech Phase II Trial Results. *Journal of Thoracic Oncology* **19**, 1297–1309 (2024).

47. Arbour, K. C. & Riely, G. J. Systemic Therapy for Locally Advanced and Metastatic Non–Small Cell Lung Cancer. *JAMA* **322**, 764 (2019).

48. Chaft, J. E. *et al.* Evolution of systemic therapy for stages I–III non-metastatic non-small-cell lung cancer. *Nat Rev Clin Oncol* **18**, 547–557 (2021).

49. Herrera-Juárez, M., Serrano-Gómez, C., Bote-de-Cabo, H. & Paz-Ares, L. Targeted therapy for lung cancer: Beyond EGFR and ALK. *Cancer* **129**, 1803–1820 (2023).

50. Imyanitov, E. N., Iyevleva, A. G. & Levchenko, E. V. Molecular testing and targeted therapy for non-small cell lung cancer: Current status and perspectives. *Crit Rev Oncol Hematol* **157**, 103194 (2021).

51. Herrera-Juárez, M., Serrano-Gómez, C., Bote-de-Cabo, H. & Paz-Ares, L. Targeted therapy for lung cancer: Beyond EGFR and ALK. *Cancer* **129**, 1803–1820 (2023).

52. Lahiri, A. *et al.* Lung cancer immunotherapy: progress, pitfalls, and promises. *Mol Cancer* **22**, 40 (2023).

53. Rosellini, P. *et al.* Clinical impact of STK11 mutation in advanced-stage non-small cell lung cancer. *Eur J Cancer* **172**, 85–95 (2022).

54. Xu, K., Lu, W., Yu, A., Wu, H. & He, J. Effect of the STK11 mutation on therapeutic efficacy and prognosis in patients with non-small cell lung cancer: a comprehensive study based on meta-analyses and bioinformatics analyses. *BMC Cancer* **24**, 491 (2024).

55. Tumeh, P. C. *et al.* PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature* **515**, 568–571 (2014).

56. Reck, M. *et al.* Pembrolizumab versus Chemotherapy for PD-L1–Positive Non–Small-Cell Lung Cancer. *New England Journal of Medicine* **375**, 1823–1833 (2016).

57. Russo, A. E. *et al.* Bevacizumab in the treatment of NSCLC: patient selection and perspectives. *Lung Cancer: Targets and Therapy* **Volume 8**, 259–269 (2017).

58. Reck, M. *et al.* Predicting and managing the risk of pulmonary haemorrhage in patients with NSCLC treated with bevacizumab: a consensus report from a panel of experts. *Annals of Oncology* **23**, 1111–1120 (2012).

59. Jaromi, L. *et al.* KRAS and EGFR Mutations Differentially Alter ABC Drug Transporter Expression in Cisplatin-Resistant Non-Small Cell Lung Cancer. *Int J Mol Sci* **22**, 5384 (2021).

60. R Core Team. R: A Language and Environment for Statistical Computing. Vienna: R Foundation for Statistical Computing. (2021).

61. Raivo Kolde. pheatmap: Pretty Heatmaps. R package Version 1.0.12. . (2019).

62. Bovari-Biri, J., Garai, K., Banfai, K., Csongei, V. & Pongracz, J. E. miRNAs as Predictors of Barrier Integrity. *Biosensors (Basel)* **13**, 422 (2023).

63. Ma, T. Y., Boivin, M. A., Ye, D., Pedram, A. & Said, H. M. Mechanism of TNF-α modulation of Caco-2 intestinal epithelial tight junction barrier: role of myosin light-chain kinase protein expression. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **288**, G422–G430 (2005).

64. Vesel, M. *et al.* ABCB1 and ABCG2 drug transporters are differentially expressed in non-small cell lung cancers (NSCLC) and expression is modified by cisplatin treatment via altered Wnt signaling. *Respir Res* **18**, 52 (2017).

65. Asem, M., Buechler, S., Wates, R., Miller, D. & Stack, M. Wnt5a Signaling in Cancer. *Cancers (Basel)* **8**, 79 (2016).

66. Housman, G. *et al.* Drug Resistance in Cancer: An Overview. *Cancers (Basel)* **6**, 1769–1792 (2014).

67. Zawadzka, I. *et al.* The impact of ABCB1 gene polymorphism and its expression on non-small-cell lung cancer development, progression and therapy – preliminary report. *Sci Rep* **10**, 6188 (2020).

68. Gao, B. *et al.* Paclitaxel sensitivity in relation to ABCB1 expression, efflux and single nucleotide polymorphisms in ovarian cancer. *Sci Rep* **4**, 4669 (2014).

69. Vaidyanathan, A. *et al.* ABCB1 (MDR1) induction defines a common resistance mechanism in paclitaxel- and olaparib-resistant ovarian cancer cells. *Br J Cancer* **115**, 431–441 (2016).

70. Fox, E. & Bates, S. E. Tariquidar (XR9576): a P-glycoprotein drug efflux pump inhibitor. *Expert Rev Anticancer Ther* **7**, 447–459 (2007).

71. Kelly, R. J. *et al.* A Pharmacodynamic Study of Docetaxel in Combination with the P-glycoprotein Antagonist Tariquidar (XR9576) in Patients with Lung, Ovarian, and Cervical Cancer. *Clinical Cancer Research* **17**, 569–580 (2011).

72. Wheate, N. J., Walker, S., Craig, G. E. & Oun, R. The status of platinum anticancer drugs in the clinic and in clinical trials. *Dalton Transactions* **39**, 8113 (2010).

73. Chen, J., Emara, N., Solomides, C., Parekh, H. & Simpkins, H. Resistance to platinum-based chemotherapy in lung cancer cell lines. *Cancer Chemother Pharmacol* **66**, 1103–1111 (2010).

74. Tsvetkova, E. & Goss, G. D. Drug Resistance and Its Significance for Treatment Decisions in Non-Small-Cell Lung Cancer. *Current Oncology* **19**, 45–51 (2012).

75. Cardama, G. A., Gonzalez, N., Maggio, J., Menna, P. L. & Gomez, D. E. Rho GTPases as therapeutic targets in cancer (Review). *Int J Oncol* **51**, 1025–1034 (2017).

76. Cheyette, B. N. R. *et al.* Dapper, a Disheveled-Associated Antagonist of β-Catenin and JNK Signaling, Is Required for Notochord Formation. *Dev Cell* **2**, 449–461 (2002).

77. Yin, X. *et al.* DACT1, an antagonist to Wnt/β-catenin signaling, suppresses tumor cell growth and is frequently silenced in breast cancer. *Breast Cancer Research* **15**, R23 (2013).

78. Sun, Y. *et al.* RETRACTED ARTICLE: SFRP2 augments WNT16B signaling to promote therapeutic resistance in the damaged tumor microenvironment. *Oncogene* **35**, 4321–4334 (2016).

79. Yao, X. *et al.* TLE1 promotes EMT in A549 lung cancer cells through suppression of E-cadherin. *Biochem Biophys Res Commun* **455**, 277–284 (2014).

80. Wang, S.-Y. *et al.* TLE4 promotes colorectal cancer progression through activation of JNK/c-Jun signaling pathway. *Oncotarget* **7**, 2878–2888 (2016).

81. Yang, R.-W. *et al.* RETRACTED ARTICLE: TLE3 represses colorectal cancer proliferation by inhibiting MAPK and AKT signaling pathways. *Journal of Experimental & Clinical Cancer Research* **35**, 152 (2016).

82. Chen, J. *et al.* WISP1 Polymorphisms Contribute to Platinum-Based Chemotherapy Toxicity in Lung Cancer Patients. *Int J Mol Sci* **15**, 21011–21027 (2014).

83. Larraguibel, J. *et al.* Wnt ligand–dependent activation of the negative feedback regulator Nkd1. *Mol Biol Cell* **26**, 2375–2384 (2015).

7. Publications

The thesis is based on the following publications:

Torok, Z; Garai, K; Miskei, JA; Bóvári-Biri, J; Adam, ZM; Kajtar, B; Sarosi, V; Pongracz, JE: Serum and serum exosome WNT5A levels as biomarkers in non-small cell lung cancer

2025. Respiratory Research 2025 26:141 https://doi.org/10.1186/s12931-025-03216-7 (IF: 4.700; Q1)

Jaromi L, Csongei V, Vesel M, Abdelwahab EMM, Soltani A, **Torok Z**, Smuk G, Sarosi V, Pongracz JE. KRAS and EGFR Mutations Differentially Alter ABC Drug Transporter Expression in Cisplatin-Resistant Non-Small Cell Lung Cancer.

2021. Int J Mol Sci. 2021 May 20;22(10):5384. https://doi: 10.3390/ijms22105384 (IF: 6.208; Q1, D1)

Summarized IF: 10.908

Manuscripts under preparation/submission:

Garai, K; Bovari-Biri, J; Torok, Z; Herczeg, R; Kajtar, B; Pongracz, JE: Serum exosome-derived miRNAs as predictive markers for bevacizumab induced adverse reactions in non-small cell lung cancer. **2025. Cancer**

Additional research articles:

Kiss, E; Abdelwahab, EMM; Steib, A; Papp, E; **Torok, Z**; Jakab, L; Smuk, G; Sarosi, V; Pongracz, JE: Cisplatin treatment induced interleukin 6 and 8 production alters lung adenocarcinoma cell migration in an oncogenic mutation dependent manner.

2020. Respiratory Research, 21:120; doi: 10.1186/s12931-020-01389-x; (IF: 5.631; Q1)

Török, Zsófia Laura; Sárosi, Veronika; Szabó, Zoltán; Szentkirályi, Éva; Bátai, István; Kerényi, Mónika: Helyi érzéstelenítők hatása a bronchoszkóppal vett bakteriológiai eredményekre

Medicina Thoracalis (Budapest) 74: 3 pp. 157-160., 4 p. (2021)

Török, Zsófia Laura; Ruzsics, István; Gódi, Szilárd; Cseke, László; Mühl, Diána; Szigeti, Nóra; Sárosi, Veronika: Hydropneumothorax és ami mögötte volt

Medicina Thoracalis (Budapest) 68: 5 pp. 332-335., 4 p. (2015)

Török, Zsófia Laura; Paraicz, Gábor; Ruzsics, István; Vékony, Ildikó; Viski, Anna; Grmela, Gábor; Hüttl, Kálmán; Sárosi, Veronika: Krónikus nekrotizáló aspergillosis végső diagnózis?: Az intervenció szerepe a pulmonológiában.

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