Summary of Professional Accomplishments

Identification of the metabolomic and proteomic molecular components associated with neoplastic disease by mass spectrometry techniques

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1. Name

Anna Wojakowska

2. Diplomas and degrees conferred in specific areas of science

- **2013:** Ph.D. degree in chemistry - in the field of biochemistry (with honors)
  Institute of Bioorganic Chemistry PAS in Poznań
  Ph.D. thesis entitled „Application of tandem mass spectrometry techniques for profiling and structural analysis of phenolic secondary metabolites in plant material".
  Supervisor: prof. dr. hab. Maciej Stobiecki

- **2008:** M.Sc in biotechnology, specialization in industrial biotechnology (with honors)
  University of Life Sciences in Poznań
  MSc thesis entitled „Application of thermoseparating polymers for extraction of lysozyme from hen egg white using aqueous two-phase systems".
  Supervisor: dr hab. Radosław Dembczyński

3. Information on employment in research institutes

- **04/2018 – at present:** Adjunct
  Institute of Bioorganic Chemistry PAS in Poznań
  Mass Spectrometry Laboratory at the Department of Biomedical Proteomics
  05/2018-05/2019: maternal and parental leave

- **11/2013 – 04/2018:** Senior specialist
  Maria Skłodowska-Curie Institute - Oncology Center in Gliwice
  (currently the National Research Institute of Oncology)
  Center for Translational Research and Molecular Biology of Cancer
  09/2015-09/2016: maternal and parental leave

- **02/2013 – 10/2013**: Assistant
  Institute of Bioorganic Chemistry PAS in Poznań
  Laboratory of Proteomics and Metabolomics IBCh PAS

4. Description of the achievements, set out in art. 219 para 1 point 2 of the Act

4.1. Title of the scientific achievement

Identification of the metabolomic and proteomic molecular components associated with neoplastic disease by mass spectrometry techniques.

4.2. List of publications included as the scientific achievement

H1. Wojakowska A, Marczak Ł, Jelonek K, Polanski K, Widlak P, Pietrowska M*. An Optimized Method of Metabolite Extraction from Formalin-Fixed Paraffin-Embedded Tissue for GC/MS Analysis. *PLoS One*. **2015**;10(9):e0136902.
DOI:10.1371/journal.pone.0136902
IF_{2014}=3.234; $MNiSW_{2014}$=40; $MNiSW_{2021}$=100; C=30

My contribution to the publication consisted of formulating the research problem and the concept of the work, planning the experiments (with the corresponding author), developing the research methodology, performing the experiments, data analysis, data interpretation (with the corresponding author), writing the first version of the manuscript, final edition and revising the manuscript in response to reviews (with the corresponding author).

H2. Wojakowska A, Chekan M, Marczak Ł, Polanski K, Lange D, Pietrowska M, Widlak P[*]. Detection of metabolites discriminating subtypes of thyroid cancer: Molecular profiling of FFPE samples using the GC/MS approach. *Molecular and Cellular Endocrinology*. **2015**;417:149-57. DOI:10.1016/j.mce.2015.09.021
IF_{2014}=4.405; $MNiSW_{2014}$=30; $MNiSW_{2021}$=100; C=31

My contribution to the publication consisted of: formulating the research problem and the concept of the work (together with the corresponding author), planning and executing all experiments, analysis of the obtained data, data interpretation (with the corresponding author), writing the first version of the manuscript, editing and the final version of the manuscript (with the corresponding author).

H3. Wojakowska A, Chekan M, Widlak P, Pietrowska M[*]. Application of Metabolomics in Thyroid Cancer Research. *International Journal of Endocrinology*. **2015**;258763. DOI:10.1155/2015/258763
IF_{2014}=1.948; $MNiSW_{2014}$=20; $MNiSW_{2021}$=70; C=37

My contribution to the publication consisted of: formulating the concept of the work (together with the corresponding author), collecting and analyzing source materials, writing the first version of the manuscript, editing the final version of the manuscript, and correcting it in response to reviews (including the corresponding author).

H4. Wojakowska A, Cole LM, Chekan M, Bednarczyk K, Maksymiak M, Oczko-Wojciechowska M, Jarząb B, Clench MR, Polańska J, Pietrowska M, Widlak P[*]. Discrimination of papillary thyroid cancer from non-cancerous thyroid tissue based on lipid profiling by mass spectrometry imaging. *Endokrynologia Polska*. **2018**;69(1):2-8. DOI:10.5603/EP.a2018.0003
IF_{2017}=1.059; $MNiSW_{2016}$=15; $MNiSW_{2021}$=70; C=15

My contribution to the publication consisted of: formulation of the research problem and the concept of the work (together with the corresponding author), planning and execution of experiments, analysis of the obtained data, interpretation of the obtained data (with the corresponding author), writing the first version of the manuscript, editing, and revision the final version of the manuscript (with the corresponding author).

H5. Gawin M[#], **Wojakowska A[#]**, Pietrowska M, Marczak Ł, Chekan M, Jelonek K, Lange D, Jaksik R, Gruca A, Widłak P[*]. Proteome profiles of different types of thyroid cancers. *Molecular and Cellular Endocrinology*. **2018**;472:68-79. DOI:10.1016/j.mce.2017.11.020
IF_{2017}=3.563; $MNiSW_{2016}$=35; $MNiSW_{2021}$=100; C=15

My contribution to the publication consisted of: formulation of the research problem and the concept of the work (together with the corresponding author), participation in the planning and execution of

experiments, participation in data analysis, and editing the final version of the manuscript (with the corresponding author).

H6. Wojakowska A[#], Zebrowska A[#], Skowronek A, Rutkowski T, Polanski K, Widlak P, Marczak L[*], Pietrowska M[*]. Metabolic Profiles of Whole Serum and Serum-Derived Exosomes Are Different in Head and Neck Cancer Patients Treated by Radiotherapy. *Journal of Personalized Medicine*. **2020**;10(4):229.
DOI:10.3390/jpm10040229
IF_{2019}=4.433; $MNiSW_{2019}$=70; $MNiSW_{2021}$=70; C=10

My contribution to the publication consisted of: participation in the development of research methodology and supervision of experiments, analysis of the obtained data, data interpretation (with the corresponding authors), writing the first version of the manuscript (with the participation of an equal first author), editing and improvement of the final version of the manuscript (with corresponding authors and other co-authors).

H7. Strybel U, Marczak L, Zeman M, Polanski K, Mielańczyk Ł, Klymenko O, Samelak-Czajka A, Jackowiak P, Smolarz M, Chekan M, Zembala-Nożyńska E, Widlak P, Pietrowska M, **Wojakowska A[*]**. Molecular composition of serum exosomes could discriminate rectal cancer patients with different responses to neoadjuvant radiotherapy. *Cancers*. **2022**;14(4):99.
DOI: 10.3390/cancers14040993
IF_{2020}=6.639; $MNiSW_{2021}$=140; C=0

My contribution to the publication consisted of: formulation of the research problem and the concept of the work, planning and direct supervision of all experiments, selection of research methodology, analysis and interpretation of the obtained data, writing the first version of the manuscript, editing and correcting the final version of the manuscript (with the participation of other co-authors).

The name of the Applicant is in bold;[*] corresponding author;[#] authors contributed equally

Table 1. The scientometric data concerning the habilitation achievement are given according to the Web of Science Core Collection database, April 22nd, 2022

Total	**Cycle**	**All publications from with IF (38)**
IF	25.281	119.599
MNiSW	350	1794
$MNiSW_{2021}$	650	3430
Number of citations (excluding self-citations)	138 (135)	846 (821)
H-index	(6)	17

IF: Impact Factor for the year preceding the date of publication;
MNiSW: Points awarded by the Ministry of Science and Higher Education for the year preceding the date of publication;
$MNiSW_{2021}$: Points awarded by the Ministry of Science and Higher Education (according to the latest "List of scientific journals and peer-reviewed materials from international conferences" published on 01 Dec 2021).

All publications included in the habilitation achievement are the result of the implementation of grants, in which I was a PI.

Statements confirming the contribution of the Applicant in the creation of the above-mentioned papers are included in Appendix No. 7. Copies of the above publications are included in Appendix No. 8.

4.3. Discussion of the habilitation achievement

4.3.1. Introduction

After cardiovascular diseases, **neoplastic diseases** are the second cause of death in highly developed countries. Early diagnosis and proper cancer classification are key factors in selecting an appropriate treatment method. Failures in cancer treatment are primarily the result of disease spread and metastasis to other tissues and organs. Despite the progress made in oncological diagnostics, it is not always possible to predict the course of the disease and thus start appropriate treatment. For this reason, molecular prognostic factors are beginning to play an increasingly important role as a prognostic supplement filling the gap left by imprecise prognostic factors. Reliable identification of molecular prognostic factors would allow the identification of a group of patients requiring more aggressive treatment, thus improving the survival rate. In the classic approach, the main prognostic factors are clinical parameters determined based on histopathological analysis. They include classification according to TNM (including the depth of infiltration, invasion into the blood and lymphatic vessels, lymph node involvement, and degree of tumor differentiation) [1]. Unfortunately, the determination of the clinical cancer stage does not always enable proper disease prognosis and assessment of the risk of recurrence and metastasis. This difficulty is due to the significant biological heterogeneity of cancer. Therefore, it is necessary to fully understand the processes responsible for their dissemination and to develop reliable molecular indicators of their invasiveness and progression, helpful for the development of new diagnostic and therapeutic schemes.

Cancer biomarkers are molecules related to neoplastic processes taking place at the level of the cell, tissue, and whole organism, including mutations, cell migration, proliferation, and angiogenesis. Potential cancer biomarkers include genes undergoing altered expression, proteins, metabolites, and lipids [2]. Two approaches are used in the search for molecular markers of cancer progression: finding a new combination of known tumor markers (molecular signature) or the discovery of new molecular factors useful in diagnosis, classification, prognosis, and monitoring of cancer progression and its response to therapy.

Until recently, searching for potential tumor biomarkers was conducted mainly using immunohistochemical techniques and gene expression methods. Currently, newly developed high-throughput **multi-omics tools** are used to search for new tumor-specific molecular signatures. These tools are based on next-generation sequencing techniques, mass spectrometry techniques, and high-throughput assays using antibodies, in combination with advanced computational and data processing methods (computer modeling, machine learning, artificial intelligence). The proteomic and metabolomic approach significantly broadens the scope of identification of molecular factors and allows, together with the achievements in the field of genomics and transcriptomics, to look at the problem of cancer biomarkers in a holistic way, consistent with the concept of systems biology. Proteins and metabolites (including lipids) are the

final products of biochemical processes, therefore they most reliably reflect the molecular changes taking place in a cell, tissue, and the whole organism. Metabolomics and proteomics, which are one of the fastest-growing areas of the omics family, are widely used in cancer-oriented research. Particularly noteworthy is the least-known metabolomics, which directly reflects the neoplastic phenotype. Cancer-related processes significantly affect changes in basic metabolic pathways, hence cancer cells are characterized by altered metabolism compared to normal differentiated cells. The main difference between cancer and normal cells concerns the pathways involved in energy production. The research conducted in the last decade allowed the identification of a wide group of small molecules undergoing changes in metabolic pathways related to the development of cancer disease. These include, among others, organic acids, amino acids, sugars, nucleotides, and other low-molecular compounds, that are intermediate products of the metabolism of amino acids, purines and pyrimidines, glycolysis, gluconeogenesis, and the citric acid cycle [3]. A special group of compounds connected with the neoplastic disease are lipids, which play an important role in many cellular processes, such as energy metabolism, cell signaling, proliferation, apoptosis, and cancer differentiation. It has been proven that disturbances in lipid metabolism and function contribute to the development of neoplastic disease and can be used to assess the cancer prognosis [4].

In recent years, researchers have been increasingly interested in the possibility of using **exosomes** as a source of cancer biomarkers. Exosomes are a group of small extracellular vesicles of endosomal origin with a 30-150 nm diameter. These nanovesicles are released by almost any type of cell under both physiological and pathological conditions and can therefore be detected in most body fluids. They are enriched with many classes of bioactive molecules, including nucleic acids, proteins, lipids, and metabolites that reflect the phenotype of the parent cell. They play an essential role in cancer biology, being the key mediators of communication between cells [5]. The schematic structure of exosomes and the role they play in cancer-related processes are presented in **Figure 1**.

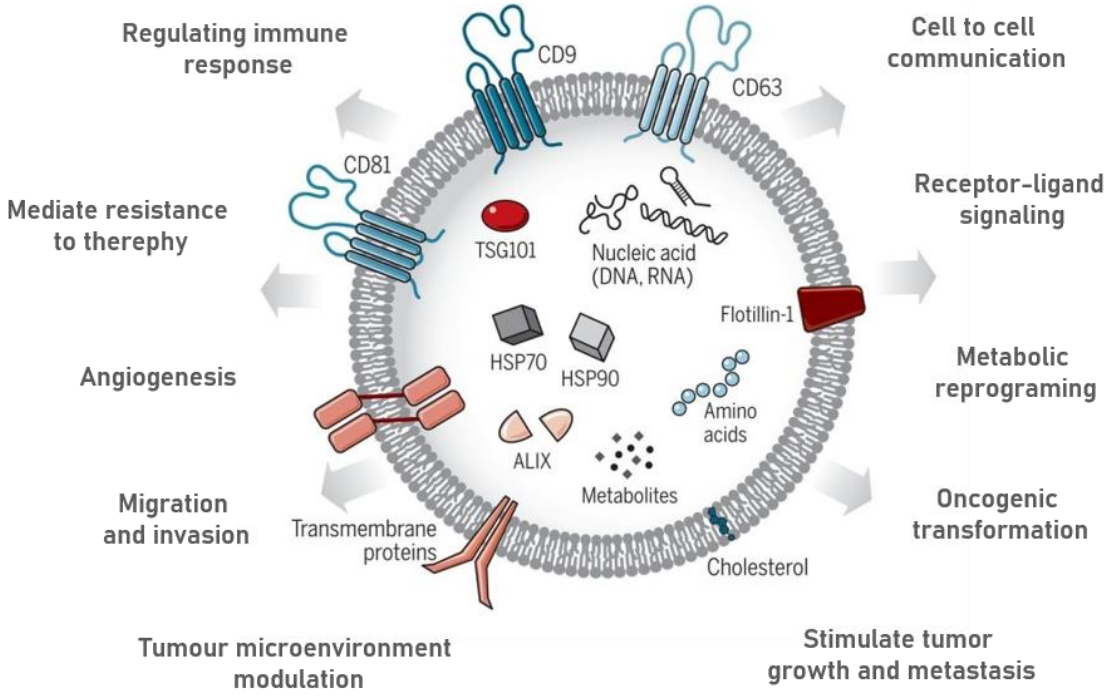

Figure 1. The schematic structure of exosomes and the role they play in the cancer-related processes (based on *Kalluri and LeBleu* [5]).

Exosomes secreted into the blood by cancer cells and other cells present in the tumor microenvironment, act as mediators in angiogenesis, immune response modulation, metabolic reprogramming, metastasis, and response to therapies. For this reason, the exosomes circulating in the blood of patients constitute a new source of tumor biomarkers in liquid biopsy [6].

The search for exosomal cancers biomarkers based on the proteomic and metabolomic approach using **mass spectrometry techniques** is undoubtedly the future of holistic clinical research. The most frequently used systems in this type of study are based on liquid and gas chromatography coupled with tandem mass spectrometry. Comprehensive analysis of the molecular components associated with cancer, ranging from small molecules such as metabolites to protein macromolecules, enables the identification of signaling and metabolic pathways connected with neoplastic processes. Such an approach allows not only a systemic assessment of the molecular processes taking place in complex biological systems, but also enables the identification of potential diagnostic and therapeutic targets [7]. However, because these techniques are still improved, both in terms of technological analytical solutions and advanced computational tools, a study using omics approaches is a significant research challenge. Studies using multi-omics approaches are usually complex interdisciplinary projects on the frontiers of chemistry, biology, medicine, and bioinformatics, requiring conscious collaboration between researchers and clinicians. Projects about searching for molecular markers of cancer using proteomic and metabolomic approaches need proper planning of the research experiment and strict adherence to analytical procedures at every stage of the research. The most important stages of research projects in the field of clinical proteomics and metabolomics are presented in **Figure 2**. These stages include (1) proper planning of the experiment, (2) collection and preservation of clinical material and collection of full clinical data, (3) sample preparation and selection of the appropriate isolation method, (4) instrumental analysis with the use of chromatography and mass spectrometry techniques - profiling and targeted analysis, (5) preliminary analysis of the obtained data, including ion filtering, normalization, deconvolution, scaling, (6) statistical analysis, (7) identification of potential biomarkers, (8) bioinformatic and chemometric analysis, (9) biological interpretation, (10) validation of potential biomarkers.

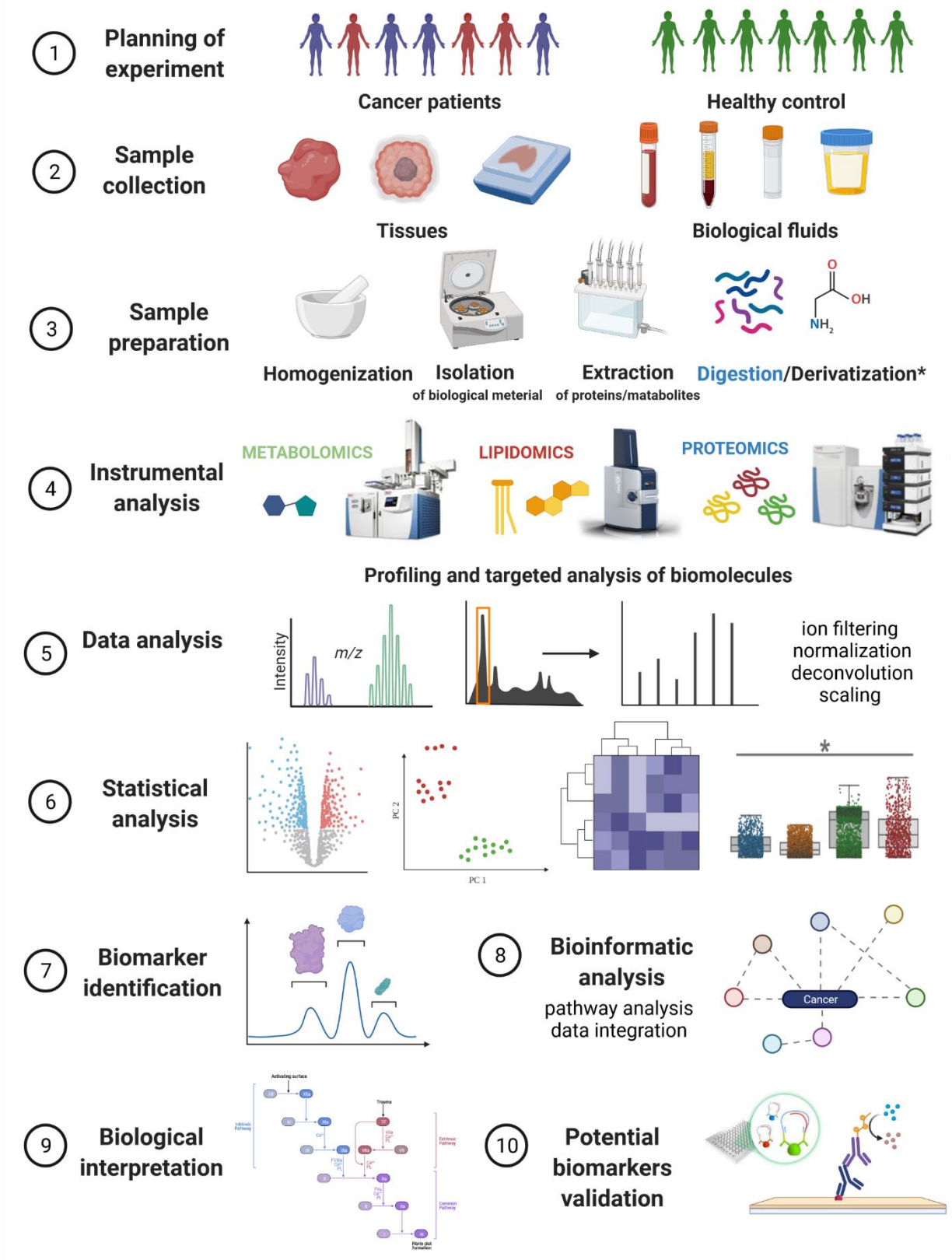

Figure 2. Schematic overview of the different research steps for the comprehensive analysis of the molecular components associated with cancer using modern approaches in proteomics and clinical metabolomics. * *Depending on the selected approach*

4.3.2. Motivation and purpose of research

The presented scientific achievement is a cycle of seven publications, which includes 6 experimental papers and 1 review. All works were created as a result of the implementation of scientific grants, in which I was a PI. In 6 works I am the first author, while in 1 I am a corresponding author. All papers have been published in journals included in the Journal Citation Reports (JCR) database with a total impact factor (IF) of 25.281 and a number of citations of 135 (according to the Web of Science Core Collection, 22.04.2022). The presented work is a series about the identification of metabolomic and proteomic molecular components associated with cancer using mass spectrometry techniques.

Publications **H1-H5** were created as a result of the implementation of the PolishNational Science Center (NCN) FUGA research grant on the use of mass spectrometry techniques for profiling and identification of proteomic and metabolomic components specific for each type of thyroid cancer (https://ncn.gov.pl/finansowanie-nauki/przyklady-projektow/wojakowska). I carried out my research as part of the national postdoctoral internship at the Center for Translational Research and Molecular Biology of Cancer, headed by prof. dr hab. Piotr Widłak, at the Maria Skłodowska-Curie Institute - Oncology Center in Gliwice (currently the Maria Skłodowska-Curie National Research Institute of Oncology, NRIO). The major part of my research was carried out in the Laboratory of Proteomics and Mass Spectrometry under the supervision of dr hab. Monika Pietrowska. Dr. Pietrowska was also the tutor of my postdoctoral fellowship. The main subject of my research was the use of mass spectrometry techniques, including LC-MS/MS, GC-MS, and MALDI-MSI, for the profiling and identification of the proteomic, lipidomic, and metabolomic components, specific for each histological type of thyroid cancer, and the correlation of the obtained data with clinical parameters (morphological and histopathological). A major challenge during the project implementation was to develop and optimize the methodology for conducting proteomics and metabolomics studies on formalin-fixed and paraffin-embedded (FFPE) thyroid cancer tissue (archival material). During the internship, I put particular emphasis on metabolomic research, which resulted in the **H1** methodical work. This work concerned the development of an original methodology for the efficient isolation of metabolites from FFPE thyroid cancer tissue for GC-MS analysis. The developed methodological approach, so far not presented in the scientific literature, I have used to conduct appropriate profiling experiments and identification of metabolites specific for particular histological types of thyroid cancer in FFPE tissue, using the GC-MS technique. The results of these studies were published in the work **H2**. At the same time, it was published the **H3** review about the use of the metabolomic approach in thyroid cancer research. This work presents in detail the methods and techniques used in metabolomic research and summarizes the current knowledge in the field of thyroid cancer metabolism. During my postdoctoral fellowship at the NRIO, I obtained another research grant, from the international STSM COST program. This short-term scientific project concerned the lipidomic study of thyroid cancer tissue. I had the opportunity to complete a monthly internship at the Mass Spectrometry Laboratory of the Biomedical Research Center Sheffield Hallam University in England, where I conducted optimization and proper lipidomic analysis of thyroid cancer tissue specimens fixed in formalin using the high-resolution MALDI-MSI molecular imaging technique. The result of this short-term project was the publication **H4** about the discrimination of thyroid cancer tissue from non-cancerous tissue based on lipid profiling by molecular mass spectrometry imaging. The last work **H5** closing the series of

publications on the MS-based molecular analysis of thyroid cancer tissue concerns protein profiling by LC-MS/MS technique. The work describes the use of the proteomic approach based on the MED FASP method for the analysis of proteins isolated from FFPE tissue. The conducted research was the first complete description of the thyroid cancer proteome and made it possible to identify the profile of proteins specific for each type of thyroid cancer. The publication refers to my previously reported metabolomic data and attempts to correlate them to obtain a global picture of molecular changes in the neoplastic thyroid tissue.

At the end of the FUGA grant, I received another research project (NCN SONATA). The main goal of the new project was to characterize the molecular factors (proteins, lipids, metabolites) associated with the progression of colorectal cancer, with particular emphasis on the participation of exosomes modulating the process of progression and metastasis. The research was carried out at the European Center for Bioinformatics and Genomics at the Mass Spectrometry Laboratory of the Institute of Bioorganic Chemistry Polish Academy of Sciences in Poznań. In works **H6** and **H7**, published as a result of this (still ongoing) project, I successfully combined knowledge and experience in the field of cancer biology and the participation of exosomes in the cancer progression, gained during the postdoctoral fellowship at the NRIO, with modern mass spectrometry techniques and computational methods, that I am still developing. The undoubted challenge of the project was the implementation and optimization of the method of isolation and evaluation of cancer patients' serum-derived exosomes, for comprehensive proteomic and metabolomic analyses using MS techniques. Once again, the object of my interest was metabolomics, which in the context of the biology of exosomes is still relatively poorly established. The developed methodology for conducting metabolomic analyzes of exosomes isolated from serum made it possible to conduct appropriate GC-MS-based experiments on serum samples of patients diagnosed with head and neck cancer (HNC) treated with ionizing radiation. Metabolic differences between patients with HNC and healthy control, as well as patients' responses to radiotherapy were presented in publication **H6**. Next, I have performed profiling and identification of proteins and metabolites in plasma and exosomes isolated from the serum of patients with rectal cancer, differing in response to neoadjuvant radiotherapy. The integration of proteomic and metabolomic profiles of exosomes, presented in the work **H7**, made it possible to identify signaling and metabolic pathways associated with different responses to neoadjuvant radiotherapy in patients with rectal cancer. The work closing the cycle presents a wide range of modern analytical approaches and techniques in the field of biochemistry, molecular biology, computational biology, bioinformatics, and biomedicine, which makes it interdisciplinary.

The main goal of my research was **the identification of the metabolomic and proteomic molecular components specific for the studied neoplasms by mass spectrometry techniques.**

<u>The specific objectives included:</u>

1. Development and optimization of analytical methods for conducting metabolomic and proteomic studies on formalin-fixed and paraffin-embedded (FFPE) tumor tissues by GC-MS and LC-MS/MS techniques.

2. Profiling and identification of metabolites and proteins present in FFPE tissues of **thyroid carcinomas** differing in histological type (PTC, FTC, MTC, ATC) in relation to benign neoplasms (FA) and healthy tissue.

3. MALDI-MSI profiling of lipids on tissue specimens to distinguish papillary thyroid cancer from adjacent non-neoplastic tissue.

4. Correlation of the molecular image with the morphological and histopathological parameters of the examined tissues of thyroid cancer.

5. Development and optimization of the methodology for comprehensive proteomic and metabolomic analysis of exosomes isolated from serum using GC-MS and LC-MS/MS techniques.

6. Metabolite profiling of serum and exosomes isolated from the serum of patients with **head and neck cancer** (HNC) treated with radiotherapy.

7. Identification of proteomic and metabolomic components of exosomes isolated from the serum of patients with **rectal cancer** that differ in response to neoadjuvant radiotherapy.

8. Integration of the results obtained from proteomic and metabolomic profiling of exosomes isolated from patients with rectal cancer differing in response to treatment, to identify signaling and metabolic pathways associated with different responses to neoadjuvant radiotherapy.

4.3.3. Discussion of the results

Development of the analytical procedures using FFPE preparations for metabolomic and proteomic studies (H1, H5)

Fixation of tissues in formalin followed by paraffin embedding is a standard procedure for the stabilization and preservation of tissue specimens commonly used in clinical practice. The fixation process allows clinical specimens to be stored at room temperature for long periods of time, while paraffin embedding facilitates the preparation of tissue for histopathological evaluation. For these reasons, FFPE tissue samples are a valuable source of clinical material for retrospective analysis, including molecular studies. Although fresh frozen tissue slides are still the gold standard for multi-omics analysis, their limited availability, the difficulty in obtaining adequate, well classified, and preserved material, and the high cost and logistical problems during storage, are major drawbacks of this type of clinical specimen. Therefore, archival FFPE tissue samples can be a good alternative to fresh frozen tissue [8]. However, it should be kept in mind that the formalin-fixation process leads to cross-linking of proteins and other molecules present in the tissue due to interactions of formaldehyde with amino acid side chains (e.g. Lys, Arg, Tyr, Asn, Gln), leading to decreased immunoreactivity of proteins in immunohistochemical assays [9]. A second limitation in molecular studies is the need to remove paraffin from the preparation without damaging or losing the biochemical molecules. For these reasons, extraction of biomolecules from FFPE tissue material for proteomic and metabolomic studies, particularly those based on mass spectrometry techniques, is methodologically challenging.

There are several papers describing the use of FFPE tissues for retrospective proteomic studies, where various methods of extracting proteins from archival material have been proposed. The proposed analytical procedures allow the identification in FFPE preparations from 40 to 90% of the proteins present in fresh frozen tissue [10]. For the proteomic studies presented below in publication **H5**, I used a modified protein extraction method according to Geoui et al [11], taking

into account the deparaffinization of tissue with n-heptane and lysis in TLB buffer, and the implemented novel MED-FASP (multienzyme digestion filter-aided sample preparation) protein isolation technique developed by Dr. Jacek Wiśniewski of the Max-Planck-Institute for Biochemistry, Martinsried, Germany [12-13]. MED-FASP method assumes protein digestion with two enzymes Lys-C and trypsin (Trp), thanks to which an increased digestion efficiency can be achieved. The obtained peptides are then fractionated on a strong anion exchanger (SAX) into six fractions (at different pH) and analyzed by LC-MS/MS using a high-resolution Q-Exactive spectrometer with an Orbitrap analyzer.

While the number of publications on the use of FFPE tissue in proteomics research has steadily increased, only a few papers on the analysis of metabolites from archival material can be found. The first studies by Kelly et al. [14] concerned the targeted analysis of polar metabolites from FFPE sarcoma tissues using the LC-MS technique. The authors were able to distinguish neoplastic tissue from non-neoplastic tissue on the basis of 106 detected metabolites. Over time, more papers confirming the possibility of using FFPE samples for the analysis of low-molecular compounds were published [15-17]. However, there were no reports on the use of GC-MS techniques for profiling metabolites from archival material. For this reason, I made an attempt to develop a method of extracting small molecules from FFPE tissue for GC-MS-based analysis. In the work **H1,** I presented a method of isolation of primary metabolites from FFPE tissue, assuming the dewaxing of the tissue in xylene followed by a two-step extraction of compounds in a mixture of water with methanol (1:1 ratio) and methylene chloride with methanol (3:1 ratio). The extraction protocol was developed based on the metabolite isolation method from fresh tissue proposed by Denkert et al. [18] from Oliver Fiehn's group (Davis University, USA). I conducted comparative studies on three types of mouse kidney tissue preparations: (1) fresh-frozen, (2) formalin-fixed, and (3) FFPE. The proposed approach allowed to identify nearly 80 metabolites (including amino acids, sugars, carboxylic acids, fatty acids, and their esters, sterols, sugar, and others), 75% of which were present in all types of the examined tissue. The most numerous group among all identified classes of compounds, present in each type of analyzed tissue, were fatty acids and their esters. Increased levels of lipid species in the FFPE tissue suggest that the identified metabolites are the organic solvent-resistant part of the protein-lipid membrane complexes. Moreover, I observed that both the process of tissue fixation in formalin itself and the fixation time showed no significant effect on the composition of the identified metabolites. In contrast, embedding the fixed tissue in paraffin affects the extraction efficiency, resulting in a reduction of the relative amount of identified compounds. Nevertheless, I have demonstrated the feasibility of using FFPE tissue samples for untargeted analysis of metabolites based on the GC-MS technique, which is important in the reliable analysis of low-molecular compounds carried out with the use of retrospective clinical material.

The use of metabolomic and proteomic techniques for the profiling and identification of molecular components specific to different histological types of thyroid cancer (H2-H5)

Thyroid cancer is the most common malignancy of the endocrine glands. Although it represents only 1% of all diagnosed cases of thyroid tumors, the incidence of this type of cancer is constantly increasing. There are four main types of thyroid cancers: papillary (PTC), follicular (FTC), medullary (MTC), and anaplastic (ATC) [19]. The most common and also the most benign of thyroid malignancies is papillary cancer, which contributes to about 70% of all malignant

thyroid neoplasms. Its diagnosis is based on the characteristic features of the nuclei. The follicular variant of papillary carcinoma (PTC-FV) shares some features with follicular carcinoma. Follicular carcinoma is the second most common malignant thyroid neoplasm and accounts for about 15% of all thyroid cancers. It is characterized by slow growth and its diagnosis is based on macro- and microscopic features. There are many variants of FTC that share some features with other follicular lesions, hence the diagnosis and classification of these neoplasms are one of the most demanding and controversial issues in thyroid pathology. One of the key issues in the diagnosis of thyroid cancer is the discrimination between benign follicular adenoma (FA), follicular carcinoma (FTC), and follicular variant of papillary carcinoma (PTC-FV). Medullary carcinoma (MTC) is placed between differentiated carcinomas (PTC and FTC) and poorly differentiated carcinoma (ATC) in terms of its malignancy and clinical course. MTC (comprising 3-5% cases), unlike other thyroid carcinomas arising from the follicular cells, is derived from the parafollicular C cells and reveals neuroendocrine features. Undifferentiated ATC, constituting only 1-2% of thyroid cancers, is characterized by rapid dynamics of growth and a high degree of malignancy [20]. Proper determination of the thyroid cancer type is a key factor in the assessment of prognosis and selection of an appropriate strategy for oncological therapy, which largely translates into an improvement of the patient's survival rate. Currently, preoperative differentiation of thyroid tumors is mainly based on the morphological image of fine-needle aspiration biopsy, and postoperative on the results of histopathological examination. Unfortunately, in many cases, an unambiguous classification of cancer based on histopathological analysis is not possible, which creates serious problems when making the right therapeutic decisions. Therefore, there is a need to search for reliable molecular markers supporting the classic methods of thyroid cancer classification. Identification of such a marker (s) could minimize the number of unnecessarily performed thyroidectomies and constitute another advancement in the diagnosis of thyroid tumors after the discovery of fine-needle aspiration biopsy.

After the optimization of metabolomic and proteomic analyzes on FFPE tissue (the methodical part of the **H1** and **H5** works), I performed appropriate experiments on various histological types of thyroid cancer tissue (PTC, FTC, MTC, ATC) with the use of different mass spectrometry technics. The main goal of my research was to search for a molecular signature that would allow to distinguish different types of thyroid cancer and, in particular, to distinguish benign thyroid lesions (FA) from malignant neoplasms (PTC-FV, FTC). Metabolomic, proteomic, and lipidomic experiments were carried out using the newest techniques of mass spectrometry, based on GC-MS, LC-MS/MS, and MALDI-TOF molecular imaging, respectively. The obtained molecular images of the examined thyroid tissues were correlated with their morphological and immunohistopathological parameters. The results of the conducted research were published in the works **H2, H4,** and **H5**. The scheme of the conducted research is presented in **Figure 3**.

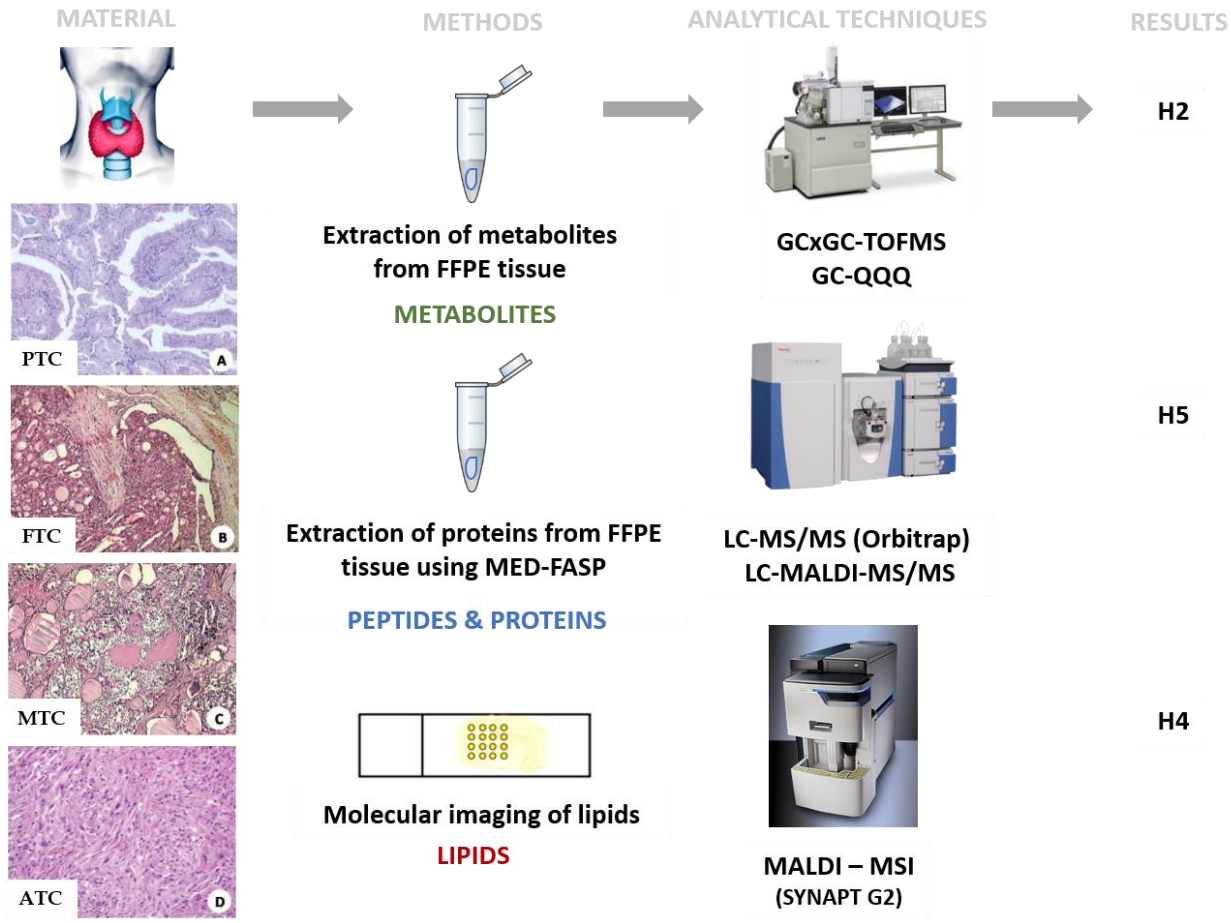

Figure 3 Research approaches used in the search for molecular markers of different histological thyroid cancer types

Before starting the particular part of my research, I have made an extensive review of the literature about the known molecular markers of thyroid cancer, with particular emphasis on the problems in thyroid cancer diagnosis and the description of omics techniques and approaches used in searching for molecular biomarkers. The least known issue so far, and also the most interesting to me, was the use of metabolomics in thyroid cancer research. The different aspects of this research I described in the review **H3**, paying particular attention to the characteristic changes in the metabolism of a neoplastic cell, including, i.a. the Warburg effect, changes in the metabolism of amino acids, fatty acids, and lipids. I also had the opportunity to look closer at pathologists' work as well as to know the dilemmas and nuances in the histopathological diagnosis of resected thyroid tumors. I have undergone initial training on the preparation and evaluation of thyroid tissues, courtesy of Dr. Mycola Chekan, who specializes in the histopathological evaluation of thyroid cancers, with whom I have been cooperating on further research projects.

The research material was FFPE thyroid cancer tissue (papillary carcinoma (PTC), classical variant (CV) and follicular variant (FV), follicular carcinoma (FTC), medullary carcinoma (MTC), anaplastic carcinoma (ATC), and benign follicular adenoma (FA)) and normal thyroid tissue as control (Ctr). The samples were characterized by the percentage of neoplastic cells. Samples selected for analyses contained at least 90% of tumor cells and no necrotic areas. For metabolomics research, I used the protocol previously developed and described at work **H1**. To compare the efficiency of the proposed extraction method, I analyzed the frozen thyroid tissue

using the same analytical platform (including the same extraction procedure without the dewaxing step), which showed that about 90% of the metabolites detected were in frozen tissue could also be identified in FFPE material. To the best of our knowledge, it was the first published successful profiling of metabolites from archival FFPE tissue based on the GC-MS technique, to identify the metabolomic signatures distinguishing between normal tissue and different types of thyroid neoplastic lesions (publication **H2**). The applied approach allowed the identification and relative quantification of 81 metabolites present in all types of thyroid tissue, including amino acids, sugars, carboxylic acids, fatty acids, and their esters. We identified 28 metabolites (mainly fatty acids, carboxylic acids, and sugars), the levels of which showed a statistically significant difference between different types of thyroid tumors and non-cancerous tissue. We have proposed several metabolic signatures to distinguish thyroid cancer from non-cancerous tissue (including increased levels of lactic acid and decreased levels of fatty acids and their esters). Increased levels of myo-inositol phosphate, succinic acid, and some fatty acids and their esters allowed to distinguish malignant thyroid neoplasms from benign follicular adenoma. In addition, the identified metabolomic components allowed to distinguish the classical variant of papillary carcinoma and follicular thyroid lesions (decreased levels of gluconic acid and increased levels of citric acid) and distinguished between follicular and papillary carcinoma (increased levels of decanoic acid ester). Hence the conclusion that the applied metabolite profiling approach in thyroid FFPE samples, based on the GC-MS technique, can potentially be used as an auxiliary diagnostic tool supporting the classification of thyroid cancers.

Moreover, the conducted research allows the identification of overrepresented metabolic pathways related to metabolites differentiating the studied types of thyroid tissue. We have shown that the metabolic pathways involved in thyroid carcinogenesis mainly involved processes related to energy production, e.g. tricarboxylic acid cycle (TCA), electron transport chain, pentose phosphate pathway (PPP), glycolysis, gluconeogenesis, galactose and pyruvate metabolism. It should be emphasized that the level of lactic acid was elevated in all neoplastic lesions of the thyroid in relation to normal tissue. This phenomenon is related to the Warburg effect, which is characteristic of cancer cells and favors glycolysis over the TCA cycle and oxidative phosphorylation [21]. In addition to reprogrammed energy metabolism, another characteristic feature of cancer cells is altered lipid and fatty acid metabolism. In our studies, we have shown that altered fatty acid metabolism is associated with metabolites that differentiate studied types of thyroid cancer and normal tissue. Earlier studies [22] showed that changed levels of lipids in neoplastic cells are associated with inflammatory and proliferative processes, which may have a significant prognostic value in the diagnosis and progression of cancer. Importantly, our research revealed several metabolites (including citric, succinic, and gluconic acid, as well as myo-inositol phosphate) that significantly differentiated thyroid follicular changes (follicular adenoma, follicular carcinoma, and a follicular variant of papillary carcinoma) and a classic variant of papillary carcinoma. These compounds were mainly related to energy metabolism (citric and succinic acid included in the TCA cycle and gluconic acid as an intermediate product of the pentose phosphate pathway) and the metabolism of inositol, which plays an important role in signaling pathways related to apoptosis, proliferation, and progression of cancer cells [23]. In summary, the identified signatures that distinguished normal from neoplastic tissue, as well as benign and malignant thyroid lesions, and the associated metabolic processes, correspond to the general known metabolic characteristics of a neoplastic cell. Detailed metabolic processes characteristic for neoplastic cells, including thyroid cells, are described in the review **H3**.

In order to obtain a more complete, systemic picture of the molecular features and corresponding biological processes characteristic for particular types of thyroid cancer (FTC, PTC-CV, PTC-FV, MTC, ATC), and to distinguish them from benign lesions (FA), we conducted a global comprehensive analysis of the proteomic profiles of the studied FFPE tissues using the shotgun LC-MS/MS approach (publication **H5**). This type of comprehensive approach to the analysis of protein profiles in thyroid cancer tissue has not been presented in the literature. so far. We profiled FFPE tissue samples representative for the same types of thyroid neoplasms that we analyzed using the metabolomic approach. For the sample preparation, we used the previously mentioned modified method of protein extraction from FFPE tissue according to Geoui et al. [11] and the MED-FASP isolation method of proteome components [12-13]. First, we analyzed all types of thyroid cancer tissue in the LC-MALDI-MS/MS and Orbitrap LC-MS/MS systems, after digesting the protein with trypsin alone. Due to the number of fractions obtained from one sample after multi-enzymatic (trypsin and Lys-C) digestion of proteins with the MED-FASP method, in the next stage, we analyzed only the follicular lesions (FTC, PTC-FV, FA), interesting for us from the point of view of a significant diagnostic problem, and the classical variant of papillary carcinoma (PTC-CV). These analyzes were performed in the Orbitrap LC-MS/MS system.

An approach using two analytical systems (Orbitrap and MALDI-TOF) enabled the identification of protein products of 3,700 unique genes and revealed significant differences between medullary, anaplastic, and epithelium-derived differentiated cancers (papillary and follicular). Proteins characteristic for medullary and anaplastic carcinomas were related to neuroendocrine functions (MTC) and factors directly related to advanced malignancies (ATC), respectively. It should be emphasized that distinguishing undifferentiated anaplastic carcinoma from epithelium-derived differentiated carcinomas (FTC and PTC) on the basis of histopathological features is not a diagnostic challenge, while knowledge of the proteomic differences of these tumors may help in understanding their evolution. Proteomic components specific to anaplastic cancer have been associated with features of advanced malignancies, including proteins associated with inflammation, immune response, cell adhesion, and migration (including integrins, adhesive proteins, and matrix metalloproteinases). On the other hand, medullary thyroid cancer can frequently show a morphological landscape resembling features of other differentiated thyroid neoplasms, hence assessment of tissue and/or serum level of calcitonin might be a good complementary biomarker of MTC [24]. In some rare cases, negative calcitonin is found in MTC, hence the search for an additional molecular marker is justified [25]. As expected, proteins involved in neuroendocrine functions and the processing of neurohormones were among the components of the proteome characteristic of medullary carcinoma.

The subsequent profiling of epithelium-derived differentiated carcinomas (FTC and PTC) and follicular adenoma (FA) using multi-enzymatic digestion and analysis in the Orbitrap LC-MS/MS system, enabled the identification of protein products of 4,800 unique genes. Comparative analysis of the examined thyroid tumor profiles showed a general similarity of follicular cancers to both variants of papillary carcinoma. Moreover, follicular adenoma showed a higher overall similarity to follicular cancer than to either variant of papillary cancer. Based on the enrichment of GO (Gene Ontology) terms analysis, which was applied to the gene equivalents of proteins differentiating epithelium-derived differentiated carcinomas (FTC and PTC), over-represented processes related to lipid metabolism, hormone metabolism, regulation of gene expression and maintenance of DNA structure were observed.

An extremely interesting aspect of our research was the ability to compare the obtained metabolomic and proteomic profiles of various types of thyroid cancer. We observed changes in the levels of proteins involved in the metabolism of lipids, nucleic acids and related to the functions of mitochondria, which corresponded to the characteristics of metabolic profiles differentiating particular types of thyroid neoplasms, including differentiated cancers. For example, the decreased level of citric acid observed in ATC compared to PTC/FTC corresponded to decreased levels of enzymes involved in the TCA cycle (ACO1, ACO2, CS, IDH1, IDH2), which was probably associated with increased glycolysis in undifferentiated cancer. We also observed decreased levels of fatty acids in benign lesions (FA) in relation to differentiated malignant neoplasms (PTC and FTC), which corresponded to differences in the levels of proteins involved in the maintenance of homeostasis and triglyceride metabolism. In addition, the decreased levels of myo-inositol phosphate characteristic of FA corresponded generally to the decreased expression of several phosphatases and kinases involved in the metabolism of inositol and phosphatidylinositol observed in FA as compared to malignant thyroid neoplasms. Further, the increased levels of succinic acid observed in malignant tumors may be related to the overall increased mitochondrial activity and the increased amino acid synthesis, characteristic of these lesions, which correspond to our observed elevated levels of many proteins involved in amino acid metabolism. In the case of differentiated thyroid neoplasms (PTC and FTC), we observed increased levels of citric acid and an increased amount of the key enzymes involved in citrate metabolism (ACLY, ACO1/2, CS, IDH1/2) in PTC compared to FTC. Importantly, the registered proteomic profiles corresponded to other transcriptomic and metabolomic profiles of thyroid carcinomas presented in the literature, which completed the molecular picture of these neoplasms from the systems biology point of view and confirmed the high potential of multi-omic tools in the classification of thyroid neoplasms. In addition, we proposed proteomic signatures specific to a particular type of thyroid cancer, which can be used for further validation studies to identify specific biomarkers in the future.

Another challenge in the context of searching for molecular classifiers of thyroid cancer tissue samples was the use of the lipidomic approach based on the MALDI molecular imaging technique. Lipids, which are the main components of cell membranes, play an important role in cell signaling, inflammation, and tissue differentiation, and therefore have a potentially high value in cancer diagnostics [26]. The applied MALDI-MSI molecular imaging technique allows the detection of the spatial distribution of bioactive compounds, such as peptides, proteins, lipids, and metabolites, in tissue specimens. With the help of appropriate bioinformatics tools, it is possible to correlate molecular profiles with the morphological and clinical features of the examined tissues, as well as to define molecular cancer biomarkers. My research in this area was carried out as part of the short-term COST research project "Lipidomics in thyroid cancer research", carried out at the Mass Spectrometry Laboratory of the Biomedical Research Center Sheffield Hallam University (England), under the supervision of prof. Malcolm Clench. The results of this project were published in paper **H4** concerning the discrimination of papillary thyroid cancer from non-cancerous thyroid tissue based on lipid profiling by mass spectrometry imaging. The research was carried out on tissue fixed in formalin and compared with the results obtained for frozen tissue. I also made an attempt to analyze the lipids from FFPE tissue. However, the paraffin removal step with toluene significantly contributed to the washing out of most lipid species from the studied tissue. The results obtained from the optimization work suggest that it is possible to detect some types of lipids present in a tissue sample after dewaxing, but their amount is negligible compared

to the number of lipids found in a frozen or formalin-fixed specimen. Preparation of frozen/formalin-fixed tissue was relatively simple and involved washing the specimen twice with distilled water and covering it with a CHCA matrix (α-cyano-4-hydroxycinnamic acid). The lipid profiling was carried out using a high-resolution HDMS SYNAPT TM G2 spectrometer with the ion mobility technology that enables the separation of ions from isomeric and isobaric compounds, which is particularly important in lipid analyses. I performed lipid identification by comparing the exact molecular mass and fragmentation pattern of protonated ions, registered during MS/MS experiments, with the LIPID MAPS database.

Based on the conducted analysis, I identified lipids belonging to the classes of phosphatidylcholine (PC), sphingomyelin (SM), and phosphatidic acids (PA), and their level was significantly higher in cancer tissue compared to non-neoplastic thyroid. Eight phospholipids, including PC(32:0), PC(32:1), PC(34:1), PC(36:3), PA(36:2), PA(36:3), SM(34:1), and SM(36:1), were upregulated in PTC and could be used to discriminate this malignancy from non-malignant thyroid tissue, either "normal" or benign neoplasm. This observation validated and further extended previous reports [27-29]. I detected the same lipid components in formalin-fixed as well as frozen tissue, with $[M + Na]^+$ ions being predominantly present in formalin-fixed tissue, while $[M + K]^+$ ions predominated in frozen tissue. It should be noted that the intensities of the registered ions derived from lipid compounds were higher in the frozen material compared to the tissue fixed in formalin. The presented results confirm the possibility of using the MALDI-MSI technique in the analysis of lipid distribution directly in formalin-stabilized tissues and give hope for their potential use in the classification of thyroid diseases.

Identification of proteomic and metabolomic components of exosomes present in cancer patients' serum by mass spectrometry techniques (H6, H7)

All types of cells and tissues, including neoplastic ones, release a mixture of extracellular vesicles that vary in size, origin, and content. Small extracellular vesicles (sEV) which include endosomal-derived exosomes, are a population of vesicles with a diameter of 30–150 nm, carrying many classes of bioactive molecules, including fatty acids, proteins, metabolites, and lipids, which reflect the phenotype of parental cells. The sEV present in the blood of cancer patients is a mixture of tumor-derived vesicles and vesicles produced by non-malignant cells, mostly immune cells [6]. Both sEV released by cancer cells and other cells present in the tumor microenvironment are key mediators in cell-to-cell communication, involved in various aspects of cancer development, including growth, migration, angiogenesis, extracellular matrix degradation (ECM), epithelial-mesenchymal transition (EMT) as well as tumor escape from immune surveillance. Numerous studies on exosomes postulate their significant role in cancer progression, metastasis, and resistance to therapies [30]. Hence, exosomes, present in the body fluids, are considered promising candidates for liquid biopsy, which represents a new source of potential prognostic and predictive tumor biomarkers.

Transcriptomics and proteomics approaches are used for searching for potential exosomal biomarkers, yet knowledge about metabolomic components of such vesicles is very limited. Before performing the appropriate experiments on serum-derived exosomes to look for proteins and small molecules related to cancer progression or treatment response (publications **H6** and **H7**), it was necessary to implement and optimize the method of isolation and characterization of exosomes for the purpose of conducting comprehensive proteomic and metabolomic analyses

using mass spectrometry techniques. Exosomes were isolated using the size exclusion chromatography (SEC) technique based on the protocol developed by Smolarz et al. [31] and Ludwig et al. [32], optimized for the strict requirements of the MS methods. The SEC method allowed to isolate the fraction of vesicles (fraction # 4) which in terms of morphology, size (~ 50 nm), and the presence of specific biomarkers fit in the category of exosomes. The applied isolation method allowed to obtain high-quality, non-aggregated, functionally active exosomes free from contamination with high-copy serum proteins (mainly albumin). The evaluation of exosomal quality according to the requirements of MISEV 2018 [33] (Minimal Information for Studies of Extracellular Vesicles) was carried out with the use of various analytical techniques. The vesicular size and morphology were assessed by dynamic light scattering (DLS) and transmission electron microscopy (TEM), respectively. The presence of the exosome-specific markers CD9, CD63, CD81, TSG101, and ALIX was analyzed by the Western blot technique. The isolation efficiency was estimated to be approximately $1x10^{10}$ sEV/1ml of serum by measurement with the most modern Amnis Imagestream x MkII flow cytometer of the labeled fraction of exosomes. We further developed a procedure for extracting proteins (using sodium deoxycholate, SDC) and metabolites (sequential elution with solvents of increasing polarity) for MS analyses, which are detailed described in publication **H7**. The simple metabolites extraction method (with methanol) described in work **H6**, resulted in a slightly lower yield than that used in the publication **H7**. We conducted metabolomic studies with the use of the GC-MS (QQQ) system, while proteomic studies were carried out using LC-MS/MS QExactive Orbitrap system. The analysis of the obtained data included their initial processing (i.a. ion filtering, normalization, deconvolution, alignment, scaling), identification of compounds, statistical and bioinformatic analysis, including integration of multi-omics data and biological interpretation. The main steps of research carried out to search for proteomic and metabolomic features of exosomes related to cancer and the response to the therapy are presented in **Figure 4**.

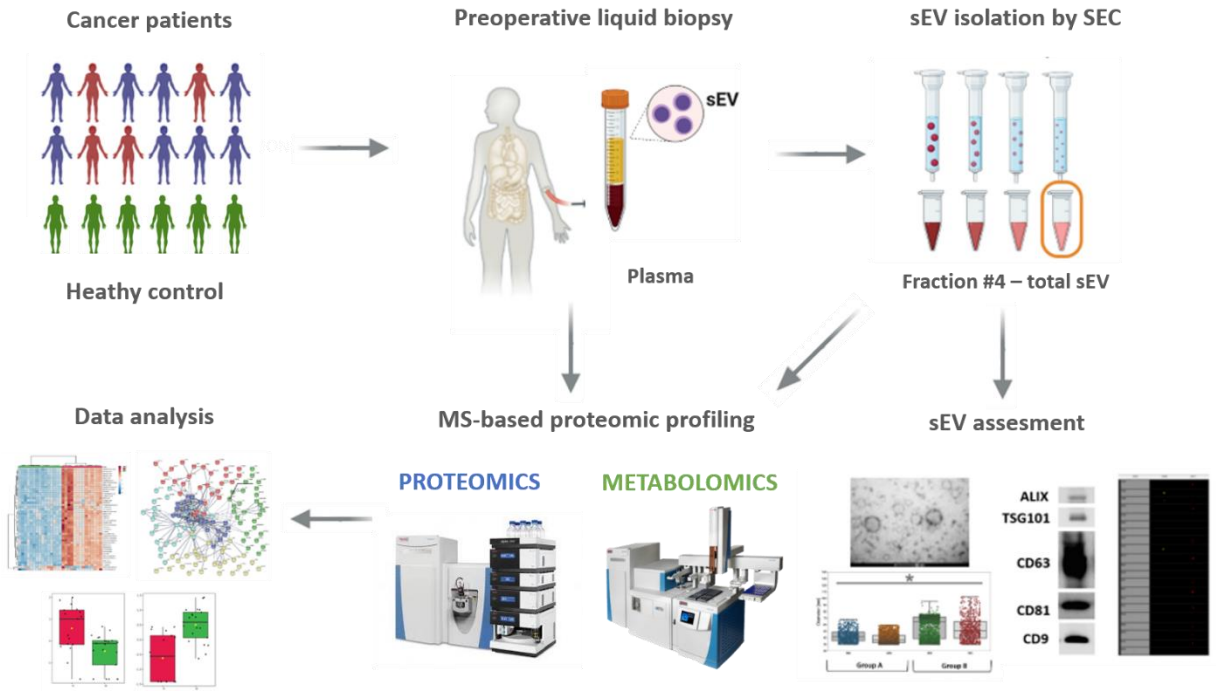

Figure 4. Workflow of proteomic and metabolomic study to search for exosomal components connected with neoplastic disease and response to therapy.

It should be emphasized that the last stage, including a detailed and global analysis of the obtained data, was particularly time-consuming and required a lot of involvement in exploring methods allowing for the integration of the obtained data, which so far has not been widely presented in the literature. Detailed parameters of the instrumental and computational analyzes are presented in works **H6** and **H7**.

The use of the GC-MS technique for the metabolomic profiling of serum and exosomes isolated from serum of head and neck cancer (HNC) patients treated with radiotherapy (H6)

Head and neck cancer (HNC) is the sixth most common malignancy worldwide and accounts for approximately 6% of all cancer cases worldwide [34]. Radiotherapy (RT), used either alone or in combination with other treatment modalities (surgery, chemotherapy, or immunotherapy) is the major modality in the HNC treatment. The major benefit of RT is a well-established local control of the tumor. However, ionizing radiation induces damage to the adjacent healthy tissues, which is reflected at the systemic level in body fluids [35]. Hence, detection in the patient's blood of a molecular fingerprint of the body's response to the treatment is another important aspect of HNC diagnostics, which could potentially enable the monitoring and prediction of radiation toxicity.

Various types of "omics" studies (genomics, transcriptomics, proteomics, metabolomics) using different sources of samples (blood, urine, saliva, tissues) uncovered molecules and genes of potential use as clinical biomarkers in terms of diagnostics, assessment of prognosis, or treatment selection. However, knowledge regarding radiation-induced changes in the metabolome and related molecular mechanisms remains limited. Assuming that the serum metabolome directly reflects the patient's response to both the disease state and the implemented treatment, we decided to assess radiation-induced metabolomic changes and metabolomic differences between patients with head and neck cancer and healthy individuals, in two types of material. i.e. whole serum and serum-derived exosomes. In our pilot study, described in publication **H6**, we conducted comprehensive metabolomic profiling of serum and exosomes isolated from patients with squamous cell carcinoma located in the pharynx region, immediately before and one month after radiotherapy (accelerated irradiation scheme with a daily fraction dose of 1.8 G to the total dose 64-72 Gy), in order to assess changes caused by the applied treatment. At the same time, we analyzed the metabolome of serum and exosomes from patients before radiotherapy and from healthy volunteers, in order to assess the metabolomic components related to the HNC. On the basis of the GC-MS-based untargeted analysis, we observed that the metabolomic profile of exosomes, dominated mainly by fatty acids, carboxylic acids, and sugar acids, is less complex than that of whole serum. However, it should be emphasized that cancer-related features of energy metabolism were detected in both types of specimens, which confirmed the feasibility of cancer biomarkers based on exosomes. In head and neck cancer, as in many other types of cancer, cancer cells can alter their energy metabolism, switching from the TCA cycle to glycolysis and fatty acid oxidation as a backup mechanism for energy production (the previously mentioned Warburg effect). Our study confirmed that metabolites associated with processes involved in energy metabolism, including glycolysis, gluconeogenesis, Warburg effect, TCA cycle, pyruvate metabolism, and mitochondrial electron transport chain showed different levels in samples of HNC patients and healthy controls. Importantly, features associated with this characteristic cancer phenotype were observed in both whole serum and serum-derived exosomes. Nevertheless,

pathways associated with metabolites specific for serum-derived exosomes of cancer patients included oxidation of fatty acids and ketone body metabolism as a result of increased lipolysis. Such processes, which are probably a backup mechanism of energy production for cancer cells, have been previously reported in research using the NMR technique in patients with head and neck cancer [36]. Moreover, previous research suggests that exosomes (rich in fatty acids) may mediate the intercellular transport of fatty acids across cell membranes. Hence, also confirmed in our study, the potential role of small extracellular vesicles as mediators associated with neoplastic processes and increased lipid metabolism deserves further attention.

Our study revealed that RT affected the serum levels of several amino acids, biogenic amines, sugars, nucleotides, lipids, and fatty acids, which mirrored potential RT-induced changes in a plethora of metabolic pathways ongoing in a patients' body. It is noteworthy that different radiation-related mechanisms might contribute to metabolic changes observed in samples collected one month after the end of RT, including toxicity induced by radiation in normal tissues and a reduced number of cancer cells. According to recent studies, the metabolism of amino acids, in particular alanine, whose decreased serum level was correlated with the effect of acute radiation toxicity associated with weight loss in patients after radiotherapy, plays a particularly important role in the response of patients with HNC to RT [37]. Moreover, the whole-body response to irradiation frequently involves molecules associated with oxidative stress and inflammation [38]. Hence, it is noteworthy that hypotaurine, which is involved in protection against oxidative stress as an effect of RT, was significantly elevated in post-RT serum samples. Moreover, changes in phospholipid levels associated with the inflammatory response, that we observed in the serum of patients after RT, confirmed the overall metabolic phenotype associated with radiation therapy. On the other hand, in contrast to the characteristic changes in serum metabolome induced by radiation, our study revealed no specific changes after RT in the composition of the exosome metabolome. This was in contrast to the significant radiation-induced changes observed at the level of the proteome and miRNome of exosomes released by HNC cells. Exosomes released by irradiated cells are known mediators of the radiation bystander effect and other aspects of radiation-related cell-to-cell signaling [39-41]. Therefore, there is a need for further research to verify the role of exosome metabolites that they can potentially play in radiation-related intercellular signaling.

Proteomic and metabolomic composition of exosomes differentiates rectal cancer patients depending on the response to preoperative radiotherapy (H7)

The first line of therapy for patients with locally advanced rectal cancer is total mesorectal excision supplemented with neoadjuvant treatment. In the group of rectal cancer (RC) patients with a suspected increased risk of local recurrence or metastasis (i.e., $T \geq 3$ or N+), it is advisable to use neoadjuvant radiotherapy (neo-RT) as a component of radical treatment [42]. Therapeutic response evaluation according to the tumor regression grading (TRG) system is essential for formulating treatment and survival forecasting, yet the actual prediction of tumor regression remains a challenge. Moreover, despite the benefits of preoperative neo-RT, generally leading to a reduction in tumor mass, such treatment may also result in radiation toxicity and other adverse effects [43]. Hence, a proper selection of patients who require a more aggressive preoperative treatment would be a desired component of tailored therapy. However, molecular markers which

could be used for the prediction of efficacy and toxicity of neo-RT in locally advanced rectal cancer are still missed and searched for.

Literature reports suggest that exosomes can be used as biomarkers to predict and monitor treatment response, but knowledge in this area regarding rectal cancer remains limited [44]. Nevertheless, it is generally accepted that radiation has an effect on the molecular cargo of the exosomes, and these vesicles are involved in the transmission of radiation resistance. In our study presented in publication **H7**, we used a novel integrated proteomics and metabolomics approach to reveal exosome components associated with the different responses of rectal adenocarcinoma patients to neoadjuvant radiotherapy (total dose 39-54 Gy) and related changes in metabolic and signaling processes. In addition, we analyzed the composition of serum-derived exosomes and whole plasma in parallel in order to compare the potential of the biomarkers identified in both tested samples. We conducted the study in two groups of patients, classified as "good responders" (radiosensitive tumors: TRG 0-1) and "poor responders" (radioresistant tumors: TRG 2-3) to preoperative radiotherapy.

Our study showed that the proteome components of serum-derived exosomes have the relatively highest capacity to discriminate rectal cancer patients in response to neo-RT, among both types of analyzed samples (plasma, exosomes) and regarding molecular components (proteins, metabolites, lipids) identified by mass spectrometry techniques. In the case of proteomic analysis, we found significantly fewer differentiating compounds in whole plasma than in exosomes, and these differences were statistically lower than for exosome proteins. Among proteins that the most properly discriminated good and poor responders were GPLD1 (AUC = 0.85, accuracy of 74%) identified in plasma as well as C8G (AUC = 0.91, accuracy 81%), SERPINF2 (AUC = 0.91, accuracy 79%) and CFHR3 (AUC = 0.90, accuracy 81%) identified in exosomes. The differentiating proteins were functionally associated with activation of the immune response, activation of complement and platelet function, and the metabolism of lipoproteins and cholesterol. A few identified differentiating proteins have been previously reported as molecules connected with response to RT in colorectal cancer, including FGB, CD44, GLUT1/SLC2A1, and PON1. Noteworthy is the increased level of the GLUT1 enzyme (responsible for glucose transport and directly related to the Warburg effect) in the exosomes of poorly responders, which also correlated with increased glucose levels in the same exosome samples. A high GLUT1 expression was observed previously in radioresistant tumor cells, which was putatively associated with stimulation of hypoxia, and the regulation of different signaling pathways, such as MAPK and PI3K/AKT [45-46]. Interestingly, signal transduction proteins S100A8 and S100A9 upregulated in exosomes of poor responders were previously proposed as CRC tumor-specific exosomal markers, involved in migration, leucocyte recruitment, tumor-promoting inflammation, and formation of premetastatic niches [47]. Though the potential to discriminate patients with different responses to RT was higher in the case of exosome components, there were nine differentiating proteins common for plasma and exosomes. Two such molecules, galectin-3-binding protein (LGALS3BP) and CD5 antigen-like (CD5L), significantly upregulated in both plasma and exosomes of poor responders, were connected with inflammatory response and immune surveillance, which are among the key processes upregulated in response to radiotherapy [48].

Most studies on the role of exosomes in response to radiation and RT concern changes in transcriptome and proteome, yet much less is known about the metabolome component of these vesicles, and there is no data about the potential of small molecules to discriminate patients who responded differently to radiotherapy. Our studies have shown that metabolites present in

exosomes and plasma had some potential to discriminate patients with different responses to neo-RT, and a few differentially accumulated metabolites were observed in both specimens. Differentiating small molecules upregulated in the plasma of good responders were associated with lipid and amino acid metabolism. Interestingly, the levels of differentiating proteins associated with lipid metabolism were also elevated in the same group of patients. In addition, several lipids, including derivatives of triacylglycerols (TAGs), phosphatidylethanolamines (PEs), phosphatidylinositols (PIs), sphingomyelins (SMs), and diacylglycerols (DAGs), had significantly different levels in the plasma of good and poor responders. Therefore, it should be emphasized that the identified plasma components (including proteins, metabolites, and lipids), significantly differentiating both groups of patients, are generally associated with the metabolism of lipids. This is in agreement with papers documenting that radiotherapy resulted in disruption of plasma membranes and induced changes in the level of lipids potentially connected with the inflammatory response [49]. In contrast, differentiating small molecules detected in exosomes were primarily associated with energy metabolism, including glycolysis and gluconeogenesis. The observed elevated level of glucose in the exosomes of poor responders, and the increased level of glycolysis, are consistent with the general phenotype associated with the phenomenon of radiation resistance (which probably results from the induction of DNA repair pathways) [50].

The combination of proteomic and metabolomic data allowed us to reveal common pathways relevant to the response of rectal cancer patients to neo-RT. These processes included immune system response, complement activation cascade, platelet functions, metabolism of lipids, and cancer-related signaling pathways. Increasing evidence supports the role of complement in the development of cancer and the activity of the complement system is correlated with a poor prognosis of colorectal cancer [51]. Similarly, platelets and platelets-derives sEV (putatively the most abundant EVs population in plasma) serve as regulators of cancer progression, and platelets-derives EV could promote proliferation and progression, crosstalk with the tumor microenvironment, and favor metastasis formation [52-53].

Our research using an integrated proteomics and metabolomics approach has confirmed that exosomes transport the enzymes and small molecules involved in various aspects of cancer cell metabolism, and are involved in the response to ionizing radiation at the cellular level, including glycolysis, oxidative stress, immune system responses, and inflammatory processes. Moreover, we confirmed the radiation resistance-associated phenotype of cancer cells known in the literature, which is manifested by increased glycolysis. We proved that exosomes are an important mediator of metabolic reprogramming in the response of cancer cells to radiotherapy. Increased levels of glucose and phosphate as well as two key enzymes involved in glucose metabolism (GLUT1 glucose transporter and GAPDH-3-phosphate dehydrogenase) were observed in the exosomes of poor responders Furthermore, we revealed at a proteomic and metabolomic level that mechanisms associated with response to RT are associated with the metabolism of lipids. Exosomes of good responders were enriched in cholesterol and fatty acids, including polyunsaturated fatty acids (PUFA), which play an important role in cell signaling, pro-inflammatory, and antioxidant response to radiation [54]. Moreover, in the same group of patients, we observed elevated levels of paraoxonase-1 (PON1), which is an important antioxidant enzyme associated with mitochondrial membranes and high-density lipoproteins (HDL). On the other hand, two other proteins related to lipid metabolism, FABP5 and CD5L, were upregulated in exosomes of poor responders. Literature data show that CD5L, as a key regulator of lipid synthesis, decreases the content of PUFAs and limits the expression of pro-inflammatory genes.

FABP5 has been shown to deliver ligands to PPAR-β/δ in the nucleus and to increase angiogenesis through the PPAR-γ-VEGF signal transduction [55].

In summary, the multi-omics approach applied in this study allowed us to reveal several proteins and metabolites, which levels in serum-derived exosomes discriminated patients with different responses to neo-RT. Moreover, these compounds were associated with metabolic and signaling processes important in the response to the treatment, including immune system responses, the complement system, platelet function, glucose, and lipid metabolism. We showed that the protein components of exosomes showed the highest differentiation power in rectal cancer patients in response to preoperative radiotherapy. Hence, the proteomic components of the exosomes appear to be a good potential source of biomarkers for the prediction of response to neoadjuvant radiation therapy in locally advanced rectal cancer. Additionally, the integration of metabolomics and proteomics data presents a new insight into the analysis of the global response to cancer treatment.

4.3.4. Summary and perspectives

I consider the most important effects of my scientific achievement:

- Development and optimization of the methodology for conducting proteomic and metabolomic research on retrospective tissue material using mass spectrometry techniques.
- Demonstration that profiling of proteins, metabolites, and lipids by mass spectrometry techniques can serve as an auxiliary diagnostic tool supporting the classification of thyroid neoplasms.
- Development and optimization of the methodology for conducting comprehensive proteomic and metabolomic analysis of exosomes isolated from serum.
- Comparison of the metabolic profile of serum-derived exosomes and whole serum of patients diagnosed with HNC treated with radiotherapy.
- Demonstration that the molecular composition of exosomes isolated from serum can be used to predict the response to neoadjuvant radiotherapy in rectal cancer.
- Identification of disrupted metabolic and signaling processes in cancer and in response to radiation therapy.
- Demonstrate that the integration of metabolomic and proteomic data presents a novel perspective on the analysis of global response to cancer treatment from a systems biology perspective.

Research on the use of multi-omics approaches based on mass spectrometry techniques is still being continued by me as part of the NCN SONATA project entitled "Identification of biomolecules released by colorectal cancer cells and detected in serum and/or exosomes". In this project, I am looking for molecular components characteristic of colorectal cancer cells that can be released and transported to other cells and tissues via exosomes, which is related to the mechanism of invasiveness and metastasis of this tumor. The presence in the blood of tumor-derived exosomes, i.e. exosomes carrying markers specific to cancer cells, could be used in the future as biomarkers of colorectal cancer in liquid biopsy. In addition, I am going to continue research on the prognostic role of exosomes in colorectal cancer and define in detail their

relationship with immuno-modulating functions, the risk of metastasis (in particular, to regional lymph nodes), and the efficacy of neoadjuvant radiotherapy.

4.3.5. References

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5. OTHER SCIENTIFIC AND RESEARCH ACHIEVEMENTS - Presentation of significant scientific or artistic activity carried out at more than one university, scientific or cultural institution, especially at foreign institutions

The mainstream of my research is the use of mass spectrometry techniques in biochemical and biomedical projects. I conduct research using proteomic, metabolomic, and lipidomic approaches to understand the complex relationships at the molecular and cellular levels, which are of interest to systems biology. An important part of my research is the development and optimization of the methodology for conducting analyzes using mass spectrometry techniques (including LC-MS/MS, GC-MS, MALDI-MSI) on a variety of biological materials (plant tissues, serum, plasma, exosomes, human tissues, animal tissues, retrospective material). A wide analytical workshop allows me to carry out research projects on the border of biology and chemistry and medicine.

Below I present a list of scientific collaborations with research groups from Poland and abroad, which have resulted in scientific publications (their number is given in parentheses), ogether with their detailed discussion in the following subsections:

• 2020-2022: dr. hab. Justyna Gołębiewska, Medical University in Gdańsk, Poland (1);
• 2017-2022: dr. Marcin Zeman, National Research Institute of Oncology in Gliwice, Poland (1);
• 2017-2020: dr. hab. Dorota Tarnawska, Medical University of Silesia in Katowice, Poland (1);

• 2015-2022: dr. Arkadiusz Liśkiewicz's research team, Medical University of Silesia in Katowice, Poland and dr. Marta Nowacka-Chmielewska's research team, Academy of Physical Education in Katowice, Poland (5);
• 2015-2019: prof. dr. hab. Joanna Polańska's research team, Silesian University of Technology in Gliwice, Poland (7);
• 2014-2022: dr Krzysztof Polański, Wellcome Sanger Institute, Cambridge, **United Kingdom** (7);
• 2014-2018: Malcolm Clench's research team, Biomedical Research Centre Sheffield Hallam University, **United Kingdom** (1);
• 2013-2022: dr Mykola Chekan, National Research Institute of Oncology in Gliwice, Poland (6);
• 2013-2022: prof. dr. hab. Piotr Widłak's research team and dr. hab. Monika Pietrowska's research team, National Research Institute of Oncology in Gliwice, Poland (20);
• 2013-2015: dr Magdalena Grajzer, Medical University in Wrocław, Poland (2);
• 2012-2014: dr Wojciech Ostrowski, Adam Mickiewicz University in Poznań, Poland (1);
• 2012-2013: dr Agnieszka Szuba, Institute of Dendrology PAS in Kórnik, Poland (1);
• 2012-2013: prof. dr. hab. Cezary Mądrzak's research team, Poznań University of Life Sciences, Poland (1);
• 2011-2015: prof. dr. hab. Michał Jasiński's research team, Institute of Bioorganic Chemistry PAS, Poznań, Poland (3);
• 2010-2013: dr. Juana Francisco Zamora-Natera's research team, University of Guadalajara, **Mexico** (2);
• 2009-2015: prof. dr. hab. Piotr Kachlicki's research team, Institute of Plant Genetics PAS, Poznań, Poland (5).

5.1. Prior to the award of the Ph.D. degree

During my Ph.D. study at the Department of Biochemistry of Natural Products, Institute of Bioorganic Chemistry of the Polish Academy of Sciences in Poznań under the supervision of prof. Maciej Stobiecki, I conducted research on the use of mass spectrometry techniques for profiling and structural analysis of phenolic secondary metabolites in plant material. As part of the obtained NCN PRELUDIUM research project, I developed a methodology for conducting experiments with collision-induced dissociation, allowing me to obtain structural information on the studied phenolic compounds. I used the optimized methodological approach of conducting MS/MS experiments in research on the role of phenolic secondary metabolites during the infection of lupine with pathogenic fungus *C. lupini* (research project of the Ministry of Science and Higher Education - main investigator). I proved that plant defense mechanism is manifested in the level of synthesis and accumulation of phenolic secondary metabolites and the expression of genes in their biosynthesis pathway. Moreover, I demonstrated the involvement of prenylation and malonic acid acylation of isoflavones and their derivatives in pathogenesis-related processes [Wojakowska et al. *Metabolomics.* 2013;9(3):575-589]. At the same time, I conducted research on structural analysis and profiling of phenolic secondary metabolites in tissue extracts from previously unexplored plant material, including various species of lupine [Wojakowska et al. *Phytochemistry*. 2013;92:71-86], root tissues and cell suspension cultures of the model plant *Medicago truncatula* [Staszków et al. *Metabolomics*. 2011;7(4):604-613] as well as on wheat leaves (*Triticum aestivum*) [Wojakowska et al. *Journal of Mass Spectrometry*. 2013;48(3):329-39]. Using the developed MS^2 and ISCID (in source collision-induced dissociation) analysis methods,

I identified nearly 200 flavonoid derivatives. The obtained mass spectra of selected compounds were submitted to the international database of high-resolution mass spectra MassBank (http://msbi.ipb-halle.de/MassBank/). In addition, selected mass spectra were entered into the mass spectra library created by me in the Bruker Library Editor (Bruker) and were made available to interested users of Bruker LC/ESI-Qq-ToF mass systems (in Poland and worldwide).

The results of research carried out during my Ph.D. study were presented in the form of lectures and poster presentations at several national and international scientific conferences. My work in the field of plant metabolomics has been appreciated by *The Samuel Roberts Noble Foundation* and awarded the first prize for the best poster presentation at the International Metabolomics Society 2012 conference in Washington. Moreover, due to the nature of the research, the awarded dissertation was considered strategic from the point of view of the development of the Greater Poland region, as a result of which I became a scholarship holder of the Scholarship Project of the Provincial Labor Office in 2011/2012. In addition to conducting my own research, I also used my research workshop in cooperation with other research groups, including a team of prof. Michał Jasiński in their work on the role of ABCG transporters in the modulation of isoflavone levels during *M.truncatula* defense response [Banasiak et al. *Journal of Experimental Botany*. 2013;64(4):1005-15] and Dr. Agnieszka Szuba in the metabolomic research on the effect of ectomycorrhizae on the tolerance of poplars to lead, where I acted as a researcher in the NCN SONATA grant [Szuba et al. *Electrophoresis*. 2013; 34: 3234-3243]. The confirmation of the high quality of my research in the field of metabolomics of phenolic secondary metabolites is scientific publications in international journals from the JCR list with a total IF = 22.706 and a number of citations of 230, published in 2010-2013.

5.2. After the award of the Ph.D. degree

5.2.1. Metabolomics of plant phenolic compounds

After obtaining my doctorate in early 2013, I was employed as an assistant in the Proteomics and Metabolomics Laboratory of ICHB PAN, where I continued my work in the field of plant metabolomics. As a continuation of the ongoing research on the role of isoflavonoids in the pathogenesis of lupine caused by the anthracnose fungus *C.lupini*, I continued my cooperation with the research teams of prof. Piotr Kachlicki from the Institute of Plant Genetics of the Polish Academy of Sciences in Poznań and prof. Cezary Mądrzak from the University of Life Sciences in Poznań. Plant breeding was carried out in cooperation with HR Smolice Sp. z o.o. IHAR Group - Przebędowo Branch. The correlation of isoflavonoids profiles, acting as antibiotic compounds, with information on the incidence of plants against fungal infections allowed to identify lupine cultivars with increased resistance to anthracnose, which is the basic guideline for resistance breeding of new cultivars [Wojakowska et al. *Acta Physiologiae Plantarum*. 2015; 37 (8): 152]. I also started another cooperation with Dr. Magdalena Grajzer from the Medical University in Wrocław, which concerned the analysis of phenolic acids and their derivatives in extracts from cold-pressed plant oils [Grajzer et al. *Food Chemistry*. 2015;188:459-66], for which I developed a dedicated methodological approach for conducting experiments with collision-induced dissociation in the LC/ESI/MS system [Ostrowski et al. *Journal of Chromatography B*. 2014;967 21-7]. Summarizing the knowledge and experience, both in the use of LC-MS systems for the structural analysis of flavonoid derivatives [Stobiecki et al. *Phytochemistry Letters*. 2015;11:358-367] and in studies of metabolomic changes in plant response to stress [Rodziewicz et al. *Acta*

Physiologiae Plantarum. 2014;36(1);1-19], I became a co-author of two reviews which ended my journey with plant metabolomics. Post-doctoral work on the metabolomics of plant phenolic compounds was published in 2013-2015 in journals from the JCR list with a total IF = 10.643 and a number of citations of 223.

5.2.2. Metabolomics and proteomics of cancer with the role of exosomes in neoplastic disease

At the end of 2013, I started a postdoctoral fellowship at the National Institute of Oncology, Branch in Gliwice, where, apart from implementing my own NCN FUGA project, I participated in multi-omic studies on other types of cancer, including prostate [Pietrowska et al. *Proteomes*. 2015;3(3):117–131], head and neck [Jelonek et al. *Acta Biochimica Polonica*. 2015;62(2):265-72], stomach [Abramowicz et al. *Journal of Translational Medicine*. 2015;13:304], breast [Walaszczyk et al. *Protein And Peptide Letters*. 2017;24(1):37-45] and lungs [Roś-Mazurczyk et al. *Acta Biochimica Polonica*. 2017;64(3):513-518]. The research workshop, which I continued to develop and extended with new techniques used in metabolomics (mainly based on GC-MS) and proteomics (based on LC-MS/MS with an Orbitrap analyzer) I used to analyze various types of material, including clinical samples (serum and tumor tissues), as well as cells from in vitro cultures and exosomes. My analytical expertise in the analysis of metabolites and proteins with the use of MS techniques, as well as new experiences in the field of clinical biology, including the role of exosomes in cancer-related processes, allowed me to play a supporting role in the individual projects of my colleagues from the team of Dr. Monika Pietrowska and prof. Piotr Widłak, in which I had the pleasure to work. In addition to carrying out my own research, I joined the work of the Team regarding, inter alia, the influence of ionizing radiation on the composition of the metabolome [Roś-Mazurczyk et al. *Acta Biochimica Polonica*. 2017;64(1):189-193] and the proteome of the serum of cancer patients [Widłak et al. *International Journal of Radiation Oncology, Biology, Physics*. 2015;92(5):1108-1115]. Moreover, I take an important part in optimization work on the proteomic analysis of exosomes isolated from cell cultures of head and neck cancer [Abramowicz et al. *PLoS One*. 2018;13(10):e0205496] and the study of radiation-induced exosome proteome changes [Abramowicz et al. *Journal of Radiation Research*. 2019;60(3):289-297]. At this stage of my scientific career, I also became interested in the participation of metabolites carried by exosomes and their role in the process of cancer development. Finishing my adventure with NIO in Gliwice and at the same time starting the next stage of my scientific carrier after returning to ICHB with a new NCN SONATA project, I became the co-author of two reviews on the relatively poorly known metabolomics of exosomes and their participation in processes related to cancer [Wlosowicz et al. *Na pograniczu Chemii i Biologii (On the border of Chemistry and Biology)* 2017; XXXVII: 177-188 and Żebrowska et al. *International Journal of Molecular Sciences*. 2019; 20 (14): 346].

The postdoctoral fellowship at the NIO was undoubtedly a turning point in my scientific career. It resulted in co-authorship in several articles on the use of mass spectrometry in biomedical research, published in 2015-2019, whose total IF (not including works included in the scientific achievement) is 22,132 and the number of citations is 186. I took an active part in numerous conferences and scientific meetings, as a co-author of conference lectures and presentations. During the internship at the NIO, I also took other short-term research stays, e.g. at the Biomedical Research Center Sheffield Hallam University in England (under the STSM COST grant), Max-Planck-Institute for Biochemistry in Germany (under the scientific consultation of

the innovative MED-FASP technique) and the European Center for Bioinformatics and Genomics in Poznań, where I currently work. An important effect of the internship at the NIO was the establishment of friendly, successful, and lasting cooperation with outstanding scientists specializing in exosome biology, clinical proteomics, and radio-onco-biology, Dr. Monika Pietrowska, and prof. Piotr Widłak. Among the group of doctors, scientists, and specialists with whom I still cooperate in research projects on the border of chemistry, biology, medicine, and bioinformatics, I would like to emphasize the significant participation of Dr. Krzysztof Polański from the Wellcome Sanger Institute, Cambridge in England, specializing in statistical analyzes and large-scale analyzes computing. Thanks to open cooperation with people of various specialties, I had and still have the opportunity to successfully implement complex systems biology projects.

5.2.3. Metabolomics and proteomics in research on an animal model

Another project, which I supported in terms of comprehensive metabolomics analyses based on mass spectrometry techniques (GC-MS and LC-MS), concerned the study of the mechanisms of the ketogenic diet (KD) in the rat model of tuberous sclerosis. Metabolomic research on KD was carried out in cooperation with Dr. Arkadiusz Liśkiewicz from the Medical University of Silesia in Katowice. Our collaboration started during the internship at the NIO and then continued for the next years after my return to ICHB. As a result of the first collaborative research work on the rat model of tuberous sclerosis, we showed that a long-term high-fat ketogenic diet promotes the growth of mTOR and MAPK-dependent kidney tumors [Liśkiewicz A. et al. *Scientific Reports*. 2016;6:21807]. In another work, we investigated the effect of KD on liver autophagy in a mouse model. We have shown that elevated liver autophagy due to KD depends on the composition of the diet, with a particular influence on their fatty acid composition [Liśkiewicz D. et al. *Journal of Nutritional Biochemistry*. 2021;93:108620]. My participation in the preparation of the above works consisted in the optimization of the extraction method metabolites from animal tissues (rat kidney, mouse liver) and ketogenic feeds (mainly fatty acids), preparation of samples, GC-MS and data analysis.

In another project undertaken in cooperation with Dr. Liśkiewicz, we investigated the effect of physical activity on brain functions in a model of running mice [Liśkiewicz A et al. *Molecular Brain*. 2020; 13 (1): 62]. The research focused on changes in the metabolomic profiles of the hippocampus and the frontal cortex under the influence of exercise, with particular emphasis on the contribution of fatty acids. The metabolomic profiling of the examined brain structures of mice subjected to physical exercise, which I carried out, showed significant changes in the composition of the metabolites of the hippocampus and frontal cortex related to energy transformations. Particularly significant changes concerned fatty acid profiles, which were associated with reduced levels of anxiety in running mice.

As a result of my participation in metabolomics research conducted on an animal model, I started another cooperation with the team of Dr. Marta Nowacka-Chmielewska from the Academy of Physical Education in Katowice. Collaborative research focused on the effects of physical activity on changes in brain metabolism and glucose transport induced by the Western diet or chronic stress in female rats. My participation in these studies was to carry out proteomic analyzes of the brain tissues of rats subjected to physical activity, exposure to the Western diet, and chronic stress. The cooperation resulted in the co-authorship of Dr. Nowacka-Chmielewska's

team in two publications [Nowacka-Chmielewska M. et al. *Nutritional Neuroscience*. 2020;1-14 and Nowacka-Chmielewska M. et al. *Nutrients*. 2021,13,4242.].

Cooperation with teams of scientists from Katowice, conducted in the field of metabolomics and proteomics on an animal model, began during my postdoctoral fellowship in Gliwice and is still being continued. As part of the joint research conducted so far in 2016-2021, 6 publications were published in journals from the JCR list with a total IF = 10.643 and a number of citations of 41.

5.2.4. Metabolomic changes in the pathology of the cornea

The molecular etiology of keratoconus (KC-keratoconus), which is a pathological condition of the human cornea, remains unclear. In a pilot study of metabolomic profiles using GC-MS technique, I showed differences in the composition of metabolites between the keratoconus and the normal cornea [Wojakowska et al. *Molecules*. 2020;25(12):2933]. The metabolic pathways associated with the differentiating compounds were involved in energy production, lipid and amino acid metabolism. The obtained metabolomic signatures indicate a significant role of oxidative stress and inflammatory processes in the development of the studied corneal pathology, which observations are consistent with previous reports on cellular processes involved in the development of KC. Work carried out in cooperation with prof. dr hab. Dorota Tarnawska, an ophthalmologist specializing in corneal diseases, is the first application of GC-MS-based metabolomic profiling, which showed differences in metabolite composition between keratoconus and normal cornea. Due to the unique and difficult to analyze material, the obtained results are all the more valuable. However, research in this area needs to be continued in order to obtain a complete picture of the metabolic changes that play a key role in the etiopathology of keratoconus.

5.2.5. Involvement of exosomes in the mechanism of graft rejection

In addition to the known functions that exosomes act in cancer-related processes, they may also play an immunomodulatory role in cellular processes related to the mechanism of transplanted organs rejection. The influence of exosomes on the development of rejection or tolerance of the transplanted organ and attempts to use small extracellular vesicles as a non-invasive method of monitoring the organ status after transplantation and predicting the risk of rejection were described in the review by Gołębiewska et al. [*Cells*. 2021;10:2989], of which I am a co-author. Recently started cooperation with dr hab. Justyna Gołębiewska from the Medical University of Gdańsk has also resulted in the submission of a joint NCN OPUS 22 grant application (in which I act as the head of the consortium member on behalf of ICHB), which is currently in the second stage of substantive evaluation. Together with dr hab. Justyna Gołębiewska (MUG) and dr hab. Monika Pietrowska (NIO), we started proteomic research in the context of searching for mechanisms of transplanted kidney rejection, with particular emphasis on the participation of exosomes in this process. The ability to quantify the level of exosome-specific components released by a transplanted organ may in the future serve as a non-invasive method of monitoring organ status after transplantation and predicting the risk of transplant rejection.

6. Presentation of teaching and organizational achievements as well as achievements in the popularization of science

6.1. Teaching Achievements

Scientific supervisor in diploma theses and student internships:

- **2019-2022**: M.Sc. Urszula Strybel; Institute of Bioorganic Chemistry PAS; admitted into a Ph.D. program – direct research tutor and auxiliary supervisor.
  Published articles: Strybel U. et al. *Cancers*. 2022;14(4):99 (H7).

- **2021**: Igor Karasiński: 3rd year of Forensic and forensic analytics, Medical University of Karol Marcinkowski in Poznań; 1 month of student internships in the Laboratory of Mass Spectrometry IBCh PAS - scientific supervisor.

- **2021**: Katarzyna Wilczyńska: 3rd year of Chemical Technology, Poznan University of Technology; 1.5 months of student internships in the Laboratory of Mass Spectrometry IBCh PAS - scientific supervisor.

- **2018**: Agata Włosowicz/Skowronek: 3rd year of Ph.D. study, National Research Institute of Oncology, Gliwice Branch; 2 months of student internships in the Laboratory of Mass Spectrometry IBCh PAS - scientific supervisor.
  Published articles: Wojakowska et al. *Journal of Personalized Medicin*e. 2020;10(4):229 (H6).

- **2013**: M.Sc. Magdalena Grajzer: 2nd year of Ph.D. study, Medical University in Wrocław; 2 months of student internships in the Laboratory of Proteomics and Metabolomics IBCh PAS - scientific supervisor.
  Published articles: Ostrowski et al. *Journal of Chromatography B*. 2014;967:21-7 and Grajzer et al. *Food Chemistry*. 2015;188:459-66.

- **2013:** Wojciech Smułek: 5th year of Chemical and Process Engineering, Poznan University of Technology; 1 month of student internships in the Laboratory of Proteomics and Metabolomics IBCh PAS - scientific supervisor.

- **2013**: Marta Nolka: 5th year of Chemistry, Adam Mickiewicz University in Poznań; 2 months of student internships in the Laboratory of Proteomics and Metabolomics IBCh PAS - scientific supervisor.

- **2013**: dr. Marta Olech, M.Sc. Marta Drozd, M.Sc. Wioleta Pietrzak, Medical University of Lublin. 1 month of student internships in the Laboratory of Proteomics and Metabolomics IBCh PAS - scientific supervisor.

- **2012**: M.Sc. Wojciech Ostrowski: 2nd year of Ph.D. study, Adam Mickiewicz University in Poznań; 2 months of student internships in the Laboratory of Proteomics and Metabolomics IBCh PAS - scientific supervisor.
  Published articles: Ostrowski et al. *Journal of Chromatography B*. 2014;967:21-7.

6.2. Organizational achievements and popularization of science

6.2.1. Research project management

- **04/2018-04/2022: Principal investigator** in National Science Center (NCN) research project SONATA, No. 2017/26/D/NZ2/00964.
  Project entitled: Identification of biomolecules released by colorectal cancer cells and detected in serum or/and exosomes.
  Direct supervision over the work of a Ph.D. student, M.Sc. Urszula Strybel.
  Opublikowane prace: H6, H7

- **11/2013-04/2018: Principal investigator** in National Science Center (NCN) research project FUGA, No. 2013/08/S/NZ2/00868.
  Project entitled: The use of mass spectrometry techniques for profiling and identification of proteomic and metabolomic components specific for each type of thyroid cancer.
  Published articles: H1- H5

- **01-31/10/2014: Principal investigator** in international grant STSM COST No. BM1104-011014-04633.
  Project entitled: Lipidomics in thyroid cancer research.
  Published articles: H4.
- **05/2013-10/2013: Principal investigator** in the internal grant from IBCH PAS for the implementation of a research task aimed at the development of young scientists or participants of doctoral studies.
  Project entitled: Application of the nanoLC-ESI-IT-MS technique for the analysis of phenolic secondary metabolites present in complex plant extracts.
  Published articles: Wojakowska et al. *Journal of Mass Spectrometry*. 2013;48(3):329-39.

- **12/2011-06/2013: Principal investigator** in National Science Center (NCN) research project PRELUDIUM, No. 2011/01/N/NZ2/00025.
  Project entitled: Application of tandem mass spectrometry with collision-induced dissociation for profiling and structural analysis of phenolic secondary metabolites present in complex plant extracts.
  Published articles: Wojakowska A. et al. *Acta Physiologiae Plantarum*. 2015;37(8):152; Wojakowska A. et al. *Phytochemistry*. 2013;92: 71-86; Wojakowska A et al. *Metabolomics*. 2013;9(3):575-589; Wojakowska et al. *Journal of Mass Spectrometry*. 2013;48(3):329-39.

6.2.2. Membership in the organizing committees of international conferences

- Scientific seminar "Applications of proteomics and data analysis methods in biological research", organized by the Polish Proteomics Society and the Association for the Support of Cancer Research, 4-5.06.2014, Gliwice, Poland – a member of the organizing committee.

- Joint Conference of Polish Mass Spectrometry Society and German Mass Spectrometry Society, 4-7.03.2012, Poznań, Poland – a member of the organizing committee.

- 2nd Conference of Polish Mass Spectrometry Society, 24-26.03.2010, Poznań, Poland – a member of the organizing committee.

6.2.3. Membership in international or national scientific organizations and societies with information about their functions

• Polish Proteomic Society, Founding member and Secretary, 2019-2022.
• International Society for Extracellular Vesicles, Member, 2020-2022.
• Metabolomics Society, Member, 2010-2022.

7. Other professional career information

Detailed information related to my scientific activity, including:
- a full list of articles published in scientific journals (38), post-conference publications (8), lectures (11), and conference presentations (17 as the author of the presentation and 19 as a co-author);
- information on participation in research projects as a team member-researcher (5);
- list of training and workshops held in Poland and abroad (11);
- information on peer-reviews of scientific papers (13);
- information on participation in European programs (2);
- awards and distinctions (5);

are included in Appendix 6. List of achievements.

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(Applicant's signature)