

SUMMARY OF PROFESSIONAL ACCOMPLISHMENTS

Germline and somatic loss-of-function mutations in the *TSC1* and *TSC2* genes – role in tumorigenesis and association with human phenotype

Katarzyna Klonowska, PhD

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Poznan, 2025

1. Name

Katarzyna Klonowska

2. Diplomas and degrees conferred in specific areas of science

- 2017 **PhD degree in chemistry in the field of biochemistry
(with honors)**
Institute of Bioorganic Chemistry PAS in Poznan
Title of PhD thesis: 'Searching for genes associated with breast cancer predisposition – focus on copy number variants as a risk factor'
Supervisor: prof. dr hab. Piotr Kozlowski
- 2013 **MSc in biotechnology; biotechnology conducted in English
(with honors)**
Faculty of Biology, Adam Mickiewicz University, Poznan
MSc thesis performed in the Institute of Bioorganic Chemistry PAS in Poznan
Title of MSc thesis: 'Analysis of large mutations in *BARD1* gene in patients with breast and/or ovarian cancer'
Supervisor: prof. dr hab. Piotr Kozlowski
- 2011 **Bachelor's degree in biotechnology**
Faculty of Biology, Adam Mickiewicz University, Poznan
Title of Bachelor's thesis: 'Analysis of microRNA precursors in different developmental stages in plants: application of a high-throughput qPCR platform'
Supervisor: prof. dr hab. Artur Jarmolowski

3. Information on employment in research institutes

- 03.2024 – present **PI of the Polish National Science Centre grant OPUS25**
Department of Cancer Genetics
Institute of Bioorganic Chemistry PAS in Poznan
- 01.2024 – present **Head of Department/Assistant Professor**
Department of Cancer Genetics
Institute of Bioorganic Chemistry PAS in Poznan
- 04.2023 – 12.2023 **Assistant Professor**
Department of Molecular Genetics
Institute of Bioorganic Chemistry PAS in Poznan
- 12.2020 – 11.2022 **PI of the TSC Alliance grant 'Postdoctoral Fellowship Grant Award 2020'**
Cancer Genetics Laboratory

Brigham and Women's Hospital and Harvard Medical School in Boston, USA

- 04.2018 – present **Post-doc/affiliated member**
Broad Institute of MIT and Harvard in Cambridge, USA
- 03.2018 – 03.2023 **Post-doc**
Cancer Genetics Laboratory
Brigham and Women's Hospital and Harvard Medical School in Boston, USA
- 01.2018 – 02.2018 **Researcher biologist**
Department of Molecular Genetics
Institute of Bioorganic Chemistry PAS in Poznan
- 08.2016 – 08.2019 **PI of the Polish National Science Centre grant PRELUDIUM10**
Department of Molecular Genetics
Institute of Bioorganic Chemistry PAS in Poznan
- 01.2014 – 11.2017 **PhD student**
Department of Molecular Genetics
Institute of Bioorganic Chemistry PAS in Poznan

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

4.1. Title of the habilitation achievement

Germline and somatic loss-of-function mutations in the *TSC1* and *TSC2* genes – role in tumorigenesis and association with human phenotype.

4.2. List of publications included in the habilitation achievement

Declarations of the doctor habilitated candidate and the corresponding author(s) specifying the doctor habilitated candidate's contribution to the preparation of publications included in the habilitation achievement are included in 'Attachment 6'.

Formatting and abbreviations:

- My name - **bold**; Name(s) of the first author(s) - underlined; Equal contribution - #; Name(s) of the corresponding author(s) - *
- **MNiSW**: the MNiSW score from the year preceding the publication
- **MNiSW₂₀₂₄**: the MNiSW score according to the most up-to-date MNiSW journal score list from 5.01.2024
- **Impact Factors (2y-IF – 2-year Impact Factor, 5y-IF – 5-year Impact Factor)** from the year preceding the publication
- **Citations**: The total number of citations (according to Web of Science), from: 16.04.2025

- H1. Salussolia CL, Klonowska K, Kwiatkowski DJ, Sahin M*.**
Genetic Etiologies, Diagnosis, and Treatment of Tuberous Sclerosis Complex.
Annual Review of Genomics and Human Genetics, 2019, 20:217-240.
doi: 10.1146/annurev-genom-083118-015354
2y-IF₂₀₁₈=7.914; 5y-IF₂₀₁₈=9.290
MNI_{SW}=140, MNI_{SW}₂₀₂₄=140
Citations =108 (102 without self-citations)
- H2. Klonowska K*, Giannikou K, Grevelink JM, Boeszoermenyi B, Thorner AR, Herbert ZT, Afrin A, Treichel AM, Hamieh L, Kotulska K, Jozwiak S, Moss J, Darling TN, Kwiatkowski DJ***
Comprehensive genetic and phenotype analysis of 95 individuals with mosaic Tuberous Sclerosis Complex
American Journal of Human Genetics, 2023, 110(6):979-988.
doi: 10.1016/j.ajhg.2023.04.002
2y-IF₂₀₂₂=9.8; 5y-IF₂₀₂₂=11.8
MNI_{SW}=200, MNI_{SW}₂₀₂₄=200
Citations =18 (18 without self-citations)
- H3. Klonowska K, Thiele EA, Grevelink JM, Thorner AR, Kwiatkowski DJ*.**
Sporadic facial angiofibroma and sporadic angiomyolipoma mimicking tuberous sclerosis complex.
Journal of Medical Genetics, 2022, 59(9):920-923.
doi: 10.1136/jmedgenet-2021-108160.
2y-IF₂₀₂₂=5.945; 5y-IF₂₀₂₂=6.451
MNI_{SW}=140, MNI_{SW}₂₀₂₄=140
Citations =1 (1 without self-citations)
- H4. Ogórek B, Hamieh L, Hulshof HM, Lasseter K, Klonowska K, Kuijf H, Moavero R, Hertzberg C, Weschke B, Riney K, Feucht M, Scholl T, Krsek P, Nabbout R, Jansen AC, Benova B, Aronica E, Lagae L, Curatolo P, Borkowska J, Sadowski K, Domańska-Pakieła D, Janson S, Kozłowski P, Urbanska M, Jaworski J, Jozwiak S, Jansen FE, Kotulska K; EPISTOP Consortium members, Kwiatkowski DJ*.**
TSC2 pathogenic variants are predictive of severe clinical manifestations in TSC infants: results of the EPISTOP study.
Genetics in Medicine, 2020, 22(9):1489-1497.
doi: 10.1038/s41436-020-0823-4
2y-IF₂₀₁₉=8.904; 5y-IF₂₀₁₉=10.435
MNI_{SW}=200, MNI_{SW}₂₀₂₄=200
Citations =62 (57 without self-citations)

H5. Klonowska K*, Grevelink JM, Giannikou K, Ogorek BA, Herbert ZT, Thorner AR, Darling TN, Moss J, Kwiatkowski DJ*.

Ultrasensitive profiling of UV-induced mutations identifies thousands of subclinical facial tumors in tuberous sclerosis complex.

Journal of Clinical Investigation, 2022, 132(10):e155858.

doi: 10.1172/JCI155858

2y-IF₂₀₂₁=19.477; 5y-IF₂₀₂₁=19.232

MNiSW=200, MNiSW₂₀₂₄=200

Citations =4 (3 without self-citations)

H6. Giannikou K*, Klonowska K, Tsuji J, Wu S, Zhu Z, Probst CK, Kao KZ, Wu CL, Rodig S, Marino-Enriquez A, Zen Y, Schaefer IM, Kwiatkowski DJ*.

TSC2 inactivation, low mutation burden and high macrophage infiltration characterise hepatic angiomyolipomas

Histopathology, 2023, 83(4):569-581.

doi: 10.1111/his.15005

2y-IF₂₀₂₂= 6.4 ; 5y-IF₂₀₂₂= 5.1

MNiSW=100, MNiSW₂₀₂₄=100

Citations =2 (2 without self-citations)

H7. Adib E[#], Klonowska K[#], Giannikou K, Do KT, Pruitt-Thompson S, Bhushan K, Milstein MI, Hedglin J, Kargus KE, Sholl LM, Tsuji J, Hyman DM, Sisk A, Shapiro GI, Vargas HA, Harding JJ, Voss MH, Iyer G, Kwiatkowski DJ*.

Phase II Clinical Trial of Everolimus in a Pan-Cancer Cohort of Patients with mTOR Pathway Alterations.

Clinical Cancer Research, 2021, 27(14):3845-3853.

doi: 10.1158/1078-0432.CCR-20-4548

2y-IF₂₀₂₀=12.531; 5y-IF₂₀₂₀=12.836

MNiSW=200, MNiSW₂₀₂₄=200

Citations =27 (27 without self-citations)

Table 1. Scientometric data on publications included in the habilitation achievement and all my publications (from 16.04.2025).

	Habilitation achievement (n=7 publications)	All publications from <i>Web of Science Core Collection</i> (n=21 publications)
2y-IF (5y-IF)	71.0 (75.1)	155.3 (154.9)
2y-IF (5y-IF) / publication	10.1 (10.7)	7.4 (7.4)
MNiSW₂₀₂₄	1180	2920
citations (without self-citations)	222 (210)	523 (499)
H-index	-	12

2y-IF: total 2-year Impact Factor from the year preceding the publication

5y-IF: total 5-year Impact Factor from the year preceding the publication

2y-IF (5y-IF) / publication: average Impact Factor values per publication

MNiSW₂₀₂₄: total MNiSW score according to the most up-to-date list from 5.01.2024

Citations (without self-citations): total citations numbers (according to Web of Science), from 16.04.2025

4.3. Discussion on the habilitation achievement

My habilitation achievement entitled '*Germline and somatic loss-of-function mutations in the TSC1 and TSC2 genes – role in tumorigenesis and association with human phenotype*' includes a cycle of 7 publications, including 6 publications summarizing the results of original research and 1 review publication.

All publications included in this cycle were prepared in the years 2019-2023 during my long-term 5-year postdoctoral fellowship at Brigham and Women's Hospital and Harvard Medical School in Boston, in the research group of Dr. David Kwiatkowski, a leading expert in the genetics of Tuberous Sclerosis Complex (TSC) and cancer genetics (member of the Broad Institute of MIT and Harvard; one of the leaders of The Cancer Genome Atlas Project (TCGA); leader of the Cancer Genetics program at Dana-Farber/Harvard Cancer Center).

Publications included in my habilitation achievement were published in renowned prestigious journals in the field of human genetics, including 4 out of 7 publications in journals with a 5-year Impact Factor (IF) of >10 and the maximum MNiSW score (=200 points). The average 5-year IF per publication in the cycle was 10.7. I am the first author in 4 of the 7 presented publications, in 2 of which I am also the corresponding author. 3 publications from this cycle were prepared as part of the *Postdoctoral Fellowship Grant Award 2020*, funded by TSC Alliance (Maryland, USA), which I obtained and completed during my postdoctoral fellowship in Boston.

4.3.1. Introduction

My research included in the habilitation achievement was focused mainly on exploring novel aspects of the genetic pathogenesis of TSC.

TSC is a hereditary autosomal dominant syndrome with an incidence of $\sim 1/6000$ in the general population, which occurs due to loss-of-function variants* in tumor suppressor *TSC1* and *TSC2* genes. TSC is characterized by tumors/lesions in multiple organs, including the brain, heart, skin, lung, and kidney (Crino, 2006; Henske, 2016; Salussolia, 2019) (**Figure 1**).

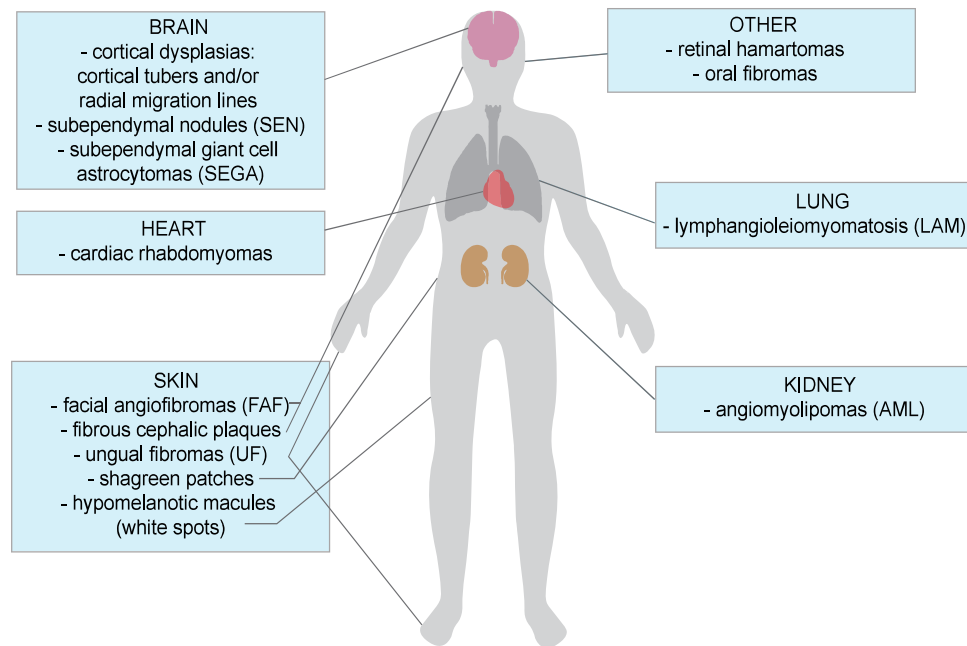


Figure 1. Tumors/lesions affecting different organs in TSC patients.

Studies performed in Dr. David Kwiatkowski laboratory revealed that genetic mosaicism is a relatively common phenomenon in individuals with TSC (Tyburczy, 2015; Giannikou, 2019; Treichel, 2019). Individuals with mosaicism bear cell populations with different genetic make-up. Mosaicism in TSC occurs due to a postzygotic *TSC1* or *TSC2* variant, resulting in a clonal population of cells with the variant mixed in with a larger fraction of cells that do not have the variant.

* It is worth noting that in the field of genetics, there are various standards and customs regarding the naming of changes in DNA sequence. In the literature in the field of human genetics, the term 'variant' is often used in the context of germline changes, while the term 'mutation' is used in the context of somatic changes. Therefore, in my summary of professional accomplishments, I have decided to (i) use the term 'variant' to describe germline changes, as well as mosaic changes that occur systemically, i.e., in the blood and/or other body fluids/tissues (which, like germline changes, serve as 'first hits' in the pathogenesis of TSC, according to the Knudson model), and (ii) use the term 'mutation' in the context of somatic changes (including 'second hits' in TSC and somatic changes in sporadic tumors and other tissues in individuals without TSC).

Mosaicism in TSC can be either generalized/systemic, i.e., seen in blood and/or other bodily fluids and tissues, or limited to a single tissue/organ (restricted mosaicism). Next Generation Sequencing (NGS)/Massively Parallel Sequencing (MPS)** analyses performed in Dr. Kwiatkowski laboratory revealed that systemic mosaicism is seen in 10-15% of individuals with TSC and frequently occurs among patients for whom the pathogenic *TSC1/TSC2* variant was not identified in prior conventional genetic testing in clinical labs (Giannikou, 2019).

Further comprehensive analysis of mosaicism in TSC, including the analysis of the spectrum of mosaic variants in the *TSC1/TSC2* genes in different 'normal' tissues and tumors in TSC patients, along with the analysis of their impact on the phenotype, would provide an important compendium of knowledge on mosaicism for TSC patients.

The pathogenesis of tumors occurring as a manifestation of TSC is consistent with the Knudson two-hit model (Knudson, 1971), where inactivation of one allele of *TSC1* or *TSC2* ('first hit': germline (variant allele frequency=50%) or systemic mosaic variant) is followed by a somatic 'second-hit' in tumor cells, which inactivates the second allele of the same gene, leading to activation of mammalian target of rapamycin complex 1 (mTORC1). 'Second-hit' in *TSC1* and *TSC2* occurs through different genetic mechanisms that are specific for tumors in the respective organs/body parts. 'Second hit' mechanisms in TSC tumors include: (i) copy-neutral loss of heterozygosity (cnLOH). i.e., exchange of the 'normal' allele of the gene and the surrounding region of the chromosome as a result of uniparental disomy or gene conversion, and (ii) point mutations, including single nucleotide variants (SNVs), dinucleotide variants (DNVs), and 1-several nucleotide insertions and deletions (indels) (**Figure 2**). Previous analyses have shown that the major mechanism of the 'second hit' in SEGA tumors in the brain and AML tumors in the kidneys is cnLOH (Henske, 1995; Henske, 1996; Au, 1999; Niida, 2001; Giannikou, 2016; Giannikou, 2021). Analysis of the second-hit mechanisms in some tumors associated with TSC, including skin tumors, constitutes a significant challenge due to the low tumor cell content in biopsies. A small fraction of tumor cells results in a situation where the observed 'second-hit' mutations occur at a very low variant allele frequency (VAF), often well below 2% (Tyburczy, 2014; Tyburczy, 2015; Giannikou, 2019; Treichel, 2019). Therefore, the application of an ultrasensitive MPS method is highly sought after to comprehensively characterize the spectrum and frequency of second-hit somatic mutations in tumor biopsies with low tumor cell content in individuals with TSC.

Somatic loss-of-function *TSC1* and *TSC2* mutations, leading to the activation of mTORC1 which is a key regulator of cell growth and division, are observed not only in tumors in TSC patients but also in multiple cancer types. Somatic mutations in *TSC1* are particularly frequently observed in bladder cancer (6%), while somatic mutations in *TSC2* are most frequent in uterine sarcoma (5%). Somatic mutations in both genes are also relatively frequently observed in

** The terms Next Generation Sequencing (NGS) and Massively Parallel Sequencing (MPS) can be considered synonymous and are used interchangeably in the English-language literature in the field of genetics. In the publications included in my habilitation achievement, as well as in the summary of my professional accomplishments, I will use the term MPS.

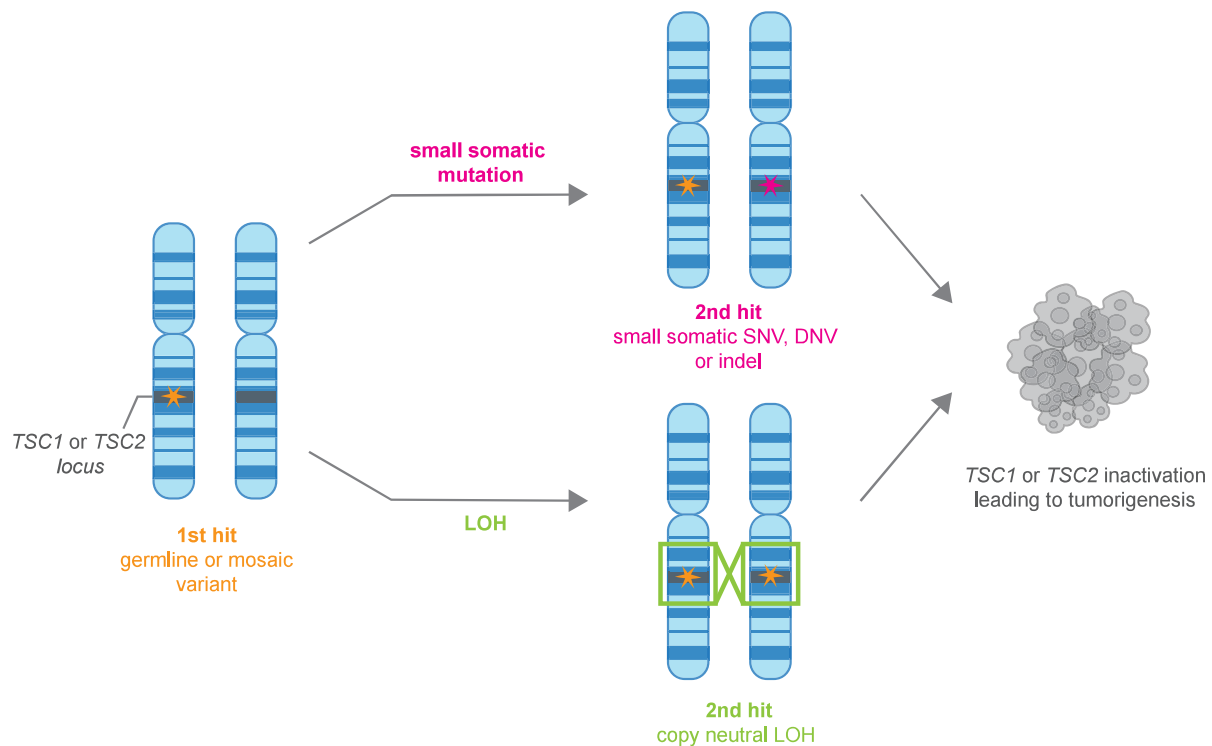


Figure 2. Genetic mechanisms leading to tumor development frequently observed in TSC patients.

Abbreviations: LOH – loss of heterozygosity; SNV – single nucleotide variants; DNV – dinucleotide variants; indel – small insertions/deletions.

Renal Cell Carcinoma (RCC) (*TSC1* - 5%, *TSC2* - 3%) (<https://www.oncokb.org/gene/TSC1>; <https://www.oncokb.org/gene/TSC2> (Chakravarty, 2017; Zehir, 2017; Suehnholz, 2024)).

It has been suggested that everolimus therapy may be beneficial for patients with cancer with somatic loss-of-function mutations in *TSC1/TSC2*. Everolimus is a derivative of rapamycin acting as an allosteric inhibitor of mTORC1, which has been approved by Food and Drug Administration (FDA) for treatment of RCC (Hudes, 2007), pancreatic neuroendocrine tumors (Yao, 2011), and hormone- positive HER-2–negative breast cancer in postmenopausal women (Baselga, 2012), as well as tumors in TSC patients (Bissler, 2013; Franz, 2013; Kwiatkowski, 2015). A study on the potential benefit of everolimus therapy in patients with bladder cancer, performed by Gopa Iyer and coworkers from Memorial Sloan Kettering Cancer Center in New York, reported an exceptional responder, i.e., a patient with advanced bladder cancer with somatic mutations in *TSC1* and *NF2* who achieved a durable (>2 years) and ongoing complete response to everolimus (Iyer, 2012). Several other individual cases of exceptional responses to everolimus therapy in patients with tumors with somatic *TSC1/TSC2* mutations have also been published (Wagle, 2014; Kwiatkowski, 2015; Alsidawi, 2018). These observations highlighted the need for a systematic assessment of the effectiveness of everolimus therapy in patients with various types of cancers with somatic loss-of-function mutations in *TSC1/TSC2*.

4.3.2. Motivation and research aim

The general aim of the research included in my habilitation achievement was to characterize germline and somatic loss-of-function variants/mutations in *TSC1* and *TSC2* in patients with TSC and patients with various cancer types, as well as to determine their association with the phenotype.

The abovementioned aim was achieved through the following specific objectives:

- Comprehensive analysis of the germline and mosaic variants in *TSC1/TSC2* in patients with TSC and analysis of their association with the phenotype (**H1, H2, H3, H4**).
- Development and optimization of the ultrasensitive MPS method and its application for characterization of the spectrum of *TSC2* somatic mutations at low allele frequencies in TSC tumors (**H5, H6**).
- Exome analysis in clinical trial of everolimus therapy in patients with different types of cancers with mutations in *TSC1/TSC2* (**H7**).

4.3.3. Discussion of the results

- **Comprehensive analysis of the germline and mosaic variants in *TSC1/TSC2* in patients with TSC and analysis of their association with the phenotype (**H1, H2, H3, H4**)**

My work on habilitation achievement began with a review of the literature on the genetic background of TSC. Within my first project, initiated during my postdoctoral fellowship in Dr. David Kwiatkowski laboratory in Boston, I was involved in preparing a paper in the form of a systematic review in collaboration with Dr. Mustafa Sahin, a leading expert in the field of TSC molecular neurology, from Boston Children's Hospital and Harvard Medical School (publication **H1**).

During the preparation of this paper, I gathered and described scientific reports on TSC clinical genetics, which became the subject of subsequent experimental analyses included in my habilitation achievement (**H1**: p. 222-226). I have performed an analysis of the spectrum and frequency of pathogenic variants in *TSC1* and *TSC2* in the general population of TSC patients using data gathered in the publicly available Leiden Open Variation Database (LOVD) (Fokkema, 2011). The content and annotation of the LOVD database of *TSC1/TSC2* variants in individuals with TSC provides an excellent resource for the community of patients, physicians, and researchers in the field of TSC. The results of my analysis using LOVD data were summarized on schemes which are maps of germline variants, visualizing the frequency and distribution of pathogenic SNVs and indels, in *TSC1* (**H1**: 'Figure 2') and *TSC2* (**H1**: 'Figure 3'). I later used the *TSC1* and *TSC2* variant maps many times as a reference in my experimental work, including the studies summarized in publications **H2** and **H5**, which are part of my habilitation achievement.

In the framework of expanding my interest in genetic variation in the *TSC1/TSC2* genes, my experimental work has focused on a comprehensive analysis of mosaicism in *TSC1/TSC2* in 95 patients with TSC. This study has been summarized in a publication in one of the most prestigious journals in the field of human genetics, *American Journal of Human Genetics* (5y-IF 2022=11.8) (publication **H2**), in which I am both the first and corresponding author. The study was largely conducted in the framework of the grant funded by *TSC Alliance*, which I have led as a PI.

This study was one of the first experimental projects initiated as part of my habilitation achievement and lasted throughout the entire duration of my 5-year postdoctoral fellowship in Dr. David Kwiatkowski laboratory (2018-2023). This project required a lot of commitment due to the need to collect a representative group of unique samples from individuals with TSC with suspected mosaicism. The samples were collected from individuals with TSC for whom the *TSC1/TSC2* pathogenic variant was not identified as part of the prior conventional genetic testing in diagnostic laboratories, usually performed using methods with limited sensitivity (such as Sanger sequencing or MPS with low depth of coverage). The project was performed in collaboration with experts in the field of TSC, dermatologists Dr. Joannes Grevelink (Massachusetts General Hospital, Boston, USA) and Dr. Thomas Darling (Uniformed Services University, Bethesda, USA), and neurologists prof. Katarzyna Kotulska and prof. Sergiusz Jóźwiak (Children's Memorial Health Institute, Warsaw).

In this project, I have performed a large-scale MPS analysis of 330 DNA samples derived from multiple different tissues and bodily fluids (e.g., skin, saliva, blood, semen) and different tumors associated with TSC derived from 95 TSC patients with mosaicism (**H2**: 'Graphical abstract' and 'Figure 1'). FAF facial skin tumors were the tumors most commonly included in the analysis due to their greatest availability and relative ease of biopsy collection. This study included genetic analysis of the largest cohort of patients with mosaic TSC ever reported and, at the same time, the largest cohort of mosaic patients ever reported for any hereditary syndrome predisposing to tumors associated with tumor suppressor gene inactivation (tumor suppressor syndromes).

In the project we have applied a MPS strategy with deep sequencing coverage of the entire genomic extent of *TSC1* and *TSC2* (covering both exons and introns). Initial sequencing analysis with a median read depth of at least 500x was performed on several (1-5) samples per patient. The identified pathogenic loss-of-function *TSC1* or *TSC2* variant was validated, predominantly using amplicon MPS with a very high median read depth of 100000x, using all 'normal' and tumor DNA samples available for each patient (**H2**: 'Figure 1'). I used our in-house computational pipeline, which enables the detection of variants at low frequency (below 1%), 'large' (multiexonic) deletions, and rare complex genome rearrangements for the analysis of the sequencing results.

The pathogenic systemic mosaic variants in *TSC2* were identified in 91% of the analyzed patients, whereas in *TSC1* in the remaining 9%. The distribution is different from the

distribution of germline variants observed in the general population of TSC patients (data from LOVD database (**H1**)), where *TSC1* variants are much more common and observed in 26% of TSC patients (**H2**: 'Figure 2A'). The distribution of mosaic variants in the *TSC1* and *TSC2* gene sequence was similar to that seen for pathogenic germline variants in general for TSC patients (**H2**: 'Figure 2B-C').

In this study, I have performed a comprehensive analysis of the systemic mosaic variant allele frequencies in matched (derived from the same patient) samples from different 'normal tissues'/bodily fluids and tumors (**H2**: 'Figure 3A' and 'Figure S1A'). The analysis revealed that variant allele frequencies in FAF skin tumors were significantly higher than variant allele frequencies in matched 'normal' skin and blood samples, which suggests that the inclusion of FAF samples in genetic testing performed in diagnostic laboratories can facilitate the identification of *TSC1/TSC2* mosaic variants. The median allele frequency of the systemic mosaic variant in blood was very low, ~3%, which is well below the level of variant allele frequency detection/reporting by the majority of diagnostic laboratories (which is mostly variant allele frequency of 10-15%), what may lead to the situation where most of the mosaic *TSC1/TSC2* variants in TSC are missed, using the conventional genetic testing in these laboratories. Moreover, the analysis revealed that in almost 20% of the analyzed mosaic TSC patients, the systemic mosaic *TSC1/TSC2* variant was not present in the blood, what highlights the need for the analysis of multiple samples from different tissues and tumors to enable the systemic mosaic variant detection (**H2**: 'Figure S2').

The analysis also showed that the mosaic variant allele frequency is significantly higher in *TSC1* than in *TSC2*, both in blood and saliva and in samples from FAF tumors. This is consistent with the observation that the phenotype (i.e., number and severity of the TSC clinical manifestations) in patients with germline *TSC1* variants is milder than in patients with *TSC2* variants (Dabora, 2001). In light of this observation, our results may suggest that a higher allele frequency of the mosaic variant in *TSC1* is needed than in *TSC2* for diagnostic clinical symptoms to appear. As a result, a significant fraction of individuals with low levels of *TSC1* mosaicism (variant allele frequency <3% in blood/saliva) will not be diagnosed as having insufficient TSC clinical symptoms to meet diagnostic criteria.

In the next step, I performed a comprehensive genotype-phenotype analysis to compare the spectrum and number of clinical manifestations in TSC patients with *TSC1/TSC2* mosaicism. Remarkably, I observed a positive correlation between the number of TSC-related clinical symptoms and variant allele frequency in blood and/or saliva in *TSC1* and *TSC2* genes (**H2**: 'Figure 4'). Furthermore, the analysis showed that the number of TSC clinical features in different organs, including skin, brain, kidneys, and lungs, is similar for individuals with mosaicism in the *TSC1* and *TSC2* genes (**H2**: 'Figure S3', 'Table S2').

In addition, I compared the phenotype (i.e., the prevalence of 14 different hallmark TSC clinical features) (i) in patients with mosaic *TSC1/TSC2* variant and (ii) in patients with heterozygous germline variant (allele frequency =50%) in *TSC1/TSC2*, using two independent cohorts with

heterozygous germline variants, i.e., a cohort characterized in our laboratory (n=39 patients) and a cohort characterized in the Natural History Database led by *TSC Alliance* (n=272 patients). The analysis revealed significant differences, including (i) less common occurrence of the majority (12 of 14) of clinical features in mosaic patients cohort and (ii) significantly more frequent occurrence of 2 symptoms, including FAF and AML tumors, in patients with mosaicism, in comparison to the cohort with heterozygous germline mutations from the Natural History Database (**H2**: 'Figure 5').

In this project, I have also identified a total of 11 novel variants that were not reported in LOVD, including an interesting case of a large inversion (221 kb) encompassing part of the *TSC2* gene identified in a sample from amniotic fluid.

In conclusion, the performed analysis significantly expands the current knowledge on the spectrum and frequency of mosaic *TSC1/TSC2* variants and clinical manifestations in TSC patients with mosaicism. The results indicate that in the analyses of TSC mosaicism, there is a need for (i) application of the MPS strategy of a very high read depth (500x) of the entire extent of *TSC1/TSC2* genomic sequence and (ii) application of a specialized computational analysis method that enables detection of low-level mutations and rare, complex genomic rearrangements that are missed by conventional exon sequencing or low sequencing depth genome sequencing. Our results are also highly relevant in the context of other human genetic diseases, including the investigation of mosaicism in other tumor suppressor syndromes, where mosaicism is not well studied, primarily due to the limited number of samples available from various tissues and some methodological limitations (Chen, 2022).

In the group of patients with suspected TSC mosaicism, whose samples were studied by MPS during my postdoctoral fellowship in Boston, I identified a patient for whom the genetic results were particularly interesting. The obtained results became the subject of my separate first-author *case report* publication which is also included in my habilitation achievement (publication **H3** in *Journal of Medical Genetics*, 5y-IF₂₀₂₂=6.451). The publication was prepared as part of a grant funded by *TSC Alliance*, which I led as a PI.

This study reports on a patient with a mild TSC phenotype, in whom pathogenic *TSC1/TSC2* variants were not identified in the prior conventional genetic testing in the clinical lab. I performed a high read depth MPS analysis (average coverage >700x) of the entire extent of the *TSC1* and *TSC2* gene sequence (including all exons and introns) for two samples derived from the patient, including skin FAF tumor and kidney AML tumor. In the subsequent validation analysis, I analyzed all samples (including FAF, AML, and 'normal' bodily fluids and tissues such as blood, saliva, and 'normal' skin) available from the patient using amplicon MPS (average coverage of >19000x). The MPS analysis led to the identification of 4 different loss-of-function *TSC2* mutations, constituting two different mutation pairs in each of the FAF and AML tumors, leading to the independent inactivation of both *TSC2* alleles in each of the tumors (**H3**: 'Figure 1C' oraz 'Table 1'). None of the identified mutations were observed in the 'normal' samples. This suggests that the two different lesions (FAF and AML) were not due to the same

underlying germline or systemic mosaic variant, but rather both are likely sporadic events, i.e., are not TSC-related. Taking into account the frequency of occurrence of sporadic AML (~0.1-0.2%) (Fujii, 1995; Flum, 2016) and sporadic FAF (8%) (Asgharian, 2004) in the general population, it can be estimated that co-occurrence of sporadic FAF and AML tumors in one individual can be observed with a frequency of only about 1 in 10000.

To sum up, the case presented here is an extremely extraordinary situation that I have not observed previously in studying over a hundred patients with clinical manifestations consistent with mosaic TSC. This case highlights the relevance of the accurate clinical examination, comprehensive deep-coverage MPS of various tumors and matched 'normal' samples, as well as appropriate genetic counseling for patients with mild TSC phenotype and possible *TSC1/TSC2* mosaicism.

I have also performed an analysis of *TSC1* and *TSC2* variants in TSC patients in another thematically related project included in my habilitation achievement, which has been summarized in a paper published in *Genetics in Medicine* (5y-IF₂₀₁₉=10.435), i.e., the official journal of the prestigious organization *American College of Medical Genetics and Genomics* (ACMG) (publication H4). I also participated in this project during my postdoctoral fellowship in Boston. It was performed in an international, multi-center collaboration of the leading scientific institutions from Poland, Germany, the Netherlands, Great Britain, Italy, Austria, Belgium, the Czech Republic, Australia, and the United States, as part of the EPISTOP project (the European Union 7th Framework Programme for Research, PI of the project: prof. Sergiusz Jozwiak, Children's Memorial Health Institute in Warsaw). In the project, a comprehensive analysis of variants in *TSC1* and *TSC2* and a genotype-phenotype study in a group of 94 children with TSC were performed. The obtained results demonstrated that loss-of-function *TSC2* variants are associated with more severe phenotype than *TSC1* variants in infants with TSC (H4: 'Fig.2') and constitute an important source of knowledge regarding the differences in observed clinical manifestations in patients with *TSC1/TSC2* mutations. It has to be noted that in this publication, I was the fourth author, and I was responsible for only part of the analyses performed in the project, i.e., the analysis of large deletions using Multiplex Ligation-dependent Probe Amplification (MLPA). Results of the analysis were summarized in H4: 'Supplementary Figure S2'. In the analysis, I had the opportunity to use my deep expertise in experimental work with the MLPA method and in the analysis and interpretation of the MLPA results, which I developed earlier during the preparation of my PhD thesis. In addition, I was involved in preparing the text and editing the manuscript.

- **Development and optimization of the ultrasensitive MPS method and its application for characterization of the spectrum of *TSC2* somatic mutations at low allele frequencies in TSC tumors (H5, H6).**

In addition to characterizing germline and mosaic variants in the *TSC1/TSC2* genes in patients with TSC, in the research included in my habilitation achievement I also focused on the analysis of somatic 'second hit' mutations in the *TSC2* gene in tumors associated with TSC.

‘Second hit’ mutations analysis on some lesions associated with TSC, including facial angiofibroma (FAF) skin tumor, is a big challenge due to the small fraction of tumor cells in the collected biopsies. FAF biopsy (average diameter ~2mm) contains a small fraction of the FAF tumor cells, i.e., fibroblasts with *TSC1/TSC2* inactivation, which occur in the dermis in the surrounding of an abundance of ‘normal’ cells, including inflammatory cells and blood vessels, as well as keratinocytes in the overlying epidermis. Due to the low content of FAF tumor cells in biopsies, the observed ‘second hit’ mutations occur with a very low allele frequency, often well below 2% (Tyburczy, 2014; Tyburczy, 2015; Giannikou, 2019; Treichel, 2019).

Conventional methods typically used in genetic analyses have limited sensitivity. One example is Sanger sequencing, which does not detect mutations with an allele frequency of <10%. Another example is the standard MPS sequencing method, where the detection of mutations with low allele frequency (<1%) is a challenge due to high sequencing error rates. This highlights the need for ultrasensitive MPS for comprehensive analysis of somatic ‘second-hit’ mutations in FAF and potentially in biopsies of other tumors with low content of tumor cells derived from individuals with TSC.

Therefore, in this project, I developed a new ultrasensitive strategy, Multiplex High-sensitivity PCR Amplification (MHPA), and applied it to the analysis of somatic mutations in the *TSC2* gene in FAF skin tumors from patients with TSC. I conducted this project during my postdoctoral fellowship in Boston as part of the *TSC Alliance* grant, which I led as a PI. The project was conducted in collaboration with TSC experts, dermatologists, Dr. Joannes Grevelink (Massachusetts General Hospital, Boston, USA), and Dr. Thomas Darling (Uniformed Services University, Bethesda, USA). The results of the project were summarized in a publication in the renowned *Journal of Clinical Investigation* (5y-IF2021=19.232) (publication **H5**), in which I am both the first and corresponding author.

MHPA is an ‘amplicon MPS’ sequencing method in which barcoding of single DNA molecules with unique molecular identifiers (UMIs), i.e., 14-nucleotide segments of random sequence, is applied. UMI labeling enables the elimination of errors (‘artifacts’) generated in the PCR reaction during library preparation or during sequencing, and the detection of mutations at a very low allele frequency of 0.01-0.05%. The MHPA test provides 10-fold greater sensitivity in mutation detection compared to MPS methods previously used to detect mutations in TSC (Giannikou, 2019; Treichel, 2019). MHPA enables the analysis of any regions in the genome with a very high depth of coverage (depth of coverage after data compression using UMI: ~30,000X). MHPA is based on a multiplex PCR reaction, enabling simultaneous amplification of many selected short genomic regions (amplicons up to ~120 nt) analyzed in the project. During the optimization of newly designed MHPA assays, MHPA amplicons are labeled with a fluorescent marker FAM, which enables the assessment of the level of PCR amplification of individual amplicons using capillary electrophoresis; this allows to ensure that amplification of individual genomic regions occurs at a comparable level (**H5**: ‘Figure 1’, ‘Figure 2A-B’).

Using the previously developed map of germline variants in *TSC2* collected in the LOVD database (**H1**), I designed the *TSC2*-MHPA assay consisting of 40 pairs of primers, enabling the amplification of short, 48-117 nucleotide long amplicons (**H5**: 'Figure 2C', 'Supplemental Table 1.1'). *TSC2*-MHPA amplicons are located in exons and exon-intron junctions of *TSC2* and cover the majority (74%) of pathogenic *TSC2* variants previously published in the LOVD database, including all hot-spot regions. Additionally, I developed an MHPA assay for the analysis of *TP53*, comprising 12 pairs of primers enabling the amplification of short fragments (90-126 nucleotides) located in exons and exon-intron junctions of the *TP53* gene. The *TP53*-MHPA set covers all hot-spot regions and 93% of somatic nonsynonymous mutations in *TP53*, identified in 'normal' skin and skin cancers in previously published analyses (**H5**: 'Supplemental Table 1.2.', 'Supplemental Figure 1'). *TP53* was included in this project as a reference gene, most frequently mutated in various cancers, including skin cancers.

The MHPA assays were used for the ultrasensitive analysis of somatic mutations in a set of 81 samples from different tissues (**H5**: 'Table1'). *TSC2*-MHPA and *TP53*-MHPA analyses were performed using samples from patients with TSC and from individuals without TSC ('non-TSC' samples); among others, the analyzed sample sets included (i) a panel of 24 biopsies from FAF skin tumors from patients with TSC and (ii) a control panel of 28 biopsies from individuals without TSC, including 8 samples of normal skin adjacent to basal cell carcinoma (BCC) (high sun exposure), 10 samples of normal skin from the inner arm (limited sun exposure), and 10 samples of neonatal foreskin (non-sun exposed area).

TSC2-MHPA analysis performed on a panel of 24 FAF samples from patients with TSC led to the identification of 112 somatic SNV, DNV, and indel mutations, with extremely low allele frequency (0.01-8%, median: 0.08%). CC:GG>TT:AA mutations with a known association with UV radiation accounted for 34 of 112 (30%) *TSC2* mutations identified in FAF. SNVs accounted for 60 of 112 (54%) *TSC2* mutations, of which 51 of 60 (85%) also occurred as a result of UV radiation, as previously indicated in the literature (including 34 C>G>T:A mutations and 17 G>C>T:A mutations) (Martincorena, 2015; Bonilla, 2016; Alexandrov, 2020; Fowler, 2021; Wei, 2021). Remarkably, an average of 5 *TSC2* somatic mutations per 2 mm diameter FAF biopsy were identified. Two or more somatic *TSC2* mutations were identified in most of the analyzed FAFs (19 of 24, 79%), suggesting that the majority of these small FAF biopsies contained at least two independent FAF clones (**H5**: 'Figure 3A', 'Figure 4', 'Supplemental Table 2.1.').

In contrast to sun-exposed TSC facial FAF biopsies, the number of *TSC2* mutations observed in non-sun- exposed non-TSC normal skin samples from the upper inner arm and foreskin was very low (average of 0.7 and 0.5 *TSC2* mutations, respectively). As expected, none of the non-TSC foreskin DNA samples showed a CC:GG>TT:AA mutation, and just a single such mutation was seen in one of the ten non-TSC normal skin samples from the inner upper arm (**H5**: 'Supplemental Figure 9').

In *TP53*-MHPA analysis, an average of 10.1 *TP53* mutations were identified per FAF whole skin punch biopsy (likely occurring in keratinocytes). I have also observed a positive correlation

between the number of *TP53* and *TSC2* mutations in the same FAF samples, reflecting the probable role of UV in the generation of both genes' mutations in these samples (**H5**: 'Figure 3', 'Supplemental Table 5').

Mutation signature analysis of SNVs identified by MHPA in both *TSC2* and *TP53* showed that TSC FAF and non-TSC normal skin adjacent to BCC had patterns similar to the canonical mutation signature SBS7b (COSMIC) (Alexandrov, 2020), known to be caused by UV radiation, with predominance of C>T substitutions. The DNV signature matched very well with the DBS-1 canonical UV signature, with CC:GG>TT:AA substitutions accounting for >90% of all DNVs detected in FAF and non-TSC BCC adjacent normal skin samples exposed to sunlight (**H5**: 'Figure 8').

Moreover, I identified a recurrent novel complex mutation signature pattern in *TP53*, consisting of both an SNV or DNV and a deletion *in cis*, likely occurring due to UV radiation (**H5**: 'Figure 10').

In summary, my MHPA analysis led to the discovery of highly prevalent low allele frequency mutations generated by sunlight UV radiation in the facial skin of individuals with TSC. The prevalence of somatic mutations in TSC facial skin likely represents multiple individual fibroblast clones (which we call 'micro-FAF') that were present in these small (2mm diameter) FAF biopsies. The analysis results enabled us to estimate that hundreds of thousands of independent clonal fibroblast proliferations ('micro-FAF') due to 'second hit' mutations in *TSC2* occur in the skin of TSC patients, a small proportion of which develop into observable FAF lesions (**H5**: 'Figure 7').

In the next project, I used the developed MHPA method for ultrasensitive analysis of somatic mutations in *TSC2* in samples derived from hepatic angiomyolipomas (hAML). hAML tumors occur in about 25% of patients with TSC, but are also observed as sporadic lesions in the general population (Black, 2012; Jozwiak, 2018). The general aim of the project was to analyze the genetic basis and tumor microenvironment of sporadic hAMLs. I performed this project during my postdoctoral fellowship in Boston. The results of the project were summarized in a publication in the journal *Histopathology* (5y-IF₂₀₂₂= 5.1) (publication **H6**), in which I was one of the main authors of the article (with 2nd position among co-authors). The graphical abstract of our article was appreciated by the editors and published on the *Histopathology* journal cover (vol.83, no.4, 2023).

In this project, a comprehensive analysis of the genetic basis of sporadic hAML tumors was performed for the first time. The whole exome sequencing strategy was used to identify the spectrum of somatic mutations in hAML. My role in this project was to use a deep, ultrasensitive MHPA sequencing strategy to characterize *TSC2* mutations in hAML. Genetic analysis using whole exome sequencing and MHPA revealed the presence of somatic loss-of-function mutations in *TSC2* in 10 of 12 (83%) analyzed hAML cases (median allele frequency of 13.6%) (**H6**: 'Table S4'). Whole exome sequencing analysis revealed a low frequency of

mutations in genes other than *TSC1/TSC2*, i.e., 5–18 somatic mutations per analyzed sample (median: 9; median mutation allele frequency: 21%), mostly with unknown pathogenicity status (variants of unknown significance, VUS).

- **Exome analysis in clinical trial of everolimus therapy in patients with different types of cancers with mutations in *TSC1/TSC2* (H7)**

In the next project included in my habilitation achievement, I focused on the characterization of loss-of-function mutations in the *TSC1/TSC2* genes and co-occurring somatic mutations in the exome of cancer tumors and determining their association with the response to everolimus therapy in patients with different types of cancer.

This project was carried out during my postdoctoral fellowship in Boston as part of a phase II clinical trial of everolimus therapy in patients with any type of advanced cancer ('pan-cancer' project) with loss-of-function mutations in *TSC1/TSC2* or gain-of-function mutations in *MTOR* (study ID in NIH clinicaltrials.gov: NCT02201212, <https://clinicaltrials.gov/study/NCT02201212?term=NCT02201212&rank=1>).

This study was performed in collaboration with physicians and scientists from the Dana-Farber Cancer Institute (Boston, MA), Memorial Sloan Kettering Cancer Center (MSKCC, New York, NY), and Novartis. The project was summarized in an article published in *Clinical Cancer Research* (5y-IF₂₀₂₀=12.8) (publication **H7**), in which I am the co-first author.

The main objective of the project was to identify factors that play a role in the response to everolimus therapy, including the cancer type, clinical and demographic characteristics of patients, *TSC1/TSC2* or *MTOR* somatic mutations, as well as co-occurring somatic mutations in the exome of cancer tumors. As part of the project, I performed a comprehensive analysis of somatic mutations (including SNVs, indels, and large deletions/amplifications (copy number alterations, CNA)) in cancer exomes of patients with a previously detected somatic loss-of-function *TSC1* or *TSC2* mutation or a gain-of-function mutation in the *MTOR* gene (**H7**: 'Table 1', 'Table 2', 'Figure 2', 'Supplementary Table S4'). The results of the exome analysis were compared with previously obtained results of somatic mutation profiling performed in diagnostic laboratories using MPS tests covering panels of 300–500 candidate genes with a known association with oncogenesis (OncoPanel (Brigham and Women's Hospital, Boston), MSK-IMPACT (MSKCC, New York), or Foundation One (Foundation Medicine) panels) (**H7**: 'Supplementary Table S1', 'Supplementary Table S5', 'Supplementary Table S6'). I also performed an analysis of the association between response to everolimus therapy and (i) tumor mutation burden (TMB) and (ii) the occurrence of somatic mutations in cancer-related genes from the OncoKB database maintained by MSKCC in New York (**H7**: 'Figure 3').

The study demonstrated that everolimus therapy had a beneficial effect in 4 of 30 (13%) patients included in this clinical trial, including 2 patients with uterine leiomyosarcoma with concurrent biallelic loss-of-function mutations in *TSC2* and *NF1*.

A small fraction of patients showed a good response to therapy (only 13%), what indicates that loss-of-function mutations in *TSC1/TSC2* are not a universal prognostic biomarker of response to everolimus therapy in advanced cancers of any histology. However, the study suggests that concurrent somatic mutations leading to inactivation of the *TSC2* and *NF1* genes may be associated with a good response to everolimus therapy in some types of cancers, including uterine leiomyosarcoma.

4.3.4. Summary

The most important effects and practical applications of the research included in my habilitation achievement are as follows:

- The maps/schemes visualizing the frequency and distribution of pathogenic variants in *TSC1* and *TSC2*, which I created as part of my habilitation achievement (**H1**: 'Figure 2', 'Figure 3') have been used many times as a reference in my experimental work and are also a useful source of knowledge for the community of scientists, patients and physicians in the TSC field.
- Analysis of TSC mosaicism performed in the largest group of samples/patients analyzed to date (>300 samples from 95 patients) (**H2**) significantly enriches the knowledge on (i) the spectrum and frequency of *TSC1/TSC2* mosaic variants in various tissues and (ii) clinical symptoms in mosaic TSC patients. The obtained results provide a valuable compendium of knowledge, contributing to the reduction of the risk of inheriting variants and the introduction of preventive measures that alleviate TSC symptoms.
- The abovementioned analysis of mosaicism (**H2**), as well as the case of a patient with a mild TSC phenotype with concurrent sporadic AML and FAF tumors (**H3**), highlights the great importance of the accurate examination of clinical manifestations, comprehensive deep-coverage MPS of various tumors and matched 'normal' samples, as well as appropriate genetic counseling for patients with suspected *TSC1/TSC2* mosaicism.
- The MHPA analysis in FAF skin tumors in individuals with TSC (**H5**) led to the discovery of a 'shower' of highly prevalent somatic mutations generated by the UV radiation in the *TSC2* gene in TSC patients' facial skin. The mutations likely represent thousands of subclinical precursor FAF lesions, a small proportion of which develop into clinically observable tumors. These observations highlight the importance of sunblock and other measures to limit facial UV exposure for TSC individuals of all ages.
- The ultrasensitive MHPA method developed as part of this habilitation achievement (**H5**) is a universal method and can be further applied in profiling mutations of low allele frequencies in TSC patients and individuals with other genetic diseases in various contexts, for instance in the analyses of (i) low-level mosaicism, (ii) somatic mutations of low allele frequency in biopsies derived from tumors with low tumor cell content, or (iii) mutations in the cell-free DNA (cfDNA).

- Comprehensive exome analysis in the everolimus clinical trial in patients with different cancer types with *TSC1/TSC2* mutations (**H7**) suggests that the somatic mutations leading to the concurrent biallelic inactivation of *TSC2* and *NF1* may be associated with a good response to the everolimus therapy in patients with uterine leiomyosarcoma.

4.3.5. Perspectives

The MHPA method, which I developed during my postdoctoral fellowship in Boston, has become the main research tool in my current research being performed within the Polish National Science Centre OPUS25 grant entitled '*Ultrasensitive profiling of mutations driving tumorigenesis in hereditary syndromes associated with tumor suppressor genes inactivation*', carried out in the Department of Cancer Genetics at ICHB PAN in Poznań, which I lead as a PI. The aim of the project is to identify the spectrum of somatic mutations and the mechanism of tumor development observed in patients with various syndromes associated with tumor suppressor genes inactivation, including TSC, Birt-Hogg-Dubé syndrome, and hereditary leiomyomatosis and renal cell cancer (HLRCC).

5. Other scientific and research achievements: description of professional career, presentation of significant scientific activity carried out at more than one university or scientific institution, especially at foreign institutions.

*In the description below, I refer to my publications marked as **N1-N15**, listed in section 'II. Information on scientific activity', in the document 'Attachment_5_List_of_scientific_achievements'.*

Over the last 15 years of my research work, I have developed a deep expertise in the field of human genetics. My research has led to discoveries of high scientific value and applicative potential, uncovering previously unrecognized aspects of the pathogenesis of human diseases of genetic origin.

5.1. Research carried out before obtaining the PhD degree

My interest in scientific research was initiated in high school when I attended the '*medicus*' class of biology and chemistry profile at Karol Marcinkowski High School No. 1 in Poznań, Poland. In 2009, I began my higher education in biotechnology at the Faculty of Biology of Adam Mickiewicz University (UAM) in Poznań. I completed my bachelor's thesis in the field of plants molecular biology, entitled '*Analysis of microRNA precursors in different stages of plant development: application of high-throughput qPCR platform*' under the supervision of prof. Artur Jarmołowski in the Department of Gene Expression.

In the next stages of my education and scientific career, I focused on research topics oriented toward biomedical research, especially research in the field of human genetics. In 2011-2013, I attended studies in biotechnology with a biomedical profile conducted in English at the Faculty of Biology, Adam Mickiewicz University in Poznań. I gained additional experience in

the field of human genetics during a 3-month (July-September 2012) internship in Germany, during the scholarship 'The Lifelong Learning Programme – Erasmus' in the Division of Molecular Biology of Breast Cancer, Heidelberg University Women's Clinic/German Cancer Research Center in Heidelberg, under the supervision of Dr Harald Surowy and prof. Barbara Burwinkel, experts in the field of breast cancer genetics and epidemiology.

In 2011-2017, I initiated my research work in the field of human genetics as part of my Master's and PhD theses under the supervision of prof. Piotr Kozłowski, in the Department of Molecular Genetics, in the Institute of Bioorganic Chemistry, Polish Academy of Sciences in Poznań. I was involved in ambitious scientific projects focused on the genetic basis of breast cancer predisposition. The research was partially funded by the Polish National Science Centre PRELUDIUM10 grant, which I was awarded during the preparation of my PhD thesis.

In the first project, carried out in cooperation with prof. Janusz Limon (Department of Medical Biology and Genetics, Medical University of Gdańsk) group, I developed a new Multiplex Ligation-dependent Probe Amplification (MLPA) strategy for large variants (multi-exon deletions/amplifications) genotyping in the *BARD1* gene. I used the developed strategy in the analysis of large variants in the *BARD1* gene in a large group of women with breast and/or ovarian cancer (>800 DNA samples from blood). The obtained results allowed us to exclude the previously suggested significant contribution of large variants in *BARD1* to predisposition to breast and/or ovarian cancer (publication **N4**). In parallel, I also participated in the assessment of the pathogenicity of several selected small variants in the *BARD1* gene using *in silico* methods (publication **N6**).

In the next project, I performed an analysis of the structure and function of the *APOBEC3B* gene deletion and characterized its contribution to the predisposition to breast cancer in the European population in collaboration with (i) the research group of prof. Jan Lubinski (Department of Genetics and Pathomorphology, Pomeranian Medical University in Szczecin), (ii) dr Magdalena Ratajska (Department of Biology and Medical Genetics, Medical University of Gdańsk), and (iii) dr Danuta Vasilevska (Department of Gynecology at the Centre of Obstetrics and Gynecology, University Hospital Santaros Klinikos in Vilnius). The analysis allowed us to define the exact structure of the *APOBEC3B* deletion and confirmed its homogeneity. It also showed for the first time that the *APOBEC3B* deletion affects the expression of all covered genes, including the hybrid gene *APOBEC3A/APOBEC3B*. Association analysis performed in three independent panels of DNA samples (in total: >6000 samples) showed a lack of association of the *APOBEC3B* deletion with a predisposition to breast cancer in the European population, which was additionally confirmed in the performed meta-analysis (publication **N9**).

The reports on the genetic factors associated with familial breast and/or ovarian cancer and candidate genes with a suggested role in breast cancer predisposition were gathered in the systematic literature review and summarized in a review article, which was also included as part of my PhD thesis (publication **N1**).

During the preparation of my PhD thesis, I also developed various genetic assays that can be used in further genetic studies. The developed assays/strategies include: (i) an MLPA strategy for genotyping multi-allelic copy number changes in *loci* associated with common human diseases (publication **N2**), and (ii) an MLPA-based, Multipoint Test for Targeted-enrichment Efficiency (MTTE) assay for validation of DNA libraries prepared for exome sequencing (publication **N8**).

During my PhD studies, I also contributed to other projects on somatic genetic variation in human cancers (publications **N3**, **N5**, **N7**).

I have presented the abovementioned results at multiple oral and poster presentations at >10 local and international conferences in Poland, Canada, Denmark, and Great Britain. My scientific achievements during my studies and doctoral research have been recognized with 9 awards/stipends, including the 'Award for the best PhD dissertation defended in 2017/2018', awarded by the Scientific Council of the City of Poznan, and 'Scholarship of the president of the Polish Academy of Sciences for the best PhD students'.

Summing up, my research work during doctoral studies resulted in co-authorship in a total of 9 publications (**N1-N9**), 5 of which I am the first author (total 5y IF: 36.4; total citations: 204). 6 of the publications (**N1**, **N2**, **N4**, **N6**, **N8**, **N9**) were combined in my PhD thesis as a collection (so called 'pin') of thematically related scientific papers, which I have defended with honors in the Institute of Bioorganic Chemistry of Polish Academy of Sciences in Poznan, in 2017.

5.2. Research carried out after obtaining the PhD degree

5.2.1. Analysis of the association of the variants in chosen candidate genes with breast cancer risk

After my PhD dissertation defense, during my employment as a biologist at the Department of Molecular Genetics at the IBCH PAS in Poznan (01-02.2018) and in the subsequent period, I was involved in three projects focused on determining the role of selected putative candidate genes in breast cancer susceptibility. As part of these projects, I had the opportunity to apply my expertise in the genetic basis of breast cancer predisposition, which I had acquired earlier during my doctoral studies.

In the first project, together with dr Malwina Suszynska, I did a large-scale meta-analysis (~120,000 breast cancer cases and ~120,000 controls) of variants identified in 37 candidate genes commonly included in diagnostic panels for the identification of variants predisposing to breast and/or ovarian cancer. The meta-analysis allowed for the first time for precise assessment of the breast and/or ovarian cancer risk associated with specific genes and supported proper knowledge-based interpretation of identified variants in clinical diagnostics practice. The study has been summarized in an article published in *Gynecologic Oncology* (5y-IF₂₀₁₈=4.3), in which I am the second author (publication **N10**).

In the second project, I was involved in the analysis carried out in collaboration with the research group of prof. Jan Lubinski (Department of Genetics and Pathomorphology, Pomeranian Medical University in Szczecin) and dr Magdalena Ratajska (Department of Biology and Medical Genetics, Medical University of Gdansk). The aim of the project was to analyze the founder variant p.Gln564* in *BARD1*, which occurs relatively often in the Polish population. Genotyping of samples from a group of ~14,000 patients with breast cancer and ~5,900 control individuals from the Central European population was performed, which led to the discovery of an association of the variant with a moderate risk of breast cancer (odds ratio: OR=2.3, p=0.04). An additional increase in breast cancer risk (OR=3.6, p=0.02) was observed in carriers of the p.Gln564* variant with triple-negative cancer, i.e. an aggressive subtype of breast cancer characterized by the lack of expression of steroid receptors (estrogen and progesterone) and the lack of overexpression of the HER2 receptor. The results were published in *Cancers* (2y-IF₂₀₁₈=6.162) (publication **N11**).

In addition, I contributed to a large-scale analysis of copy number variants (CNVs) in genes associated with breast and ovarian cancer predisposition in a group of >4000 patients, which was performed in collaboration with Dr. Corinna Ernst's team from the Family Center for Breast and Ovarian Cancer at the University Hospital Cologne in Germany. In this project, the *BARD1*-MLPA test, which I developed earlier during my doctoral studies, was used to independently validate CNVs in *BARD1* previously identified using MPS sequencing. The results were summarized in the journal *Cancers* (5y-IF₂₀₂₀=6.99) (publication **N13**).

In 2018-2023, I did a long-term postdoctoral fellowship with Dr. David Kwiatkowski at Brigham and Women's Hospital and Harvard Medical School in Boston. Dr. Kwiatkowski is a world-leading expert in the genetics of Tuberous Sclerosis Complex (TSC) and its clinical manifestations and cancer genetics (member of Broad Institute of MIT and Harvard and leader of The Cancer Genome Atlas (TCGA) program; leader of Cancer Genetics in Dana-Farber/Harvard Cancer Center). Since 2018, I have also held the position of post-doc/affiliated member at the Broad Institute of MIT and Harvard in Cambridge in the 'cancer program,' which has enabled me to collaborate with leading experts in the field of bioinformatics and acquire expertise in the bioinformatics analysis of MPS data.

During my post-doc fellowship and the ongoing collaboration with Dr. David Kwiatkowski, I was/am involved in over 10 research projects whose aim is to characterize novel aspects of TSC and cancer genomics using state-of-the-art MPS technologies. In 2020-2023, I was awarded and carried out a prestigious grant *TSC Alliance Postdoctoral Fellowship Award* funded by *TSC Alliance* (<https://www.tscalliance.org/tsc-matters/tsc-researcher-spotlight-katarzyna-kasia-klonowska/>). The *TSC Alliance Postdoctoral Fellowship Award* is aimed at supporting basic and translational research on TSC.

Most of the projects that I have performed during my post-doc in Boston have been summarized in a cycle of publications that are included in my habilitation achievement (**H1-H7**). Other published scientific achievements/projects that I was involved in during my post-doc in Dr. David Kwiatkowski's lab are described below in paragraphs **5.2.2.** and **5.2.3.**.

5.2.2. I phase clinical trial of celecoxib therapy in patients with lymphangioleiomyomatosis (LAM)

Lymphangioleiomyomatosis (LAM) is a pulmonary disease that affects almost exclusively women. LAM is characterized by the proliferation of abnormal smooth muscle-like cells with mutations in *TSC2*, rarely in *TSC1*. LAM can occur sporadically or as a symptom of TSC. In one of the projects, I was involved in a clinical trial of celecoxib therapy in patients with LAM, in collaboration with experts in clinical LAM manifestations, Dr. Souheil El-Chemaly and Dr. Elisabeth Henske from Brigham and Women's Hospital. In this project, I was responsible for performing a high-depth MPS analysis (average read depth: >1000x) to detect mutations in *TSC1/TSC2* in cfDNA samples isolated from plasma of patients with LAM. The results of the project were summarized in the prestigious *European Respiratory Journal* (5y-IF₂₀₁₉=11.706) (publication **N12**).

5.2.3. EPISTOP – comprehensive multi-omic analysis in search of epilepsy biomarkers

The analysis was performed in an international, multi-center collaboration of the scientific institutions from Poland, Germany, the Netherlands, Great Britain, Italy, Austria, Belgium, the Czech Republic, Australia, and the United States, as part of the EPISTOP project (the European Union 7th Framework Programme for Research, PI of the project: prof. Sergiusz Jozwiak, Children's Memorial Health Institute in Warsaw). EPISTOP is a study of early childhood epilepsy in TSC patients aimed at explaining the mechanisms of epilepsy and finding ways to prevent it in children. It is the first program in the world in which epilepsy was studied from the stage of the appearance of changes in the brain before epileptic seizures are observable.

In the part of the project that we call 'molecular EPISTOP' and that I was involved in, a comprehensive multi-omic analysis was performed using integrative analysis of gene expression, genomic, metabolomic, and proteomic data. The analysis was done on blood samples from 93 children with TSC (age 0-2 years) included in a clinical trial of presymptomatic vigabatrin therapy for the prevention of epileptic seizures. The aim of the analysis was to identify biomarkers of the development of epileptic seizures. My participation in this project encompassed the selection and characterization of single nucleotide polymorphisms (SNPs) associated with epilepsy. The selected SNPs were included in the analysis, which led to the identification of integrative multi-omic models predicting the development of epileptic seizures in infants with TSC. The project was summarized in the renowned journal *Nature Communications* (5y-IF₂₀₂₂= 17.0) (publication **N14**).

In addition to the above-mentioned projects, during my post-doc in Boston, I had a minor role in preparing a case study article in collaboration with Laura Farach from UTHealth Houston and Children's Memorial Hermann Hospital in Houston (publication in the journal *Gene* (5y-IF₂₀₂₂= 3.3), **N15**), in which I participated in the assessment of the pathogenicity of the c.4255_4256delCA mutation in *TSC2*.

It is worth noting that based on the experience gained during my post-doc, I was also employed as a consultant in the field of cancer genetics in the pharmaceutical company AADi Bioscience Inc (California, USA) (May 2021 - July 2021 and October 2021 - February 2023), in a clinical trial of nab-sirolimus therapy for cancers with mutations in *TSC1/TSC2* (clinicaltrials.gov id: NCT05103358, <https://clinicaltrials.gov/search?cond=NCT05103358>). My role was to visualize the patient's genetic data (preparation of lollipop and co-mutation plots) and to advise and prepare expert opinions on the assessment of the pathogenicity of mutations in *TSC1 /TSC2* in patients included in this clinical trial in the context of the applied therapy. I was also responsible for recording and characterizing genetic data for patients included in the study, using the online database Medidata Rave for Electronic Data Capture (EDC) System.

Following my postdoctoral fellowship, in April 2023, I started working as an assistant professor at the Department of Molecular Genetics, and since January 2024, I have held the position of Head of the Department of Cancer Genetics at IBCH PAS in Poznan. Since March 2024, I have been carrying out the Polish National Science Centre grant OPUS25 entitled 'Ultrasensitive profiling of mutations driving cancer in hereditary syndromes associated with inactivation of suppressor genes', which I lead as a PI. We are working on the project in a 3-person team (including a post-doc and a PhD student) in international collaboration with leading experts in clinical genetics and syndromes associated with tumor suppressor genes inactivation, including:

- (i) Dr. David'em J. Kwiatkowski, Cancer Genetics Laboratory, Brigham and Women's Hospital, Boston, United States;
- (ii) Dr. Neil Rajan, Translational and Clinical Research Institute, Newcastle University, Newcastle upon Tyne, United Kingdom;
- (iii) Dr. Kuniaki Seyama, Division of Respiratory Medicine, Faculty of Medicine, Juntendo University, Tokyo, Japan.

In addition, I continue my scientific collaboration with Dr. David Kwiatkowski, participating in projects related to (i) somatic genetic variation in TSC tumors, and (ii) the search for genes/mutations that modify the phenotype in patients with TSC.

In the future, I plan to expand the research activity of our Department, focusing on ultrasensitive analysis of low-level mutations in various skin cancers, including BCC.

My research achievements after obtaining the PhD degree have been recognized with 6 awards/stipends; out of these, I consider the following two awards to be the most prestigious:

(i) the 'Research Excellence Award 2020 and 2021' for outstanding scientific achievements, awarded by The Brigham Research Institute's Research Oversight Committee (Brigham and Women's Hospital, Boston, USA), and (ii) a stipend in the habilitation category in the 'L'Oreal-UNESCO For Women in Science 2024' program.

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

6.1. Teaching achievements

- Supervision of 6 students (high school, college, and Master's/graduate students, including students from Massachusetts Institute of Technology (MIT) and Boston University), 1 PhD student, and 3 post-docs, including:
 - Mentoring Master's student Klaudia Wieczorek (4th and 5th year student of biotechnology at Poznan University of Life Sciences) during my doctoral studies in the Department of Molecular Genetics at the Institute of Bioorganic Chemistry Polish Academy of Sciences in Poznan
April 2015 – July 2016
Master's Thesis supervisor: prof. dr hab. Piotr Kozłowski
Title of Master's Thesis: *'Analysis of genetic variants associated with hereditary predisposition to breast cancer.'*
 - Mentoring intern Karly Hamilton (1st-year high school student, Brimmer School in Chestnut Hill, MA, USA) during my postdoctoral fellowship in Cancer Genetics Laboratory, Brigham and Women's Hospital/Harvard Medical School (Boston, USA)
July – August 2019
Intern's article: <https://bwhclinicalandresearchnews.org/2019/09/06/insights-from-a-summer-of-science/>
Subject of the internship: experimental research in the field of TSC genetics
 - Mentoring intern Jenna Smith (2nd-year college student, University of Minnesota) during my postdoctoral fellowship in Cancer Genetics Laboratory, Brigham and Women's Hospital/Harvard Medical School (Boston, USA)
July – August 2019
Subject of the internship: assistance in developing a bioinformatics script for analyzing MPS data
 - Mentoring intern Mariana Meza Vazquez (at the moment of internship initiation, 5th year student of Molecular Medicine at Norwegian University of Science and Tech (NTNU) in Trondheim, Norway); mentoring initiated during my postdoctoral fellowship in Cancer Genetics Laboratory, Brigham and Women's Hospital/Harvard Medical School (Boston, USA)
July 2020 – present

Subject of the internship: remote research, currently participating in preparing a review article summarizing the spectrum of somatic mutations in human skin

- Mentoring intern Victoria Dziecioł (at that time 2nd year college student, Massachusetts University of Technology (MIT), Cambridge, USA) during my postdoctoral fellowship in Cancer Genetics Laboratory, Brigham and Women's Hospital/Harvard Medical School (Boston, USA)

September 2020 – May 2021

Subject of the internship: Analysis of genomic data in EPISTOP project – searching for genes modifying TSC patients phenotype

- Mentoring intern John Lee (at the moment of internship initiation, 2nd year student of college, Boston University, Boston, USA) initiated during my postdoctoral fellowship in Cancer Genetics Laboratory, Brigham and Women's Hospital/Harvard Medical School (Boston, USA)

June 2021 – present

Subject of the internship: analysis of the genome sequencing data from TSC patients skin tumors

- Supervision of a post-doc Victoria-Elisabeth Gruber during my postdoctoral fellowship in Cancer Genetics Laboratory, Brigham and Women's Hospital/Harvard Medical School (Boston, USA)

October 2021 – July 2022

Subject: experimental research in the field of human genetics; analysis of somatic mutations in lung cysts in patients with lymphangioleiomyomatosis (LAM)

- Supervision of a post-doc Elias Bou Farhat during my postdoctoral fellowship in Cancer Genetics Laboratory, Brigham and Women's Hospital/Harvard Medical School (Boston, USA)

July 2022 – March 2023

Subject: experimental research in the field of human genetics; analysis of somatic mutations in lung cysts in patients with lymphangioleiomyomatosis (LAM)

- Mentoring a PhD student, mgr inż. Marcin Drzewiecki in the framework of grant OPUS25, which I lead as a PI in the Department of Cancer Genetics at the Institute of Bioorganic Chemistry Polish Academy of Sciences in Poznan

April 2024 – present

Subject: experimental research in the field of human genetics; Ultrasensitive profiling of mutations driving tumorigenesis in hereditary syndromes associated with tumor suppressor genes inactivation

My current role: co-supervisor. After obtaining the habilitation degree, I would like to become his supervisor.

- Mentoring post-doc dr Izabela Broniarek in the framework of grant OPUS25, which I lead as a PI in the Department of Cancer Genetics at the Institute of Bioorganic Chemistry Polish Academy of Sciences in Poznan
January 2025 – obecnie
Subject: experimental research in the field of human genetics; Ultrasensitive profiling of mutations driving tumorigenesis in hereditary syndromes associated with tumor suppressor genes inactivation
- Lecture for Kitasato University students– Exchange Program 2022, organized in collaboration between the Department of Biosciences, School of Science, Kitasato University (Tokyo, Japan) and Cancer Genetics Laboratory, Brigham and Women's Hospital/Harvard Medical School (Boston, USA)
The program aims to encourage students to do scientific work and conduct research in the international scientific environment at foreign fellowships.
- I gained additional knowledge and skills in mentoring students thanks to participation in workshops 'Science Mentoring Workshop Intensives 2020-2021' organized by Harvard University.

6.2. Organizational achievements

- Since January 2024, I have held the position of Head of Department of Cancer Genetics in the Institute of Bioorganic Chemistry Polish Academy of Sciences in Poznan.
 - I gained organizational skills and experience in research projects management in Poland and USA, as a PI of Polish National Science Centre grants PRELUDIUM and OPUS, and prestigious *TSC Alliance Postdoctoral Fellowship*. Additional knowledge on management of research team and projects thanks to participation in 'Leadership for the Researchers 2022' organized by Harvard University.
 - Since 02.2024, I have been a member of the advisory board of the Director of the Institute of Bioorganic Chemistry of Polish Academy of Sciences in Poznan, on the development of young scientists employed in the Institute.
 - I participated in the organization of two conferences, including:
 - Conference 'PHYSICIAN-PATIENT-SCIENTIST: collaboration in rare diseases 2024' 28.02.2024, online, celebration of the International Day of Rare Diseases (funded by a grant from the Ministry of Science and Higher Education under the 'Excellent Science II' Program)
- Role:
- member of the scientific committee
 - member of the organizing committee

- chair of the lecture session 'PHYSICIAN' with the participation of distinguished experts in the field of genetics and molecular biology of rare diseases
- Conference '15th International Conference of Contemporary Oncology', 17-20.04.2024, online, Poznan, Poland

Role:

Chair of the session 'Genetics and Cancer' (<https://www.termedia.pl/Konferencja-15th-International-Conference-of-Contemporary-PROGRAM-ENG,2217,29375.html>)

- At the 151st Session of the General Assembly of PAS, I was elected as a new member of the prestigious Polish Young Academy of PAS (term 2024-2029).

Members of the Polish Young Academy PAS are distinguished by outstanding research achievements and science popularization activities. Polish Young Academy PAS was established to promote scientific research conducted by young scientists. Its goal is to motivate the community of young researchers through different activities, including presenting opinions and programs related to scientific matters; organizing debates, discussions, and scientific conferences, and promoting ethical standards among young scientists.

- Member of a poster committee ('poster judge') at a conference '18th Annual Broad Institute Scientific Retreat 2022' organized by Broad Institute of MIT and Harvard, Boston, USA

6.3. Popularization of science

- Participation in organizing 'Researcher's Night' (2014-2017) and 'Art and Science Festival', organized in the Institute of Bioorganic Chemistry PAS in Poznan

Both events promote science among students and the general public through observation and participation in research experiments.

- Lecture at 'Women in Medicine & Science 2021' symposium, promoting research achievements of women scientists and physicians in Brigham and Women's Hospital (Boston, USA)

'Women in Medicine & Science' symposium annually organized at Brigham and Women's Hospital in Boston, supports and promotes the scientific achievements of talented women in research to encourage the next generation of women to scientific work.

- Lecture at the conference 'Young Science Beyond Borders 2024'
<https://amu.pan.pl/ysbb/programme-outline-2024/>

Conference organized by the Polish Young Academy of PAS, promoting scientific work in Poland among the international community of students and young researchers.

- Activity in the Events Team of the Polish Young Academy of PAS (term 2024-2029)

Participation in the organization of conferences and events promoting science, including:

- *Participation in the organization of 'Forge of Young Talents 2025' (scheduled event date: September 2025, <https://amu.pan.pl/kmtamu/>), educational workshops for PhD students covering topics related to preparing research grant applications, statistics and data analysis in research practice, as well as international cooperation in the scientific community*

My role: Deputy Chair of the Program Council, lecturer.

- *Participation in conducting 'Mine of Young Scientists 2025' (scheduled initiation of the mentoring part of the event: kwiecień 2025, <https://amu.pan.pl/kmn/>), mentoring program for high school students, aimed at promoting scientific achievements and scientific work, as well as encouraging young people to pursue a career in science*

My role: mentor.

7. Other important information on professional career

My scientific achievements have been recognized with 15 awards/stipends in Poland, United States, and the United Kingdom, including:*

**The most prestigious marked in blue*

2024 Stipend in the habilitation category in 'L'Oreal-UNESCO for Women in Science 2024' program awarded by L'Oreal in collaboration with UNESCO, Ministry of Science and Higher Education, Polish Academy of Sciences and UN Global Compact Network Poland

2024 Joining the prestigious Polish Young Academy of the Polish Academy of Sciences as a member for the 2024-2029 term, based on the election at the 151st PAS General Assembly

2022 One Minute Abstract Award awarded by the organizers of International LAM Research Conference & Lamposium 2022, Chicago, USA

2021 Early Career Researcher Award awarded by the organizers of International Tuberous Sclerosis Complex Research Conference 2021 (virtual conference, UK)

2021 Research Excellence Award 2021 for outstanding scientific achievements, awarded by The Brigham Research Institute's Research Oversight Committee (Brigham and Women's Hospital, Boston, USA)

2020 Research Excellence Award 2020 for outstanding scientific achievements, awarded by The Brigham Research Institute's Research Oversight Committee (Brigham and Women's Hospital, Boston, USA)

2019 Award for the best PhD dissertation defended in 2017/2018, awarded by the scientific council of the city of Poznan

2018 Award from the Poznań branch of the Polish Academy of Sciences for the best research paper, with the PhD student as the lead author, for Klonowska et al., Oncotarget, 2017, 8(44): 76357-76374

2017 Travel award by the European Society of Human Genetics for participation in 'The European Human Genetics Conference 2017: 1967 - 2017: 50th Anniversary of the ESHG' in Copenhagen, Denmark

2016 Stipend for talented young scientists from Poznan area, awarded by the scientific council of the city of Poznan

2016 Conference scholarship from the National Scientific Leadership Center (KNOW), Poland funding, for participation in "The American Society of Human Genetics 2016 Annual Meeting" in Vancouver, Canada

2016 Scholarship of the president of the Polish Academy of Sciences 2016 for the best PhD students

2015 Travel award by the Wellcome Trust Sanger Institute and Nature Genetics for participation in "The Genomics of Common Diseases 2015" conference in Hinxton, UK

2015-2017 Scholarships for the best PhD students awarded by the Institute of Bioorganic Chemistry of Polish Academy of Sciences in Poznan

2012-2013 Adam Mickiewicz University Rector Scholarship for the best students

Detailed information on my scientific activities, including:

- full list of published articles in scientific journals (n=22);
 - full list of oral lectures (n=17) and conference posters (n=15 as presenting author, and n=7 as coauthor);
 - information on participation in grants/scientific projects (n=11);
 - list of foreign fellowships (n=2);
 - information on reviewed scientific articles (n=12) and other
- can be found in 'Attachment_5_List_of_scientific_achievements'.*

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The image shows an electronic signature stamp. On the left is a square icon containing a stylized signature. To the right of the icon, the text reads: "PODPIS ZAUFANY" (Trusted Signature) in a dark box, followed by "KATARZYNA KLONOWSKA" in bold, the timestamp "06.05.2025 11:42:53 [GMT+2]", and the phrase "Dokument podpisany elektronicznie podpisem zaufanym" (Document signed electronically with a trusted signature).

PODPIS ZAUFANY
KATARZYNA
KLONOWSKA
06.05.2025 11:42:53 [GMT+2]
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podpisem zaufanym

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