SPATIOTEMPORAL SCRUTINY OF CLONAL EVOLUTION FOR TREATMENT PERSONALIZATION IN CHRONIC LYMPHOCYTIC LEUKEMIA

FINAL REPORT
(K119950, K_16, 2016.12.01 - 2021.05.29)

Introduction

In 2016, our group established the Hungarian Ibrutinib Resistance Analysis Initiative with the specific aim to collect clinical samples and annotations of chronic lymphocytic leukemia (CLL) patients receiving targeted therapies in Hungary. This framework greatly facilitated the nationwide collaboration between the oncohematological centers and the host laboratory located in the 1st Department of Pathology and Experimental Cancer Research, Semmelweis University. To date, 1,298 specimens from 576 patients have been collected and stored in our biobank, with 455 of these patients treated with ibrutinib, 29 patients with acalabrutinib and 92 patients receiving venetoclax. Due to a change in the indication of ibrutinib treatment in Hungary in 2016, we have received a massively reduced number of bone marrow and lymph node samples as compared to the initially expected figures. This shortcoming was however, greatly compensated by the large number of peripheral blood samples from which we archived mononuclear cell fraction and circulating cell-free DNA in our national registry.

Major scientific accomplishments

DNA copy number analysis by multiplex ligation-dependent probe amplification (MLPA) has been performed on a subset of CLL patients using the SALSA P037 and P038 MLPA probemixes, allowing for the copy number assessment of 41 and 35 disease-relevant genomic loci, respectively. Diagnostic samples as well as specimens collected before and during ibrutinib treatment were analyzed and the results were successfully validated by fluorescence *in situ* hybridization (FISH). Data acquired by the investigation of an initial cohort of patients provided basis for a paper reporting the first CLL focused MLPA study in Hungary (*Kiss et al. Hematology-Transfusiology 2018*). By complementing traditional methods, MLPA can provide a more comprehensive picture of disease-associated genetic complexity and contribute to the efficient prognostic classification of patients in the country. Along this line, we also published a Hungarian review paper on the application of MLPA in oncohematology (*Kiss et al. Orv Hetil 2018*).

In 20 CLL patients receiving ibrutinib monotherapy, mutation screening covering all recurrently affected (>2%), disease-relevant genes was performed by ultra-deep next-generation sequencing (NGS) analysis (Fig. 1). Our collection of serial samples allowed for a time-resolved investigation, providing insight into clonal evolution, subclonal dynamics and treatment resistance mechanisms associated with ibrutinib treatment. Besides the canonical mutations, four novel *BTK* mutations and three previously unreported *PLCG2* variants were identified. A subset of aberrations was confirmed with Sanger sequencing and/or digital droplet PCR. *BTK* and *PLCG2* mutations were backtracked in five patients using digital droplet PCR and were detectable on average 10.5 months before the clinical relapse. With a median follow-up time of 36.5 months, all but one (7/8) patients harboring *BTK* mutations showed disease progression based on clinical and/or laboratory features. Intriguingly, analysis of the subclonal architecture identified a novel alternating pattern of clonal dynamics of *BTK* and *TP53*

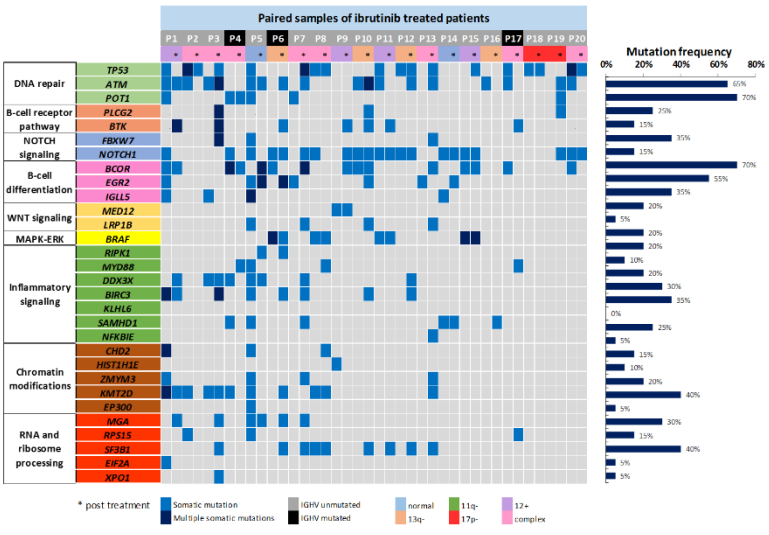

Figure 1. *Heat map displaying somatic variants detected in sequential samples of 20 patients treated with ibrutinib.*

mutations. The majority of *BTK* mutations were detectable following reduction or elimination of *TP53* mutated subclones and conversely, were never acquired on the ground of persisting or expanding *TP53* mutated subclones. (*Gángó et al. Int J Cancer 2020, Q1 journal*) Our group also published an extensive Hungarian review paper on ibrutinib resistance (*Aczél et al. Hematology-Transfusiology 2019*).

Acquired mutations emerging at position C481 in the *BTK* tyrosine kinase domain are the predominant genetic alterations associated with secondary ibrutinib resistance in patients with CLL. To assess the correlation between disease progression, and the emergence and temporal dynamics of the most common resistance mutation BTK^{C481S}, in an extended study, sensitive time-resolved molecular screening was performed in peripheral blood samples of 83 relapsed/refractory (R/R) CLL patients during single-agent ibrutinib treatment. With a median follow-up time of 40 months, BTK^{C481S} was detected in 40/83 patients, with 32/40 of them showing disease progression during the

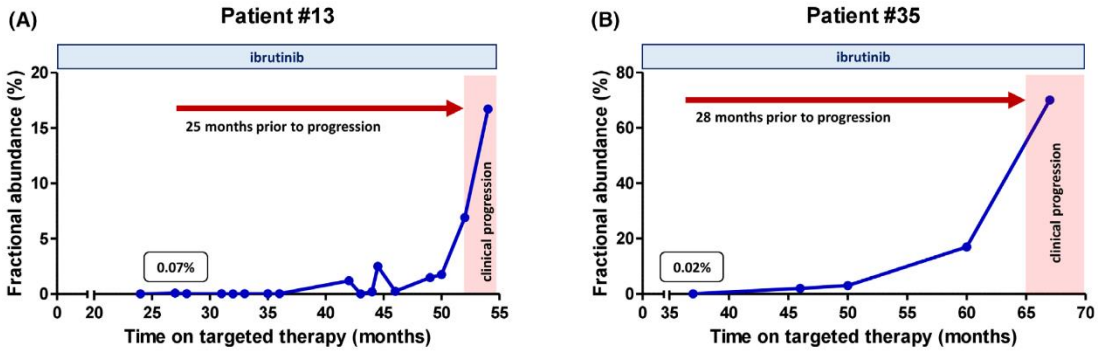

Figure 2. *Temporal dynamics of BTK^{C481S} resistance mutation in two relapsed CLL patients receiving ibrutinib therapy.*

examined period. In these 32 cases representing 72.7% (32/44) of all patients experiencing relapse, emergence of the BTK^{C481S} mutation predated the symptoms of clinical relapse with a median of 9 months (range: 0-28 months) (Fig. 2). Subsequent Bcl-2 inhibition therapy applied at 28/32 patients harboring BTK^{C481S} and progressing on ibrutinib conferred clinical and molecular remission ubiquitously across the patients. This study provided real-life evidence for the clinical value of sensitive BTK^{C481S} monitoring, which allows for the prediction of an impending relapse in ibrutinib treated R/R CLL patients well before the first clinical signs of disease progression (*Bödör et al. Br J Haematol 2021, Q1 journal*).

In-depth scrutiny of spatial genomic heterogeneity and targeted treatment associated subclonal dynamics was performed in an index patient with CLL undergoing ibrutinib treatment and subsequent venetoclax therapy followed by autologous stem cell transplantation (Fig 3). Lymph node and blood samples including peripheral blood mononuclear cells and isolated blood plasma fraction were available at multiple time points before and during targeted therapy, providing an opportunity for the spatiotemporal screening of driver mutations in the nucleated cell compartment as well as in circulating cell-free DNA specimen. In the paper summarizing the results, we documented for the first time an example of ibrutinib driven spatial convergent evolution leading to disease progression and transformation (*Kiss et al. Haematologica 2018, D1 journal*).

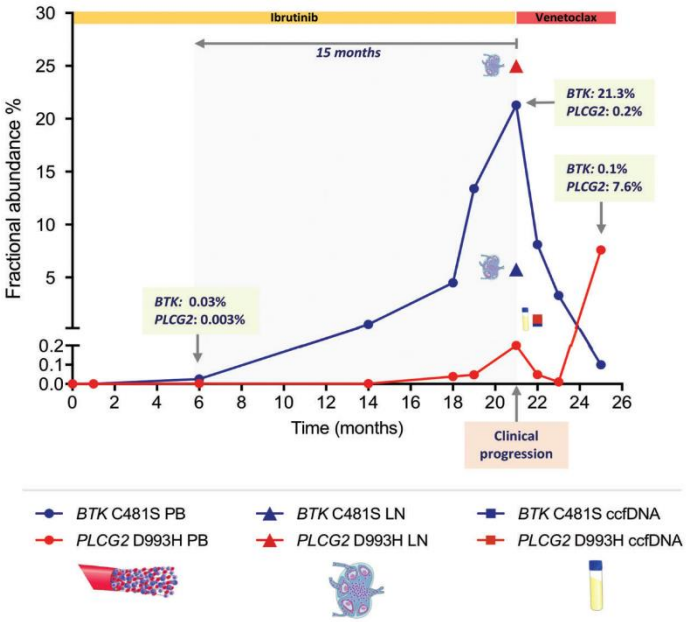

Figure 3. *Spatiotemporal dynamics of BTK and PLCG2 mutations.*

In collaboration with Christoph Bock's group at CeMM (Vienna), high-resolution time courses of ibrutinib treatment have been investigated in patients with CLL, combining immunophenotyping/FACS sorting, large-scale single-cell transcriptome profiling, and genome-wide chromatin mapping (Fig 4). We identified a consistent regulatory program starting with a sharp decrease of NF-κB binding in CLL cells, which is followed by reduced activity of lineage-defining transcription factors, erosion of CLL cell identity, and acquisition of a quiescence-like gene

signature. Patient-to-patient variation in the speed of execution of this program was observed and we exploited this phenomenon to predict patient-specific dynamics in the response to ibrutinib based on the pre-treatment patient samples. This study utilizing a highly complex bioinformatic analysis including machine learning described time-dependent cellular, molecular, and regulatory effects for therapeutic inhibition of B cell receptor signaling in CLL, and established a broadly applicable method for epigenome/transcriptome-based treatment monitoring (*Rendeiro et al. Nat Commun 2020, D1 journal*).

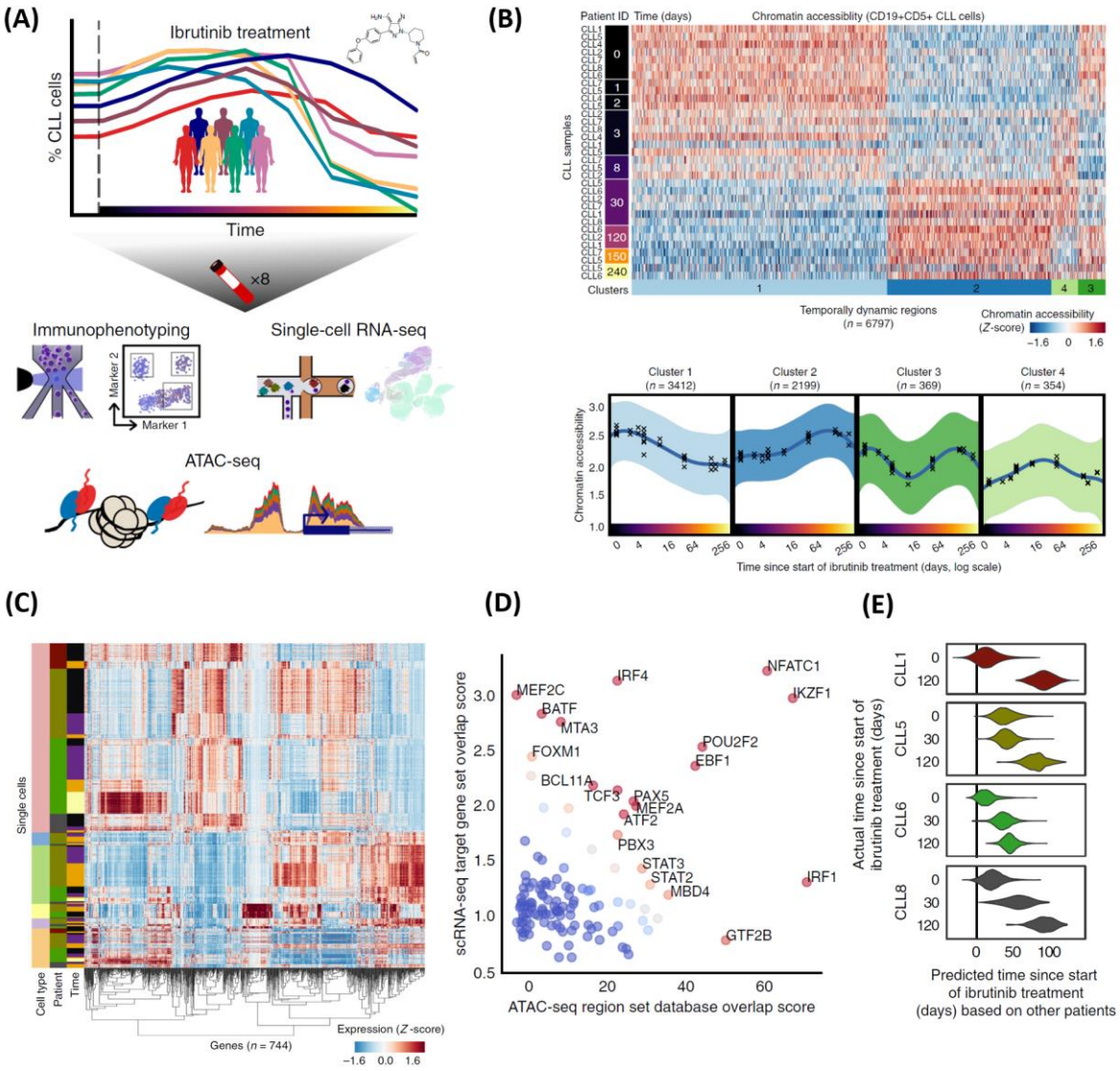

Figure 4. *Multiomics analysis of ibrutinib time courses. (A) Schematic representation of the study design. Peripheral blood from patients with CLL undergoing single-agent ibrutinib therapy was collected at defined time points and assayed by flow cytometry (cell composition and immunophenotype), single-cell RNA-seq (gene expression), and ATAC-seq (chromatin accessibility). (B) Heatmap showing changes in chromatin accessibility for CLL cells over the time course of ibrutinib treatment, based on ATAC-seq data. (C) Clustered single-cell transcriptome heatmap for the most differentially expressed genes between time points. (D) Scatterplot showing differential regulation of transcription factors. (E) Violin plots showing the predicted (x-axis) and actual (y-axis) number of days under ibrutinib therapy in each patient. Predictions were derived from regression models.*

Richter syndrome (RS) represents the development of high-grade lymphoma in patients with CLL or small lymphocytic lymphoma (SLL) and presents a diagnostic and therapeutic challenge with an adverse prognosis. To understand the morphological and molecular changes leading to RS in CLL patients treated with ibrutinib and venetoclax, sequential samples from six CLL/SLL patients undergoing RS were characterized including detailed immunophenotypic analysis as well as detection of immunoglobulin heavy chain gene (IGH) rearrangements, *TP53* mutations, drug-induced resistance mutations in the *BTK* and *BCL2* genes and various copy number changes and point mutations identified by MLPA. Rare, non-diffuse large B cell lymphoma phenotypes of RS were observed in 3/6 cases, including plasmablastic lymphoma and a transitory entity between diffuse large B cell lymphoma and

classical Hodgkin lymphoma. The majority of cases were clonally related and harbored an unmutated variable region of the *IGH* gene. Abnormalities affecting the *TP53* gene occurred in all patients, and every patient carried at least one genetic abnormality conferring susceptibility to RS. In the background of RS, 2/5 patients treated with ibrutinib showed a *BTK*^{C481S} resistance mutation. One patient developed a *BCL2*^{G101V} mutation leading to venetoclax resistance and RS (*Gángó et al. Pathology 2021, Q1 journal*).

Project related technology developments

During the study period, several technologies have newly been established and optimized in our laboratory, including MLPA, digital MLPA, digital droplet PCR and a high number of NGS based applications. Beyond CLL, these techniques have efficiently been utilized to the investigation of other hematological cancers such as myelodysplastic syndrome, acute lymphoblastic leukemia, multiple myeloma, primary central nervous system lymphoma and follicular lymphoma. Most of these applications have also been incorporated into the routine diagnostic workflow of the host department.

Studies beyond CLL using the newly established technologies

MLPA

A pedigree with two siblings and their parents diagnosed with myelodysplastic syndrome was analyzed using MLPA. Partial deletion within the *TERC* gene was identified in samples of the two siblings, with the same aberration also detected in the father's DNA. Multiple occurrences of this genetic abnormality in the family underlined the importance of screening for DNA copy number alterations in patients with myelodysplastic syndrome as well as assembling a properly detailed anamnesis for the whole family. The genetic variant and its recognition also influence the further therapeutic management of the family, as predisposition syndromes may well be present in individuals displaying no specific phenotype (*Kotmayer et al. Hematology-Transfusiology 2018*).

In collaboration with Frederik W. van Delft and Mel F. Greaves (London), genomic characterization was undertaken on a retrospective discovery cohort of 80 patients aged 15–26 years with primary or relapsed T-cell acute lymphoblastic leukemia (T-ALL), using a combination of SNP Array, targeted gene mutation and promoter methylation analyses. Findings were confirmed by MLPA, real-time quantitative PCR, and FISH. Whole Exome Sequencing was performed in 4 patients with matching presentation and relapse samples to model clonal evolution. Genomic characterization of our cohort of TYA T-ALL patients identified recurrent isochromosome 7q i(7q) (n = 3). Analysis of 6 pairs of matched presentation relapsed T-ALL established that all relapses were clonally related to the initial leukemia. Whole exome sequencing analysis revealed recurrent, targetable, mutations disrupting NOTCH, PI3K/AKT/mTOR, FLT3, NRAS as well as drug metabolism pathways. All genetic aberrations in TYA T-ALL occurred with an incidence similar or intermediate to that reported in the pediatric and adult literature, demonstrating that overall TYA T-ALL exhibits a transitional genomic profile. Analysis of matched presentation relapse samples supported the hypothesis that relapse is driven by the Darwinian evolution of sub-clones associated with drug resistance (NT5C2 and TP53 mutations) and re-iterative mutation of known key T-ALL drivers, including *NOTCH1* (*Mansur et al. Cancer Med 2021, D1 journal*).

Digital MLPA

Besides MLPA, our group gained an early access to digital MLPA via its collaboration with the MRC-Holland (Amsterdam). This recently developed technique combines the advantages of MLPA and NGS. The method is highly scalable and allows for the analysis of an approximately ten times higher number of genomic loci as compared to conventional MLPA. One of the first digital MLPA probemixes was designed for screening genomic aberrations including copy number aberrations and a point mutation in plasma cell myeloma and our group had the opportunity to use this newly established technique for studying mature B-cell malignancies for the first time (*Kosztolanyi et al. J Mol Diagn 2018, D1 journal*). Alterations identified using digital MLPA were successfully confirmed by conventional MLPA, FISH and digital droplet PCR.

Our second digitalMLPA study was focused on pediatric acute lymphobastic leukemia (ALL). Bone marrow samples of 91 children with B- or T-cell ALL were analyzed. Aberrations were observed in 96% of diagnostic patient samples, and increased numbers of copy number aberrations were detected at the time of relapse as compared with diagnosis. Comparative scrutiny of 24 matching diagnostic and relapse samples from 11 patients revealed three different patterns of clonal relationships: (*i*) recurrence of the original leukemic clone, (*ii*) clonal evolution and (*iii*) presence of a common ancestral cell compartment. Clonal relationship between B-cell precursor cell populations prevailing at different time points during the disease course was also investigated by deep-sequencing of the immunoglobulin heavy chain (*IGH*) gene rearrangements and the results supported our conclusions drawn based on the digitalMLPA profiles. In addition, we used digitalMLPA for determining copy number aberration based risk groups which were then also combined with karyotyping and molecular (cyto)genetic data in order to establish an extended prognostic classifier for patients with B-cell precursor ALL. This novel risk assessment distinguished four combined genetic risk groups showing significantly different 5-year event-free survival rates (*Kiss et al. Mod Pathol 2020, D1 journal*).

NGS

Primary central nervous system lymphomas (PCNSL) are aggressive non-Hodgkin lymphomas affecting the central nervous system (CNS) and showing activated B-cell (ABC) or germinal center (GC) origin. We determined the molecular subtype of 77 PCNSL and 17 secondary CNS lymphoma patients, using the novel NanoString Lymphoma Subtyping Test (LST), a gene expression-based assay representing a more accurate technique of subtyping compared with standard immunohistochemistry (IHC). In addition, mutational landscapes of 14 target genes were determined by ultra-deep NGS. Using the LST-assay, a significantly lower proportion of PCNSL cases displayed ABC phenotype compared with the IHC-based characterization (80% vs 95%). The most frequently mutated genes included *MYD88*, *PIM1*, and *KMT2D*. Among patients with ABC-type PCNSL, aberrations predominantly occurred in the *PIM1*, *IRF4*, *CD79B*, *MYC*, *CARD11*, *CSMD2* and *CSMD3* genes, while defects in the *TP53*, *PAX5* and *CCND3* genes were more frequent in GC-type PCNSL. Our results provide a more precise patient stratification potentially applicable in the diagnostic algorithm of PCNSL (*Bödör et al. JNEN 2020, Q1 journal*).

Digital droplet PCR

We demonstrated on four follicular lymphoma patients that circulating tumor DNA based *EZH2* mutation analysis performed by a highly sensitive droplet digital PCR method may be a valuable treatment monitoring approach in *EZH2* mutant follicular lymphoma. *EZH2* variant allele frequencies changed in parallel with the volume of metabolically active tumor sites observed with PET-CT scans. Variant allele frequencies of *EZH2* mutations decreased or were eliminated rapidly upon successful treatment, with treatment failure being associated with elevated *EZH2* variant allele frequencies. We also demonstrated spatial heterogeneity in a patient with two different *EZH2* mutations in distinct anatomical sites, with both mutations simultaneously detected in the liquid biopsy specimen. (*Nagy et al. Genes 2020, Q2 journal*). Our group also published a Hungarian review paper on the potential applications of liquid biopsy analyses in oncohematology (*Nagy et al. Hematology-Transfusiology 2020*)

Deviation from the original plan

Scientific plan

Bone marrow and lymph node biopsies were typically not received during the study period despite the consent and supporting letter provided by all oncohematological centers before the commencement of the project. This was mainly caused by a modified financing scheme of ibrutinib treatment introduced in Hungary in 2016, which commonly allowed for the administration of this targeted therapy before the first signs of lymphadenopathy or bone marrow failure, diminishing the significant clinical need for invasive biopsy. These circumstances limited our opportunity to explore the spatial heterogeneity of CLL cell populations in patients with ibrutinib and targeted therapies. Nevertheless, we still could publish a D1 paper with a special focus on spatiotemporal heterogeneity.

In vitro compound screening has eventually not been performed due to the reduced niche conferred by high-profile papers of competitor groups (*e.g. Schmidl et al. Nat Chem Biol 2019*). Instead, we performed a detailed multiomic

profiling of molecular dynamics in CLL cells and other immune cell populations upon ibrutinib treatment using flow cytometry, ATAC-seq and large-scale RNA-seq, that altogether allowed for a robust prediction of patient-specific molecular responses.

Financial plan

The originally planned purchase of a -80°C freezer was covered by another budget. The corresponding amount has been spent on a Bio-Rad Thermal Cycler that we used for NGS library preparation and for validation studies.

Outlook

Building upon our large biobank and advanced molecular technologies presented above, we aim to continue our nationwide leukemia research program. The host department, as the largest pathology and molecular oncohematology center in Hungary will provide a fully supportive environment for all upcoming studies.

Dissemination of the results

Scientific papers in peer-reviewed journals and PhD theses

In total, **17 papers** were published and **2 PhD theses** were defended during the study period. **Five** research papers in international peer-reviewed **D1** journals (*Kosztolányi et al. J Mol Diagn 2018, Kiss et al. Haematologica 2019, Kiss et al. Mod Pathol 2020, Rendeiro et al Nat Commun 2020 and Mansur et al. Cancer Med 2021*), **four** research papers in international peer-reviewed **Q1** journals (*Gángó et al. In J Cancer 2020, Bödör et al. J Neuropathol Exp Neurol 2020, Bödör et al. Br J Haematol 2021 and Gángó et al. Pathology 2021*), **one** research paper in an international peer-reviewed **Q2** journal (*Nagy et al. Genes 2020*), one research paper and one review paper in the leading Hungarian medical journal (*Kiss et al. Orv Hetil 2018, Kosztolányi et al. Orv Hetil 2019*), as well as two research papers and three review papers in the official journal of the Hungarian Society of Haematology and Transfusion (*Kiss et al. Hematology-Transfusiology 2018, Kotmayer et al. Hematology-Transfusiology 2018, Fésüs et al. Hematology-Transfusiology 2019, Aczél et al. Hematology-Transfusiology 2019, Nagy et al. Hematology-Transfusiology 2020*).

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Conference presentations by group members

Sensitive screening and monitoring of BTK C481S mutations in chronic lymphocytic leukemia using droplet digital PCR. ESH 1st Translational Research Conference: Chronic Lymphocytic Leukaemia. Online, 2020.

Genomic analysis of pediatric acute lymphoblastic leukemia using targeted next-generation sequencing. Semmelweis University - Students' Research Conference. Budapest, Hungary, 2020.

Liquid biopsy-based monitoring of EZH2 mutations in follicular lymphoma: implications for non-invasive disease monitoring and targeted therapy. ESH 2nd How to Diagnose and Treat Lymphoma. Online, 2020.

Comprehensive profiling of disease-relevant copy number aberrations improves risk assessment and unveils the cloncal origin of relapse in pediatric acute lymphoblastic leukemia. 61st ASH Meeting, Orlando, 2019.

Early and sensitive detection of therapy associated BTK resistance mutations in chronic lymphocytic leukemia using droplet digital PCR. Invited Bio-Rad webinar. 2019.

Early detection of ibrutinib resistance associated BTK C481S mutation in chronic lymphocytic leukemia. XXVII. Congress of the Hungarian Society of Haematology and Transfusion, Pécs, 2019.

Detection of venetoclax resistance associated BCL2 G101V mutation in chronic lymphocytic leukemia. XXVII. Congress of the Hungarian Society of Haematology and Transfusion, Pécs, 2019.

Identification of the cell of origin in diffuse large B-cell lymphoma – the first Nanostring results in Hungary. XXVII. Congress of the Hungarian Society of Haematology and Transfusion, Pécs, 2019.

TP53 and IGHV mutation analyses in chronic lymphocytic leukemia. XXVII. Congress of the Hungarian Society of Haematology and Transfusion, Pécs, 2019.

Investigation of genomic copy number alterations in a family with myelodysplastic syndrome using multiplex ligation-dependent probe amplification. XXVII. Congress of the Hungarian Society of Haematology and Transfusion, Pécs, 2019.

Droplet digital PCR based monitoring of EZH2 mutations in liquid biopsy of patients with follicular lymphoma. XXVII. Congress of the Hungarian Society of Haematology and Transfusion, Pécs, 2019.

Detection and monitoring of EZH2 mutations in follicular lymphoma. XXVII. Congress of the Hungarian Society of Haematology and Transfusion, Pécs, 2019.

Spatial convergent clonal evolution leading to ibrutinib resistance and disease progression in chronic lymphocytic leukemia. 60th ASH Meeting, San Diego, 2018.

Personalized diagnostics and therapy in hematological malignancies. International Semmelweis Symposium, Budapest, 2018.

Dissection of clonal evolution by temporal mutation profiling in chronic lymphocytic leukemia patients treated with ibrutinib. Hungarian Medical Association of America, Balatonfüred, 2018.

Rapid and scalable genome-wide profiling of clinically relevant genetic aberrations in multiple myeloma. 23rd Congress of the European Hematology Association, Stockholm, 2018.

NGS platform and liquid biopsy in lymphoma diagnostics. XXI. Lymphoma Conference of the Hungarian Society of Haematology and Transfusion, Debrecen, 2018.

Analysis of the immunoglobulin heavy chain variable region mutational status in chronic lymphocytic leukemia. XXI. Lymphoma Conference, Debrecen, 2018.

Screening for DNA copy number alterations in chronic lymphocytic leukemia by multiple ligation-dependent probe amplification. XXI. Lymphoma Conference, Debrecen, 2018.

Molecular screening for clinically relevant genetic aberrations in myeloma multiplex. XXI. Lymphoma Conference, Debrecen, 2018.

Investigation of ibrutinib induced clonal evolution in chronic lymphocytic leukemia by next generation sequencing. XXI. Lymphoma Conference, Debrecen, 2018.

Identification of novel therapeutic targets and analysis of clonal evolution in hematological malignancies using next generation sequencing. Eurolife Spring Symposium, Budapest, 2018.

Dissection of clonal evolution by temporal mutation profiling in chronic lymphocytic leukemia patients treated with ibrutinib, PhD Scientific Days, Budapest, 2018.

Rapid and comprehensive screening for DNA copy number abnormalities in plasma cell myeloma using a next-generation sequencing based approach. PhD Scientific Days, Budapest, 2018.

Identification of novel biomarkers in B-cell lymphomas using next generation sequencing. Future Research Challenges Conference, Budapest, 2018.

Dissection of clonal evolution by temporal mutation profiling in chronic lymphocytic leukemia patients treated with ibrutinib. 59th ASH Meeting, Atlanta, 2017.

<u>Outreach activities</u>

Quest for the secrets of a dreaded disease - the mysteries of leukemia. Researchers' Night, Semmelweis University, Budapest, 2020.

Leukemia caught in the crosshairs: from diagnosis to targeted therapy. Researchers' Night, Semmelweis University, Budapest, 2019.

Darwin's Finches in Leukemia Research. Researchers' Night, Semmelweis University, Budapest, 2018.

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