Final Report of OTKA (NKFI) 115861K project:

"Extracellular vesicle studies in pediatric malignant hematology"

Extracellular vesicles (EVs) and their relevance in life sciences have recently started unfolding. The aim of this OTKA project was to study if EVs in peripheral blood carry information applicable in disease monitoring and in detection or differential diagnosis of adverse drug reactions in childhood leukemia and lymphoma.

The project has been successful, relevant research has been conducted, the publication targets have been achieved: 3 original articles were published in D1, 3 in Q1 international journals. Two review articles in Hungarian medical journal, one original article is in preparation. Ten lectures (out of this, international: 5) and 13 posters (out of this, international: 9) were presented at conferences. Out of all our findings, CSF miR investigations are the most promising for clinical utilization, we could organize an international consortium and plan to apply for European research funds to further explore this topic. Investigations on circulating miRs in blood plasma as MRD markers in hematological malignancies made sense, but, in view of our results, following further this track is not worthwhile, is not likely to yield clinical benefit.

DESCRIPTION AND RESULTS OF PROJECT ELEMENTS

Sample and databank

In December 2015, we gained approval from the Ethics Committee of the Hungarian Medical Research Council (60106-1/2015/EKU and 6886/2019/EKU). In 2018, we requested addition of control samples to the project and received approval (6886/2019/EKU). The structure of the databank was created; a laboratory was put into shape for sample processing and storage. Nurses, Ph.D. students and undergraduate students were trained for sample collection and processing.

We collected bone marrow (BM), peripheral blood (PB) and cerebrospinal fluid (CSF) samples from pediatric patients with acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), Hodgkin's disease (HD), lymphoblastic lymphoma, diffuse large B-cell lymphoma (DLBCL), Burkitt's leukemia and lymphoma, at sequential time points as description in the original application. Platelet free plasma (PFP) was prepared from the PB and BM samples within 2 hrs of sampling and CSF samples were centrifuged to get rid of cells, both to make the analysis of free circulating miRs possible. All samples were stored at -80 °C. See the detailed description of procedures in our published article: **Rzepiel et al., J Transl Med. 2019; 17: 372**. Besides finished work detailed in this report, aliquots of these samples are available for further planned studies. Data collection went on swiftly with the help of the Hungarian Childhood Cancer Registry and the eMedSol system of the university. The total number of collected samples are demonstrated in Table 1.

Table 1: Samples collected within the framework of NKFI 115861K project

Patient Group	Period of Time					Total
	21/12/2015* - 31/08/2016	01/09/2016 - 31/08/2017	01/09/2017 - 31/08/2018	01/09/2018 - 31/08/2019	01/09/2019 - 31/08/2020	
ALL	25	31	22	24	21	**123**
AML	5	1	6	4	1	**17**
HD	3	4	4	10	1	**22**
NHL	1	2	2	2	5	**12**
Other	5	2	3	3	2	**15**
Total	**39**	**40**	**37**	**43**	**30**	**189**

**Date of ethical authorization, Abbreviations: ALL: acute lymphoblastic leukemia, de novo and relapsed; AML: acute myeloid leukemia, de novo, secondary and relapsed; HD: Hodgkin disease; NHL: non-Hodgkin lymphoma: Burkitt's lymphoma, lymphoblastic lymphoma or diffuse large B-cell lymphoma; Other: Mixed-phenotype acute leukemia and non-malignant conditions*

Flow cytometry studies

When our research grant application was written, two contracts were in process on ultra-flow-cytometers at the Department of Genetics, Cell- and Immunobiology. Their arrival was then delayed year by year. We had to start our work using traditional flow cytometers. Due to the expected size range measurable, we focused on the relatively larger apoptotic bodies.

Altogether 142 PB and BM samples were analyzed by a FACSCalibur (BD) cytometer for extracellular vesicles (EVs), using CellQuest Pro software (BD). We selected 2-2 cell surface markers that we hypothesized might be found on the surface of blast-derived microvesicles or apoptotic bodies; CD10 and CD19 for preB-cell, CD7 and CD99 for T-cell patient samples. We could not identify blast derived apoptotic bodies or microvesicles from fresh plasma samples and from the archived platelet-free plasma (PFP) samples, respectively. Thus, flow cytometry yielded no useful results to be detailed. We concluded that the conventional flow cytometers available for us at the time were not suitable to measure EVs 100-500 nanometres in diameter. We had no results that could have been published. As the new generation, more sensitive flow cytometers designed for EV research were still outstanding, their arrival uncertain, we redirected our focus on circulating micro-RNAs which are mostly transported inside EVs in the PB and BM plasma.

Plasma circulating microRNAs in ALL

We investigated the clinical value of microRNAs (miRs) as biomarkers of residual disease or minimal residual disease (MRD) at early time points of therapy in pediatric ALL, AML and Hodgkin's lymphoma. The largest sample set was available and so the most extensive work done in ALL: total RNA extraction, reverse transcription; selection of target miRs and normalizing RNA. Custom Taqman Low Density Array Card (TLDA) experiment with 48 miRs; statistical evaluation, selection of 4 most promising miRs for further analyses; qPCR experiments with 4 selected miRs; statistical analyses in the whole ALL cohort and in cytogenetic subgroups, correlation testing with standard prognostic factors; publication. Our

novel findings in summary: expression of miR-128-3p, miR-181a-5p, miR-181b-5p and miR-222-3p in PB PFP (peripheral blood derived platelet free plasma) is significantly higher in childhood ALL compared to controls, and higher at the time of ALL presentation compared to later time points in patients under therapy (Figure 1). Trends are similar among cytogenetic subgroups. These miRs behave as residual disease biomarkers in ALL. However, the sensitivity of circulating miRs falls behind that of standard methods and this hampers their clinical utilization. See more details in: **Rzepiel et al., J Transl Med. 2019; 17: 372.**

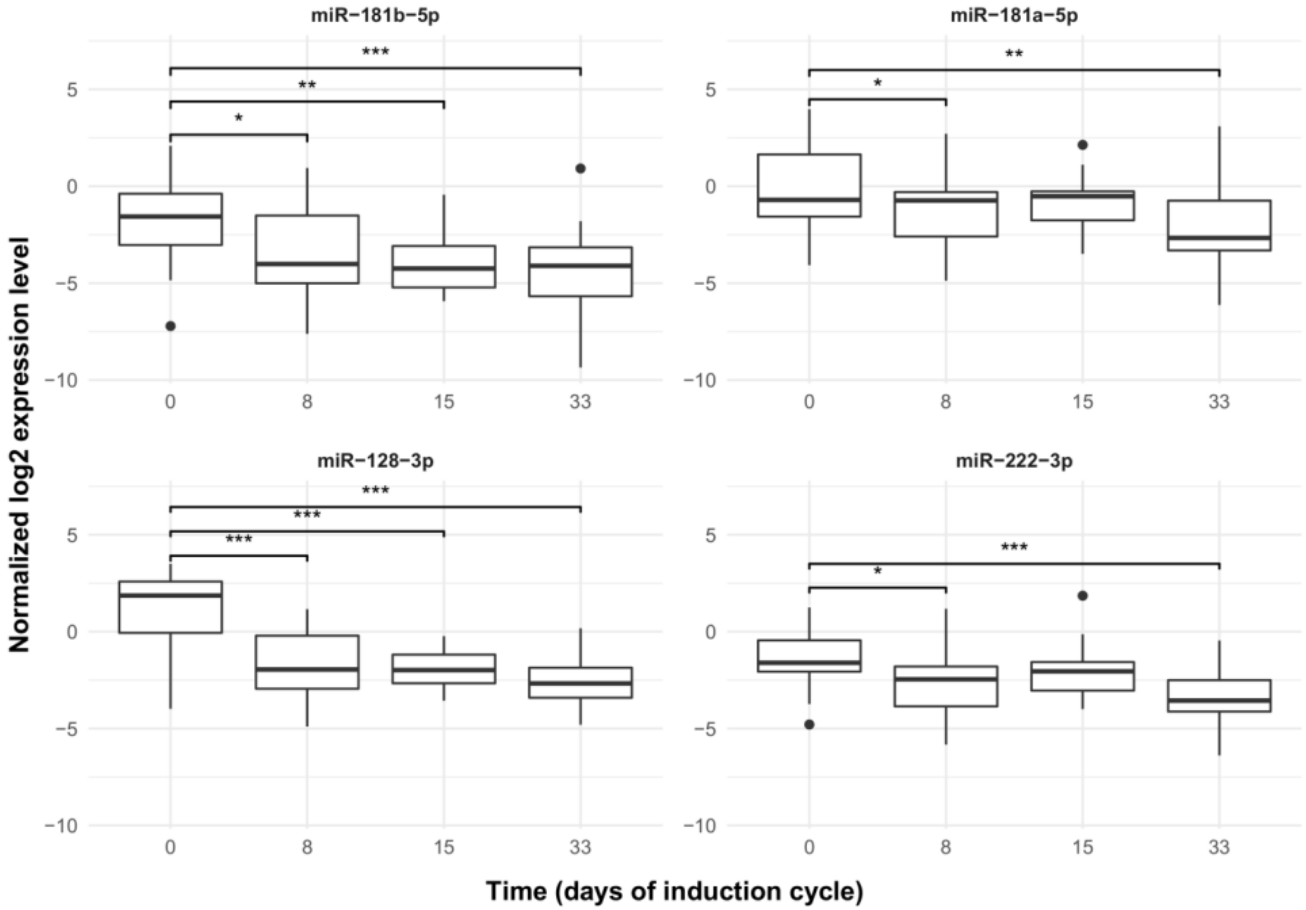

Figure 1.: *MiR expressions in PB PFP during the first month of therapy (by TaqMan Advanced qPCR). Box: the 2nd and 3rd quartiles; thick line in the box: median; whiskers: minimum and maximum values if there are no outrange values, or Q1 − 1.5*IQR; dots: outliers, lines above the boxes: significant correlation ($p < 0.05$); ***p value: $0 \leq p < 0.001$, **p value: $0.001 \leq p < 0.01$, *p value: $0.01 \leq p < 0.05$*

MiRs in cerebrospinal fluid in ALL and AML

Leukemic involvement of the central nervous system (CNS) represents an outstanding therapeutic problem. We lack tools with high sensitivity regarding the CNS niche. The most widespread contemporary methods for the assessment of CSF are conventional cytologic examination of a cytocentrifuge smear and flow cytometry. We hypothesize that some non-cellular biomarkers could identify undiagnosed CNS leukemia, and can be used for monitoring the leukemic invasion to this compartment.

The relative expressions of 47 circulating miRNAs in CSF samples of patients with ALL were determined using the custom-made TLDA card described above. Regarding the discovery study, principal component analysis identified the role of miR-181-family (miR-181a-5p, miR-

181b-5p, miR-181c-5p) in clustering CNS-positive (CNS^+) and CNS-negative (CNS^-) CSF samples. As a validation step, a candidate miR family was further studied with TaqMan Advanced miRNA Assays on serial CSF, BM and PB samples with different acute leukemia subtypes. Furthermore, small EV-rich fractions were isolated from CSF and the samples were processed for immunoelectron microscopy with anti-CD63 and anti-CD81 antibodies, simultaneously. We were able to confirm the high expression of miR-181a-5p in diagnostic CSF samples in a partly independent set of 8 CNS^+ ALL patients compared with 10 CNS^- ALL patients by conventional qPCR (Figure 2). However, similar results were not found when also including AML patients in the analysis. In those patients with ALL, miR-181a-5p expression levels conferred a more than 52-fold increased risk for CNS leukemia (CNS+ vs. CNS−patients: ΔFC=52.30, p=1.49E−4). See more details in: **Egyed et al. J Transl Med (2020) 18:250.**

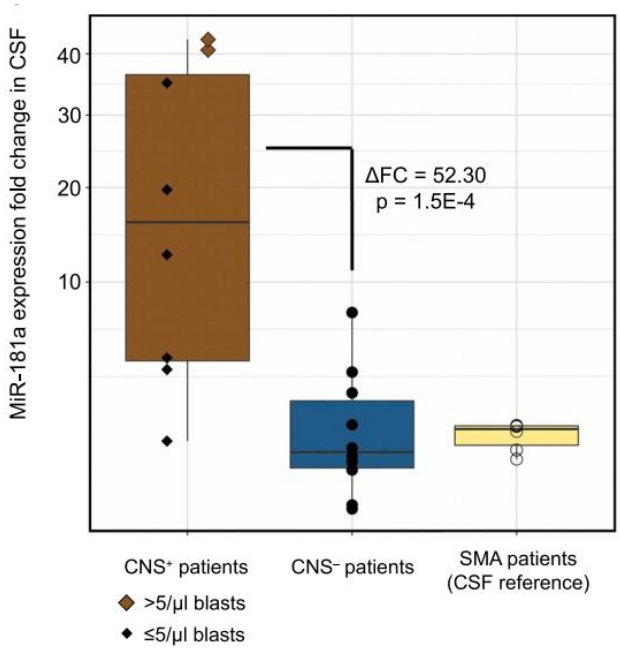

Figure 2.: *MiR-181a-5p level is a central nervous system involvement indicator in pediatric acute lymphoblastic leukemia. Each dot indicates the miR-181a-5p expression of a patient. Horizontal line in box plots represents median fold change (FC), extent of boxes indicates upper and lower quartiles, whiskers show maximum and minimum values. Abbreviations: CSF: cerebrospinal fluid, CNS: central nervous system, SMA: spinal muscular atrophy, ΔFC: difference in fold change*

Exosome-enriched fractions in ALL

We investigated the clinical potential of miRs in exosome-enriched fraction (EEF) as biomarkers of MRD during the induction therapy in pediatric ALL and compared to our previous results. We used the same samples of patients with normal karyotype or t(12;21) ALL and the same methodology was used as described previously apart from the EEF isolation (Rzepiel et al., J Transl Med. 2019; 17: 372).

For EEF isolation three commercially available isolation kits were tested according to the manufacturer's instruction: The METM kit (Plasma) (New England Peptide), qEV Single/70nm (Izon) Exosome isolation column (Size Exclusion Chromatography) and Macherey/Nagel Precipitation Solution (Serum/Plasma) (MN kit). On extended cohort ultracentrifugation (UC) was used (for 52 archived PFP samples of 13 patients with ALL for exosome isolation).

Correlation was found between the decrease of miR-x concentration in PFP and EEF by day 8 of chemotherapy, and the BM MRD measured by routine diagnostics in the middle of induction chemotherapy (Rho_{PFP} = 0.99, p_{PFP} < 0.001 and Rho_{EEF} = 0.99, p_{EEF} < 0.001, respectively). Furthermore, the decrease of miR-x in EEF by day 15 of treatment correlated with BM MRD on day 15 (Rho = 0.96; p <0.001), however the same relation was not seen in PFPs. Our results show that circulating miRs are potential biomarkers of ALL MRD. MiR-x level in both PFP and EEF predicts day 15 BM flow cytometry MRD 7 days earlier. In addition, the assessment of the EEF gave a more precise result. (Article in preparation.)

Biomarkers of adverse drug reactions

Association of allergy to native E. coli asparaginase with genotypes located at chromosome 5q33 and HLA types (as DNA toxicity-biomarkers) in pediatric ALL was investigated, a project finished partly within the framework of the K-115861 project, falling under its chemotherapy toxicity subtopic, done by the only full time researcher paid from this grant. Our results confirmed the role of the *GRIA1* variants in asparaginase hypersensitivity, but we observed significant differences between the subgroups of patients studied (**Kutszegi et al., PLoS One. 2015 10(10): e0140136**). We found that patients carrying the *HLA-DRB1*07:01* and *HLA-DQB1*02:02* alleles had significantly higher risk of developing hypersensitivity reactions. We first identified the *HLA-DRB1*07:01-DQB1*02:02* haplotype in asparaginase allergy (**Kutszegi et al., Haematologica. 2017 Sep;102(9):1578-1586**). Later, we identified a combination of 2 SNPs - rs28383172 and rs7775228 - as a tag for *HLA-DRB1*07:01-DQA1*02:01-DQB1*02:02* haplotype (**Kutszegi et al., Br J Clin Pharmacol. 2021 Jun;87(6):2542-2548**). In ALL, cardiotoxicity is related mainly to the use of anthracyclines and might have genetic risk factors. We tested the association between left ventricular function and genetic variations of candidate genes. Our results indicate that variations in *ABCC2*, *CYP3A5*, *NQO1*, *SLC22A6* and *SLC28A3* genes might influence the left ventricular parameters (**Ságі et al., BMC Cancer. 2018; 18: 704**)

Plasma circulating miRs in Hodgkin's disease

We performed an MRD study of patients with Hodgkin's disease. Altogether, PB PFP samples were tested on TLDA cards. In case of miR-532-5p, weak significance was observed compared to control samples. We carried out a qPCR study on an extended cohort using all of the available samples (14 patients, day of diagnosis and on day before the 2nd block) testing the expression of miR-155, miR-21-5p and miR-19-3p. For normalization, we used cel-miR-39 and miR-16. The methodology was the same as in Rzepiel et al., J Transl Med. 2019; 17: 372. We did not find any significant correlation in this set up either. The two assumed (based on previous studies) miRs for normalization did not work and we decided not to try any further miRs for this purpose considering our limited available samples. (Unpublished.)

Plasma circulating miRs in AML

We investigated the role of miR-146a-5p, miR-10a-5, miR-1228-3p, miR-129-5p, miR-155-5p, miR-181b-5p, miR-210-3p and miR-320d in 13 patients with acute myeloid leukemia (AML)

and 8 healthy controls. The same methodology was used as described in Rzepiel et al., J Transl Med. 2019; 17: 372.

The levels of miR-10a and miR-320d were found to be elevated significantly in PFP samples of patients with AML compared to controls (p_{adj} = 0.0331 and p_{adj} = 0.00661, respectively) (Figure 3). The level of miR-181b-5p showed a marginally significant difference (p_{adj} = 0.0465). No significant differences were found between treatment time-points of patients with AML or between those with ALL and those with AML. The usefulness of the above biomarkers for monitoring therapy could not be established because the number of patients was too small, and we finally decided that since we would not be able to enroll significantly more patients, we would not do further studies. The existing results were used to produce an MSc thesis in biology, but they were not sufficient for publication in an international journal.

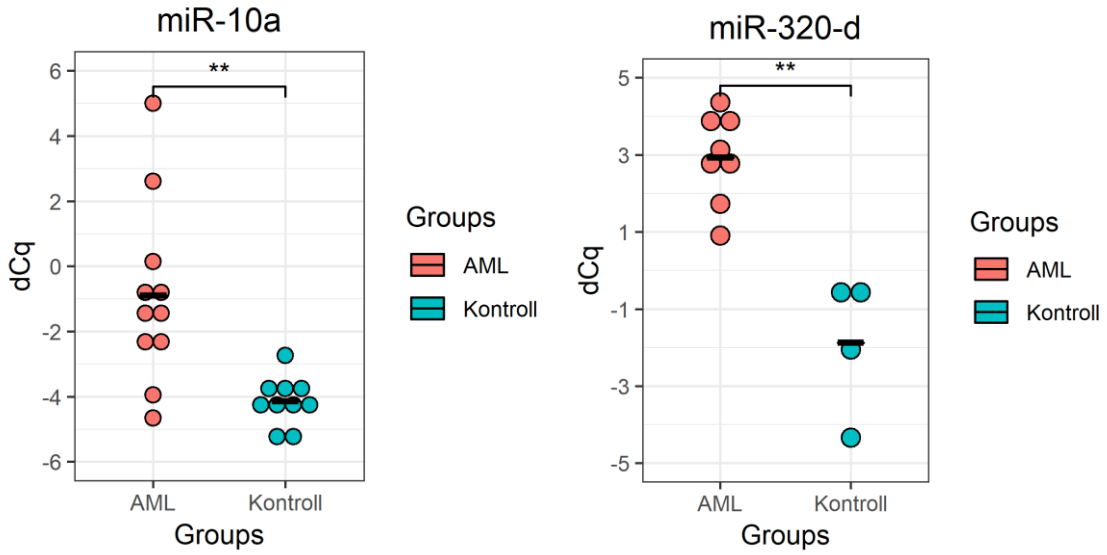

*Figure 3: Significantly different RNA levels in PFP from patienst with AML before treatment versus healthy controls. Each point indicates the normalized mean of the duplicate measurement result for a sample and ** indicates significance.*

PhD and undergraduate students whose work benefited from NKFI K-115861:

- Andrea Rzepiel (defended PhD dissertation; focused on miRNAs of blood plasma)
- Bálint Egyed (majority of Ph.D. work performed; with focus on miRNAs of CSF),
- Judit Csányiné Sági (defended PhD dissertation, some part of her Ph.D. work was performed within the framework of this OTKA project),
- Laura Almási (started PhD studies, project is based on CSF samples stored in this grant).
- Nóra Kutszegi (defended PhD dissertation; some part of her Ph.D. work was performed within the framework of this OTKA project)
- Eight students wrote all together 5 thesis and gave a total of 13 presentations at Students' Scientific Association (TDK) university and national conferences (five 1st places, one 2nd place)

COMPARISON OF PLANS AND ACHIEVEMENTS

These are the differences between our plans as per the 2015 grant application and the final materialisation of the project at present.

- Obtained sample numbers turned out lower than estimated originally despite the fact that yearly new case numbers were roughly as predicted at the 2nd Department of Paediatrics.
- Lack of detection of organ-specific EVs and low case (sample) numbers made investigating toxicities according to the original plans futile. For these reasons, we have not performed such analyses with EVs or miRs.
- We put much effort in investigating a subtopic of residual leukaemia biomarkers: measuring low level CNS leukemic involvement in CSF. This was not specifically mentioned among the original plans but is an area within minimal residual disease monitoring.

The project was hampered or forced to change so by the following issues:

- Unforeseen moving of all inpatient wards of 2nd Dept. Paediatrics from the original building (Tűzoltó utca 7-9, Budapest, 1094) to the Korányi building of Semmelweis University (Üllői út 78, Budapest, 1083) from July 2017 till Dec. 2017 and again from July 2018 till Aug. 2019. Before these, the Haematology ward already moved within the Tűzoltó utca building to temporal wards in Apr. 2017 and again in June 2017. Around times of moving, research projects were paused and so was sample collection.
- Coronavirus epidemic, spring 2020: Ph.D. students and undergraduate students were not allowed to enter hospital premises for months, we had to halt sample collection.
- Due to building works, the lab we equipped for sample processing was also closed in repeated goes between 2017 and 2019 at Tűzoltó utca.
- Lack of ultra-flow-cytometers expected for autumn 2015.
- Staffing issues: maternity leave of dr. Márta Pilissyné Hegyi during the whole study period, working time reduction of her replacement, dr. Nóra Borszékiné Kutszegi from spring 2019 onwards and later maternity leave of both her and dr. Ágnes Félné Semsei. The lead financial officer of the 2nd Department of Paediatrics, also the designated financial officer of this research project, dr. Csaba Tuncsik left in mid-2019 and his deputy, Mária Nagy was overwhelmed with other tasks for months.

To keep our project running, to fulfil our contract and to make the research project fruitful, the following steps were taken:

- Heim Pál Children's Hospital was invited to join sample and data collection and a useful cooperation was started.
- Laboratory work was repeatedly relocated to the Dept. of Genetics, Cell- and Immunobiology, Semmelweis University at Nagyvárad tér 4., Budapest, 1089.
- Change to part time employment of the full time researcher paid from the grant (dr. Nóra Borszékiné Kutszegi) was initiated with extended time frame and the whole project time period was extended to July 2020 (originally August 2019).

- Total spending on nurses' remuneration for sample collection was increased partly by increased study time period, partly by involvement of another hospital. We could divert here sums from material budget lines as low case numbers made reduced lab spending possible.
- We changed focus from flow cytometry analysis of EVs to PCR based investigations of miRs regarding MRD of all diseases and sample types. These are related topics as the largest part of extracellular miRs are found packed in EVs in the plasma and CSF. We wrote about the possibility of this shift already in our grant application in 2015.
- Regarding chemotherapy toxicities, instead of EV or miR biomarker investigations we turned to DNA-based toxicity biomarkers (association of genetic variants and allergic reactions to native E. coli asparaginase and cardiotoxicity).
- Spending related to conferences and publication were higher than predicted initially, and indeed, the team gained important knowledge for planning the experiments and fine tune research at these conferences. Over the last year, spending on these issues were reduced partly by COVID-related cancellation of many conferences, partly by Semmelweis University contracts with publishers and subsidies for publications.

Budapest, 28th June 2022,

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