THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigation of liquid biopsy biomarkers by immune-based methods in malignant diseases

by Eszter Judit Szánthó MD

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DOCTORAL SCHOOL OF KALMAN LAKI

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The examination takes place at the Library of the Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, 11 a.m. on Monday 31 May 2021 (online).

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Members of the Defense Committee:	Ilona Kovács MD, PhD
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The PhD Defense takes place at the Library of the Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, 1 p.m. on Monday 31 May 2021 (online).

Public access is provided online. If you wish to participate in the debate, please send an e-mail to koteles.julianna@med.unideb.hu by 10 a.m. on Monday 31 May 2021.

1. Introduction

1.1. Definition and history of immuno-analytical methods

Functions in living organisms are determined by interactions between molecules. One of these interactions is the antigen-antibody reaction. The immuno-analytical methods are based on the specificity of these reactions. Turbidimery and nephelometry are designed for quantification of large antigen-antibody comlexes (immunocomplexes) since the 1930s. These methods measure the amout of immunocomplexes directly, hence are called direct immuno-analytical methods. Turbidimetry with latex particles are used widely in automated clinical laboratories for detecting various plasma proteins.

Production of the specific antibodies needed for the immuno-analytical methods was carried out using various animal species; these antibodies were polyclonal and were available only in limited quantities. Kohler and Milstein developed a novel method in 1975: after immunizing mice with an antigen, isolated B cells from their spleens were fusionated with cells of a mouse myeloma cell line, creating hybridomas, then expanding the certain hybridoma cells that produced the appropriate antibody. These antibodies were monoclonal, specific for a certain epitope of the antigen.

1.1.1. Immunochemistry methods

In vitro ligand assays are especially capable of measuring small molecules. Detectability is ensured by a tracer that produces well-measurable signal. The method is sensitive enough to meausre analytes present in the sample in as low as 10^{-10} - 10^{-21} mol/L concentration, is specific and may easily be automated. The principle of ligand assays relies on examining the immune complex formation as an index of antigen-antibody reaction.

Immunoassays may be competitive or non-competitive. Competitive assays have limited amount of specific antigen binding sites. Non-competitive assays have the reagent (antibody) in excess amount, there is no competition for the binding sites, the labeled compound is the antibody. These assays are also named immunometric ones. Most commonly used is the sandwich technique: the analyte is bound by two (capturing and signalling) antibodies specific for two different epitopes. This method is more specific than others using a single antibody.

Immunoassays can also be classified as homogeneous or heterogeneous. Homogeneous assays contain all the components of the reaction in one fluidic phase, separation is not necessary. When bound by the antibody a change happens in the enzyme activity or in the fluorescence (luminescence) of the labeled antigen, hence the concentration of the analyte can be determined. Homogeneous immunoassays are fast, cost-effective methods for measuring small

molecules, with the sensitivity of 10^{-10} mol/L concentration. In heterogeneous immunoassays the antibody is usually bound to the surface of a solid phase within the liquid phase of the reaction. The antibody-antigen complex bound to this solid phase must be separated from the unbound fraction before measurement. Aside from a few precipitation methods most of the heterogeneous immunoassays use the mentioned solid phase method. The solid phase may vary: polystyrene tube surface, surface of a microplate cavity, latex particles, paper disc, glass bead, paramagnetic particles, etc.

1.1.2. Principals of flow cytometry and its use in clinical practice

Flow cytometry is an analytical method for examining cells in suspension. Detection of cell surface and intracellular markers is an objective and fast method, now indispensable in leukemia diagnostics. Its main point is to identify certain CD (Cluster of Differentiation) markers specific for the cell type and maturational stage on the pathological cells, with the help of specific monoclonal antibodies labeled with various fluorescent dyes. The main purpose in the diagnostics of acute leukemias is to describe the blast cells according to their cell line and maturational stage, along with detecting aberrant marker expressions (e. g. lymphoid and myeloid markers appearing on the same cell). This characterization gives the possibility of seeking residual leukemic blast after chemotherapy (minimal residual disease, MRD). For being able to recognize pathological patterns the examiner must have stable knowledge of the normal antigen patterns and intensity levels of the certain CD markers found on the different cell types and their maturational stages.

1.2. Liquid biopsy in malignant diseases

In the diagnostics of malignant diseases the principal method is the histopathologic examination of the cancerous tissue from the (solid) tumor and nearby lymph nodes, to evaluate the stage of the disease. During the course of a malignant disease tumor cells may appear in various body fluids. Liquid biopsy means the sampling and analysis of non-solid biological material. Advantage of this method is that it is less invasive for the patient than ordinary biopsies or surgical methods.

An immunology-based magnetic separation method for isolating circulating tumor cells was described by Racila et al. in 1998, still frequently used nowadays. As circulating tumor cells are present in the peripheral blood in very low numbers, several methods have been published for their separatipon and enrichment, based on either their physical properties (size, electric charge, density, deformability) or specific cell surface marker expression. The method for

separation and enrichment can be either positive or negative selection. Negative selection means the removal of CD45-positive hematopoietic cells. Advantage of this method is that no antibody is bound to the tumor cells, but the cell suspension is much less pure than one can achieve by positive selection. Disadvantages of the positive selection are that the antibodies bound on the tumor cells are hard to remove and choosing the most appropriate antibody is difficult, especially that the tumor cells become more and more heterogenous during the course (and therapy) of a malignant disease.

Besides intact tumor cells nucleic acids originating from them may also appear in body fluids, either in free form or in extracellular vesicles. Detecting and examining them may give a more precise insight of tumor heterogeneity, it is less invasive and more cost-effective than traditional histopathology, which is not always possible or easy to perform.

Disadvantage of fluid biopsy is the difficulty to distinguish tumor-originated cells, extracellular vesicles, nucleic acids from those with a benign origin. Another hurdle is that these are present in body fluids in very low amounts. Only recent technological developments made their detailed investigation possible.

1.3. Laboratory procedures and their characteristics

Laboratory results play a crucial role in establishing a diagnosis and in the follow-up of patients. Two-thirds of the clinical decisions are based on laboratory findings. The complex system of laboratory procedures are composed of the preanalytical, analytical and postanalytical phases. In recent years certain authors describe pre-preanalytical (choosing the most adequate laboratory tests for a patient) and post-postanalytical (evaluation of the laboratory findings by the clinician) separately.

1.3.1. Preanalytical phase

Preanalytical phase is composed of all the actions done before the actual laboratory test. Before taking the sample the clinician must decide which laboratory tests to have performed, and inform the laboratory personnel about what is significant from the patient's history in regard of the chosen laboratory test (e.g. time of taking certain medicine, chemotherapy, *de novo* or follow-up test). During sampling proper attention must be paid to identify the patient correctly, and to draw the necessary amount of sample in the appropriate sampling tube (e.g. bone marrow aspirate if multiple myeloma is suspected, peripheral blood is paroxysmal nocturnal hemoglobinuria is suspected; CSF must be drawn into special sampling tubes, containing fixative if flow cytometry is requested). Samples must be labeled correctly, test requests must be denoted precisely. Quality of the sample (clotted, hemolysed, lipemic, icteric) is one of the error sources in the preanalytical phase. Circumstances of transportation (e.g. pneumatic tube; temperature during transportation – room temperature, on ice, frozen) may have a significant effect on the results or on the performability of the requested tests. Flow cytometry may have special preanlytical demands as some tests can be performed only from certain samples taken at a designated time point, e.g. the Day 15, Day 33 or Day 78 bone marrow samples in childhood ALL for MRD detection. In the context of flow cytometry hemodilution of a bone marrow sample or a traumatic tap for obtaining CSF sample are among the possible preanalytical errors, along with the low viable cell count in a sample, making the evaluation limited or even impossible.

1.3.2. Analytical phase

Part of the analytical phase in the flow cytometry laboratory is labeling the sample with the appropriate antibody panels in accordance with the clinicians's request. To be able to achieve the best results the antibody clones, dilution ratio, fluorochromes must be optimized, the quality of other reagents, fixatives, permeabilizing agents, lysing agents must meet high standards. The correct flow cytometer setup, calibration with beads, compensation, usage of internal and external quality control are also crucial in obtaining adequate results. After measuring the sample the raw data are analyzed by a qualified expert by applying gates (either by automated gating or manually). Results may be quantitative (e.g. percentage and absolute count of T, B and NK cells, percentage of blast cells) or qualitative (describing the immunophenotype of blast cells). Defining sensitivity is important especially in regard of examining the follow-up samples during the course of a hematological malignancy; this data (limit of detection, limit of quantitation) must be reported.

1.3.3. Postanalytical phase

Postanalytical phase is composed of the procedures after obtainig the results, e.g. reporting the results, comparison with reference ranges and evaluation, notifying the clinician or writing comments. Important quality measurement factor for a test and the whole laboratory is turnaround time, the time between reqesting a laboratory test and obtaining the result. Local regulations decide the necessary duration of storing the sample (for possible further tests), or the data files, analysis files. Interpreting flow cytometry measurement data requires special education, the responsible person must know the technical details of the flow cytometer, antibody panels and controls, along with the details for the diagnostics and follow-up of hematological diseases.

1.3.4. Possible errors and their indicators of the laboratory procedures

During the laboratory procedures, 46-68% of errors occur in the preanalytical, 7-13% in the analytical and 18-47% in the postanalytical phase. Due to strict quality control and modern, accurate instruments errors in the analytical phase are rare, however, error rates in the extra-analytical phases are high. Moreover, the laboratory is unable to affect most of these errors. Recognising this, numerous researchers evaluated the background of pre- and postanalytical errors, developing indicators for recognising and registering these errors. Several workgroups made suggestions for internationally harmonising these indicators. According to the current consensus the preanalytical phase has the highest number of indicators (28), analytical phase has 6, postanalytical phase has 11. By registrating and storing these indicators in the laboratory information system laboratories are able to monitor the number and percentage of errors occurring in the certain phases of laboratory procedures, and to improve their performance and assessment by clinicians. In recent years all of the errors during the phases of the laboratory procedures are considered diagnostic errors, suggesting that in whatever phase the error occurs, it is going to have an effect on interpreting the result and establishing the diagnosis.

1.4. Preanalytical considerations about samples from pediatric patients with acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is the most common cancer among children. ALL can be either B-cell or T-cell type with 85% of the cases being B-cell precursor ALL (BCP-ALL) and 15% T-ALL. The core of modern-day treatment of ALL is the administration of combined chemotherapy. The intensity of chemotherapy and further therapeutic decisions depend on several prognostic factors. Sample quality is crucial in the correct risk assessment. A

common preanalytical error can be the contamination of either the bone marrow (BM) or the cerebrospinal fluid (CSF) sample with peripheral blood (PB). The sample must also contain enough viable cells for analysis, carried out either by morphological or by flow cytometric studies. According to the AIEOP BFM 2009 protocol evaluation of minimal residual disease (MRD) in the PB on day 8 and in the BM on day 15 and day 33 is important for adjusting treatment intensity. The possibility of diluting the BM specimen with PB should be minimized as a diluted sample may result in underestimating residual blast percentage and wrong risk assessment. According to the ALL IC-BFM 2009 Flow MRD SOP a day 15 BM cannot be used for risk stratification if the percentage of the erythroid precursors is below 2% as PB contamination in this case is highly suspected. However, those inadequate samples containing more than 10% pathological blasts can still be reported and used for risk stratification as a Flow High Risk case. If blast percentage is less than 10% in a BM sample contaminated with peripheral blood, no classification into flow risk groups (0.1% - 10%: Flow Medium Risk and <0.1%: Flow Low Risk) can be performed and the sample must be reported as „Inadequate for MRD assessment".

CNS involvement requires administration of intrathecal chemotherapy, however, PB contamination can lead to misdiagnosing CNS involvement as the origin of a malignant population detected in a contaminated CSF sample remains unclear. Another pitfall can be the rapid decay of the cells in a CSF sample especially if the CSF sample has to be transferred to a central laboratory e.g. for flow cytometry. As the degradation rate is different in each cell type, long transfers of the sample will affect the qualitative analysis and pathological cell populations might be missed.

Low viable cell count is usually not a problem with PB and BM samples but CSF samples are frequently paucicellular, further hampering the analysis. Another hurdle is that there is no consensus on either the minimal number of cells needed for adequate analysis (varying between 100 and 1000 in literature), or on the precise definition of a traumatic tap (PB contamination).

1.5. Analytical considerations about detecting disseminated tumor cells with flow cytometry and immunohistochemistry

Neuroblastoma, rhabdomyosarcoma, Ewing sarcoma, and retinoblastoma are those pediatric tumors that most commonly inflitrate the bone marrow (BM). Neuroblastoma is derived from neural crest cells of the adrenal gland medulla or sympathetic ganglia. The "International Ne-

uroblastoma Staging System" (INSS) stratifies neuroblastoma according to its anatomic presence and BM involvement at diagnosis. Park et al proposed the use of immunohistochemistry (IHC) in detecting BM metastases of neuroblastoma with staining of CD56, chromogranin A, and synaptophysin. In adults, metastases of prostate, breast, and lung cancers can be detected in the BM. Disseminating cells of a primary solid tumor may represent the origin of metastases and relapses. Tumor cell detection in BM is important in the staging of malignant diseases and also in therapy planning. BM aspirates and trephine biopsies provide morphologic information of possible tumorous infiltration. Kaur et al stated bilateral trephine biopsy to be the gold standard method when searching for infiltrating tumor cells in the marrow.

Flow cytometry has been used for the measurement of DNA content of malignant cells since the 1980s. Nowadays, the main application of multicolor flow cytometry (MFC) is to diagnose and classify hematopoietic disorders and malignancies, but it has a role in solid tumor diagnostics and follow-up as well. However, only few studies are available focusing on the phenotypic characteristics of solid tumors as determined by MFC. Ferreira-Facio et al studied the immunophenotype of pediatric cancers and concluded MFC to be a fast diagnostic and screening tool in childhood cancer. The most useful markers they found were CD45, CD56, CD81, CD99, CD271, epithelial cell adhesion molecule (EpCAM), GD2, nMYOD, and n-myogenin along with other T-cell-specific and B-cell-specific markers. In some rare cases, the primary tumor cannot be found, and flow cytometric BM examination becomes indispensable for the diagnosis. Only a few studies have been published on the comparison of morphology/IHC results with flow cytometric findings of tumor-infiltrated BM.

1.6. Postanalytical considerations about thymidine kinase as a tumor marker in chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia in the developed countries, affecting individuals primarily in advanced ages, with a mild male dominance. Staging is based on mainly the Rai scoring system in Hungary. According to the Rai scoring system CLL has five stages: Rai 0 stage is characterized by lymphocytosis, usually detected by accident, the patient has no specific symptoms in this stage. Rai 1 stage means lymphocytosis with lymphadenomegaly, in Rai 2 stage hepato-splenomegaly is also present. In Rai stage 3 lymphocytosis and anemia are found, either with or without lymphadenomegaly and hepato-splenomegaly. Rai stage 4 means lymphocytosis with thrombocytopenia, with or without anemia, lymphadenomegaly and hepato-splenomegaly.

Many prognostic factors, mainly chromosomal aberrations, genetic alterations were revealed in the previous decades in the context of CLL. As well as genetic alterations, certain biomarkers found in the circulation can provide prognostic information. One of these biomarkers is thymidine kinase 1. Distinguishing from a few tumor specific biomarkers (PSA, AFP, HCG etc), thymidine kinase 1 (TK1, ATP; thymidine 59-phosphotransferase; EC.2.7.1.21) is one of the non-specific tumor markers that can be measured in continuously dividing cells. TK1 is a key cellular enzyme in the so-called salvage pathway of DNA synthesis. It phosphorylates thymidine into thymidinemonophosphate. The enzyme has two isoforms, TK1 is found in the cytoplasm, while TK2 remains in the mitochondria. The activity of TK1 is cell cycle-dependent, its level begins to rise in G1/S phase and reaches its peak in the G2/M phase. TK1 can be isolated from many different cell and tissue types, and elevated serum TK1 activity is documented in many types of cancer, nonetheless, the mechanism of TK entering the systemic circulation is not well understood. Furthermore, TK has been reported as a prognostic marker in chronic lymphocytic leukemia (CLL) and in non-Hodgkin's lymphoma.
Hallek et al found that a serum TK level of 7.1 U/L was the cutoff value that could differentiate shorter (high TK levels) and longer (low TK levels) progression-free survival subgroups in Binet A stage disease. In the French-German CLL7 trial, one of the adverse risk factors to identify high-risk Binet A patients was a serum TK level >10 U/L. Magnac et al examined the relationship between serum TK levels and IGHV mutational status and found that TK levels above 15 U/L correlated well with the IGHV unmutated status of the patients.

2. Aims

The aim of our study was to evaluate the preanalytical, analytical and postanalytical phase of laboratory methods applied in the special fields of laboratory medicine used in the laboratory diagnosis of malignant disorders and to investigate the effects of errors on the interpretation of laboratory results.

1. Investigation of preanalytical phase: the basis of patient stratification and treatment modification is the percentage of blast cells in predefined timepoints from peripheral blood, bone marrow aspirate or cerebrospinal fluid in pediatric ALL. In order to perform a correct blast count by flow cytometry, an adequate sample quality is required. Our aim was to determine the percentage of samples with inadequate quality when no blast percentage result was reported.

2. Investigation of analytical phase: detection of disseminated tumor cells in liquid biopsy samples is traditionally performed by morphological/immunohistochemical methods. We aimed to compare the sensitivity, specificity and diagnostic efficacy of the traditional techniques with flow cytometry in the detection of solid tumor cells.
3. Investigation of postanalytical phase: for the correct interpretation of thymidine kinase enzyme activity results we aimed to establish age-related reference ranges with the measurement of large number of healthy individuals. Since CLL is predominantly a disease of the elderly we wanted to investigate the thymidine kinase activity of untreated CLL patients and the correlation of enzyme activity with the stage of the disease and other laboratory parameters. We also aimed to establish a cut-off value that could disciminate CLL patients from age-and sex-matched healthy individuals.

3. Patients, materials and methods

Antibodies, instruments and softwares are listed at the appropriate study.

3.1. Preanalytical phase: Methodology of the study evaluating sample inadequacy

3.1.1. Examining bone marrow samples from pediatric patients with ALL

Cohort 1: data of patients with childhood ALL between 2011 and 2018 were analyzed retrospectively. Day 15 bone marrow samples were obtained from 104 patients, 59% of whom were male, 41% were female. 23 patients (20 male, 3 female) had T-ALL (22%), 81 patients (41 male, 40 female) had BCP-ALL. Average age at sampling time of the whole population was 83 months, with a range between 1 and 201 months.

Cohort 2: day 33 bone marrow samples were analyzed from 90 patients (56% male, 44% female), 13 (14%) T-ALL (11 male, 2 female), 77 (86%) BCP-ALL (39 male, 38 female). Average age in this population was also 83 months, range between 2 and 202 months.

3.1.2. Examining CSF samples from pediatric patients with ALL

In the mentioned time period a total of 26 CSF samples were analyzed by flow cytometry from 20 pediatric patients with ALL. The average age was 75 months, range between 7 and 214 months. Twelve patients were male (60%), eight were female (40%). One patient had T-ALL (5%), the others had BCP-ALL (95%). More than one sample was sent from five patients, 3 samples from the T-ALL patient and two samples each from the other four patients.

3.1.3. Comparing native and stabilized CSF samples

Fifty-one CSF samples from 47 patients (adults and children) were evaluated and viable cell percentage in native and stabilized samples (TransFix®; Ref. No. TF-CSF-5-25, Caltag Medsystems, Buckingham, UK) were compared. Nineteen of these 47 patients were female (40%), 28 were male (60%). Average age in this group was approximately 43 years with a range of 10 months and 79 years. 29 samples were stabilized, 22 were native.

3.1.4 Flow cytometric studies

Flow cytometric measurements were carried out in a FACSCanto II flow cytometer, data were analyzed by FACSDiva 8.0.2 software (both by Beckton Dickinson Biosciences, San Jose, CA, USA). Pediatric ALL samples before March, 2013 (regarding BCP-ALL) and September, 2013 (regarding T-ALL) were examined in a 4-colour setting, all samples afterwards were examined by 8-colour setting. CSF samples from the ALL patients were stained with antibodies based on these panels; due to sample shortage in most cases the whole panels could not be applied. To make the results comparable, the flow cytometer was calibrated daily, using Cytometer Setup and Tracking fluorescent microbeads (Cat No. 641319, Becton Dickinson Biosciences, San Jose, CA, USA) and Autocomp software as recommended by the manufacturer.

Viable cell count in bone marrow samples was evaluated by syto-staining. Duplicates were excluded from all samples before the evaluation of the percentage of syto+ viable cells. Bone marrow samples containing <2% erythroid precursors were considered contaminated by PB according to the ALL IC-BFM 2009 Flow MRD SOP. In CSF samples viable cell count was evaluated either by syto+FSC/SSC sequential gating or by FSC/SSC gating if syto was not used. CSF samples containing <30% of viable cells were considered inadequate for analysis and PB contamination was concluded if the CSF sample contained >100 red blood cells / microliter (RBC/μL).

3.2. Analytical phase: methodology of the study designed for the detection of disseminated tumor cells

In total, 72 samples consisting of 63 BM specimens, 6 cerebrospinal fluid (CSF) samples, 2 bronchoalveolar lavage fluid (BAL) samples, and 1 peripheral blood (PB) sample from 50 patients were investigated retrospecively. IHC data were available in 48 cases. The study population was divided into 2 groups. In the first cohort, 36 samples derived from 34 patients with various forms of suspected and proven solid tumors (16 children, 18 adults) were studied, and, in the second cohort, 36 samples of 16 children with suspected or proven neuroblastoma were analyzed at diagnosis or during follow-up.

3.2.1. Study group with various forms of suspected and proven solid tumors

In 13 patients (3 pediatric and 10 adults) no malignant disease was proved, whereas in 21 patients (13 pediatric, 8 adults) various types of solid tumors were detected. In the subgroup with verified malignant diseases, 13 were male patients (8 adults, 5 children) and 8 were female patients (all children). The average age in the pediatric subgroup was 8.95 years (range, 3 mo and 15 y), whereas for the adult subgroup it was 58 years (range, 21 to 94 y).

3.2.2. Study group with suspected and proven neuroblastoma

Thirteen of the 16 neuroblastoma patients were male individuals and 3 were female individuals, with an average age of 27.4 months (range, 2 to 83 mo) at the time of diagnosis.

As of November 2015, 6 of them were alive, 9 deceased, and 1 patient's state was unknown. All deceased patients were older than 12 months at the time of diagnosis.

3.2.3. Flow cytometry immunophenotyping studies

Surface staining of whole blood and BM cells were carried out according to standard procedures. In total, 50 μL of whole blood or BM sample adjusted to leukocyte count of $10x10^9$/L by phosphate-buffered saline (PBS) was incubated by saturating concentrations of directly conjugated antibodies for 15 minutes at room temperature in the dark with antibodies against different cell surface epitopes. The non-hematopoietic tumor cell panel contained CD7-FITC, EpCAM-PE, CD45-PerCP, and CD33-APC, whereas the neuroblastoma panel was a 4-color combination of CD81-FITC, CD117-PE, CD45-PerCP, and CD56-APC. When Ewing sarcoma or histiocytosis were suspected, we included CD99-FITC and CD1a-APC, respectively. In some cases, CD71-FITC was also used for the better identification of erythroid precursor cells. Red cells were lysed by FACS lysing solution and the samples were washed (300g, 5 min) in PBS and finally resuspended in PBS containing 1% paraformaldehyde. Paraformaldehyde–fixed samples were kept at 4 °C for a maximum of 24 hours. Flow cytometric measurements were carried out on a FACSCalibur or on a FACSCanto II flow cytometer using the same setting for all investigated samples. Data obtained on 100,000 cells in de novo cases and 300,000 cells in minimal residual disease (MRD) detection were acquired and stored in list-mode data files. Data were analyzed by FACSDiva software. Neuroblastoma cells display CD45-negative, CD56-positive, CD81-positive, and rarely CD117-positive characteristics. For detecting MRD, after gating on viable cells on the forward scatter—side scatter plot, CD56-positive/CD81-positive cells from the CD45-negative region exhibit the residual neuroblastoma cells.

3.2.4. Immunohistochemistry on trephine biopsy specimens

Trephine biopsies were fixed for 24 hours in 4% phosphate-buffered formaldehyde and decalcified for 48 hours in 1% EDTA. Following tissue processing and paraffin embedding, 4-m thick slides were cut for further stainings. Using the Bond-Max autostainer and the associated Bond Polymer Refine Detection kit, tissue slide sections were immunostained with CD56, CD99, chromogranin A, and S-100. Immunostaining was assessed by light microscopy (Leica DM2500 microscope, DFC 420 camera and Leica Application Suite V3 software).

3.2.5. Sensitivity, specificity, and diagnostic efficacy

After the classification of samples into true positive (TP), true negative (TN), false positive (FP), and false negative (FN) categories, we calculated sensitivity [100xTP/(TP+FN)], specificity [100xTN/(TN+FP)], and diagnostic efficacy [(100x(TP+TN)/(TP+TN+FP+FN)] of each method.

3.3. Postanalytical phase: Methodology of the study designed for establishing age-specific reference ranges for thymidine kinase activity and validating on a group of CLL patients

3.3.1. Healthy cohort for establishing reference ranges

Healthy volunteers (n= 369) between 18 and 86 years of age (median: 45 years, men: 157, women: 212) were recruited from blood donors and staff of the faculty with values <2x upper limit of normal for all measured laboratory parameters. Exclusion criteria were cancer, metabolic and inflammatory diseases. Due to non-confirmation with selection criteria data from 24 individuals was excluded from the final statistical analysis. To examine the influence of age, the reference population was subgrouped into the following age groups: 18–35 years (young), 35–60 years (middle-aged) and 60–86 years (elderly).

3.3.2. CLL cohort

Patients presenting with CLL between December, 2010 and November, 2012 were included in this cross-sectional, analyst-blinded, age and sex matched case-control study (n= 115). The mean age (range) of the individuals in both groups was 65.6 (46–86) years, and the male:female ratio was ~ 1:1 (61:54). As per Rai staging, 43 were in stage Rai 0, 27 were in Rai 1, 19 were in Rai 2, 15 were in Rai 3 and 11 were in Rai 4. In this cohort, 29 and 86 patients belonged to the middle-aged and elderly age groups, respectively.

3.3.3. Laboratory methods

3.3.3.1. Measurement of thymidine kinase activity

TK1 activity was measured by an indirect, modified, two-step competitive chemiluminescent immunoassay on a Liaison immunochemistry analyzer. In order to evaluate assay performance, accuracy and precision were determined from QC samples in low and high range (QC1 and QC2, respectively). Precision was expressed in CV% and accuracy was assessed as percent deviation from the nominal activity. Intra-assay precision and accuracy were determined from 9 replicates of each QC sample on a single occasion while inter-assay precision and accuracy were determined by analyzing 18 different operating days. The results were analyzed as a linear regression of the nominal vs. observed values. Linearity was evaluated by serial dilution of a patient sample with markedly elevated TK activity.

3.3.3.2. Determination of CD38 and Zap70 positivity of CLL cells by flow cytometry

White blood cell counts were measured by an Advia 120 hematology analyzer. Absolute B-cell count was calculated from the percentage of CD19+ cells and white blood cell count. Flow cytometric measurements were carried out on FACSCanto II flow cytometer, the results were evaluated by FACSDiva software. The antibodies used were: CD19-PE-Cy7, CD38-APC and Zap70 (unlabeled). The patient was considered CD38 and/or Zap70 positive if ≥20% of the CLL cells expressed the respective markers.

3.3.3.3. Fluorescence *in situ* hybridization (FISH)

Interphase fluorescence in situ hybridization (FISH) was performed on cell suspension originated from peripheral blood according to standard protocol. Cells were obtained by 72 hour culture in the presence of 12-O-tetradecanoylphorbol-13-acetate (TPA) or by direct preparation from uncultured peripheral blood. The probes used for FISH analysis included LSI Rb1(13q14)/13q34, LSI p53, LSI ATM. Cells were counterstained with DAPI (4,6-diamidino-2-phenylindole). At least 200 interphase cells were analysed for each probe in each case. The images were captured by Zeiss Axioplan2 fluorescence microscope and analysed by ISIS software.

3.3.4. Statistical analyses

Kolmogorov-Smirnov test was used for the evaluation of the normality of the data. Most parameters were non-normally distributed; therefore analyses were performed by Mann-Whitney U test. The Spearman's rho was calculated for correlation analysis. $P<0.05$ was regarded as statistically significant. All analyses, including drawing of receiver operating characteristic (ROC) curves and estimation of the area under the ROC curve (AUC), were performed using the SPSS Statistics software, version 19.0.

4. Results

4.1. Preanalytical phase: results of the study of evaluating sample inadequacy

4.1.1. Bone marrow results of the pediatric ALL patients

Cohort 1

Peripheral blood contamination was found in 16 (15%) out of 104 day15 BM samples (12 out of 81 BCP-ALL and 4 out of 23 T-ALL). Blast percentages in the BCP-ALL contaminated samples were all below the Flow High Risk limit (10%) therefore these samples were inadequate for flow risk stratification. In the T-ALL group >10% of residual blasts were found in 3 out of 4 contaminated samples, making them eligible for risk stratification as these patients belonged to the Flow High Risk group. Altogether 13 samples of day15 BMs could not be used for risk stratification out of 104 samples (12.5%) owing to PB contamination.

Cohort 2

Thirteen samples (14%) out of 90 were contaminated with peripheral blood (11 out of 77 BCP-ALL and 2 out of 13 T-ALL). All these samples contained less than 10% blasts, so they were reported as inadequate.

4.1.2. Cerebrospinal fluid results of the pediatric ALL patients

RBC count was recorded in 22 out of the 26 samples. With 100 RBC/μL as cutoff for PB contamination 10 out of 22 (45%) samples were contaminated. Despite the low (<10) white blood cell count/microliter (WBC/μL) in 11 samples, several thousands of cells (1,840 – 100,000) could be acquired and analyzed by flow cytometry. Four out of the 11 paucicellular samples contained malignant cells, one of them being PB contaminated. Malignant cells were found in 14 samples from the overall 26, eight (57%) of which were PB-contaminated.

Eleven (42%) out of the 26 CSF samples were considered inadequate for evaluation due to <30% of viable cells among the acquired nucleated cells, four of which had identifiable malignant cell population despite of the low percentage of viable cells.

4.1.3. Native and stabilized CSF samples

Viable cell count was <30% in 5 out of the 29 CSF samples (17%) in the subgroup with preservative and 9 out of 22 (41%) in the native subgroup (without stabilization), the difference is significant (p=0.05, Fisher's exact test). PB contamination (>100 RBC/μL) was found in 8 out of 22 samples (36%) in the native subgroup and in 4 out of 29 samples (14%) in the stabilized samples.

4.2. Analytical phase: results of the study of detecting disseminated tumor cells

4.2.1. Study group with various forms of suspected and proven solid tumors

In total, 36 samples (27 BM; 6 CSF; 2 BAL; 1 PB) of 34 patients with a clinical suspicion of solid tumor or transfusion-dependent anemia were investigated by MFC. Disseminated tumor cells (DTC) were revealed in 8 cases of solid tumors: CD45-/EpCAM+ carcinoma cells (n=4), CD45-/CD99+ Ewing sarcoma cells (n=1), and CD45-/CD99+/CD117+ PNET cells (n=1), and 2 cases of Langerhans cell histiocytosis with CD45-/CD1a+ cells. The remaining 13 patients with proven malignant diseases had no BM involvement. IHC results of BM samples from the same patients were not available in 14 cases. A total of 20/ 22 (91%) concordant (6 positive and 14 negative) and 2 discordant cases were observed comparing the results of MFC and that of IHC in this cohort.

4.2.2. Study group with suspected and proven neuroblastoma

In total, 36 BM samples of 16 children with suspected neuroblastoma at diagnosis and proven neuroblastoma cases during follow-up were analyzed by MFC, and 26 samples were investigated by IHC. In 17 cases results were concordant (13 positive and 4 negative), whereas in 9 cases discordant results were obtained. Among the discordant cases, 2 cases were MFC-negative/IHC-positive, both from the same patient. The 7 MFCpositive/IHC-negative discordant samples were from 6 different patients, and these were all follow-up samples with a ratio of the residual tumor cells below 1%. In the neuroblastoma group we found 65% (17/26) concordance between IHC and MFC. Overall, 77% concordance was found between MFC and IHC (37/48). We identified different patterns on the basis of concordance and discordance of the 2 methods.

4.2.3. Concordant and discordant patterns

Pattern 1: MFC-positive/IHC-positive concordant result

We had 6 MFC-positive/IHC-positive concordant results in the group of various solid tumors and 13 MFCpositive/ IHC-positive cases in the neuroblastoma group. The ratio of detected malignant cells, however, was sometimes markedly different with the 2 methods. Flow cytometry analysis of diagnostic BM aspirate was performed to rule out acute leukemia in one of the concordant neuroblastoma cases. Flow cytometry analysis revealed no signs of leukemia, however, 30% CD45-/CD56+/CD81+ neuroblastoma cells were detected. Giemsa and chromogranin staining of the same BM aspirates also detected neuroblastoma cells, whereas 90% infiltration of the trephine biopsy specimen with neuroblastoma cells was found by CD56 and chromogranin staining.

Pattern 2: MFC-negative/IHC-negative concordant results

There were 14 negative concordant results in the group of various solid tumors and 4 cases in the neuroblastoma group. Eight of the solid tumor cases were from patients with proven malignant diseases without any BM involvement.

Pattern 3: MFC-negative/IHC-positive discordant results

MFC-negative/IHC-positive discordant results were found in 2 solid tumor cases and in 2 neuroblastoma cases. One of the discordant samples was a case from a patient with Langerhans cell histiocytosis. Light microscopy images of the trephine biopsy detected 10% peritrabecular pathologic histiocytes characteristic of Langerhans cell histiocytosis by hematoxylin eosin staining, naphtol-AS-D-chloroacetate esterase, and S-100 IHC. The other discordant case was a specimen of a Hodgkin lymphoma in which flow cytometry failed to detect the characteristic Reed-Sternberg cells.

The 2 MFC-negative/IHC-positive neuroblastoma cases were 2 MRD samples from the same patient obtained at different follow-up timepoints. IHC showed about 10% of malignant neuroblastoma cells forming rosettes. As flow cytometry was carried out from the BM aspirate, we assumed that neuroblastoma cells were not removed upon aspiration in those samples. Detection of no neuroblastoma cells in the routinely stained smears by Giemsa and by chromogranin immunostaining confirmed this statement.

Pattern 4: MFC-positive/IHC-negative discordant results

In the neuroblastoma group we had 7 discordant samples obtained from 6 patients. In these cases we were able to detect a small amount of residual malignant cells (<1%) by MFC, whereas residual tumor cells were below the detection limit of the IHC method.

Pattern 5: MFC-positive/IHC not available

Overall, in 24 cases no IHC results were available: 14 in the group of various solid tumors and 10 in the neuroblastoma group. In the latter group, 9 neuroblastoma cases were positive by MFC and 1 case was negative. In the group of various solid tumors, 10 cases were negative by MFC (4 cases from patients with proven malignant disease but without BM involvement) and 4 cases were positive; one of the latter ones was false positive. One of the BM specimens infiltrated with tumor cells was taken from an elderly patient who had severe thrombocytopenia; therefore, neither tumor nor trephine biopsy could be carried out, preventing IHC analysis. May-Grunwald-Giemsa staining of the BM aspirate showed cell aggregates with low–power-field magnification and malignant cells characterized by multilobulated nuclei with rough chromatin and 1 to 2 nucleoli, and basophilic, vacuolated cytoplasm (Figs. 5B–D). MFC identified 17% of the nucleated cells in the CD45-negative region, 1% erythroid

precursors, and 16% EpCAM+/CD56+/CD117+ carcinoma cells, probably metastatic cells from the lung cancer confirmed by imaging techniques. In addition, the metastatic cells were CD117 positive and CD56 positive. Of these markers, CD56 is usually expressed by small-cell lung cancer tumor cells, and CD117 has been described as an adverse prognostic factor in small-cell lung cancer.

4.2.4. Sensitivity, specificity, and efficacy of MFC and IHC in DTC detection

We evaluated the specificity, sensitivity, and efficacy of these 2 methods in detecting tumor cells in the BM and other body fluids. For neuroblastoma cell detection, MFC was more sensitive than IHC, especially in MRD detection (90% vs. 65%), but for other solid tumors we found IHC to be more sensitive (100% vs. 82%). MFC and IHC were both 100% specific in neuroblastoma cell detection, whereas in the group of various solid tumors MFC was 96% specific. Detection of DTC in BM specimens was found to be more effective with MFC than with IHC in the diagnostic neuroblastoma samples (100% vs. 86%). The advantage of MFC was particularly pronounced when MRD was evaluated in follow-up samples (92% vs. 68%). In the group of various solid tumors, efficacy of IHC was 100%, whereas efficacy of MFC was 91%.

4.3. Postanalytical phase: results of the study of establishing age-specific TK1 reference ranges and validating the findings on a group of CLL patients

4.3.1. Analytical performance of serum thymidine kinase measurement

Reproducibility and accuracy tests gave excellent results. The nominal mean TK1 activity of QC1 was 14.8 U/L. Intra-assay measured mean was 15.0±1.58 U/L, precision CV of QC1 was 10.54%, accuracy was 101%. Inter-assay measured mean of QC1 was 14.8±2.23 U/L, precision CV was 15.03% and accuracy was 100%. The nominal mean activity of QC2 was 53.7 U/L, intraassay measured mean was 62.4±6.34 U/L, precision CV was 10.16% and accuracy was 116%. Inter-assay measured mean of QC2 was 49.15±6.27 U/L, precision CV was 12.76% and accuracy was 92% . The test was linear from 0.5 U/L to 100 U/L.

4.3.2. Establishing age-specific reference ranges

In the healthy cohort, there was no significant difference in TK1 activity between the men and women studied, as such gender was not taken into consideration when defining age groups. There was a significant difference between the TK1 activity of the young (n =95) and the elderly (n= 83), (11.85±5.7 U/L vs. 8.65±3.9 U/L; $p<0.001$) and between the young and the middle-aged (n= 191) (11.85±5.7 U/L vs. 9.79±5.2 U/L; $p<0.001$). The difference was at the

limit of statistical significance between the middle-aged and the elderly (9.79±5.2 U/L vs. 8.65±3.9 U/L; p=0.050). Overall, the lowest observed TK1 level was 0.5 U/L and the highest was 35.6 U/L. The 97.5 percentiles in the 3 age groups were 29.8 U/L (young), 25.4 U/L (middle-aged) and 19 U/L (elderly).

4.3.3 Studies on CLL patients

Results of 115 CLL patients on presentation were compared with an equal number of age- and sex-matched controls (n= 115). This age- and sex-matched control group was derived from the over-all healthy cohort (n= 393), that was used to define the reference ranges of TK1 in the different age groups.

We found significant difference in TK1 activity between the healthy control group and all Rai stages of CLL. As we had few individuals in Rai 2, Rai 3 and Rai 4 stages, we grouped them as being in a more advanced stage of the disease. The observed mean TK1 activity in the age- and sex-matched healthy group (n =115) was 9.18±5.0 U/L, which was significantly lower than that in Rai 0 (n= 43; mean: 18.58±15.6 U/L; p<0.001), Rai 1 (n= 27; mean: 28.16±32.9 U/L; p,0.001) and Rai 2 3-4 (n= 45; mean: 72.88±101.1 U/L; p<0.001). We also noticed significant difference in TK1 activity between Rai 0 and Rai 2-3-4 stages (p<0.001) and Rai 1 and Rai 2-3-4 stages (p=0.002).

CLL patients were on average 65.6 years old (range: 46–86). There was no difference in TK1 levels upon comparing those below and equal to 60 years (middle-aged, n=29) and those over 60 years (elderly, n=86) of age belonging to the different Rai stages.

The area under the receiver operating characteristic curve (ROC-AUC) for TK1 was 0.840 (95% CI: 0.787–0.892) for differentiating CLL patients from age- and sex-matched healthy controls, with a cut-off value of 10.5 U/L (sensitivity: 80.9%, specificity: 73.4%). Furthermore, at the same cut-off value, the ROC-AUC for TK1 was 0.760 (95% CI: 0.671–0.849) for differentiating Rai 0 stage CLL patients from healthy controls (sensitivity: 73.9%, specificity: 72.1%).

We also compared the TK1 results of the CLL patients with other known prognostic factors, i.e., CD38 and Zap70 expression. The mean TK1 activity in the CD38–/Zap70– group (n =52) was significantly lower than that in the CD38+/Zap70+ group (n= 31) (22.3±17.8 U/L vs. 109±130.3 U/L; p<0.001). The difference was also significant between the CD38–/Zap70+ group (n= 17) and the CD38+/Zap70+ group (26.25±33 U/L vs. 109±130.3 U/L; p=0.002); and between the CD38+/Zap70– group (n= 15) and the CD38 /Zap70– group (40.87±38 U/L vs. 22.3±17.8 U/L; p=0.022); and the CD38–/Zap70+ group and the CD38+/Zap70– group (26.25±33 U/L vs. 40.87±38 U/L; p=0.021).

With ROC analysis, the TK1 cut-off value to distinguish CD38–/Zap70– patients from those with either or both positivity was 30 U/L (ROC-AUC: 0.710, 95% CI: 0.601–0.819, Specificity: 76.6%, Sensitivity: 54.3%).

Out of 115 CLL patients, 95 had FISH data. Upon comparing those with no chromosomal alterations, del(13q), 12 trisomy (n= 76) with the group with del(17p13), del(11q22) (n= 19) no statistically significant difference was found in the TK1 values (38.7665.7 U/L vs. 55.5696.5 U/L, p = 0.347). The nonsignificance did not change when patients with 12 trisomy were excluded from the statistical analysis (33.0644.5 vs. 55.5696.5, p = 0.211). We observed a statistically significant correlation between TK1 activity and WBC (Spearman's rho: 0.367, p,0.001) and absolute B cell count (Spearman's rho: 0.369, p,0.001).

5. Discussion

5.1. Preanalytical phase: Identifying inadequate samples and their effect in result interpretation in pediatric acute lymphoblastic leukemia

The European Federation of Clinical Chemistry and Laboratory Medicine established a workgroup in 2012 for exploring and harmonising the description, coding and registration of the errors of the laboratory procedures in the preanalytical phase. The workgroup published numerous recommendations, but preanalytics of the less often obtained samples for special laboratory tests (e.g. flow cytometry of the bone marrow or CSF) were rarely mentioned in these papers. However, compared with routine samples for hematological or chemistry tests, a lot more preanalytical errors may occur in regard of these special samples, e.g. the sample is unsuitable for the requested test, is clotted or the amount of the sample is not enough, wrong anticoagulant was used, contamination with peripheral blood (bone marrow, CSF, ascites), long transportation in poor conditions, low viable cell ratio.

Examining BM and CSF samples is essential in the diagnosis and follow-up of leukemias and lymphomas, including childhood ALL. Sample quality is a very important factor in obtaining adequate results. Hemodilution of a BM sample is quite common, His et al. found that 36% of the BM samples were hemodiluted from patients with acute leukemia.

According to the ALL IC BFM 2009 Flow MRD SOP a hemodiluted BM sample obtained on day 15 with the percentage of erythroid precursors below 2% is not eligible for flow risk stratification in childhood ALL. Several other methods have been described in identifying hemodilution. Our results showed 12.5% of the Day 15 BM samples to be inadequate, hampering the decision on the most suitable therapeutic protocol. Similar results were obtained with the Day 33 samples: 14% of these were hemodiluted. To avoid hemodilution of the BM sample aspiration should be done before biopsy, and no more than 1-2 mL of BM should be aspirated.

As hematological malignancies often affect the central nervous system, examination of the CSF is frequently needed. The core of the diagnosis is the identification of malignant cells by conventional cytomorphology in a CSF sample, although up to 60% of the cases can be false-negative. Flow cytometry has great sensitivity and specificity and is recommended by the National Comprehensive Cancer Network (USA) in conjunction with cytomorphologic studies. We could also confirm the utmost importance of flow cytometry, especially in cases of CSF samples with low nucleated cell count (<10 WBC/ μL) when malignant cells could be detected without PB contamination.

The cells decay rapidly in the CSF after sampling with granulocytes and monocytes showing the highest rate of degradation. Several studies tried adding different types of cell culture media to improve cell survival with promising results. Other methods were also examined, e.g. immediate cooling, minimizing centrifugation steps, aspirating supernatant instead of decanting sample. Preservatives (e.g. TransFix®) may also be used, moreover, TransFix® is recommended for CSF by the British Committee for Standards in Hematology. TransFix has been shown to stabilise malignant haematological cells in cerebrospinal fluid, making it possible to determine lekcocyte subsets in CSF via flow cytometric analysis 72 hours after lumbar puncture. Previous studies showed that the use of TransFix/EDTA CSF Sample Storage Tubes prevents cellular loss and enhances flow cytometric detection of leptomeningeal localized hematological malignancies much better than serum-containing medium-filled tubes or untreated tubes, because scatter and antigen expression characteristics of pathological cells are preserved.

In our study, significantly better results were achieved regarding the number of reportable results since more than 30% viable cells were detected in the CSF samples when TransFix® was used. However, pathological cells still can be identified in samples with low viable cell percentage, as it happened in the case of four samples in our study, therefore it is difficult to establish a clear cutoff for viable cell percentage under which the sample is considered inadequate and results are not reported. Reporting these cases should remain the decision of the examiner.

According to Petzold et al. up to 20% of standard lumbar punctures (LPs) are traumatic taps, although there is no consensus about the precise definition. We considered the tap to be traumatic if the RBC count in the CSF was >100 per microliter. In our two CSF cohorts, 45% and 36% of the samples were PB contaminated (children with ALL and native samples of a mixed age group, respectively), which is in accordance with results found in the literature.

Limitations of the present results include the retrospective nature of the study, a prospective design would have enabled us to record all quality indicators of the preanalytical phase. Furthermore, our results concerning the percentage of reportable CSF samples with or without preservative need to be validated in higher number of samples as well. In conclusion, poor sample quality can hamper risk stratification and further therapeutic decision in childhood ALL. Despite low viable cell count malignant cell populations may still be identified in a CSF sample, therefore establishing a certain cutoff point is difficult.

5.2. Analytical phase: detecting disseminated tumor cells with flow cytometry and immunohistochemistry

For the evaluation of the analyitical phase we compared two immuno-based methods: the gold standard immunohistochemistry and flow cytometry in detecting disseminated tumor cells (concordancy, sensitivity, specificity, diagnostic efficacy).

The model that malignant cells from a primary solid tumor dissociate, enter the systemic circulation (circulating tumor cell, CTC), reach other tissues and organs, settle in a secondary organ (DTC), and generate metastases is generally accepted. Detection of CTCs and DTCs can have a prognostic value.

Flow cytometric immunophenotyping has not been used as a routine diagnostic tool for solid tumors so far. With the introduction of several nonhematopoietic tumor markers, MFC may represent a useful diagnostic aid for cancer patients, especially those lacking a suitable trephine or tumor biopsy sample. Chang et al. have already suggested to apply CD99, myogenin, and CD56 in addition to EpCAM to indentify DTCs by MFC. The transmembrane glycoprotein EpCAM — expressed on epithelial cells — is involved in cell signaling, migration, proliferation, and differentiation. Several cancer cells express EpCAM. In lung cancer, EpCAM is usually overexpressed and acts as an oncogene. Detection of a CD45-/CD71-/EpCAM+ cell population by MFC may open a new path in the diagnosis of solid tumors and DTCs originating from these tumors.

Few studies have been published on comparing IHC results with MFC findings of tumor-infiltrated BM. Back in 1991, with the application of 1 tumor-specific monoclonal antibody, Molino et al found immunocytochemistry to be far more superior to flow cytometry and morphology in detecting breast cancer cells in the BM. Sah et al. compared MFC and morphology of BM biopsy and BM aspirates in chronic B-cell disorders. They found BM biopsy morphologic examination and flow cytometry to be complementary. Very few discrepancies were reported between these 2 methods, mainly in cases with evaluation of MRD. Graf et al. examined 141 BM samples in a comparative study focusing primarily on hematologic malignancies. They found 80.5% concordance between MFC and IHC, which was close to the 77% overall concordance figure found in our cohort.

On the basis of concordant/discordant results, we classified our cases into 5 different patterns. In the solid tumor cohort we had 22 samples investigated with both MFC and IHC. In 91% of these cases, results of the 2 different methods were concordant. MFC, however, has its limitations, as we also had MFCnegative/IHC-positive samples. One of these samples was from a

Hodgkin lymphoma patient. It has already been documented that MFC is not sensitive enough to detect Hodgkin cells even in lymph nodes. The other discordant case was from a patient with Langerhans cell histiocytosis in which tumor cells were located paratrabecularly, wherein it was difficult to "catch" them by BM aspiration.

In the neuroblastoma cohort 26 samples were investigated in parallel by the 2 methods and 65% gave concordant results. In the 2 MFC/IHC-positive cases tumor cells were embedded in stromal environment. As MFC is carried out on BM aspirate, these tumor cells anchored to BM stroma might have not been present in the aspirate. In these cases 5% and 36% erythroid precursors were detected by MFC in the samples, respectively, that excluded pronounced contamination by PB. MFC has several advantages to routine histopathology, especially if the ratio of residual pathologic cells is very low. Malignant cells below the detection limit of classic histopathology may unequivocally be detected by MFC, as it was shown by 7 MFC-positive/IHC-negative samples in the neuroblastoma-MRD cohort. When detecting residual neuroblastoma cells in the BM, Swerts et al found a strong correlation (χ^2=6.4, P=0.011) between MFC and IHC. Shen and colleagues examined serous cavity effusion samples from children with suspected malignancy. They found MFC to be 88% sensitive and 98% specific in detecting malignant cells in these samples. In our study MFC had 82% sensitivity in DTC detection in various solid tumors and 93% in neuroblastoma, whereas specificity was 100%.

Our results also showed that MFC was more sensitive and more effective in detecting the residual pathologic cells of neuroblastoma in the BM. For other types of solid tumors, MFC was less effective than IHC (91% vs. 100%). This may be due to the adherent nature of tumor cells, the preanalytical procedure of MFC that can destroy vulnerable malignant cells, and the lack of specific antibodies for detecting certain solid tumors. However, increasing the number of tumor-specific antibodies in 1 tube, labeled by 8 or 10 colors, and acquisition of 0.5 to 1.0 million cells can increase the accuracy of MFC in solid tumor detection. In conclusion, MFC and BM morphology/IHC represent complementary methods for the detection of DTCs. Detection of DTCs by MFC may provide a diagnostic aid for patients with suspected malignancies who do not have a proper tumor biopsy sample. In the detection of MRD in patients with neuroblastoma, MFC is superior to IHC.

5.3. Postanalytical phase: establishing age-specific reference ranges for thymidine kinase activity and validating the results on a group of CLL patients

The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) established a workgroup (Laboratory Errors and Patient Safety) in 2008. This workgroup is dedicated especially for dealing with the extra-analytical (pre- and postanalytical) procedures and their errors, the effects of these errors on result interpretation. Postanalytical phase consists of interpretating the result, comparing it with the reference range.

According to the 2008 guideline of the Clinical Laboratory Standards Institute, it may be necessary to carry out a minor reference value study to validate a reference interval given by the test manufacturer or a donor laboratory. Soon after we started our TK1 measurements with the test manufacturer's reference range (2–7.5 U/L) it turned out that it was not applicable to our population, as we often observed higher TK1 activity than the suggested upper reference limit even in healthy individuals. The interpretation of these results was not always convincing and caused some unnecessary anxiety for physicians and patients. The observed elevated TK1 activity in our healthy population and the significant differences in TK1 activity between the age groups encouraged us to define our own reference ranges for TK1. Although TK1 measurements are widely used in malignant diseases for prognostic purposes and even in routine health screening, to our knowledge no age-related reference ranges have been published so far for TK1 activity.

Based on our 97.5 percentile results, the suggested reference values are <30 U/L for young (18–35 years), <25 U/L for middle-aged (36–60 years) and <19 U/L for elderly (60–86 years) adults. The age-specific reference ranges may be crucial in the context of chronic lymphocytic leukemia, where most of the affected are older individuals. We also identified a cutoff point of 10.5 U/L distinguishing CLL patients in the RAI 0 stage from healthy individuals. This result correlates well with the finding of Letestu et al, who found a serum TK1 activity of 10 U/L to be an independent predictor of progression-free survival with a hazard ratio of 2.98, $p<0.0001$.

It is also known that CD38+ CLL has a more adverse prognosis. Hus et al showed that CD38 combined with Zap70 expression amplified the prognostic power of both markers. They found that Zap70+/CD38+ patients had shorter event-free survival than CD38–/Zap70– patients. In our study, we found significantly higher TK1 activity in CD38+/Zap70+ as compared to CD38–/Zap70– patients and there was no significant difference between CD38–/Zap70– and CD38–/Zap70+ patients. Higher serum TK1 activities of CD38+ CLL patients can be

explained by the intense proliferation of cells, as both TK1 and CD38 are proliferation markers.

Furthermore, in line with published data, del(13q), 12 trisomy, del(17p13) and del(11q22) FISH aberrations did not influence TK1 values in our patients. TK1 activity correlated with WBC and absolute B cell count in our study, this finding supports the data reported previously by Hallek et al.

Limitations of the present results include the cross-sectional nature of the CLL study, a prospective design would have enabled us to examine the utility of TK1 as a prognostic marker. Although the CLL population was represented by a cohort of 115 patients, the non-similar number of patients in the different Rai stages, and as per their CD38 and Zap70 characteristics may have distorted the statistical findings. Furthermore, our age-specific reference ranges need to be validated in other healthy populations as well. In conclusion, serum TK1 levels decline significantly with age in the healthy, and this finding warrants the use of age-related reference ranges. Furthermore, it may distinguish CLL patients in the Rai 0 stage from healthy individuals in the diagnostic work-up of CLL suspect cases.

6. Summary

The complex system of laboratory procedures are composed of the preanalytical, analytical and postanalytical phases. Numerous studies have been published about the analysis of the certain phases and searching for possible errors in the context of automatized laboratory methods. The special diagnostic procedures, however, are much less represented in such studies. Our aim was to evaluate the preanalytical, analytical and postanalytical phase in special, immunology-based laboratory methods by examining liquid biopsy samples in malignant diseases, and to examine their effect on result interpretation.

In our studies of the preanalytical phase we found hemodilution in 12.5% of the Day 15, 14% of the Day 33 BM samples from pediatric ALL patients, inadequate for risk stratification. CSF samples from children with ALL were contaminated with preipheral blood in 45% of the samples. In 54% of the mentioned CSF samples the viable cell ratio among all the collected events was <30%. Half of these low viable cell ratio samples, however, did have a recognizable malignant cell population, which means that no clear cutoff can be established in regard of the evaluability of such samples. Using stabilized samples the ratio of these low viable cell count samples dropped from the observed 41% in the native samples to 17%.

In the studies of the analytical phase we found 91% concordancy of flow cytometry and immunohistochemistry in detecting disseminated tumor cells. The concordancy was 65% in the context of neuroblastoma. Flow cytometry had better performance in detecting neuroblastoma cells in *de novo* samples (100% vs. 86%), and its advantage was even more clear in detecting minimal residual disease (92% vs. 68%) in the hypoplastic bone marrow. In other solid tumors immunohistochemistry had better performace than flow cytometry (100% vs. 91%).

In regard of the postanalytical phase we established age-specific reference ranges of thymidine kinase activity. Reference limit in the young (18-35y) population was determined as <30 U/L, <25 U/L in the middle-aged (36-60y) and <19 U/L in the elderly (>60y) population. In the CLL group patients in advanced Rai stages had significantly higher TK levels. We found correlation between TK levels and WBC and B cell count. ROC analysis determined TK cutoff value as 10.5 U/L between CLL patients and healthy individuals. This result is in accordance with data in literature.

7. Main new scientific findings and their clinical significance

1. Preanalytical phase findings:

   a) In pediatric ALL 12.5% of day15 and 14% of day33 bone marrow samples were contaminated with peripheral blood, thus being inadequate for flow cytometric risk stratification.

   b) In pediatric ALL 45% of cerebrospinal fluid samples were contaminated with peripheral blood while in 54% the percentage if living cells was below 30%, thus these samples were considered inadequate for evaluation. Despite low viable cell count malignant cell populations may still be identified in a CSF sample, therefore establishing a certain cutoff point is difficult.

   c) Ratio of samples with less than 30% viable cells significantly decreased when a stabilizer was used (41% vs 17%; p=0,05).

2. Analytical phase findings:

   a) Concordance between immunohistochemistry and flow cytometry in the detection of disseminated solid tumor cells was 91%, in case of neuroblastoma cells it was 65%.

   b) Diagnostic efficacy of flow cytometry is better than immunohistochemistry in the detection of *de novo* neuroblastoma cells (100% vs 86%), this difference is even more pronounced in minimal residual disease detection (92% vs 68%), especially in hypoplastic bone marrow environment.

   c) Diagnostic efficacy of immunohistochemistry is better than flow cytometry in the detection of solid tumor cells (100% vs 91%).

3. Postanalytical phase findings:

   a) Serum TK1 levels show no difference in men and women, however, decline significantly with age in the healthy, and this finding warrants the use of age-related reference ranges.

   b) In a healthy population we determined the reference ranges: <30 U/L for young (18–35 years), <25 U/L for middle-aged (36–60 years) and <19 U/L for elderly (60–86 years) adults.

   c) We also identified a cutoff point of 10.5 U/L distinguishing CLL patients in the RAI 0 stage from healthy individuals.

8. List of publications

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Registry number: DEENK/278/2020.PL
Subject: PhD Publication List

Candidate: Eszter Szánthó
Doctoral School: Kálmán Laki Doctoral School

List of publications related to the dissertation

1. **Szánthó, E.**, Kárai, B., Ivády, G., Baráth, S., Széles, M., Kappelmayer, J., Hevessy, Z.: Evaluation of sample quality as preanalytical error in flow cytometry analysis in childhood acute lymphoblastic leukemia.
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List of other publications

4. Kárai, B., Gyurina, K., Ujfalusi, A., Sędek, Ł., Barna, G., Jáksó, P., Svec, P., **Szánthó, E.**, Nagy, A. C., Müller, J., Simon, R., Vojczek, Á., Szegedi, I., Tiszlavicz, L. G., Kowalczyk, J. R., Kolenova, A., Kovács, G. T., Szczepański, T., Dworzak, M., Schumich, A., Attarbaschi, A., Nebral, K., Haas, O. A., Kappelmayer, J., Hevessy, Z., Kiss, C.: Expression Patterns of Coagulation Factor XIII Subunit A on Leukemic Lymphoblasts Correlate with Clinical Outcome and Genetic Subtypes in Childhood B-cell Progenitor Acute Lymphoblastic Leukemia.
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Total IF of journals (all publications): 14,44
Total IF of journals (publications related to the dissertation): 5,097

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

01 October, 2020