Protocol

Prognostic Significance of Circulating Tumor DNA in Hodgkin Lymphoma

Multicentric, prospective, non-interventional study

Brief Title: Circulating tumor DNA in Hodgkin Lymphoma

Sponsor: Department of Hematology, Faculty Hospital Kralovske Vinohrady, Prague

Principal investigator and coordinator of the study

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Synopsis

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Brief Title: Circulating tumor DNA in Hodgkin Lymphoma

Official Title: Prognostic Significance of Circulating Tumor DNA in Hodgkin Lymphoma

Brief Summary: Specific somatic mutations using ctDNA will be analyzed in predefined subgroups of cHL (e.g., age <60 and ≥60 years, EBV). These mutations will be correlated with response to the treatment in the first line, in the relapse, during brentuximab vedotin and/or nivolumab treatment. Circulating tumor DNA will be correlated with the extent of tumor mass and chemo/radiotherapy.

Detailed Description: Samples of plasma from peripheral blood will be taken for investigational ctDNA examination during the specific timepoints: at diagnosis, after 2 cycles of initial chemotherapy, at the end of chemotherapy, 3 months after radiotherapy, at the diagnosis of the first relapse, after salvage chemotherapy before ASCT, 3 months after ASCT, at the diagnosis of second relapse and every 3 months during brentuximab vedotin treatment or during nivolumab treatment until progression. The buccal swab for germline DNA extraction will be performed at the time of enrollment into the study. Samples of peripheral blood for EBV-DNA analysis will be obtained from the EBV-positive cHL patients to measure EBV load at the same time-points as ctDNA. Microdissected HRS cells from fresh frozen biopsies at the diagnosis and at the relapse will be used for tumor cells next generation sequencing.

Study Type: Observational, multicentric.

Study Design: Time perspective: Prospective non-interventional study.

Biospecimen: Samples with DNA. Description: frozen plasma.

Sampling Method: Non-probability sample.

Observational Model: Cohort.

Study Population: Male or female adults 18 years or older with a documented diagnosis of Hodgkin lymphoma.

Condition: Hodgkin lymphoma.

Estimated Enrollment: 400 newly diagnosed Hodgkin lymphoma patients.
100 relapsed Hodgkin lymphoma patients.

Study Start Date: January 2, 2022

Estimated Primary Completion date: December 31, 2025

Estimated Study Completion Date: December 31, 2027

Outcome Measures:

1. Identification of tumor specific mutation profiles at diagnosis of cHL based on ctDNA analysis in correlation to specific characteristics:

a) Age at diagnosis <60 years in comparison to patients with age at diagnosis ≥ 60 years;

b) EBV-negative vs. EBV-positive cases;

c) Correlation with the first line treatment outcome;

2. Quantitative analysis of ctDNA level during the first line chemotherapy:

a) Correlation to the type of chemotherapy: BEACOPP escalated vs. ABVD;

b) Dynamics of ctDNA decline in correlation with treatment response;

3. Identification of tumor specific mutation profiles at relapse of cHL:

a) Detection of newly developed mutations in comparison to the initial diagnosis;

b) Characteristics of mutations in cHL tumors refractory to brentuximab vedotin;

c) Characteristics of mutations in HL tumors refractory to nivolumab;

4. In vitro functional characterization of identified DNA variants and/or mutations:

a) Variants with unknown impact on cHL development;

b) Variants identified as associated with features analyzed in above mentioned measures.

Eligibility Criteria:

- Male or female adults 18 years or older;
- Documented diagnosis of classical Hodgkin lymphoma newly diagnosed or relapsed;
- Willing and able to comply with the scheduled study procedures;
- Evidence of a signed informed consent.

Exclusion Criteria:

- Other diagnosis than classical Hodgkin lymphoma (newly diagnosed or relapsed);
- Noncompliance to undergo the scheduled study procedures;
- Refusal to sign the informed consent.

Sex/Gender: Sexes eligible for study: all.

Ages: 18 years or older.

Contents

1.Responsibilities for management of the study

1.1. Sponsor and coordinating center of the study: Department of Hematology, Faculty Hospital Kralovske Vinohrady, Prague

To become an active center, the process of authorization must take place. The minimum documentation required for authorization includes:

- investigator's CV

- local ethics committee approval

- multicentric ethics committee (or any equivalent) approval

- approval of the hospital, where the study is planned

- each study center is responsible for: data entry to the Czech Hodgkin Lymphoma Registry

Note: this is a non-interventional (observational) study and the approval of the State Institute for Drug Control (SÚKL) is not required.

1.2. Investigators and collaborators

Principal investigator (PI): Heidi Mocikovaa, M.D. Ph.D. (Faculty Hospital Kralovske Vinohrady, Prague) is responsible for the design of the study. Her role is to coordinate the work of all co-investigators, collaborators, and data managers in all centers. Investigators closely cooperate in resolving medical problems associated with this study. PI is responsible for the correct enrollment of patients and their treatment. PI is responsible for the correct method of acquisition of the study samples and their storage. PI is responsible for the study medical data recording and for collection of study data from all centers and their submission for statistical analyses. Pi and co-investigators are responsible for interpretation of acquired statistical data and for publication of articles in scientific journals.

Main co-investigator: Ondrej Havranek, M.D. Ph.D. (The First Faculty of Medicine, Charles University, Prague and BIOCEV Institute) is a leader of the laboratory team. He is responsible for the ctDNA laboratory analyses and follow-up experiments. He contributes to the overall

management of the research, data interpretation, statistical analyses, and publication of the results.

Investigators: Jan Koren M.D. (General University Hospital in Prague), prof. Vit Prochazka, M.D. Ph.D. (the University Hospital Olomouc), Alice Sykorova, M.D. Ph.D. (the University Hospital Hradec Kralove). Investigators are responsible for providing information to the patients, enrollment of eligible patients, treatment of patients, acquisition, storage and shipping of the study samples, restaging procedures in regular intervals defined by this study, medical data recording, registering patients into the Hodgkin Lymphoma Registry, submission of study medical records for statistical analyses, and coordination of the work of all included physicians, nurses, and data managers within their centers.

Collaborators: prof. Tomas Kozak, M.D. Ph.D. (Faculty Hospital Kralovske Vinohrady, Prague), Maria Maco M.D. (Faculty Hospital Kralovske Vinohrady, Prague), Lukasová Marie M.D. (the University Hospital Olomouc), Pavla Stepankova M.D. (the University Hospital Hradec Kralove). Collaborators are involved in treatment of patients, acquisition, storage and shipping of the study samples, restaging procedures in regular intervals defined by this study, medical data recording, registering patients in the Hodgkin Lymphoma Registry, and submission of study medical records for statistical analyses.

Collaborators: Kristyna Kupcova, MSc. Ph.D. (Biocev Institute) and Iva Ondeckova, M.D. Ph.D. (BIOCEV Institute) are involved in the ctDNA sequencing as well as data processing.

Collaborators: Zuzana Prouzova, M.D. (Faculty Hospital Kralovske Vinohrady, Prague), Patrik Flodr, M.D. (the University Hospital Olomouc) and Marketa Kalinova, M.D. assoc.prof. (Faculty Hospital Kralovske Vinohrady, Prague) are responsible for examination of pathology samples and microdissection of HRS cells from frozen biopsies and preparation for exome sequencing.

Collaborator: Petr Hubacek, M.D. (University Hospital Motol) is responsible for EBV DNA analyses from the peripheral blood.

Data manager: Ing. Katerina Klaskova (Faculty Hospital Kralovske Vinohrady, Prague) is responsible for the study medical data records of all patients from all centers. She is responsible for submission of all electronic documentation for statistical analyses.

2. Background

The incidence of Hodgkin's lymphoma (HL) in the Czech Republic is relatively low with 2.7 new cases per 100,000 inhabitants per year. Majority of HL patients (80%) can be cured with conventional chemoradiotherapy. However, 20% of patients younger than 60 years and almost 50% of patients older than 60 years relapse after the first line treatment. Relapse of HL is generally associated with a poor prognosis. Multiple systems of predictive factors and scoring systems were developed for patient stratification and therapy outcome prediction to guide selection of the most appropriate type and intensity of chemotherapy and radiotherapy. The unfavorable prognostic factors in early stages of HL are: high sedimentation rate, massive mediastinal tumor, extranodal involvement and involvement of three or more lymph node areas. The Hasenclever International Prognostic Score (IPS) is used in advanced stages. Lack of our ability to accurately separate patients at high risk of relapse does not allow rational treatment intensity adjustment at diagnosis. Several studies concluded, that the interim positron emission tomography/computed tomography (PET/CT) after two cycles of chemotherapy (PET-2) is a stronger predictor of outcome than any pretreatment prognostic factor, however, the exact positive and negative prognostic significance in early and advanced stages are still discussed.

Despite the success of the current therapy, there is an unmet need to identify risk factors related to the initial treatment response, as well as to the relapse after ASCT or responsible for brentuximab vedotin and/or nivolumab treatment resistance. From a large number of novel biomarkers, the latest advances suggest that circulating tumor DNA (ctDNA) based approach might represent novel and non-invasive method for classical HL (cHL) risk stratification and disease monitoring.

It has been shown that DNA could be detected in various non-cellular body compartments and/or fluids. This non-cellular DNA, termed cell-free DNA (cfDNA), is detectable in all healthy individuals. It is generated during multiple physiological cellular processes including apoptosis, necrosis or active secretion of extracellular vesicles and exosomes. One of the main sources of cfDNA are hematopoietic cells, cfDNA is a double stranded and naturally fragmented DNA (<200 base pairs) presented in low concentrations, between 10 and 30 ng per ml of plasma. Circulating tumor DNA (ctDNA) is a fraction of cfDNA that originates in the tumor cells. The capability to detect and analyze this tumor-specific DNA from plasma, serum or other body fluids

led to the concept of a "liquid biopsy". In comparison to the “classic” tumor biopsy, the main advantages of the ctDNA analysis are: minimal invasivity, reflection of the tumor heterogeneity across all affected tumor sites and easy repetitive sampling allowing longitudinal assessment in various timepoints. Depending on the stage and location of lymphoma, ctDNA can be found in a variety of body fluids, including blood plasma, urine, sputum, cerebrospinal fluid, pleural fluid and saliva, but low levels of ctDNA may represent a substantial issue for its analysis. The amount of required ctDNA is dependent on the particular technique with the highest yields of ctDNA achievable from plasma. In contrast to other cancers, the Hodgkin and Reed-Sternberg (HRS) malignant cells represent less than 5% of the total number of cells within the HL tumors. The remaining part is formed by tumor infiltrating immune cells forming inflammatory microenvironment. This issue complicates studies of frequency and types of HRS tumor associated somatic mutations, therefore, making the concept of liquid biopsy to analyze the tumor genome even more advantageous in this disease. Following initial studies of mutation status of individual genes, HL somatic mutation patterns were analyzed by next generation sequencing (NGS) methods. The most affected pathways and genes implicated in HL development include: JAK-STAT signaling (e.g. STAT6 and SOCS1), NF-kB pathway regulation (e.g. NFKBIE, TNFAIP3), antigen presentation (B2M), PI3K/AKT signaling regulation (ITPKB, PIK3CA), signaling form G-protein-coupled receptors (GNA13, a G-proteins subunit), DNA repair and its regulation (e.g. ATM, TP53), chromatin modifying genes (KMT2D) or nuclear export protein XPO1. Despite this progress, there are many unanswered questions in HL, e.g. an association of EBV-positive HL with a certain spectrum of mutations that is different from EBV-negative HL. EBV is a known factor in HL development with unfavorable prognosis. In patients with EBV-positive HL the cell-free EBV-DNA is derived from apoptotic or necrotic EBV- infected cells. EBV-DNA in plasma correlates with EBV-positive tumor status in HL before therapy and during the follow-up. According to the data from the Hodgkin Lymphoma Registry, the incidence of EBV-positive HL is 20% in the Czech Republic.

The first NGS based analysis of ctDNA was performed in 10 HL patients showing that ctDNA in HL could be used to monitor disease related genomic imbalances. Spina et al. performed a larger analysis of 80 untreated and 32 relapsed/refractory patients using NGS of predesigned panel of 77 genes relevant to HL and B-cell lymphomas. They showed that ctDNA profiles largely mirrored the mutational status of HRS cells and that the rate of ctDNA level decreased after the

first two cycles of chemotherapy and correlated with treatment outcome. Analysis of relapsed patients detected largely similar mutation spectrum as in the untreated patients, which could have been caused by limited number of genes analyzed in their panel not allowing detection of mutations in unpredicted genes and low number of relapsed/refractory patients. The correlation of ctDNA levels decrease and treatment outcome was confirmed in the study of Camus et al. They analyzed 60 cHL patients and panel of nine commonly mutated genes in HL. The above-mentioned studies show the proof of concept that ctDNA analysis in HL has a potential for treatment monitoring and disease course prediction, however, additional, and larger prospective studies are needed to confirm the concept of ctDNA levels monitoring during the treatment and answer additional questions. Could ctDNA analysis before treatment predict the treatment outcome in the first line therapy or in relapsed/refractory patients? Could the spectrum of somatic DNA mutations correlate with therapy resistance at any stage of the treatment? Could ctDNA analysis-based DNA mutations be used in HL diagnostics or provide more information about the HL biology? The overall aim of the proposed study is to answer those questions using qualitative and quantitative NGS based ctDNA analyses to identify tumor derived DNA mutations, clonality and changes in the ctDNA levels.

3. Definitions of study end points

1. Identification of tumor specific mutation profiles at diagnosis of cHL based on ctDNA analysis in correlation to specific characteristics:

a) Age at diagnosis <60 years in comparison to patients with age at diagnosis ≥ 60 years;

b) EBV-negative vs. EBV-positive cases;

c) Correlation with the first line treatment outcome;

2. Quantitative analysis of ctDNA level during the first line chemotherapy:

a) Correlation to the type of chemotherapy: BEACOPP escalated vs. ABVD;

b) Dynamics of ctDNA decline in correlation with treatment response;

3. Identification of tumor specific mutation profiles at relapse of cHL:

a) Detection of newly developed mutations in comparison to the initial diagnosis;

b) Characteristics of mutations in cHL tumors refractory to brentuximab vedotin;

c) Characteristics of mutations in HL tumors refractory to nivolumab;

4. In vitro functional characterization of identified DNA variants and/or mutations:

a) Variants with unknown impact on cHL development;

b) Variants identified as associated with features analyzed in above mentioned measures.

4. Design of the study

4.1.Design of the study

This is a prospective non-interventional observational study. Patients ≥ 18years with newly histologically confirmed classical Hodgkin lymphoma (cHL) will be enrolled into this study after signing the informed consent. All patients will undergo standard diagnostic procedures including EBV status, clinical stage and evaluation of risk factors. Patients <60 years will ve divided into early, intermediate or advanced risk group based on the risk factors and treated according to the standard treatment recommendations. Patients≥ 60 years will be treated according to the regimens for older patients. Younger relapsed patients will be treated with salvage platinum-based chemotherapy followed by high-dose chemotherapy and autologous stem cell transplantation (ASCT). Relapses after ASCT in younger patients or after at least two lines of chemotherapy in older patients will be treated with nivolumab until progression or brentuximab vedotin.

4.2.Start date: January 2, 2022

4.3.Primary completion date: December 31, 2025

4.4. Study completion date: December 31, 2027

5. Study population

5.1. Study population

Male or female adults 18 years or older with a documented diagnosis of Hodgkin lymphoma.
Estimated enrollment: 400 newly diagnosed Hodgkin lymphoma patients and 100 relapsed Hodgkin lymphoma patients.

5.2. Eligibility criteria:

Male or female adults 18 years or older.

Documented diagnosis of classical Hodgkin lymphoma newly diagnosed or relapsed.

Willing and able to comply with the scheduled study procedures.

Evidence of a signed informed consent.

5.3.Exclusion criteria:

Other diagnosis than classical Hodgkin lymphoma (newly diagnosed or relapsed).

Noncompliance to undergo the scheduled study procedures.

Refusal to sign the informed consent.

6. Schedule of procedures

6.1. Informed consent

Written informed consent (Appendix A) must be obtained from each subject prior to entering the study. The patient and the investigator must personally date and sign two originals of informed consent forms. The patient shall receive one original of a fully completed informed consent form and the second completed original will be archived as a part of the medical documentation.

6.2.Sampling time-points

Samples of plasma from peripheral blood will be taken for investigational ctDNA examination during specific timepoints: at diagnosis, after 2 cycles of initial chemotherapy, at the end of

chemotherapy, 3 months after radiotherapy, at the diagnosis of the first relapse, after salvage chemotherapy before ASCT, 3 months after ASCT, at the diagnosis of second relapse and every 3 months during brentuximab vedotin treatment (up to16 cycles) or during nivolumab treatment until progression. The buccal swab for germline DNA extraction will be performed at the time of enrollment into the study. Samples of peripheral blood for EBV-DNA analysis will be obtained from the EBV-positive cHL patients to measure EBV load at the same time-points as ctDNA. Biopsies are usually performed at the diagnosis and at the relapse. Microdissected HRS cells from fresh frozen biopsies at the diagnosis and at the relapse will be used for tumor cells next generation sequencing.

6.3. Number of patients and general strategy of ctDNA analysis

Overall, 400 patients with newly diagnosed HL, 50 relapsed HL patients and 50 patients treated with brentuximab vedotin or nivolumab using a custom designed panel of 400 genes relevant to the HL and/or lymphoma biology will be analyzed. Twenty relapsed patients with the worst treatment outcome will be analyzed by whole exome sequencing as a discovery set for more focused analysis in the relapsed/refractory patients. To validate the accuracy of panel based ctDNA sequencing, the exome sequencing on microdissected HRS cells will be performed in 20 frozen biopsies/sorted cells with adequate numbers of HRS cells.

6.4.Collection and ctDNA extraction

We will use the Cell-Free DNA BCT tubes (Streck) containing cell lysis preservative to prevent lysis of white blood cells and contamination of plasma with genomic DNA and to allow storage of samples at the room temperature for up to 7 days before initial processing. Samples with frozen plasma will be delivered to the BIOCEV Institute for further processing. The cfDNA will be extracted using the QIAamp Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer‘s protocol. The cfDNA concentration will be measured using Quant-iT Pico Green dsDNA Assay kit (ThermoFisher Scientific).

6.5. NGS based sequencing procedures

As a general sequencing approach, we will use the methodology of CAncer Personalized Profiling by deep Sequencing (CAPP-Seq). Lymphoma specific panel of 400 genes based on the

previously published studies of lymphoma tumor genome will be used. In a limited number of patients (e.g., primary refractory patients, subset of relapsed patients), a deep whole exome sequencing will be performed.

Standard procedures will be used for library preparation, capture and sequencing. The gDNA will be shared by sonication (Covaris) to obtain 200 bp long fragments. Libraries construction will be done using KAPA Hyper Prep library preparation kit (KAPA Biosystems), hybrid selection will be done by custom SeqCap EZ Choice Library (Roche) for each panel of genes or SeqCap EZ Exome Probes (Roche) for exome sequencing. Multiplexed libraries will be then sequenced using 150-bp paired-end runs on NovaSeq instrument (Illumina) with targeted coverage of 2000 in at least 80% of the design panel positions.

6.6.NGS data processing pipeline

Circulating tumor DNA and germline paired samples analysis will include the following steps: 1) deduplication using Fastuniq, 2) reads alignment using BWA, 3) indexing sorting and Mpileup file creation using SAMtools, 4) variants calling using VarScan246 (min-var-freq 0.003, min-var-freq-for-hom 0.75) for nucleotide changes and Mutect2 (Broad Institute) for longer deletions and insertions identification. In the next step, identified variants will be filtered to correct for multiple comparisons, strand bias and sequencing errors. The VarScan2 generated p value of each individual variant will be corrected by Bonferroni correction for multiple comparisons, specifically divided by the number of the base positions in a particular panel design multiplied by 4 (as each of the individual 4 bases could be detected at each position). For each variant, Fisher's exact test will be applied to compare the number of wt forward and wt reverse vs. mutated forward and mutated reverse reads. Variants will be filtered to be present in at least 4 forward and 4 reverse reads. Sequencing errors also repeat in different samples, therefore, the last step to filter out sequencing errors will be filtering of variants identified with high frequency across all sequenced samples (cfDNA as well as gDNA), taking into account hot-spot mutations. The quantity of ctDNA in plasma will be expressed as a haploid genome equivalents per milliliter of plasma (hGE/mL). The ctDNA proportion of the total cfDNA concentration in each sample will be calculated based on the mean allele fraction of tumor associated somatic mutations

Filtered variants will be annotated for their functional impact and explored by pathway analysis.

Identified variants as well as the dynamics of ctDNA levels will be correlated with proposed and all available clinical and pathological characteristics of the patients and tumors as outlined in individual aims of the proposed project. The most significant mutations and genes will be further explored in-vitro to confirm their pathogenic role in HL development and/or treatment resistence.

6.7.EBV DNA load analysis

In agreement with EU regulation 2017/746, EBV DNA will be the detected using commercial detection kits. DNA extraction will be performed using Qiagen QIAamp DNA Blood Mini kits and for quantitative detection, Geneproof Epstein-Barr Virus (EBV) PCR kit. Results will be expressed in the IU/ml.

6.8.Molecular biology analyses

We will use well-defined STR-verified, and commercially available HL cell lines (e.g., SUPHD1, L540, KMH2, L-428, L1236). We will knock out (KO) genes of interest (GOI) using the CRISPR/Cas9 system, specifically the pSpCas9(BB)-2A-GFP (PX458) plasmids. To transiently transduce the cell lines with Px458 plasmid, electroporation will be used (Neon Transfection System, ThermoFisher Scientific), which will be followed by single cell sorting of GFP positive cells and clone's expansion. To avoid artifacts of possible non-specific off target effects (e.g., KO of other gene), GOI will be targeted at three independent sites and individual different KO clones will be evaluated in parallel. The GOI KO status will be verified by western blotting. For overall analysis of GOI KO phonotype, modified and control cells will be interrogated for changes in general cellular functions (e.g., growth, cell cycle, apoptosis, metabolism, drug resistance).

6.9. Proposed study schedule and expected results

Year	Planned study progress in individual aims	Presentation of results

1	Samples of peripheral blood, clinical, laboratory and imaging data will be collected from 100 newly diagnosed and treated patients including 20 EBV-positive patients, 10 relapsed patients treated with ASCT and 10 relapsed patients treated with brentuximab vedotin or nivolumab. Laboratory ctDNA and EBV-DNA analyses will be initiated. Exome sequencing from 10 frozen biopsies will be performed on microdissected HRS cells.	Annual report
2	Samples of peripheral blood, clinical, laboratory and imaging data will be collected from 150 newly diagnosed patients including 30 EBV-positive patients, 20 relapsed patients treated with ASCT and 20 relapsed patients treated with brentuximab vedotin or nivolumab. Laboratory ctDNA and EBV-DNA analyses will be performed. Exome sequencing from 10 frozen biopsies will be performed on microdissected HRS cells.	Annual report
3	Samples of peripheral blood, clinical, laboratory and imaging data will be collected from 150 newly diagnosed patients including 30 EBV-positive patients, 20 relapsed patients treated with ASCT and 20 relapsed patients treated with brentuximab vedotin or nivolumab. Analyses of ctDNA and EBV-DNA will continue. Follow-up molecular biology experiments will be initiated.	Annual report
4	Final ctDNA analyses and follow-up molecular biology experiments will be performed. Database lock and data cleaning will be performed. Final statistical analyses will be performed.	Final report and submission of manuscripts

7. Premature withdrawal of a subject

Circumstances that lead to a premature withdrawal of a subject from the study must be clearly recorded by the investigator in the medical documentation. Criteria for subject withdrawal include death, non-compliance, voluntary withdrawal of patient or responsible physician, failure to meet the eligibility criteria, etc. Patients are free to withdraw from the study at any time without affecting their standard treatment. When the patient interrupts any contact with the study center, the patient is considered lost for follow-up.

8.Data acquisition and statistical analyses

8.1. Working hypothesis of the study

We hypothesize, that specific HL associated somatic mutations detectable by the analysis of HL ctDNA might be associated with the specific subgroups (e.g., age <60 and 60 years, EBV status), might predict response to the initial and relapse treatment, might correlate with brentuximab vedotin and nivolumab treatment response and/or might be responsible for resistance. We also hypothesize, that ctDNA correlates with the extent of tumor mass and treatment sensitivity and can be used for monitoring of the treatment.

8.2. Planned number of recruited subjects and collected samples

The anticipated number of recruited newly diagnosed patients is 400 and each patient will provide samples of peripheral blood at diagnosis, after 2 cycles of chemotherapy and at the end of treatment (2022-2025). After the first line treatment the number of complete and partial remissions, stable diseases and progressions evaluated by PET/CT will be recorded. The overal estimated number of EBV-positive patients indicated for EBV DNA load analysis is 80. Gene mutations will be analyzed on microdissected HRS cells from 20 representative frozen biopsies.

The anticipated number of relapsed patients undergoing ASCT (without further relapses) is 50 and each patient will provide samples of peripheral blood at the diagnosis of relapse, after salvage chemotherapy and 3 months after autologous stem cell transplantation (2022-2025). Responses evaluated by PET/CT will be recorded. The estimated number of patients with multiple relapses treated with brentuximab vedotin is 25 and with nivolumab is 25 (2022-2025). Each of these patients will provide samples of peripheral blood before treatment with brentuximab vedotin or nivolumab and every 3 months thereafter until confirmed relapse.

8.3. Description of statistical methods

Unsupervised hierarchical clustering according to the mutational status of individual genes in individual patients will be performed to identify eventual subgroups of patients. These subgroups will be tested for differences in survival (by Kaplan-Meyer method) and correlation with clinical or pathological features: categorical variables by χ-square and Fisher's exact tests, continuous

variables by Mann-Whitney test. Similar correlation will be performed for patients divided into two groups based on: 1) the presence or absence of mutations in the most frequently mutated genes, for each gene individually, 2) the absolute levels of ctDNA (testing multiple thresholds), 3) the degree of drop of ctDNA levels after two cycles of chemotherapy setting the threshold at the proposed 100-fold decrease. To compare the survival, we will use the progression free survival defined as a time from treatment initiation to the time of progression, death from any cause, or last follow-up. All tests will be performed as two sided, with significance set at $p < 0.05$. Results will be also corrected for multiple comparisons.

9. Ethical and regulatory principles

9.1 Ethical principles

This protocol is created in accordance with the principles laid down by the 18th World Medical Assembly (Helsinki, 1964) and its amendments laid down by the 29th (Tokyo, 1975), the 35th (Venice, 1983) and the 41st (Hong Kong, 1989) World Medical Assemblies.

9.2 Laws and regulations

This protocol is performed in accordance with laws and regulations of the Czech Repubic as well as with other standards of good clinical practice.

10. Administrative procedures

10.1 Secrecy agreement

All materials, information (oral or written) and unpublished documentation provided to the investigators including this protocol are the exclusive property of the sponsor of the study. They may not be given or disclosed by the investigator or by any person without the prior written formal approval of the study coordinator. The investigator shall consider the study confidential and shall take all necessary measures to ensure that there is no break of confidentiality in respect

of all information accumulated, acquired or deduced in the course of the study, other than that information to be disclosed by law.

10.2. Record retention in investigating centers

Laws and regulations of the Czech Republic determine the maximal period of data retention. Studies performed across the European Union should retain study data of patients for 15 years after the completion or discontinuation of the study. Each center will notify the sponsor before destroying any data or records.

10.3. Ownership of data and use of the study results

Sponsor has the ownership of all data and results collected during this study. Sponsor has the right to use the data for presentation of the study.

10.4. Publication policy

All study data and publications are the property of the sponsor. Study results will be published after data collection and evaluation. Interim results can be published before the final analysis. Any presentation of data - oral, poster or publication must be approved by the sponsor. The final report will include at least coordinator of the study, investigators according to the of number of enrolled patients. Names of co-authors should change if several publications are performed.

10.5. Final report of the study

Study coordinator is responsible for preparation and processing the final report.

10.6. Protocol amendments

Protocol amendments are integral parts of the protocol. Changes or amendments of this protocol may be made only after discussion and agreement of all investigators, study coordinator and the sponsor. Any changes agreed upon will be recorded in written form, the written amendment will be signed by the investigators and by the sponsor and the signed amendment will be added to the protocol.

11.Literature

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Appendix A. Information for a Patient

Prognostic Significance of Circulating Tumor DNA in Hodgkin Lymphoma

Dear patient,

We would like to invite you to participate in a laboratory research focused on Hodgkin lymphoma: its early diagnostics, and prediction of eventual tumor progression. This research is based on the analysis of circulating tumor DNA. Upon signing an informed consent, we would collect additional samples of your peripheral blood that will be taken together with regular blood tests related to your treatment in order to estimate the extent of the tumor and assess the prognosis.

Providing a sample of peripheral blood for molecular-genetic testing does not mean any additional medical risk, as it will be taken together with other routine diagnostic peripheral blood samples at the diagnosis and during the treatment.

Research of Hodgkin lymphoma is necessary for better understanding of its origin and behavior and critical for development of new therapeutic options in order to further improve its diagnostics and treatment.

Information obtained within this research will not be relevant for your current treatment, however, it could potentially improve early diagnostics or it could enhance development of new treatment options in patients with Hodgkin lymphoma in the future.

Providing a sample for molecular-genetic testing is entirely voluntary. If you do not wish to provide a sample, it would not negatively impact your medical care in any way. Analyses of samples and subsequent publications of results related to this research will be strictly anonymous and could not lead to individual patient identification.

Participation in this research does not entitle you to any compensatory payment.

All research data will be handled in accordance with regulations of the Czech Republic for protection of personal data and with corresponding EU legislation - the new General Data Protection Regulation (abbreviated GDPR) that was adopted and became effective on 25 May 2018. It replaces the Data Protection Directive (1995.

We ask you to give us a consent to process your selected disease related data. Only authorized staff involved in your regular health care would have an access to your medical records with your

full identification data. This authorized staff has a duty of confidentiality based on the GDPR for personal data of patients.

Samples will be stored for research purposes during several years based on the advances of the research and related to research field. Samples will be labelled using codes, stored and processed anonymously in order to protect the personal data.

Informed Consent

Prognostic Significance of Circulating Tumor DNA in Hodgkin Lymphoma

I agree that the sample of peripheral blood can be used anonymously for research purposes.
This consent is given entirely voluntarily and I was informed that I can withdraw this consent at any time.
I acknowledge that no payment can be claimed by providing the sample of peripheral blood.
I agree that the results of blood samples analyses can be published in a scientific journal and can be potentially used for the development of new diagnostic procedures and for treatment follow-up. All results will be published anonymously in accordance with the relevant EU legislation- the General Data Protection Regulation that became effective on 25 May 2018.

I declare that I was informed about the research objective related to blood sample testing and that I received all additional information based on my questions.

Name and Surname of the patient: ……

Signature of the patient: ……

Place and date:

Name and Surname of the physician providing information:……

Signature of treating the physician providing information:……

Place and date:

Department: