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Keywords:	Hemophagocytic Lymphohistiocytosis (HLH), Hemophagocytic syndrome (HS), Macrophage activation syndrome (MAS), Sepsis, biomarker, intensive care unit (ICU)

Study protocol: Diagnostic Biomarkers for Adult Hemophagocytic Lymphohistiocytosis in Critically Ill Patients (HEMICU)

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Abstract

Background: Hemophagocytic Lymphohistiocytosis (HLH) in adults is characterized by toxic immune activation and a sepsis-like syndrome leading to high numbers of undiagnosed cases and mortality rates up to 68%. Early diagnosis and specific immune suppressive treatment is mandatory to avoid fatal outcome. Though, diagnostic criteria (HLH-2004) are adopted from pediatric HLH, and have not been validated in adults. Experimental studies suggest biomarkers to sufficiently diagnose HLH. However, investigation of biomarkers for diagnosis of adult HLH has not been labored, yet.

Methods: The Diagnostic Biomarkers for Adult Hemophagocytic Lymphohistiocytosis in Critically Ill Patients study (HEMICU) aims to estimate the incidence rate of adult HLH among suspected adult intensive care unit (ICU) patients. Screening for HLH will be performed in 16 ICUs of the Charité – Universitätsmedizin Berlin. Inclusion criteria are bicytopenia, hyperferritinemia (≥500 µg/L), fever, or when HLH is suspected. Over a period of two years, we expect inclusion of about 100 patients with suspected HLH. HLH will be diagnosed if at least 5 HLH-2004 criteria are fulfilled together with an expert review, all other included patients will serve as controls. Secondarily, a panel of potential biomarker candidates will be explored. DNA, plasma and serum will be stored in a biobank.

Results: Primary endpoint of the study is the incidence rate of adult HLH among suspected adult patients during ICU stay. Out of a variety of measured biomarkers, this study furthermore aims to find highly potential biomarkers for diagnosis of adult HLH in ICU.

Conclusion: The HEMICU study is the first study to estimate the incidence rate of adult HLH among suspected patients during ICU stay. Furthermore, we aim to

investigate biomarkers for diagnosis of adult HLH in ICU patients. Results of this study will contribute to improved recognition and patient outcome of adult HLH in the clinical routine.

Keywords: Hemophagocytic Lymphohistiocytosis (HLH), Hemophagocytic syndrome (HS), Macrophage activation syndrome (MAS), sepsis, biomarker, intensive care Unit (ICU)

Background

Hemophagocytic Lymphohistiocytosis (HLH) is a hyperinflammatory syndrome due to toxic immune activation associated with multiple organ failure and high mortality in intensive care unit (ICU) patients [1-3]. Primary HLH due to genetic causes has been subject of extensive research in pediatric medicine resulting in an advanced understanding of its pathophysiology including identification of underlying genetic defects related to cytotoxic granule exocytosis [4]. However, much less is known about HLH in adults where the secondary form triggered by infections, autoimmune diseases, malignancies or immunosuppressive therapy is more common. Both hereditary primary and reactive secondary HLH are characterized by impaired immune function, i.e. impaired natural-killer (NK) or cytotoxic-T-cell function leading to abnormal activation of cytokine-releasing macrophages and T-cells and finally to an uncontrolled inflammatory condition known as cytokine storm [5].

Currently, diagnosis is based on the HLH-2004 criteria (Table 1) derived from the pediatric HLH-2004 protocol which has not been validated in adult HLH patients [6, 7]. Moreover, diagnosis of HLH in ICU-admitted patients is hampered by its sepsis-like presentation. Clinical features include repetitive fever, hepato- and/or splenomegaly and antibiotic-refractory infections as well as pulmonary and renal involvement with consequent multiple organ failure [7]. Lab findings may reveal cytopenia, hypertriglyceridemia, ferritin ≥500 µg/L, and hypofibrinogenemia. Timely diagnosis is crucial to initiate adequate treatment and thus to improve the prognosis. As demonstrated by Jordan et al. [8], early therapy reduces mortality to 30-35%. However, up to 78% of all HLH cases remain undiagnosed leading to mortality rates as high as 68% [2, 9]. Given the lack of specific diagnostic tests and the established use of invalidated diagnostic criteria in adults, we aim to identify a biomarker panel of high sensitivity and specificity to allow early detection of HLH in critically ill patients.

This biomarker panel might help to differentiate HLH from sepsis between these two types of critical illnesses for ICU patients.

Methods

Design and screening

Over a two-year period, screening of all patients admitted to 16 ICUs of the Charité - Universitätsmedizin Berlin will be performed. As part of the process, electronic patient charts will be screened daily for bicytopenia by an automated script. All patients with bicytopenia are daily searched for HLH-2004 criteria (Table 1), the HScore [10] and suspected HLH by the study team. If the patient is highly suspective for HLH, patients will be enrolled after informed consent by the patient itself or the legal representative. Immediately after study enrolment and before initiation of specific HLH treatment, whole blood samples will be obtained for all analyses. Additionally, plasma and serum samples as well as DNA will be stored in a biobank (Figure 1). HLH will be diagnosed if at least 5 HLH-2004 criteria are fulfilled and finally be confirmed after a case-by-case review by two HLH experts. The study aims to include at least 100 patients with suspected HLH. Patients in whom HLH is not confirmed will serve as controls.

Table 1. HLH-2004 diagnostic criteria [6]

HLH-2004 diagnostic criteria of which at least 5 must be fulfilled
Ferritin ≥500 µg/L
Fever (≥38.2 °C)
Splenomegaly
Cytopenias in ≥2 lines (Hemoglobin <9 g/dL, platelets <100 /nL, neutrophils <1.0 /nL)
Hypertriglyceridemia and/or hypofibrinogenemia (fasting triglycerides ≥265 mg/dL, fibrinogen <1.5 g/L)
Hemophagocytosis in bone marrow or spleen or lymph nodes

Low or absent NK activity
Soluble CD25 (soluble IL-2 receptor (sIL-2R)) ≥2,400 U/mL

Study Population and eligibility criteria for patients

Inclusion criteria:

- Intensive care patients of at least 18 years old
- Suspected or diagnosed HLH
- Eligible to informed consent by the patient itself or the legal representative

Exclusion criteria:

- Participation in an interventional study
- Female patients with pregnancy or breastfeeding

Setting

Participating ICU include anesthesiological, surgical, medical, mixed and neurological ICUs. In total, 16 ICUs of the Charité – Universitätsmedizin Berlin will contribute patients.

Objectives and hypotheses

This study aims to investigate critically ill patients for adult HLH and estimate its incidence rate among suspected patients during ICU stay. Secondarily, a panel of various biomarkers candidates from experimental and pediatric studies will be measured to explore diagnostic potential to diagnose HLH in adult ICU-admitted

patients. As a result, HLH might be detected earlier leading to an improved outcome. Highly sensitive biomarkers may also help to distinguish HLH from sepsis.

Statistical analyses

Incidence rate of HLH among suspected adult patient during ICU stay will be calculated with 95% confidence intervals. Investigation of potential biomarker will be exploratory. Descriptive statistics between patient groups with confirmed HLH and controls will incorporate mean and standard deviation or absolute and relative frequencies depending on each variable's scale. Uni- and multivariable logistic regression models with confirmed HLH diagnosis as outcome will be calculated for different combinations of influencing variables and biomarkers. Receiver operator characteristic (ROC) analysis will be performed to determine discrimination ability as measured by the area under the curve (AUC) for each continuous biomarker. Sensitivity and specificity within our patient sample will be given for cut-off points with highest Youden index. Highly potential markers are found when sensitivity and specificity each reach 90%.

Outcome measures

Primary Endpoint

- Incidence rate of adult HLH among suspected patients during ICU stay

Secondary Endpoints

- Identification of highly sensitive and highly specific biomarkers to safely diagnose adult HLH in ICU
- Therapy of HLH by clinicians
- ICU and hospital length of stay
- Mortality and survival after 6 months

- Quality of life questionnaire 36-item short form health survey (SF-36) after 6 months [11]
- Human immunodeficiency virus (HIV) antibodies and -antigen
- Epstein Barr Virus (EBV) and Cytomegalovirus (CMV) viral loads
- Inflammatory markers (ferritin, C-reactive protein (CRP), procalcitonin (PCT), interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-18, IL-33, tumor necrosis factor (TNF)-α, interferon (IFN)-ɣ, sCD25, sCD163, presepsin) [12]
- Perforin and CD107a [13]
- Fibrinogen, triglycerides, bilirubin, lactate dehydrogenase (LDH), liver transaminases (ALAT and ASAT)
- Sodium, serum albumin, serum protein electrophoresis
- Detailed immune status (differential blood count, T cells (CD3+), B cells (CD19+), NK cells (CD16+), T helper cells (CD4+), cytotoxic T cells (CD8+), CD4 / CD8 ratio, HLA-DR of CD8+, CD11a of CD8-, CD57 of CD8-, CD28 of CD8+, HLA-DR of monocytes, CD56bright and CD69 of NK cells)
- Glycosylated ferritin [14] and microRNAs (miR-205-5p, miR-194-5p and miR-30c-5p) [15]
- Chemokines CCL2 (MCP-1), CCL3, CCL4, CCL5 (RANTES), CCL11 (Eotaxin), CCL19, CCL20, CXCL1 CXCL8 (IL-8), CXCL10 (IP-10) , CXCL12 (SDF1A) [16]
- HLA Typing
- Biobanking for future research questions (e.g. genetic polymorphisms and gene expression of PRF1, UNC13D, STX11, STXBP2 [7])

Data collection

Data on all outcome measures will be collected prospectively. Further data including patient demographic data, past medical conditions, physicians' reports and routine laboratory results will be abstracted from the hospital´s database. For follow-up six months after study inclusion, patients will be contacted either by telephone or by mail as indicated upon enrolment to assess health-related quality of life and mortality, respectively.

Immunological measurements

Plasma concentrations of soluble IL-2R (sCD25) will be determined with the IMMULITE™ semi-automatic chemiluminescent immunoassay (Siemens Healthcare GmbH, Erlangen, Germany). Additional soluble factors IL-1β, IL-6, IL-8, IL-10, IL-18, IL-33, TNF-α, IFN-ɣ will be measured by Meso Scale Discovery® (Meso Scale Diagnostics, Maryland USA). The kit provides all reagents, together with a 96-well plate with specific pre-coated spots, the detection antibodies and assay diluent. The standard will be reconstituted with assay diluent to obtain a lotspecific concentration which differs for all cytokines. The vials are inverted multiple times for mixing and, after vortexing, the vials will be kept for 5–10 min at RT and then on ice until use. Preparation of further serial 1:5 dilution of Cytokine Standard is performed. Quality Controls (low and high) are components of the kits and respective Quality Control ranges are provided by the manufacturer. The Quality Controls (low and high) will be reconstituted with 250 µl of deionized water. The vials are inverted multiple times for mixing and, after vortexing, kept for 5–10 min at RT and then on ice until use. Plasma concentrations of soluble CD163 protein levels from plasma will be determined with the Quantikine® ELISA human CD163 Immunoassay (R&D Systems, Minneapolis, USA). The minimum detectable dose ranged from 0.058-0.613 ng/mL. For measurement of presepsin, Presepsin (Human) ELISA Kit will be used (BioVision, California, USA) with a detection range of 0.156-10 ng/mL.

Flow cytometric analysis of human lymphocyte subsets in EDTA whole blood will be performed, as described recently [17]. Briefly, the following mouse anti-human fluorescently-labelled monoclonal antibodies (mAb) are used for quantification of lymphocytes subsets and analysis of T and NK cell activation markers: cluster of

differentiation (CD)3 Allophycocyanine-Alexa Fluor 750 (APC-A750), CD4 energy coupled dye (ECD), CD8 APC, CD11a Fluorescein isothiocyanate (FITC), CD14 FITC, CD16 Phycoerythrine (PE), CD19 PE-Cy5.5, CD28 PC5, CD45 Krome-Orange (KrO), CD56 PE or CD56 APC, CD57 Pacific Blue (PB), CD69 PE, HLA-DR PE, (all from Beckman Coulter). Functional analysis of NK cells will be performed using the CD107a degranulation assay according to a protocol published by Bryceson et al. [18]. Briefly, peripheral blood mononuclear cells (PBMC) will be isolated by density gradient centrifugation and incubated overnight in the presence or absence of 3600 IU/mL recombinant interleukin-2 (Peprotech). PBMC will then be incubated with the target cell line K562 (ATCC) in a 1:1 ratio for 3 hours. Subsequently, CD107a expression on NK cells is assessed by staining samples with fluorescently-labelled antibodies against lineage markers (see above) and an anti-CD107a FITC-labelled mAb (eBioscience). Perforin expression in NK cells will be assessed by intracellular staining in unstimulated NK cells using the PerFix-nc reagent (Beckman Coulter) for cell permeabilization and an APC-labelled anti-perforin mAb (eBioscience) according the manufacturer's instructions.

Expression of human leukocyte antigen-DR (HLA-DR) on monocytes (mHLA-DR) will be determined by flow cytometry using a highly standardized quantitative assay, as described earlier [19]. In short, whole blood in Vacutainer tubes (BD Biosciences, San Jose, CA, USA) containing EDTA are stained with 20 µl of monoclonal phycoerythrin-conjugated anti–HLA-DR and PerCP-Cy5.5–conjugated anti-CD14 antibodies (Quantibrite HLA-DR/monocyte™; BD Biosciences) in the dark at room temperature for 30 min. Erythrocyte lysis will be done with 0.5 ml of lysing solution (BD Biosciences) for another 30 min at room temperature. Finally, the cells are washed with 1ml PBS buffer containing 2% FCS and analyzed on a Navios flow cytometer (Beckman Coulter, Krefeld, Germany). HLA-DR surface expression on

monocytes will be calculated as monoclonal antibodies bound per cell (mAb/cell) using the QuntiBRITE™ PE calibration beads. All flow cytometric analyses will be performed on a ten-color Navios flow cytometer using the Navios Software (Beckman Coulter). Human glycosylated ferritin will be determined by Enzyme-linked Immunosorbent Assay (MyBioSource, San Diego, USA). The sensitivity of this kit is 2.0 ng/mL.

The expression of miR-205-5p, miR-194-5p and miR-30c-5p will be determined in whole blood, plasma and serum using a protocol published by Balcells et al. [20]. Briefly, after RNA isolation, miRNAs are polyadenylated and then reverse transcribed with a special primer (RT-primer). For quantitative real time PCR (qPCR), two specific primers for each miRNA are designed using a software tool from Busk [21]. All primers are tested for specify and efficiency. The qPCR will be performed with SYBR Green using the QuantStudio5® (Thermo Fisher, Darmstadt, Germany). The quantification of chemokines involved in cell trafficking and effector functions of lymphocytes, granulocytes, and mononuclear cells from the CC subfamily (CCL2, CCL3, CCL4, CCL5, CCL11, CCL19, and CCL20) as well as the CXC subfamily (CXCL1, CXCL8 (IL-8), CXCL10, and CXCL12) will be performed with the LUNARIS™ Human 11-Plex Chemokine Kit (AYOXXA Biosystems GmbH, Cologne, Germany).

Determiniation of human leukocyte antigen (HLA) typing will be performed by reverse sequence-specific oligonucleotide (rSSO) assay LABType® (One Lambda, Canoga Park, CA, USA). Typing will be assessed on an intermediate resolution level for HLA-A, -B, -C, -DRB1, -DQA1 and -DQB1. The assay will be performed according to the manusfacturer's instructions and data will be acquired on a Luminex® FlexMAP 3D machine (Luminex, Austin, TX, USA).

Informed consent in critically ill patients

Written informed consent will be obtained from all patients or their legally authorized representatives. Consent for genetic analyzes in future projects will be obtained separately.

Sample size

The true incidence of adult HLH in ICU is unknown. According to our own research [9] and the annual number of patients admitted to our ICU, we expect to see about 200 patients with diagnosed HLH over two years, and about 400 with suspected HLH Of these, we hope to include at least 100 patients with suspected HLH into the study, of whom about 50 patients are expected to be diagnosed with HLH. When the sample size is 100, a two-sided 95.0% confidence interval for a single proportion using the large sample normal approximation will extend 0.1 from the observed proportion for an expected proportion of 0.5 (nQuery Advisor 7.0).

Ethics

The institutional ethics committee approved this study on August 1, 2018 (EA4/006/18). The data protection commissioner also approved the study (91-SP-18), which was registered with clinicaltrials.gov (NCT03510650) on April 27, 2018.

Discussion

HLH is a rare condition in adults with poor prognosis. Due to the paucity of data available on adult HLH, recognition remains low resulting in delayed diagnosis and

treatment and finally, fatal outcome. This is the first prospective study to systematically investigate routine and non-routine parameters for biomarker development. Importantly, the daily systematic screening will help to identify HLH patients at an early stage of the syndrome which ultimately will improve patient care, patients' safety and outcome. Moreover, describing a distinct pattern of biomarkers generates new hypothesis for future research thereby potentially providing targets for therapy development. With regard to clinical practice, the HEMICU study seeks to inform clinicians about HLH and ICU therapies to improve outcomes for HLH patients. However, it is of note that this study does not seek to advice the clinician in charge to change therapy. No change in routine management is intended due to the observational study design and final decisions are left to the discretion of the responsible clinician.

Strengths and Limitations

This study might be limited in that it only includes ICU patients and findings will not be generalizable to non-ICU patients. In addition, patients might have developed HLH before ICU admission and will thus be detected and measurements obtained at an advanced stage of the disease possibly limiting comparability of the results. Moreover, we will not assess longitudinal parameters preventing us from describing dynamics of biomarkers over time. However, as this study aims to develop a tool facilitating diagnosis at the earliest possible time point, study endpoints will not be affected by lack of repeated measurements. Possible advances of this study include comprehensive lab testing of parameters, which have previously been suggested to be associated with HLH [12-16]. Previous studies, most of which are retrospective in

nature, aimed at identifying associations to detect risk factors [2, 22]. Therefore, the HEMICU study is the first prospective study of its kind.

Conclusion

HLH is a life threatening but poorly investigated condition in adult ICU patients. The HEMICU study is the first prospective study in adult ICU patients and aims to estimate the incidence rate of adult HLH among suspected patients during ICU stay. Furthermore, we aim to investigate biomarkers for diagnosis of adult HLH in ICU patients. Results of this study will contribute to improved recognition of adult HLH in the clinical routine. Potentially earlier diagnosis and thus more effective treatment of adult HLH could lead to improved patient outcome. Moreover, our study will provide a better understanding of adult HLH pathophysiology and the biobanking of DNA, plasma and serum will generate a data base to investigate future research questions.

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Competing interests

The authors declare no conflicts of interest.

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Authors´contributions

Study concept: GL. Conceived and designed the experiments: GL, CvH, NP, CM, PL, TS, NL, HDV, DK. Performing the experiments: GL, CK, CvH, NP, CM, PN, FSS, GV, FB, NU, UK, NL, LA. Analyzing the data: GL, CK, PN, FSS, SKP, JK, PL, TS. Wrote the manuscript: GL, CK, CvH, NP, CM, SKP, NL, LA, DK. Commented on the manuscript: all authors.

Data statement

Due to legal restrictions imposed by the ethics committee of the Charité – Universitätsmedizin Berlin and the data protection commissioner of the Charité – Universitätsmedizin Berlin, public sharing of study data with other researchers or entities is not allowed. Requests may be sent to dai-researchdata@charite.de.

Figure legends

Figure 1. Screening protocol and blood sampling.

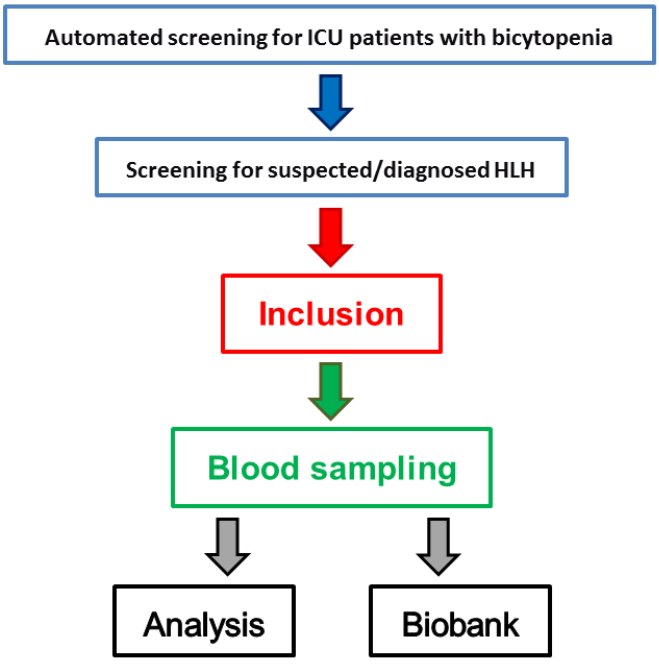

Figure 1

Figure 1 / Screening protocol and blood sampling.

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Trial registration: data set	#2b	All items from the World Health Organization Trial Registration Data Set	14
Protocol version	#3	Date and version identifier	14
Funding	#4	Sources and types of financial, material, and other support	16
Roles and responsibilities: contributorship	#5a	Names, affiliations, and roles of protocol contributors	1

Roles and responsibilities: sponsor contact information	#5b	Name and contact information for the trial sponsor	16
Roles and responsibilities: sponsor and funder	#5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	16
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Interventions: description	#11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	7
Interventions: modifications	#11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving / worsening disease)	7
Interventions: adherance	#11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return; laboratory tests)	7
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Participant timeline	#13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	7
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Allocation concealment mechanism	#16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	7
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Methods: Data collection, management, and analysis			
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Data collection plan: retention	#18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	7
Data management	#19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	7
Statistics: outcomes	#20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	9
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Statistics: analysis population and missing data	#20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	9
Methods: Monitoring			
Data monitoring: formal committee	#21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	7
Data monitoring: interim analysis	#21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	7
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Protocol amendments	#25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC / IRBs, trial participants, trial registries, journals, regulators)	14
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Dissemination policy: authorship	#31b	Authorship eligibility guidelines and any intended use of professional writers	14
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Diagnostic Biomarkers for Adult Hemophagocytic Lymphohistiocytosis in Critically Ill Patients (HEMICU): Prospective Observational Study Protocol

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Keywords:	Hemophagocytic Lymphohistiocytosis (HLH), Hemophagocytic syndrome (HS), Macrophage activation syndrome (MAS), Sepsis, Biomarker, Intensive Care Unit (ICU)

Diagnostic Biomarkers for Adult Hemophagocytic Lymphohistiocytosis in Critically Ill Patients (HEMICU): Prospective Observational Study Protocol

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Abstract

Introduction: Hemophagocytic Lymphohistiocytosis (HLH) in adults is characterized by toxic immune activation and a sepsis-like syndrome leading to high numbers of undiagnosed cases and mortality rates up to 68%. Early diagnosis and specific immune suppressive treatment is mandatory to avoid fatal outcome. Though, diagnostic criteria (HLH-2004) are adopted from pediatric HLH, and have not been validated in adults. Experimental studies suggest biomarkers to sufficiently diagnose HLH. However, investigation of biomarkers for diagnosis of adult HLH has not been labored, yet.

Methods and analysis: The Diagnostic Biomarkers for Adult Hemophagocytic Lymphohistiocytosis in Critically Ill Patients study (HEMICU) aims to estimate the incidence rate of adult HLH among suspected adult intensive care unit (ICU) patients. Screening for HLH will be performed in 16 ICUs of the Charité – Universitätsmedizin Berlin. Inclusion criteria are bicytopenia, hyperferritinemia (≥500 µg/L), fever, or when HLH is suspected by the clinicians. Over a period of two years, we expect inclusion of about 100 patients with suspected HLH. HLH will be diagnosed if at least 5 HLH-2004 criteria are fulfilled together with an expert review, all other included patients will serve as controls. Secondarily, a panel of potential biomarker candidates will be explored. DNA, plasma and serum will be stored in a biobank. Primary endpoint of the study is the incidence rate of adult HLH among suspected adult patients during ICU stay. Out of a variety of measured biomarkers, this study furthermore aims to find highly potential biomarkers for diagnosis of adult HLH in ICU. Results of this study will contribute to improved recognition and patient outcome of adult HLH in the clinical routine.

Ethics and dissemination: The institutional ethics committee approved this study on August 1, 2018 (EA4/006/18). Results of the study will be disseminated in an international peer-reviewed journal and presented at conferences.

Trial registration: The study was registered with clinicaltrials.gov (NCT03510650) on April 27, 2018.

Keywords: Hemophagocytic Lymphohistiocytosis (HLH), Hemophagocytic syndrome (HS), Macrophage activation syndrome (MAS), sepsis, biomarker, intensive care Unit (ICU)

Strengths and Limitations:

- The HEMICU study is the first prospective study to investigate biomarkers for diagnosis of adult HLH in ICU patients.
- The variety of analyzed biomarkers will provide a better understanding of adult HLH pathophysiology.
- This study aims to investigate critically ill patients for adult HLH and estimate its incidence rate among suspected patients during ICU stay.- Biobanking of DNA, plasma and serum of adult HLH will generate a database to investigate future research questions.
- This study might be limited in that it only includes ICU patients and findings will not be generalizable to non-ICU patients

Introduction

Hemophagocytic Lymphohistiocytosis (HLH) is a hyperinflammatory syndrome due to toxic immune activation associated with multiple organ failure and high mortality in intensive care unit (ICU) patients [1-3]. Primary HLH due to genetic causes has been subject of extensive research in pediatric medicine resulting in an advanced understanding of its pathophysiology including identification of underlying genetic defects related to cytotoxic granule exocytosis [4]. However, much less is known about HLH in adults where the secondary form triggered by infections, autoimmune diseases, malignancies or immunosuppressive therapy is more common. Both hereditary primary and reactive secondary HLH are characterized by impaired immune function, i.e. impaired natural-killer (NK) or cytotoxic-T-cell function leading to abnormal activation of cytokine-releasing macrophages and T-cells and finally to an uncontrolled inflammatory condition known as cytokine storm [5].

Currently, diagnosis is based on the HLH-2004 criteria (Table 1) derived from the pediatric HLH-2004 protocol which has not been validated in adult HLH patients [6, 7]. Moreover, diagnosis of HLH in ICU-admitted patients is hampered by its sepsis-like presentation. Clinical features include repetitive fever, hepato- and/or splenomegaly and antibiotic-refractory infections as well as pulmonary and renal involvement with consequent multiple organ failure [7]. Lab findings may reveal cytopenia, hypertriglyceridemia, ferritin ≥500 µg/L, and hypofibrinogenemia. Timely diagnosis is crucial to initiate adequate treatment and thus to improve the prognosis. As demonstrated by Jordan et al. [8], early therapy reduces mortality to 30-35%. However, up to 78% of all HLH cases remain undiagnosed leading to mortality rates as high as 68% [2, 9]. Given the lack of specific diagnostic tests and the established use of invalidated diagnostic criteria in adults, we aim to identify a biomarker panel of high sensitivity and specificity to allow early detection of HLH in critically ill patients.

Methods and Analysis

Design and screening

Over a two-year period (ongoing since 01/09/2018), screening of all patients admitted to 16 adult ICUs of the Charité - Universitätsmedizin Berlin will be performed. As part of the process, electronic patient charts will be screened daily for bicytopenia by an automated script. All patients with bicytopenia are daily searched by the study team for HLH-2004 criteria (Table 1), the HScore [10], and suspected HLH by the clinicians. If the patient is highly suspective for HLH, patients will be enrolled after informed consent by the patient himself or the legal representative. Immediately after study enrolment and before initiation of specific HLH treatment, whole blood samples will be obtained for all analyses. Additionally, plasma and serum samples as well as DNA will be stored in a biobank (Figure 1). HLH will be diagnosed if at least 5 HLH-2004 criteria are fulfilled and finally be confirmed after a case-by-case review by two HLH experts. The study aims to include at least 100 patients with suspected HLH. Patients in whom HLH is not confirmed will serve as controls.

Table 1. HLH-2004 diagnostic criteria [6]

HLH-2004 diagnostic criteria of which at least 5 must be fulfilled
Ferritin ≥500 µg/L
Fever (≥38.2 °C)
Splenomegaly
Cytopenias in ≥2 lines (Hemoglobin <9 g/dL, platelets <100 /nL, neutrophils <1.0 /nL)
Hypertriglyceridemia and/or hypofibrinogenemia (fasting triglycerides ≥265 mg/dL, fibrinogen <1.5 g/L)
Hemophagocytosis in bone marrow or spleen or lymph nodes

Low or absent NK activity
Soluble CD25 (soluble IL-2 receptor (sIL-2R)) ≥2,400 U/mL

Study Population and eligibility criteria for patients

Inclusion criteria:

- Intensive care patients of at least 18 years old
- Suspected or diagnosed HLH: based on HLH-2004 diagnostic criteria (bicytopenia, hyperferritinemia (≥500 µg/L), fever) or suspicion by the clinicians
- Eligible to informed consent by the patient himself or the legal representative

Exclusion criteria:

- Participation in an interventional study
- Female patients with pregnancy or breastfeeding

Setting

Participating ICU include anesthesiological, surgical, medical, neurological, and mixed ICUs. In total, 16 ICUs of the Charité – Universitätsmedizin Berlin will contribute patients.

Objectives and hypotheses

This study aims to investigate critically ill patients for adult HLH and estimate its incidence rate among suspected patients during ICU stay. Secondarily, a panel of various biomarkers candidates from experimental and pediatric studies will be

measured to explore diagnostic potential to diagnose HLH in adult ICU-admitted patients. As a result, HLH might be detected earlier leading to an improved outcome. Highly sensitive biomarkers may also help to distinguish HLH from sepsis.

Patient and public involvement

Patients or public were not involved in the development of the research question, the design, the recruitment and the conduct of this study. As a regular medical care, the results of the immunological analyses will be sent to the physicians in charge. Patients will be informed of the global results of the study at their request.

Statistical analyses

Incidence rate of HLH among suspected adult patient during ICU stay will be calculated with 95% confidence intervals. Investigation of potential biomarker will be exploratory. Descriptive statistics between patient groups with confirmed HLH and controls will incorporate mean and standard deviation or absolute and relative frequencies depending on each variable's scale. Uni- and multivariable logistic regression models with confirmed HLH diagnosis as outcome will be calculated for different combinations of influencing variables and biomarkers. Receiver operator characteristic (ROC) analysis will be performed to determine discrimination ability as measured by the area under the curve (AUC) for each continuous biomarker. Sensitivity and specificity within our patient sample will be given for cut-off points with highest Youden index. Highly potential markers are found when sensitivity and specificity each reach 90%.

Outcome measures

Primary Endpoint

- Incidence rate of adult HLH among suspected patients during ICU stay

Secondary Endpoints

- Identification of highly sensitive and highly specific biomarkers to safely diagnose adult HLH in ICU
- Trigger and underlying conditions
- Therapy of HLH by clinicians
- ICU and hospital length of stay
- Mortality and survival after 6 months
- Quality of life questionnaire 36-item short form health survey (SF-36) after 6 months [11]
- Human immunodeficiency virus (HIV) antibodies and -antigen
- Epstein Barr Virus (EBV) and Cytomegalovirus (CMV) viral loads
- Inflammatory markers (ferritin, C-reactive protein (CRP), procalcitonin (PCT), interleukin (IL)-1β, IL-2, IL-6, IL-8, IL-10, IL-18, IL-33, tumor necrosis factor (TNF)-α, interferon (IFN)-ɣ, sCD25, sCD163, presepsin) [12]
- Perforin and CD107a [13]
- Fibrinogen, triglycerides, bilirubin, lactate dehydrogenase (LDH), liver transaminases (ALAT and ASAT)
- Sodium, serum albumin, serum protein electrophoresis
- Detailed immune status (differential blood count, T cells (CD3+), B cells (CD19+), NK cells (CD16+), T helper cells (CD4+), cytotoxic T cells (CD8+), CD4 / CD8 ratio, HLA-DR of CD8+, CD11a of CD8-, CD57 of CD8-, CD28 of CD8+, HLA-DR of monocytes, CD56bright and CD69 of NK cells)
- Glycosylated ferritin [14] and microRNAs (miR-205-5p, miR-194-5p and miR-30c-5p) [15]
- Chemokines CCL2 (MCP-1), CCL3, CCL4, CCL5 (RANTES), CCL11 (Eotaxin), CCL19, CCL20, CXCL1, CXCL9, CXCL8 (IL-8), CXCL10 (IP-10), CXCL12 (SDF1A) [16]
- HLA Typing

- Biobanking for future research questions (e.g. genetic polymorphisms and gene expression of PRF1, UNC13D, STX11, STXBP2 [7])

Data collection

Number of screened patients, number of patients with suspected HLH who could not be included as well as data on all outcome measures will be collected prospectively. If the patient received immunosuppressive therapy prior to inclusion, this will be documented separately. Further data including patient demographic data, past medical conditions, physicians' reports and routine laboratory results will be abstracted from the hospital's database. For follow-up six months after study inclusion, patients will be contacted either by telephone or by mail as indicated upon enrolment to assess health-related quality of life and mortality, respectively.

Immunological measurements

Plasma concentrations of soluble IL-2R (sIL-2R or sCD25) will be determined with the IMMULITE™ semi-automatic chemiluminescent immunoassay (Siemens Healthcare GmbH, Erlangen, Germany). Additional soluble factors IL-1β, IL-2, IL-6, IL-8, IL-10, IL-18, IL-33, TNF-α, IFN-ɣ will be measured by Meso Scale Discovery® (Meso Scale Diagnostics, Maryland USA). The kit provides all reagents, together with a 96-well plate with specific pre-coated spots, the detection antibodies and assay diluent. The standard will be reconstituted with assay diluent to obtain a lot specific concentration which differs for all cytokines. The vials are inverted multiple times for mixing and, after vortexing, the vials will be kept for 5–10 min at RT and then on ice until use. Preparation of further serial 1:5 dilution of Cytokine Standard is performed. Quality Controls (low and high) are components of the kits and respective Quality

Control ranges are provided by the manufacturer. The Quality Controls (low and high) will be reconstituted with 250 µl of deionized water. The vials are inverted multiple times for mixing and, after vortexing, kept for 5–10 min at RT and then on ice until use. Plasma concentrations of soluble CD163 protein levels from plasma will be determined with the Quantikine® ELISA human CD163 Immunoassay (R&D Systems, Minneapolis, USA). The minimum detectable dose ranged from 0.058-0.613 ng/mL. For measurement of presepsin, Presepsin (Human) ELISA Kit will be used (BioVision, California, USA) with a detection range of 0.156-10 ng/mL.

Flow cytometric analysis of human lymphocyte subsets in EDTA whole blood will be performed, as described recently [17]. Briefly, the following mouse anti-human fluorescently-labelled monoclonal antibodies (mAb) are used for quantification of lymphocytes subsets and analysis of T and NK cell activation markers: cluster of differentiation (CD)3 Allophycocyanine-Alexa Fluor 750 (APC-A750), CD4 energy coupled dye (ECD), CD8 APC, CD11a Fluorescein isothiocyanate (FITC), CD14 FITC, CD16 Phycoerythrine (PE), CD19 PE-Cy5.5, CD28 PC5, CD45 Krome-Orange (KrO), CD56 PE or CD56 APC, CD57 Pacific Blue (PB), CD69 PE, HLA-DR PE, (all from Beckman Coulter). Functional analysis of NK cells will be performed using the CD107a degranulation assay according to a protocol published by Bryceson et al. [18]. Briefly, peripheral blood mononuclear cells (PBMC) will be isolated by density gradient centrifugation and incubated overnight in the presence or absence of 3600 IU/mL recombinant interleukin-2 (Peprotech). PBMC will then be incubated with the target cell line K562 (ATCC) in a 1:1 ratio for 3 hours. Subsequently, CD107a expression on NK cells is assessed by staining samples with fluorescently-labelled antibodies against lineage markers (see above) and an anti-CD107a FITC-labelled mAb (eBioscience). Perforin expression in NK cells will be assessed by intracellular staining in unstimulated NK cells using the PerFix-nc reagent (Beckman Coulter) for

cell permeabilization and an APC-labelled anti-perforin mAb (eBioscience) according the manufacturer's instructions.

Expression of human leukocyte antigen-DR (HLA-DR) on monocytes (mHLA-DR) will be determined by flow cytometry using a highly standardized quantitative assay, as described earlier [19]. In short, whole blood in Vacutainer tubes (BD Biosciences, San Jose, CA, USA) containing EDTA are stained with 20 µl of monoclonal phycoerythrin-conjugated anti–HLA-DR and PerCP-Cy5.5–conjugated anti-CD14 antibodies (Quantibrite HLA-DR/monocyte™; BD Biosciences) in the dark at room temperature for 30 min. Erythrocyte lysis will be done with 0.5 mL of lysing solution (BD Biosciences) for another 30 min at room temperature. Finally, the cells are washed with 1 mL PBS buffer containing 2% FCS and analyzed on a Navios flow cytometer (Beckman Coulter, Krefeld, Germany). HLA-DR surface expression on monocytes will be calculated as monoclonal antibodies bound per cell (mAb/cell) using the QuntiBRITE™ PE calibration beads. All flow cytometric analyses will be performed on a ten-color Navios flow cytometer using the Navios Software (Beckman Coulter). Human glycosylated ferritin will be determined by Enzyme-linked Immunosorbent Assay (MyBioSource, San Diego, USA). The sensitivity of this kit is 2.0 ng/mL.

The expression of miR-205-5p, miR-194-5p and miR-30c-5p will be determined in whole blood, plasma and serum using a protocol published by Balcells et al. [20]. Briefly, after RNA isolation, miRNAs are polyadenylated and then reverse transcribed with a special primer (RT-primer). For quantitative real time PCR (qPCR), two specific primers for each miRNA are designed using a software tool from Busk [21]. All primers are tested for specify and efficiency. The qPCR will be performed with SYBR Green using the QuantStudio5® (Thermo Fisher, Darmstadt, Germany). The

quantification of chemokines involved in cell trafficking and effector functions of lymphocytes, granulocytes, and mononuclear cells from the CC subfamily (CCL2, CCL3, CCL4, CCL5, CCL11, CCL19, and CCL20) as well as the CXC subfamily (CXCL1, CXCL8 (IL-8), CXCL10, and CXCL12) will be performed with the LUNARIS™ Human 11-Plex Chemokine Kit (AYOXXA Biosystems GmbH, Cologne, Germany).

Determiniation of human leukocyte antigen (HLA) typing will be performed by reverse sequence-specific oligonucleotide (rSSO) assay LABType® (One Lambda, Canoga Park, CA, USA). Typing will be assessed on an intermediate resolution level for HLA-A, -B, -C, -DRB1, -DQA1 and -DQB1. The assay will be performed according to the manusfacturer's instructions and data will be acquired on a Luminex® FlexMAP 3D machine (Luminex, Austin, TX, USA).

Informed consent in critically ill patients

Written informed consent will be obtained from all patients or their legally authorized representatives. Consent for genetic analyzes in future projects will be obtained separately.

Sample size

The true incidence of adult HLH in ICU is unknown. According to our own research [9] and the annual number of patients admitted to our ICU, we expect to see about 200 patients with diagnosed HLH over two years, and about 400 with suspected HLH. Of these, we hope to include at least 100 patients with suspected HLH into the study, of whom about 50 patients are expected to be diagnosed with HLH. When the

sample size is 100, a two-sided 95.0% confidence interval for a single proportion using the large sample normal approximation will extend 0.1 from the observed proportion for an expected proportion of 0.5 (nQuery Advisor 7.0).

Ethics and Dissemination

The institutional ethics committee approved this study on August 1, 2018 (EA4/006/18). The data protection commissioner also approved the study (91-SP-18), which was registered with clinicaltrials.gov (NCT03510650) on April 27, 2018. Results of the study will be disseminated in an international peer-reviewed journal and presented at conferences.

Discussion

HLH is a rare condition in adults with poor prognosis. Due to the paucity of data available on adult HLH, recognition remains low resulting in delayed diagnosis and treatment and finally, fatal outcome. This is the first prospective study to systematically investigate routine and non-routine parameters for biomarker development. Importantly, the daily systematic screening will help to identify HLH patients at an early stage of the syndrome which ultimately will improve patient care, patients' safety and outcome. Moreover, describing a distinct pattern of biomarkers generates new hypothesis for future research thereby potentially providing targets for therapy development. With regard to clinical practice, the HEMICU study seeks to inform clinicians about HLH and ICU therapies to improve outcomes for HLH patients. However, it is of note that this study does not seek to advice the clinician in charge to change therapy. No change in routine management is intended due to the

observational study design and final decisions are left to the discretion of the responsible clinician.

Strengths and Limitations

This study might be limited in that it only includes ICU patients and findings will not be generalizable to non-ICU patients. In addition, patients might have developed HLH before ICU admission and will thus be detected and measurements obtained at an advanced stage of the disease possibly limiting comparability of the results. Moreover, we will not assess longitudinal parameters preventing us from describing dynamics of biomarkers over time. However, as this study aims to develop a tool facilitating diagnosis at the earliest possible time point, study endpoints will not be affected by lack of repeated measurements. Possible advances of this study include comprehensive lab testing of parameters, which have previously been suggested to be associated with HLH [12-16]. Previous studies, most of which are retrospective in nature, aimed at identifying associations to detect risk factors [2, 22]. Therefore, the HEMICU study is the first prospective study of its kind.

Conclusion

HLH is a life threatening but poorly investigated condition in adult ICU patients. The HEMICU study is the first prospective study in adult ICU patients and aims to estimate the incidence rate of adult HLH among suspected patients during ICU stay. Furthermore, we aim to investigate biomarkers for diagnosis of adult HLH in ICU patients. Results of this study will contribute to improved recognition of adult HLH in the clinical routine. Potentially earlier diagnosis and thus more effective treatment of adult HLH could lead to improved patient outcome. Moreover, our study will provide a

better understanding of adult HLH pathophysiology and the biobanking of DNA, plasma and serum will generate a database to investigate future research questions.

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Competing interests

The authors declare no conflicts of interest.

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Authors´contributions

Study concept: GL. Conceived and designed the experiments: GL, CvH, NP, CM, PL, TS, NL, HDV, DK. Performing the experiments: GL, CK, CvH, NP, CM, PN, FSS, GV, FB, NU, UK, NL, LA. Analyzing the data: GL, CK, PN, FSS, SKP, JK, PL, TS. Wrote the manuscript: GL, CK, CvH, NP, CM, SKP, NL, LA, DK. Commented on the manuscript: all authors.

Data statement

Due to legal restrictions imposed by the ethics committee of the Charité – Universitätsmedizin Berlin and the data protection commissioner of the Charité – Universitätsmedizin Berlin, public sharing of study data with other researchers or entities is not allowed. Requests may be sent to dai-researchdata@charite.de.

Figure legends

Figure 1. Screening protocol and blood sampling.

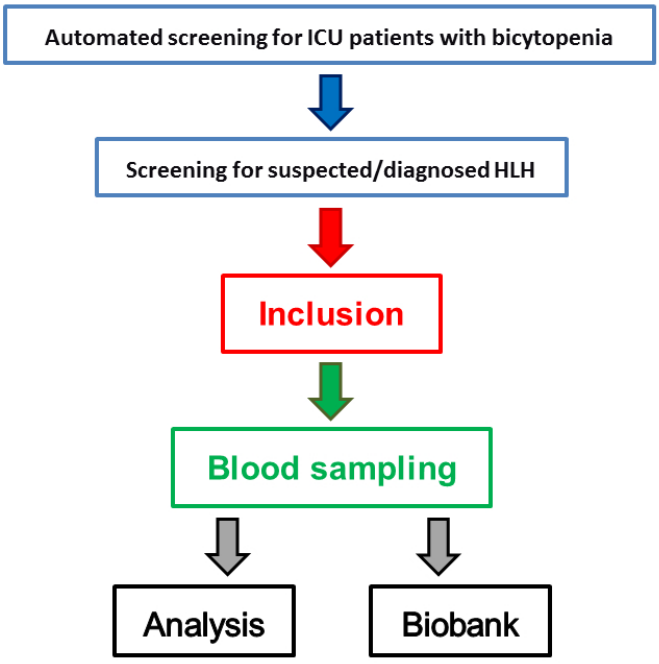

Figure 1 / Screening protocol and blood sampling.

81x60mm (300 x 300 DPI)

Reporting checklist for protocol of a clinical trial.

Based on the SPIRIT guidelines.

Instructions to authors

Complete this checklist by entering the page numbers from your manuscript where readers will find each of the items listed below.

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		Reporting Item	Page Number
Administrative information			
Title	#1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	#2a	Trial identifier and registry name. If not yet registered, name of intended registry	14
Trial registration: data set	#2b	All items from the World Health Organization Trial Registration Data Set	14
Protocol version	#3	Date and version identifier	14
Funding	#4	Sources and types of financial, material, and other support	16
Roles and responsibilities: contributorship	#5a	Names, affiliations, and roles of protocol contributors	1

Roles and responsibilities: sponsor contact information	#5b	Name and contact information for the trial sponsor	16
Roles and responsibilities: sponsor and funder	#5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	16
Roles and responsibilities: committees	#5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	16
Introduction			
Background and rationale	#6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	5
Background and rationale: choice of comparators	#6b	Explanation for choice of comparators	5
Objectives	#7	Specific objectives or hypotheses	5
Trial design	#8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, non-inferiority, exploratory)	7
Methods: Participants, interventions, and outcomes			
Study setting	#9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	7
Eligibility criteria	#10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will	8

		perform the interventions (eg, surgeons, psychotherapists)	
Interventions: description	#11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	7
Interventions: modifications	#11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving / worsening disease)	7
Interventions: adherance	#11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return; laboratory tests)	7
Interventions: concomitant care	#11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	7
Outcomes	#12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	9
Participant timeline	#13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	7
Sample size	#14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	7
Recruitment	#15	Strategies for achieving adequate participant enrolment to reach target sample size	7
Methods: Assignment of interventions (for controlled trials)			
Allocation: sequence generation	#16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	7

Allocation concealment mechanism	#16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	7
Allocation: implementation	#16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	7
Blinding (masking)	#17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	7
Blinding (masking): emergency unblinding	#17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	7
Methods: Data collection, management, and analysis			
Data collection plan	#18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	7
Data collection plan: retention	#18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	7
Data management	#19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	7
Statistics: outcomes	#20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	9
Statistics: additional analyses	#20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	9

Statistics: analysis population and missing data	#20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	9
Methods: Monitoring			
Data monitoring: formal committee	#21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	7
Data monitoring: interim analysis	#21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	7
Harms	#22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	7
Auditing	#23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	7
Ethics and dissemination			
Research ethics approval	#24	Plans for seeking research ethics committee / institutional review board (REC / IRB) approval	14
Protocol amendments	#25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC / IRBs, trial participants, trial registries, journals, regulators)	14
Consent or assent	#26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	14
Consent or assent: ancillary studies	#26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	14
Confidentiality	#27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	14

Declaration of interests	#28	Financial and other competing interests for principal investigators for the overall trial and each study site	14
Data access	#29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	14
Ancillary and post trial care	#30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	14
Dissemination policy: trial results	#31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	14
Dissemination policy: authorship	#31b	Authorship eligibility guidelines and any intended use of professional writers	14
Dissemination policy: reproducible research	#31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	14
Appendices			
Informed consent materials	#32	Model consent form and other related documentation given to participants and authorised surrogates	14
Biological specimens	#33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	14

BMJ Open

Diagnostic Biomarkers for Adult Hemophagocytic Lymphohistiocytosis in Critically Ill Patients (HEMICU): Prospective Observational Study Protocol

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Diagnostic Biomarkers for Adult Hemophagocytic Lymphohistiocytosis in Critically Ill Patients (HEMICU): Prospective Observational Study Protocol

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Abstract: 412

Introduction: 553

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Abstract

Introduction: Hemophagocytic Lymphohistiocytosis (HLH) in adults is characterized by toxic immune activation and a sepsis-like syndrome leading to high numbers of undiagnosed cases and mortality rates up to 68%. Early diagnosis and specific immune suppressive treatment is mandatory to avoid fatal outcome, but diagnostic criteria (HLH-2004) are adopted from pediatric HLH, and have not been validated in adults. Experimental studies suggest biomarkers to sufficiently diagnose HLH. However, biomarkers for diagnosis of adult HLH have not been investigated, yet.

Methods and analysis: The Diagnostic Biomarkers for Adult Hemophagocytic Lymphohistiocytosis in Critically Ill Patients study (HEMICU) aims to estimate the incidence rate of adult HLH among suspected adult intensive care unit (ICU) patients. Screening for HLH will be performed in 16 ICUs of the Charité – Universitätsmedizin Berlin. Inclusion criteria are bicytopenia, hyperferritinemia (≥500 µg/L), fever, or when HLH is suspected by the clinicians. Over a period of two years, we expect inclusion of about 100 patients with suspected HLH. HLH will be diagnosed if at least 5 HLH-2004 criteria are fulfilled together with an expert review, all other included patients will serve as controls. Secondarily, a panel of potential biomarker candidates will be explored. DNA, plasma and serum will be stored in a biobank. Primary endpoint of the study is the incidence rate of adult HLH among suspected adult patients during ICU stay. Out of a variety of measured biomarkers, this study furthermore aims to find highly potential biomarkers for diagnosis of adult HLH in ICU. Results of this study will contribute to improved recognition and patient outcome of adult HLH in the clinical routine.

Ethics and dissemination: The institutional ethics committee approved this study on August 1, 2018 (Ethics committee of the Charité – Universitätsmedizin Berlin,

EA4/006/18). Results of the study will be disseminated in an international peer-reviewed journal and presented at conferences.

Trial registration: The study was registered with clinicaltrials.gov (NCT03510650) on April 27, 2018.

Keywords: Hemophagocytic Lymphohistiocytosis (HLH), Hemophagocytic syndrome (HS), Macrophage activation syndrome (MAS), sepsis, biomarker, intensive care Unit (ICU)

Strengths and Limitations:

- The HEMICU study is the first prospective study to investigate biomarkers for diagnosis of adult HLH in ICU patients.
- The variety of analyzed biomarkers will also provide a better understanding of adult HLH pathophysiology.
- Biobanking of DNA, plasma and serum of adult HLH patients will generate a database to investigate future research questions.
- This study might be limited in that it only includes ICU patients and findings will not be generalizable to non-ICU patients.

Introduction

Hemophagocytic Lymphohistiocytosis (HLH) is a hyperinflammatory syndrome due to toxic immune activation associated with multiple organ failure and high mortality in intensive care unit (ICU) patients [1-3]. Primary HLH due to genetic causes has been subject of extensive research in pediatric medicine resulting in an advanced understanding of its pathophysiology including identification of underlying genetic defects related to cytotoxic granule exocytosis [4]. However, much less is known about HLH in adults where the secondary form triggered by infections, autoimmune diseases, malignancies or immunosuppressive therapy is more common. Both hereditary primary and reactive secondary HLH are characterized by impaired immune function, i.e. impaired natural-killer (NK) or cytotoxic-T-cell function leading to abnormal activation of cytokine-releasing macrophages and T-cells and finally to an uncontrolled inflammatory condition known as cytokine storm [5].

Currently, diagnosis is based on the HLH-2004 criteria (Table 1) derived from the pediatric HLH-2004 protocol which has not been validated in adult HLH patients [6, 7]. Moreover, diagnosis of HLH in ICU-admitted patients is hampered by its sepsis-like presentation. Clinical features include repetitive fever, hepato- and/or splenomegaly and antibiotic-refractory infections as well as pulmonary and renal involvement with consequent multiple organ failure [7]. Lab findings may reveal cytopenia, hypertriglyceridemia, ferritin ≥500 µg/L, and hypofibrinogenemia. Timely diagnosis is crucial to initiate adequate treatment and thus to improve the prognosis. As demonstrated by Jordan et al. [8], early therapy reduces mortality to 30-35%. However, up to 78% of all HLH cases remain undiagnosed leading to mortality rates as high as 68% [2, 9]. Given the lack of specific diagnostic tests and the established use of invalidated diagnostic criteria in adults, we aim to identify a biomarker panel of high sensitivity and specificity to allow early detection of HLH in critically ill patients.

Methods and Analysis

Design and screening

Over a two-year period (ongoing since 01/09/2018), screening of all patients admitted to 16 adult ICUs of the Charité - Universitätsmedizin Berlin will be performed. As part of the process, electronic patient charts will be screened daily for bicytopenia by an automated script. All patients with bicytopenia are daily searched by the study team for HLH-2004 criteria (Table 1), the HScore [10], and suspected HLH by the clinicians. If the patient is highly suspective for HLH, patients will be enrolled after informed consent by the patient himself or the legal representative. Immediately after study enrolment and before initiation of specific HLH treatment, whole blood samples will be obtained for all analyses. Additionally, plasma and serum samples as well as DNA will be stored in a biobank (Figure 1). HLH will be diagnosed if at least 5 HLH-2004 criteria are fulfilled and finally be confirmed after a case-by-case review by two HLH experts. The study aims to include at least 100 patients with suspected HLH. Patients in whom HLH is not confirmed will serve as controls.

Table 1. HLH-2004 diagnostic criteria [6]

HLH-2004 diagnostic criteria of which at least 5 must be fulfilled
Ferritin ≥500 µg/L
Fever (≥38.2 °C)
Splenomegaly
Cytopenias in ≥2 lines (Hemoglobin <9 g/dL, platelets <100 /nL, neutrophils <1.0 /nL)
Hypertriglyceridemia and/or hypofibrinogenemia (fasting triglycerides ≥265 mg/dL, fibrinogen <1.5 g/L)
Hemophagocytosis in bone marrow or spleen or lymph nodes

Low or absent NK activity
Soluble CD25 (soluble IL-2 receptor (sIL-2R)) ≥2,400 U/mL

Study Population and eligibility criteria for patients

Inclusion criteria:

- Intensive care patients of at least 18 years old
- Suspected or diagnosed HLH: based on HLH-2004 diagnostic criteria (bicytopenia, hyperferritinemia (≥500 µg/L), fever) or suspicion by the clinicians
- Eligible to informed consent by the patient himself or the legal representative

Exclusion criteria:

- Participation in an interventional study
- Female patients with pregnancy or breastfeeding

Setting

Participating ICU include anesthesiological, surgical, medical, neurological, and mixed ICUs. In total, 16 ICUs of the Charité – Universitätsmedizin Berlin will contribute patients.

Objectives and hypotheses

This study aims to investigate critically ill patients for adult HLH and estimate its incidence rate among suspected patients during ICU stay. Secondarily, a panel of various biomarkers candidates from experimental and pediatric studies will be

measured to explore diagnostic potential to diagnose HLH in adult ICU-admitted patients. As a result, HLH might be detected earlier leading to an improved outcome. Highly sensitive biomarkers may also help to distinguish HLH from sepsis.

Patient and public involvement

Patients or public were not involved in the development of the research question, the design, the recruitment and the conduct of this study. Results of the immunological analyses will be sent to the physicians in charge immediately. Patients will be informed of the global results of the study at their request.

Statistical analyses

Incidence rate of HLH among suspected adult patient during ICU stay will be calculated with 95% confidence intervals. Investigation of potential biomarker will be exploratory. Descriptive statistics between patient groups with confirmed HLH and controls will incorporate mean and standard deviation or absolute and relative frequencies depending on each variable's scale. Uni- and multivariable logistic regression models with confirmed HLH diagnosis as outcome will be calculated for different combinations of influencing variables and biomarkers. Receiver operator characteristic (ROC) analysis will be performed to determine discrimination ability as measured by the area under the curve (AUC) for each continuous biomarker. Sensitivity and specificity within our patient sample will be given for cut-off points with highest Youden index. Highly potential markers are found when sensitivity and specificity each reach 90%.

Outcome measures

Primary Endpoint

- Incidence rate of adult HLH among suspected patients during ICU stay

Secondary Endpoints

- Identification of highly sensitive and highly specific biomarkers to safely diagnose adult HLH in ICU
- Trigger and underlying conditions
- Therapy of HLH by clinicians
- ICU and hospital length of stay
- Mortality and survival after 6 months
- Quality of life questionnaire 36-item short form health survey (SF-36) after 6 months [11]
- Human immunodeficiency virus (HIV) antibodies and -antigen
- Epstein Barr Virus (EBV) and Cytomegalovirus (CMV) viral loads
- Inflammatory markers (ferritin, C-reactive protein (CRP), procalcitonin (PCT), interleukin (IL)-1β, IL-2, IL-6, IL-8, IL-10, IL-18, IL-33, tumor necrosis factor (TNF)-α, interferon (IFN)-ɣ, sCD25, sCD163, presepsin) [12]
- Perforin and CD107a [13]
- Fibrinogen, triglycerides, bilirubin, lactate dehydrogenase (LDH), liver transaminases (ALAT and ASAT)
- Sodium, serum albumin, serum protein electrophoresis
- Detailed immune status (differential blood count, T cells (CD3+), B cells (CD19+), NK cells (CD16+), T helper cells (CD4+), cytotoxic T cells (CD8+), CD4 / CD8 ratio, HLA-DR of CD8+, CD11a of CD8-, CD57 of CD8-, CD28 of CD8+, HLA-DR of monocytes, CD56bright and CD69 of NK cells)
- Glycosylated ferritin [14] and microRNAs (miR-205-5p, miR-194-5p and miR-30c-5p) [15]
- Chemokines CCL2 (MCP-1), CCL3, CCL4, CCL5 (RANTES), CCL11 (Eotaxin), CCL19, CCL20, CXCL1, CXCL9, CXCL8 (IL-8), CXCL10 (IP-10), CXCL12 (SDF1A) [16]
- HLA Typing

- Biobanking for future research questions (e.g. genetic polymorphisms and gene expression of PRF1, UNC13D, STX11, STXBP2 [7])

Data collection

Number of screened patients, number of patients with suspected HLH who could not be included as well as data on all outcome measures will be collected prospectively. If the patient received immunosuppressive therapy prior to inclusion, this will be documented separately. Further data including patient demographic data, past medical conditions, physicians' reports and routine laboratory results will be abstracted from the hospital's database. For follow-up six months after study inclusion, patients will be contacted either by telephone or by mail as indicated upon enrolment to assess health-related quality of life and mortality, respectively.

Immunological measurements

Plasma concentrations of soluble IL-2R (sIL-2R or sCD25) will be determined with the IMMULITE™ semi-automatic chemiluminescent immunoassay (Siemens Healthcare GmbH, Erlangen, Germany). Additional soluble factors IL-1β, IL-2, IL-6, IL-8, IL-10, IL-18, IL-33, TNF-α, IFN-ɣ will be measured by Meso Scale Discovery® (Meso Scale Diagnostics, Maryland USA). The kit provides all reagents, together with a 96-well plate with specific pre-coated spots, the detection antibodies and assay diluent. The standard will be reconstituted with assay diluent to obtain a lot specific concentration which differs for all cytokines. The vials are inverted multiple times for mixing and, after vortexing, the vials will be kept for 5–10 min at RT and then on ice until use. Preparation of further serial 1:5 dilution of Cytokine Standard is performed. Quality Controls (low and high) are components of the kits and respective Quality

Control ranges are provided by the manufacturer. The Quality Controls (low and high) will be reconstituted with 250 µl of deionized water. The vials are inverted multiple times for mixing and, after vortexing, kept for 5–10 min at RT and then on ice until use. Plasma concentrations of soluble CD163 protein levels from plasma will be determined with the Quantikine® ELISA human CD163 Immunoassay (R&D Systems, Minneapolis, USA). The minimum detectable dose ranged from 0.058-0.613 ng/mL. For measurement of presepsin, Presepsin (Human) ELISA Kit will be used (BioVision, California, USA) with a detection range of 0.156-10 ng/mL.

Flow cytometric analysis of human lymphocyte subsets in EDTA whole blood will be performed, as described recently [17]. Briefly, the following mouse anti-human fluorescently-labelled monoclonal antibodies (mAb) are used for quantification of lymphocytes subsets and analysis of T and NK cell activation markers: cluster of differentiation (CD)3 Allophycocyanine-Alexa Fluor 750 (APC-A750), CD4 energy coupled dye (ECD), CD8 APC, CD11a Fluorescein isothiocyanate (FITC), CD14 FITC, CD16 Phycoerythrine (PE), CD19 PE-Cy5.5, CD28 PC5, CD45 Krome-Orange (KrO), CD56 PE or CD56 APC, CD57 Pacific Blue (PB), CD69 PE, HLA-DR PE, (all from Beckman Coulter). Functional analysis of NK cells will be performed using the CD107a degranulation assay according to a protocol published by Bryceson et al. [18]. Briefly, peripheral blood mononuclear cells (PBMC) will be isolated by density gradient centrifugation and incubated overnight in the presence or absence of 3600 IU/mL recombinant interleukin-2 (Peprotech). PBMC will then be incubated with the target cell line K562 (ATCC) in a 1:1 ratio for 3 hours. Subsequently, CD107a expression on NK cells is assessed by staining samples with fluorescently-labelled antibodies against lineage markers (see above) and an anti-CD107a FITC-labelled mAb (eBioscience). Perforin expression in NK cells will be assessed by intracellular staining in unstimulated NK cells using the PerFix-nc reagent (Beckman Coulter) for

cell permeabilization and an APC-labelled anti-perforin mAb (eBioscience) according the manufacturer's instructions.

Expression of human leukocyte antigen-DR (HLA-DR) on monocytes (mHLA-DR) will be determined by flow cytometry using a highly standardized quantitative assay, as described earlier [19]. In short, whole blood in Vacutainer tubes (BD Biosciences, San Jose, CA, USA) containing EDTA are stained with 20 µl of monoclonal phycoerythrin-conjugated anti–HLA-DR and PerCP-Cy5.5–conjugated anti-CD14 antibodies (Quantibrite HLA-DR/monocyte™; BD Biosciences) in the dark at room temperature for 30 min. Erythrocyte lysis will be done with 0.5 mL of lysing solution (BD Biosciences) for another 30 min at room temperature. Finally, the cells are washed with 1 mL PBS buffer containing 2% FCS and analyzed on a Navios flow cytometer (Beckman Coulter, Krefeld, Germany). HLA-DR surface expression on monocytes will be calculated as monoclonal antibodies bound per cell (mAb/cell) using the QuntiBRITE™ PE calibration beads. All flow cytometric analyses will be performed on a ten-color Navios flow cytometer using the Navios Software (Beckman Coulter). Human glycosylated ferritin will be determined by Enzyme-linked Immunosorbent Assay (MyBioSource, San Diego, USA). The sensitivity of this kit is 2.0 ng/mL.

The expression of miR-205-5p, miR-194-5p and miR-30c-5p will be determined in whole blood, plasma and serum using a protocol published by Balcells et al. [20]. Briefly, after RNA isolation, miRNAs are polyadenylated and then reverse transcribed with a special primer (RT-primer). For quantitative real time PCR (qPCR), two specific primers for each miRNA are designed using a software tool from Busk [21]. All primers are tested for specify and efficiency. The qPCR will be performed with SYBR Green using the QuantStudio5® (Thermo Fisher, Darmstadt, Germany). The

quantification of chemokines involved in cell trafficking and effector functions of lymphocytes, granulocytes, and mononuclear cells from the CC subfamily (CCL2, CCL3, CCL4, CCL5, CCL11, CCL19, and CCL20) as well as the CXC subfamily (CXCL1, CXCL8 (IL-8), CXCL10, and CXCL12) will be performed with the LUNARIS™ Human 11-Plex Chemokine Kit (AYOXXA Biosystems GmbH, Cologne, Germany).

Determiniation of human leukocyte antigen (HLA) typing will be performed by reverse sequence-specific oligonucleotide (rSSO) assay LABType® (One Lambda, Canoga Park, CA, USA). Typing will be assessed on an intermediate resolution level for HLA-A, -B, -C, -DRB1, -DQA1 and -DQB1. The assay will be performed according to the manusfacturer's instructions and data will be acquired on a Luminex® FlexMAP 3D machine (Luminex, Austin, TX, USA).

Informed consent in critically ill patients

Written informed consent will be obtained from all patients or their legally authorized representatives. Consent for genetic analyzes in future projects will be obtained separately.

Sample size

The true incidence of adult HLH in ICU is unknown. According to our own research [9] and the annual number of patients admitted to our ICU, we expect to see about 200 patients with diagnosed HLH over two years, and about 400 with suspected HLH. Of these, we hope to include at least 100 patients with suspected HLH into the study, of whom about 50 patients are expected to be diagnosed with HLH. When the

sample size is 100, a two-sided 95.0% confidence interval for a single proportion using the large sample normal approximation will extend 0.1 from the observed proportion for an expected proportion of 0.5 (nQuery Advisor 7.0).

Ethics and Dissemination

The institutional ethics committee approved this study on August 1, 2018 (Ethics committee of the Charité – Universitätsmedizin Berlin, EA4/006/18). The data protection commissioner also approved the study (91-SP-18), which was registered with clinicaltrials.gov (NCT03510650) on April 27, 2018. Results of the study will be disseminated in an international peer-reviewed journal and presented at conferences.

Discussion

HLH is a rare condition in adults with poor prognosis. Due to the paucity of data available on adult HLH, recognition remains low resulting in delayed diagnosis and treatment and finally, fatal outcome. This is the first prospective study to systematically investigate routine and non-routine parameters for biomarker development. Importantly, the daily systematic screening will help to identify HLH patients at an early stage of the syndrome which ultimately will improve patient care, patients' safety and outcome. Moreover, describing a distinct pattern of biomarkers generates new hypothesis for future research thereby potentially providing targets for therapy development. With regard to clinical practice, the HEMICU study seeks to inform clinicians about HLH and ICU therapies to improve outcomes for HLH patients. However, it is of note that this study does not seek to advice the clinician in charge to change therapy. No change in routine management is intended due to the

observational study design and final decisions are left to the discretion of the responsible clinician.

Strengths and Limitations

This study might be limited in that it only includes ICU patients and findings will not be generalizable to non-ICU patients. In addition, patients might have developed HLH before ICU admission and will thus be detected and measurements obtained at an advanced stage of the disease possibly limiting comparability of the results. Moreover, we will not assess longitudinal parameters preventing us from describing dynamics of biomarkers over time. However, as this study aims to develop a tool facilitating diagnosis at the earliest possible time point, study endpoints will not be affected by lack of repeated measurements. Possible advances of this study include comprehensive lab testing of parameters, which have previously been suggested to be associated with HLH [12-16]. Previous studies, most of which are retrospective in nature, aimed at identifying associations to detect risk factors [2, 22]. Therefore, the HEMICU study is the first prospective study of its kind.

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Competing interests

The authors declare no conflicts of interest.

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Authors´contributions

Study concept: GL. Conceived and designed the experiments: GL, CvH, NP, CM, PL, TS, NL, HDV, DK. Performing the experiments: GL, CK, CvH, NP, CM, PN, FSS, GV, FB, NU, UK, NL, LA. Analyzing the data: GL, CK, PN, FSS, SKP, JK, PL, TS. Wrote the manuscript: GL, CK, CvH, NP, CM, SKP, NL, LA, FMB, DK, CS. Commented on the manuscript: all authors.

Data statement

Due to legal restrictions imposed by the ethics committee of the Charité – Universitätsmedizin Berlin and the data protection commissioner of the Charité – Universitätsmedizin Berlin, public sharing of study data with other researchers or entities is not allowed. Requests may be sent to dai-researchdata@charite.de.

Figure legends

Figure 1. Screening protocol and blood sampling.

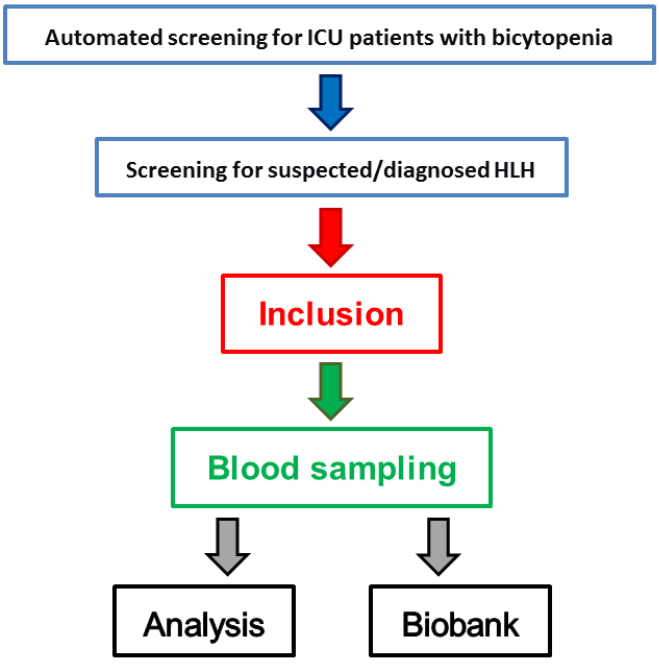

Figure 1 / Screening protocol and blood sampling.

81x60mm (300 x 300 DPI)

Reporting checklist for protocol of a clinical trial.

Based on the SPIRIT guidelines.

Instructions to authors

Complete this checklist by entering the page numbers from your manuscript where readers will find each of the items listed below.

Your article may not currently address all the items on the checklist. Please modify your text to include the missing information. If you are certain that an item does not apply, please write "n/a" and provide a short explanation.

Upload your completed checklist as an extra file when you submit to a journal.

In your methods section, say that you used the SPIRITreporting guidelines, and cite them as:

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		Reporting Item	Page Number
Administrative information			
Title	#1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	#2a	Trial identifier and registry name. If not yet registered, name of intended registry	14
Trial registration: data set	#2b	All items from the World Health Organization Trial Registration Data Set	14
Protocol version	#3	Date and version identifier	14
Funding	#4	Sources and types of financial, material, and other support	16
Roles and responsibilities: contributorship	#5a	Names, affiliations, and roles of protocol contributors	1

Roles and responsibilities: sponsor contact information	#5b	Name and contact information for the trial sponsor	16
Roles and responsibilities: sponsor and funder	#5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	16
Roles and responsibilities: committees	#5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	16
Introduction			
Background and rationale	#6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	5
Background and rationale: choice of comparators	#6b	Explanation for choice of comparators	5
Objectives	#7	Specific objectives or hypotheses	5
Trial design	#8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, non-inferiority, exploratory)	7
Methods: Participants, interventions, and outcomes			
Study setting	#9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	7
Eligibility criteria	#10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will	8

		perform the interventions (eg, surgeons, psychotherapists)	
Interventions: description	#11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	7
Interventions: modifications	#11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving / worsening disease)	7
Interventions: adherance	#11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return; laboratory tests)	7
Interventions: concomitant care	#11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	7
Outcomes	#12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	9
Participant timeline	#13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	7
Sample size	#14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	7
Recruitment	#15	Strategies for achieving adequate participant enrolment to reach target sample size	7
Methods: Assignment of interventions (for controlled trials)			
Allocation: sequence generation	#16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	7

Allocation concealment mechanism	#16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	7
Allocation: implementation	#16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	7
Blinding (masking)	#17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	7
Blinding (masking): emergency unblinding	#17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	7
Methods: Data collection, management, and analysis			
Data collection plan	#18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	7
Data collection plan: retention	#18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	7
Data management	#19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	7
Statistics: outcomes	#20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	9
Statistics: additional analyses	#20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	9

Statistics: analysis population and missing data	#20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	9
Methods: Monitoring			
Data monitoring: formal committee	#21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	7
Data monitoring: interim analysis	#21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	7
Harms	#22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	7
Auditing	#23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	7
Ethics and dissemination			
Research ethics approval	#24	Plans for seeking research ethics committee / institutional review board (REC / IRB) approval	14
Protocol amendments	#25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC / IRBs, trial participants, trial registries, journals, regulators)	14
Consent or assent	#26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	14
Consent or assent: ancillary studies	#26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	14
Confidentiality	#27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	14

Declaration of interests	#28	Financial and other competing interests for principal investigators for the overall trial and each study site	14
Data access	#29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	14
Ancillary and post trial care	#30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	14
Dissemination policy: trial results	#31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	14
Dissemination policy: authorship	#31b	Authorship eligibility guidelines and any intended use of professional writers	14
Dissemination policy: reproducible research	#31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	14
Appendices			
Informed consent materials	#32	Model consent form and other related documentation given to participants and authorised surrogates	14
Biological specimens	#33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	14