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Description and optimization of a multiplex bead-based flow cytometry method (MBFCM) to characterize extracellular vesicles in serum or culture supernatants samples from patients with hematological malignancies

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Affidavit

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Abkürzungsverzeichnis

AML	Acute myeloid leukemia
ALL	Acute lymphoid leukemia
APCs	Antigen presenting cells
allo-SCT	Allogeneic stem cell transplantation
BM	Bone marrow
CAR-T	Chimeric-Antigen Receptor-T cells
CD	Cluster of differentiation
CLL	Chronic lymphoid leukemia
CIK	Cytokine-induced killer
CTX	Cytotoxicity fluorolysis Assay
DC	Dendritic cells
DCS	DC culture supernatants
DC_{leu}	Leukemia-derived dendritic cells
DC_{mat}	Mature dendritic cells
Deg	Degranulation assay
DS	Down syndrome
EV(s)	Extracellular vesicle(s)
fNTA	Fluorescence nanoparticle tracking analysis
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H	Healthy
HSCT	Hematopoietic stem cell transplantation
ICS	Intracellular cytokine staining
IFCM	Imaging flow cytometry
LAA	Leukemia associated antigens
MRC	Myelodysplasia related changes
MBFCM	Multiplex bead-based flow cytometry
MHC	Major histocompatibility complex
MLC	Mixed lymphocyte culture
MLCS	MLC culture supernatants

MNC	Mononuclear cell
miRNA	MicroRNA
NK	Natural killer
NOS	Not otherwise specified
PB	Peripheral blood
PGE_1	Prostaglandin E1
RNA-Seq	RNA sequencing
SEC	Size exclusion chromatography
t-MN	Therapy-related myeloid neoplasms
TEM	Transmission electron microscopy
WB	Whole blood

Publikationsliste

1.1 Publications Included in This Thesis

The following publications were summarized for this cumulative medical thesis in accordance with the examination rules of the medical faculty of the LMU Munich:

Publication I and II are used for this cumulative dissertation.

Publication I:

Lin Li, André Görgens, Veronika Mussack, Elena Pepeldjiyska, Anne Sophie Hartz, Andreas Rank, Jörg Schmohl, Doris Krämer, Samir El Andaloussi, Michael W. Pfaffl, Helga Schmetzer: Description and optimization of a multiplex bead-based flow cytometry method (MBFCM) to characterize extracellular vesicles in serum samples from patients with hematological malignancies. Cancer Gene Therapy 2022; Published online:27.04.2022, https://doi.org/10.1038/s41417-022-00466-1, IF=5.987

Publication II:

Lin Li, Veronika Mussack, André Görgens, Elena Pepeldjiyska, Anne Sophie Hartz, Hazal Aslan, Elias Rackl, Andreas Rank, Jörg Schmohl, Doris Krämer, Samir El Andaloussi, Michael W. Pfaffl, Helga Schmetzer: The potential role of serum extracellular vesicle derived small RNAs in AML research as non-invasive biomarker. Accepted for publication by nanoscale advances 13.02.2023; IF=5.598

To your information:

Publication III (in addition):

Comments see in „Ihr Beitrag zu den Veröffentlichungen“: 1.3 contributions to paper III and Einleitung.

Lin Li, Veronika Mussack, André Görgens, Elena Pepeldjiyska, Anne Sophie Hartz, Hazal Aslan, Elias Rackl, Andreas Rank, Jörg Schmohl, Doris Krämer, Samir El Andaloussi, Michael W. Pfaffl, Helga Schmetzer: Role of EVs as promotors to study activation of leukemia-derived dendritic cells' (DCleu)-mediated antileukemic activation of adaptive and innate immune-reactive cells against AML-blasts

(in preparation)

1.2 Other original Publications

1, Elena Pepeldjiyska, **Lin Li**, Jincheng Gao, Christian Blasi, Erdem Özkaya, Jörg Schmohl, Christoph Schmid, Andreas Rank, Doris Kraemer, Helga Maria Schmetzer, et al: Leukemia derived dendritic cell (DCleu) mediated immune response goes along with reduced (leukemia-specific) regulatory T cells. Immunobiology; published online: 11.06.2022, https://doi.org/10.1016/j.imbio.2022.152237, IF=3.16

2, Elias Rackl, **Lin Li**, Lara Kristina Klauer, Selda Ugur, Elena Pepeldjiyska, Carina Gunsilius, Melanie Weinmann, Fatemeh Doraneh-Gard, Nina Reiter, Corinna Seidel, Caroline Plett, Daniel Christoph Amberger, Peter Bojko, Doris Kraemer, Jörg Schmohl, Andreas Rank, Christoph Schmid, Helga Maria Schmetzer: Dendritic cell triggered immune activation goes along with provision of (leukemia-specific) Integrin beta 7 expressing immune cells and improved antileukemic processes. International Journal of Molecular Sciences 2022; received 27.12.2022, IF=6.208

1.3 Manuscripts submitted for publication/in preparation:

1, Elias Rackl, Anne Hartz, Hazal Aslan, **Lin Li**, Lara Klauer, Elena Pepeldjiyska, Carina Gunsilius, Melanie Weinmann, Fatemeh Doraneh-Gard, Corinna L. Seidel, Caroline Plett, Olga Schutti, Daniel C. Amberger, Christoph Kugler, Peter Bojko, Doris Krämer, Jörg Schmohl, Andreas Rank,

Christoph Schmid and Helga Maria Schmetzer: Dendritic cell mediated provision of (leukemia specific) T-cell receptor gamma delta (TCRgd) expressing cells improve antileukemic reactions. Journal of Immunotherapy (under review)

2, Baudrexler Tobias, Boeselt Tobias, **Li Lin**, Bohlscheid Sophia, Boas Ursel, Schmid Christoph, Rank Andreas, Schmohl Jörg, Koczulla Rembert, Schmetzer Helga: Volatile profiling using an eNose allows differentiation of volatile phases derived from Serum, DC, or MLC culture supernatants from healthy or leukemic samples (under review)

3, **Lin Li**, Boeselt Tobias, Baudrexler Tobias, André Görgens, Veronika Mussack, Elena Pepeldjiyska, Anne Sophie Hartz, Hazal Aslan, Elias Rackl, Andreas Rank, Doris Krämer, Samir El Andaloussi, Michael W. Pfaffl, Helga Schmetzer: VOC pattern recognition of AML: evaluation of eNose - based miRNA strategies (in preparation)

4, **Lin Li**, Veronika Mussack, André Görgens, Elena Pepeldjiyska, Anne Sophie Hartz, Hazal Aslan, Elias Rackl, Andreas Rank, Jörg Schmohl, Doris Krämer, Samir El Andaloussi, Michael W. Pfaffl, Helga Schmetzer: Potential of extracellular vesicle (EV) derived small RNAs as non-invasive biomarker for AML monitoring? (in preparation)

5, S. Bohlscheid, E. Pepeldjiyska, **L. Li**, J. Gao, C. Seidel, C. Blasi, E. Özkaya, J. Schmohl, D. Kraemer, C. Schmid, A. Rank, H. M Schmetzer: Kit-treatment of Antileukaemic T-cell responses can be predicted by compositions of regulatory T-cell subpopulations-under hypoxic and normoxic conditions (in preparation)

1.4 Contributions to conferences (poster presentations):

1, **Lin Li**, Veronika Mussack, Elena Pepeldjiyska, Anne Hartz, Andreas Rank, Christoph Schmid, Erdem Özkaya, Selda Ugur, Michael Pfaffl, Helga Schmetzer (Germany) Role of Exosomes as promotors or biomarkers to study activation of leukemia-derived dendritic cells (DCleu)-mediated antileukemic activation of adaptive and innate immune-reactive cells against AML-blasts. ITOC7, 2-3 October 2020; P01.11. Journal for ImmunoTherapy of Cancer Oct 2020, 8 (Suppl 2) A13-A14; DOI:10.1136/jitc-2020-ITOC7.24

2, **Lin Li**, Veronika Mussack, Elena Pepeldjiyska, Anne Hartz, Andreas Rank, Christoph Schmid, Erdem Özkaya, Selda Ugur, Michael Pfaffl, Helga Schmetzer (Germany)： Role of Exosomes as promotors or biomarkers to study activation of leukemia-derived dendritic cells (DCleu)-mediated antileukemic activation of adaptive and innate immune-reactive cells against AML-blasts. EBMT, 2020, A392, Bone Marrow Transplantation 54, suppl. 1

3, **Lin Li**, Veronika Mussack, André Görgens, Elena Pepeldjiyska, Anne Sophie Hartz, Hazal Aslan, Elias Rackl, Andreas Rank, Jörg Schmohl, Doris Krämer, Samir El Andaloussi, Michael W. Pfaffl, Helga Schmetzer: The potential role of extracellular vesicle-derived small RNAs in AML research as non-invasive biomarker, EBMT online (2021)

4, **Lin Li**, Veronika Mussack, André Görgens, Elena Pepeldjiyska, Anne Sophie Hartz, Hazal Aslan, Elias Rackl, Andreas Rank, Jörg Schmohl, Doris Krämer, Samir El Andaloussi, Michael W. Pfaffl, Helga Schmetzer: The Potential role of extracellular vesicle-derived small RNAs in AML research as non-invasive biomarker, ITOC9, 2022-A-34-ITOC, P0701

5, Baudrexler Tobias, Boeselt Tobias, Bohlscheid Sophia, **Li Lin**, Schmid Christoph, Rank Andreas, Schmohl Jörg, Helga Schmetzer: Volatile profiling using an eNose allows differentiation of volatile phases derived from serum, DC, or MLC culture supernatants from healthy or leukemic samples: J. Immunotherapy of cancer 10(supp1),10.1136/jitc-2022-ITOC9.37 (2022)

Ihr Beitrag zu den Veröffentlichungen

Co-Authors'confirmations

All co-authors signed a confirmation, that Lin Li has the permission to use the publications for her PhD thesis. Furthermore, they confirmed that the publications are not part of another doctoral thesis. The Documents were submitted separately with this thesis.

1.1 Contributions to Paper I (attached)

Title: Description and optimization of a multiplex bead-based flow cytometry method (MBFCM) to characterize extracellular vesicles in serum samples from patients with hematological malignancies

Authors: Lin Li, André Görgens, Veronika Mussack, Elena Pepeldjiyska, Anne Sophie Hartz, Andreas Rank, Jörg Schmohl, Doris Krämer, Samir El Andaloussi, Michael W. Pfaffl, Helga Schmetzer.

Journal: Cancer gene therapy,2022, https://doi.org/10.1038/s41417-022-00466-1.

Contributions: Lin Li designed the study together with H.S. Lin Li conducted experiments for preparing EVs from serum. Lin Li assessed clinical reports and performed all statistical analyses. Lin Li prepared a manuscript draft (including abstract, introduction, methods, discussion and conclusion) and prepared all figures and tables. Lin Li submitted the final manuscript (prepared together with V.M, A.G and H.S) to the journal, revised it according to reviewers' comments together with H.S and resubmitted it to the journal.

1.2 Contributions to Paper II (attached)

Title: The potential role of serum extracellular vesicle derived small RNAs in AML research as non-invasive biomarker

Authors: Lin Li, Veronika Mussack, André Görgens, Elena Pepeldjiyska, Anne Sophie Hartz, Hazal Aslan, Elias Rackl, Andreas Rank, Jörg Schmohl, Doris Krämer, Samir El Andaloussi, Michael W. Pfaffl, Helga Schmetzer:

Journal: Nano advances, 2022

Contributions: Lin Li designed the study together with H.S, M.W.P and A.G. Lin Li conducted experiments for preparing EVs from serum. Lin Li assessed clinical reports and performed all statistical analyses. Lin Li prepared a manuscript draft (including abstract, introduction, methods, discussion and conclusion) and prepared all figures and tables. Lin Li submitted the final manuscript (prepared together with V.M, A.G and H.S) to the journal, revised it according to reviewers' comments together with H.S and resubmitted it to the journal.

1.3 Contributions to Paper III

Paper III is not used for cumulative dissertation, but is important for state the experimental experience.

Publication III,

Title: Role of EVs as promotors for activation of leukemia-derived dendritic cells' (DCleu)-mediated antileukemic activation of adaptive and innate immune-reactive cells against AML-blasts

Authors: Lin Li, André Görgens, Veronika Mussack, Elena Pepeldjiyska, Anne Sophie Hartz, Hazal Aslan, Elias Rackl, Andreas Rank, Jörg Schmohl, Doris Krämer, Samir El Andaloussi, Michael W. Pfaffl, Helga Schmetzer

(in preparation)

Contributions: Lin Li designed the study together with H.S, M.W.P and A.G. Lin Li conducted great parts of cellbiological and functional experiments (including dendritic cell cultures, mixed lymphocyte culture, degranulation assay (Deg), intracellular cytokine assay (ICS), cytotoxicity and other (functional) assays). Lin Li assessed clinical reports and performed all statistical analyses. Lin Li prepared a manuscript draft (including abstract, introduction, methods, discussion and conclusion) and prepared all figures and tables.

2. Einleitung

2.1 Leukemia

Leukemias are malignant disorders of the blood and bone marrow (BM) [1]. Acute myeloid leukemia (AML), Acute lymphoid leukemia (ALL) and Chronic lymphoid leukemia (CLL) are blood/BM cancers of mainly elderly patients, with uncontrolled proliferation of myeloid or lymphoid blasts [2,3]. AML is a heterogeneous disease, in which ≥20% of cells in the blood or BM are myeloblasts [4], characterized by the accumulated proliferation and survival of immature myeloid cells. According to the morphology and immunophenotype, AML is classified into categories [4]. AML is the result of genetic abnormalities, comprised of mutations and chromosomal rearrangements related with clinical, morphological and immunophenotypic features [5]. ALL develops predominantly in children and elderly patients. ALL is characterized in differentiation and proliferation of lymphoid precursor cells. Therapeutic improvements showed that, 80-90% of children with ALL can be treated and cured with combination chemotherapy [5,6]. Based on genome-wide analyses, ALL has been classified into B- and T-lineage subtypes [7]. CLL is characterized by the progressive accumulation of B cells (e.g.CD5, CD19, CD23) in the blood, bone marrow, lymph nodes and spleen[8]. Recently chlorambucil is still the standard frontline therapy for elderly CLL patients [9].

Due to high proliferation rates of leukemic blasts, symptoms mostly develop rapidly within weeks after diagnosis and may finally lead to death. Accumulation of blasts in the blood or BM results in an ineffective hematopoiesis, which leads to symptoms of neutropenia, erythrocytopenia or thrombocytopenia going along with fever, infections, hemorrhages, inappetence or degradation of performance status [10,11]. Therefore, it is essential to assess immediately diagnostic results and to evaluate treatment options. The prognosis can be estimated by a distinction of three risk groups (favourable, intermediate and adverse), based on clinical features like age, primary/secondary, comorbidities and certain cytogenetic or molecular aspects [7,11,12]. The immuno-microenvironment contributes to predict the course of leukemia. The immune evasion mechanisms mediated by tumors can inhibit T cell immunity specially [12–14].

The main efficient curative treatments of leukemia are still chemotherapy with allogeneic stem cell transplantation (allo-SCT) or Chimeric Antigen Receptor-T cells (CAR-T). Allo-SCT may survive longer or even cure more patients via an immune-mediated graft-versus-leukemia effect, however still goes along with important risks e.g. for infections, graft versus host disease and substantial non-relapse mortality [15,16]. CAR T-cell immunotherapy is a promising treatment principle, which concerns the engineering of T cells to kill tumors by targeting CD19, especially in eliminating B-cell leukemia [17,18]. The discovery of new molecular mutations might result in individualized therapies and offer new biomarkers that can be used to monitor minimal residual disease [19]. The ability to detect minimal residual disease could help to prevent relapses by early intervention.

2.2 Dendritic cells (DC) and mixed lymphocyte culture (MLC)

DCs are a portion of the innate immune system, specialized antigen-presenting cells (APCs), which stimulate immune cells by processing antigens and presenting their fragments via major histocompatibility complex (MHC) I and II. Leukemia-derived DC (DC_{leu}) can be generated from mononuclear cell (MNC) or whole blood (WB) using “DC-generating Kits” such as Kit M (GM-CSF and PGE_1) and are characterized by the expression of (patient-specific) blast markers/LAA (Leukemia associated antigens) together with DC or other immunogenic antigens [20–22].

After T cell-enriched mixed lymphocyte cultures (MLC), DC_{leu} mediate activation of immune reactive innate and adaptive immune reactive cells with the detection of increased frequencies of (leukemia specific) T and innate (especially) NK cell (subtypes) [23,24]. The innate immune system may comprise of macrophages, dendritic cells, cytokine-induced killer (CIK) and natural killer (NK) cells, which provide the first-line defense against pathogens and tumors, the adaptive immune system may comprise of T- and B- cells, which mediate tumor environment by antigen-specific responses [20,25–29].

2.3 Extracellular vesicles (EVs)

“Extracellular vesicles”, are actively or passively released from cells. EVs contain a lipid bilayer membrane that protects the encapsulated material, including proteins, nucleic acids, lipids and metabolites [30]. EVs have been classified into categories: apoptotic bodies, microvesicles and exosomes. EV types have significant differences in their biogenesis, physiochemical properties, molecular composition and biological function [31]. EVs can be harvested from all body fluids. EVs can be released by dendritic cells (DCs), B cells, T cells and others that function within the adaptive immune system and they display a plethora of functions according to their origin [32].

EVs play an important role in antigen presentation and in communication between immune cells [32] and play a role in pathological and physiological systems, in cancer or neurobiology [33]. EVs or their subtypes could generate connections with the surrounding cells, mediate cellular uptake, reform immune recognition via posttranslational modifications by the innate and adaptive immune systems [34]. Therefore, the multiple potential of EVs is to be used as biomarkers, therapeutic targets, immune evasion or immunotherapy and biologically engineered EV [35].

In hematological malignancies, EVs reorganize the environment of BM. In AML, EVs promote the communication between cells and conduct adaptation of compartmental BM function via traffic of protein, RNA and DNA [36]. Tumor-derived EVs can deliver signals, inhibit cell differentiation, enhance expansion of regulatory T cells and myeloid-derived suppressor cells [32,37]. On the other hand, EVs, as cancer monitors, can offer a minimally invasive method to carry tumor molecular information and represent collective parameters of tumor cells [38]. The collection and purification of EVs in AML might modulate disease stage, drug resistance or immune therapies [39].

Furthermore, EVs secreted by DCs mediate the mesenchymal stem or stromal cell recruitment. This was shown to support time-controlled changes to the micromilieu and could promote regenerative responses. T-cell responses are down regulated by immature DCs, the activation, proliferation and differentiation of effector T cells are promoted by mature DCs [40–42]. This DC-to-DC communication is essential to display functions, which is mediated through cell-to-cell contact, soluble mediators and interaction with apoptotic cell derived EVs [43–46].

2.4 Detection and quantification of EVs

EVs can be identified or characterized by ultra-centrifugation, precipitation, size exclusion chromatography (SEC), ultrafiltration or other methods [47,48]. EVs can be obtained by multiple methods to assess purity and/or prove specific properties of interest [48,49], e.g. transmission electron microscopy (TEM), fluorescence nanoparticle tracking analysis (fNTA), western blotting [38,50,51]. In EV research, TEM is a focused beam of electron transmitted via a thin specimen to create a sample image, [38] fNTA is particle tracking pattern to determine and identify EVs concentration and size distribution of particles [38,50], both of them are popular and useful methods. Detailed phenotyping of EV subsets can be achieved by multiplex bead-based flow cytometry analysis (MBFCM) [51] or by high resolution single vesicle imaging flow cytometry (IFCM) to quantify further EV subsets [52]. Advantages of combining such flow cytometric methods are, that subsets of EVs

can be detected and enumerated together with microvesicles with diameters above 200 nm in a multi-parametric way. The MISEV guidelines explain in detail which parameters are necessary for a reliable and valid EV isolation and characterization [47]. Precipitarom real-life clinical blood samples deliver more reproducible microRNA candidates and hence a better and more valid biomarker signature. As further advantage, the miRNA biomarker signature can be easily confirmed by orthogonal methods, like RT-qPCR, which is an essential core point in a valuable biomarker development.

MBFCM (39 capture bead populations) can reproducibly detect EV surface markers in sample types (EV-containing) via a semi-quantitative pattern [51]. According to the assay documentation, MBFCM assays focus on the 37 EV surface antigens and another two internal isotype negative controls. Particularly three bead populations, i.e., CD9, CD63, CD81 are a mixture of pan anti-tetraspanin. Other markers coated with specific capture antibodies, comprise T cell, B cell, monocyte, thrombocyte, integrin, endothelial, or MHC-associated antigens [51] and can be characterized according to their individual fluorescence intensity.

2.5 Preparation and Characterization of EV derived miRNAs

MicroRNAs (miRNAs) are short, non-coding RNAs. MiRNAs are post-transcriptional regulators of gene expression of many essential cellular processes [53]. MiRNAs can be released into body fluids in combination with lipoproteins or proteins or encapsulated in EVs [54]. It has been reported that EV-mediated transfer of surface- and intra-vesicular proteins, lipids and nucleic acids (RNA and DNA) with direct effects on recipient cells. EVs carry and deliver essential molecular information, in majority via microRNA and proteins for cell physiology and metabolism.

MiRNAs play gene-regulatory roles, generating remarkable functions in physiological and pathological processes [53]. MiRNAs can act as either tumor activating or suppressing RNAs in different subtypes of leukemia, either by targeting them on the mRNA level or by working together with concerning proteins to promote tumors, related with a series of leukemic processes, including proliferation, survival, differentiation, self-renewal, tumor development and chemotherapy resistance [55].

Huan et al. isolated total RNA derived from EVs in AML cell lines and primary AML cells [56]. However, the content of RNA derived from EVs differed significantly in cell lines and primary AML cells. Large amounts of small RNAs and miRNAs were detected in EVs [50,56,57], with 5- to 13-fold higher enrichment of miRNAs compared to parental cells found in cell lines [58]. EVs and their miRNAs are shown to play an important regulative role in nearly all physiological or immunological processes and have been connected with leukemia [59].

2.6 Outline and overview of this thesis

Leukemias are life-threatening malignant disorders of the blood and BM. For leukemia patients, the major reason of disease recurrence is the minimal residual leukemia cells after chemotherapy and/or hematopoietic stem cell transplantation (HSCT). DCs can be generated from leukemic blasts in vitro and in vivo (DC_{leu}). DC_{leu} are characterized by simultaneous expression of dendritic- and leukemic- antigens. EVs are membrane-bound particles, carrying large amounts of cargo materials including miRNA, proteins and immune-related ligands active in cell-to-cell communication. Due to their enhanced stability, EVs and EV derived miRNAs are in the focus of most biomarker and molecular studies and serve also as novel analyte in hematological neoplasms.

The aim of this thesis was to (1) isolate EVs in serum and culture supernatants from leukemic patients and healthy donors; (2) identify EVs (from serum and culture supernatant samples) by TEM and fNTA; (3) characterize and compare the EV surface antigens with MBFCM from leukemic patients versus healthy donors; (4) evaluate and compare resulting data for potential correlation with AML patients' clinical data or with immunological/functional features in culture supernatants; (5) assess EV associated miRNA profiles that could be applied as potential biomarkers comparing healthy donors' with leukemic patients' serum and culture supernatant.

Overview Publication I-Description and optimization of a multiplex bead-based flow cytometry method (MBFCM) to characterize extracellular vesicles in serum samples from patients with hematological malignancies

The **objective** of publication I was to quantify and characterize serum-derived EVs from AML, ALL, CLL and healthy controls and evaluate whether these profiles could be used as potential diagnostic markers for clinical research.

TEM, fNTA and MBFCM were used to detect and assess EVs in serum, aiming to establish a proof-of-concept workflow to characterize EVs and to potentially predict immune reactions from leukemic and healthy samples.

Typical cup-shaped imaging of serum EVs by TEM was detected and EV concentrations and size distribution profiles by fNTA were first characterized from AML, ALL, CLL patients and healthy controls. In a next step MBFCM which comprises 39 capture bead populations was adapted for serum. High signal intensities of CD133 in AML, HLA-DRDPDQ in ALL and CD11c and CD146 in CLL derived EVs were found after normalizing data from leukemic to healthy samples. Frequencies of immune cytologically detected leukemic cells as well as platelet, white blood cell counts and cellular markers (e.g.CD14, CD19, CD3 and CD56) correlated in part with frequencies of specific EV associated antigen expressions as characterized by MBFCM in leukemia samples.

In summary, MBFCM might contribute to indicate the involvement of EVs in leukemia. MBFCM is good method to detect the specificity and heterogeneity of EVs and the role of specific EVs as a suitable marker in leukemia classification and the monitoring of (leukemia-associated) EVs derived and released from cells.

Overview Publication II-The potential role of serum extracellular vesicle derived small RNAs in AML research as non-invasive biomarker

The **objective** of publication II was to evaluate the differences of EVs derived miRNAs in serum from AML compared to healthy controls and to deduce whether they could serve as biomarkers for diagnostic and disease stratification.

Therefore, the quantitative and qualitative assessment of EV surface characteristics using MBFCM and especially the characterization of the EV derived miRNA cargo by RNA-Seq were evaluated in AML samples compared to healthy controls.

According to the manufacturer's protocol, total RNA was isolated from purified EVs. 152 miRNAs were found in Healthy and 159 in AML samples, 136 of which were detected in both H and AML samples. 5 up regulated miRNAs (miR-10a-5p, miR-155-5p, miR-100-5p, miR-146b-5p, let-7a-5p) and 5 down regulated miRNAs (miR-185-5p, miR-4433b-3p, miR-199a-5p, miR-451a, miR-

151a-3p) were identified by small RNA sequencing in AML vs H samples. The largest difference in AML compared to Healthy was detected for miR-10a-5p (Log2FC=4.89).

In conclusion, EV derived miRNA dysregulation in serum by tumor activators or suppressors might have a significant impact on disease progression and prediction. MiRNA expression profiling might provide important diagnostic and prognostic information and could qualify as biomarkers in AML research.

Due to the pandemic situation, Lin was not allowed to perform any experiments in WG Pfaffl and Görgens (related with paper I and II). However, Lin could perform cellbiological and functional experiments in the WG Schmetzer. Results of this experimental work (in cooperation with WG Pfaffl and Görgens) are provided in publication III, that will be submitted to Journal in the next weeks.

Overview Publication III- Role of EVs as promotors for activation of leukemia-derived dendritic cells' (DCleu)-mediated antileukemic activation of adaptive and innate immune-reactive cells against AML-blasts (in preparation)

The **objective** of publication III was to generate and quantify DC/DC_{leu} from WB using Kit M (GM-CSF and PGE_1) (vs control) and to stimulate the patients' immune cells after MLC. The potential role of DC or MLC culture supernatants derived EVs as biomarkers in AML patients versus healthy controls as well as influences of blast/immune modulatory strategies to modify antileukemic processes should be evaluated.

Therefore, DC/DC_{leu} with blast modulating Kit M (GM-CSF and PGE_1) (vs control) from AML and healthy WB and stimulated T-cell enriched immunoreactive cells in MLC were generated. TEM, fNTA and MBFCM were used for EV detection in DC culture supernatants (DCS) and MLC culture supernatants (MLCS) from AML and Healthy samples.

DC/DC_{leu} generated with Kit M from leukemic WB (vs control) were shown to activate T-cell's (e.g.CD4+, CD8+, non-naïve T cells, central memory T cells and effector memory T cells) differentiation in AML compared to Healthy samples after MLC. Kit M pretreated WB moreover increased leukemia-specific activity after MLC (specifically intracellular IFNγ and CD107a expression) and anti-leukemic activity compared to control were evaluated and compared. EV concentrations by fNTA and EV surface antigens by MBFCM and cellular marker expressions by flow cytometry in DCS and MLCS from AML and Healthy samples were evaluated and assessed.

In summary, specific EVs may play a fundamental role in the immune modulatory responses in the AML.

Conclusion

MBFCM is generally suitable for EV characterization and can not only quantify and qualify EV surface antigens but also contribute to compare different individual surface antigens between leukemia and Healthy samples. We assume that EVs and EV derived miRNA cargo could be considered as privileged biomarkers, demonstrating their physiological features and development of the disease. These findings contribute to confirm the unique function of EVs (e.g.) also to improve antileukemic immune responses, or with respect to the role of EVs/EV derived miRNA as new biomarkers. Now we are going to analyze EVs' potential role for predicting or mediating antileukemic processes ex vivo or in vivo.

3. Zusammenfassung:

EVs sind membranöse Vesikel und ein Verständnis ihrer Rolle bei der Tumorentstehung oder Immunaktivierung könnte dazu beitragen, antileukämische Prozesse zu verstehen und zu monitorieren. EVs wurden aus dem Serum von AML-, ALL-, CLL-Patienten und gesunden Proben isoliert. Das typische becherförmige Erscheinungsbild von Serum-EVs wurde durch TEM identifiziert, und EV-Konzentrationen und Größen von Serum-EVs durch fNTA ermittelt. Hohe Anteile an EVs (positiv für CD81, CD63, CD9), die linienassoziierte EV-Marker exprimieren (CD8, CD42a, CD62P und HLA-DRDPDQ) wurden in AML-, AML-, CLL- und gesunden Serum-Proben mit Hilfe von MBFCM (unter Verwendung von 37 spezifischen Markern) detektiert. Unsere Daten zeigten Korrelationen von EV-Markerexpressionen (gemessen durch MBFCM) in Serum mit immunzytologisch nachgewiesenen Leukämiezellen, Blutplättchen- und Leukozytenzahlen in allen AML-, ALL- und CLL-Proben.

Insbesondere wurden in AML- und gesunden Proben mit MBFCM hohe Anteile an EVs (positiv für CD81, CD63, CD9) gefunden, die zusätzlich CD8, CD41b, CD42a, CD62P, HLA-DRDPDQ und SSEA-4 exprimierten. Gesamt-RNA wurde aus EVs über ‚small RNA' Sequenzierung zur qualitativen/quantitativen Charakterisierung von AML- und gesunden Proben isoliert: In AML vs gesunden Serum-EVs wurden hochregulierte miRNAs (miR-10a-5p, miR-155-5p, miR-100-5p, miR-146b-5p, let-7a-5p) und herunterregulierte miRNAs (miR-185-5p, miR-4433b-3p , miR-199a-5p, miR-451a, miR-151a-3p) detektiert.

Wir konnten mehrere vielversprechende und spannende Ergebnisse zur EV - und (aus EV präparierten) miRNA Charakterisierung erarbeiten. In den laufenden Projekten werden wir diese methodischen Strategien anwenden, um neue Ergebnisse aus Kulturüberständen zu generieren.:

Nach der Generierung von DC/DCleus mit Kit M (GM-CSF und PGE1) aus leukämischem Vollblut konnten wir zeigen, dass nach T-Zell-angereicherter MLC mit DC/DCleu-haltigen Proben leukämiespezifische/ anti-leukämischen T-zellen entstanden. Dies führte zu einer verbesserten antileukämischen Immunantwort. EVs wurden aus DC- und MLC-Kulturüberständen von AML-Patienten und gesunden Probanden isoliert. In unserer aktuellen Studie werden wir EV-Zusammensetzungen vor vs nach DC/MLC- Kultur mit oder ohne Kit M-Einfluss in den -Kulturüberständen weiter charakterisieren und vergleichen. Wir möchten herausfinden, ob EVs während der DC- oder MLC-Kultur Modulationen durchlaufen könnten.

Aus dieser Proof-of-Concept-Studie mit Serum- oder Kulturüberständen lieferte MBFCM einen intensiveren Einblick in die Expression von Oberflächenmarkern auf EVs-von Leukämiepatienten. Dies könnte ein Ausgangspunkt für zukünftige Studien sein. Darüber hinaus könnten Analysen von EVs und miRNAs aus EVs dazu beitragen neue nicht-invasive Marker zur (Sub-)Klassifizierung von Leukämien zu finden, die Vorhersagen zum Therapieansprechen oder zum Krankheitsverlauf beitragen.

4. Abstract (English)

EVs are membranous vesicles and their role in tumor or immune activation could contribute to evaluate and monitor antileukemic processes. EVs were isolated from serum of AML, ALL, CLL patients and healthy volunteers. Typical cup-shaped appearance of serum EVs was identified by TEM and EV concentrations and size distribution profiles of purified serum EVs were characterized by fNTA. EV markers (CD81, CD63, CD9) and lineage associated EV markers CD8, CD42a, CD62P and HLA-DRDPDQ were highly expressed in AML, AML, CLL and Healthy samples evaluated by MBFCM (using 37 specific markers). Our data detected correlations of serum-derived EV marker expressions (detected by MBFCM) with immune cytologically detected leukemic cells, platelet and white blood cell counts in all AML, ALL and CLL samples.

Especially, high frequencies of EVs coexpression CD81, CD63, CD9 and in addition CD8, CD41b, CD42a, CD62P, HLA-DRDPDQ and SSEA-4 (as evaluated by MBFCM) were found in AML and Healthy samples. Total RNA was isolated from EVs via small RNA sequencing for qualitative/quantitative characterization from AML and Healthy samples. Up regulated miRNAs (miR-10a-5p, miR-155-5p, miR-100-5p, miR-146b-5p, let-7a-5p) and down regulated miRNAs (miR-185-5p, miR-4433b-3p, miR-199a-5p, miR-451a, miR-151a-3p) derived from EVs in AML vs healthy samples were found.

We were able to work out several promising and exciting results in the field of EVs/EV derived miRNA characterization. In the ongoing project we will apply these methodological strategies to deduce more interesting results using culture supernatants:

After generation of DC/DC_{leu} with Kit M (GM-CSF and PGE_1) from leukemic WB, DC and DC_{leu} containing WB lead to specifically stimulated anti-leukemic (T)-cells after T-cell enriched MLC, giving rise to improved anti-leukemic immune response. EVs were isolated from DC and MLC culture supernatants of AML patients and healthy volunteers. In our ongoing study, we will precede and further characterize and compare EV compositions before or after DC/MLC culture supernatants with or without Kit M. We want to evaluate or identify, whether EVs could undergo modulations during DC or MLC culture.

From this proof-of-concept study using serum or culture supernatants, MBFCM provided more insight into general expression of EV surface markers from leukemia patients and could support some starting points for future studies. Moreover, analyses of EVs and EV derived miRNAs, as novel non-invasive markers, could contribute to (sub)classify leukemia and to predict outcome and response to treatment for leukemia.

5. Paper I

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Description and optimization of a multiplex bead-based flow cytometry method (MBFCM) to characterize extracellular vesicles in serum samples from patients with hematological malignancies

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Extracellular Vesicles (EVs) are membranous vesicles produced by all cells under physiological and pathological conditions. In hematological malignancies, tumor-derived EVs might reprogram the bone marrow environment, suppress antileukemic immunity, mediate drug resistance and interfere with immunotherapies. EVs collected from the serum of leukemic samples might correlate with disease stage, drug-/immunological resistance, or might correlate with antileukemic immunity/immune response. Special EV surface protein patterns in serum have the potential as noninvasive biomarker candidates to distinguish several disease-related patterns ex vivo or in vivo. EVs were isolated from the serum of acute myeloid leukemia (AML), acute lymphoid leukemia (ALL), chronic lymphoid leukemia (CLL) patients, and healthy volunteers. EVs were characterized by transmission electron microscopy and fluorescence nanoparticle tracking analysis, and EV surface protein profiles were analyzed by multiplex bead-based flow cytometry to identify tumor- or immune system-related EVs of AML, ALL, CLL, and healthy samples. Aiming to provide proof-of-concept evidence and methodology for the potential role of serum-derived EVs as biomarkers in leukemic versus healthy samples in this study, we hope to pave the way for future detection of promising biomarkers for imminent disease progression and the identification of potential targets to be used in a therapeutic strategy.

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INTRODUCTION

Leukemia

Leukemia and lymphoma are blood malignancies that affect people of all ages and result in approximately 23,000 deaths in the United States per year [1]. Acute myeloid (AML) [2], lymphoid (ALL) [3], or chronic lymphoid leukemia (CLL) [4] are clonal diseases with uncontrolled proliferation of myeloid or lymphoid leukemic cells, that can be identified and characterized by flow cytometry. Rates of complete remission (CR), prognosis and survival depend on the grade of anemia, thrombocytopenia, white blood cell expansion and karyotypes. Risk-adapted therapies for AML, ALL, and CLL patients consist of chemotherapy with/without stem cell transplantation (SCT), but the rate of early failures and relapses is still unsatisfying. Since relapse rates in successfully treated AML, ALL, and CLL patients are high, new therapy options are needed [5, 6].

Immune surveillance

Effective immune surveillance of patients with hematologic malignancies such as leukemia is mediated by cellular and noncellular arms of the innate and adaptive immune system. The innate immune system includes macrophages, dendritic cells (DC), and natural killer (NK) cells, which respond quickly to an immunological threat. The adaptive immune system includes T and B cells, which mediate tumor immunity by antigen-specific responses and provide long-lasting protection by effector-memory responses [6, 7]. Furthermore, other cells at the interface of the innate and the adaptive immune system (e.g cytokine-induced killer cells (CIK) or invariant natural killer T-cells (iNKT)) are important mediators in antitumor-, autoimmune-, and antimicrobial responses and tumor surveillance. Moreover, soluble key players and mediators of immune reactions trafficking through the body like hormones, (B-cell-derived) antibodies, cytokines, che-

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mokines, and several macromolecules, membranous vesicular entities, such as extracellular vesicles (EVs), circulating nucleic acids and their derivatives appear equally relevant in immunomodulatory mechanisms [6–9].

Tumor and immune monitoring

Regularly, anergy of T cells or other immune cells can be regularly reverted to anti-leukemic functionality. It is important to understand leukemia-related as well as antileukemic processes. Therefore analyzing and monitoring the involvement of different (activating or inhibitory) cells, soluble or even the smallest molecules in antileukemic processes is necessary: qualitative and quantitative flow cytometric evaluations and monitoring of leukemic- and immune-reactive cells and their subtypes in a tumor-, inflammatory or infectious immunological context (in vivo or in various cell cultures) is important and informative to evaluate and monitor inhibitory or activating (antigen specific) cell populations [6, 8, 9].

In recent years, previously unrecognized influences of physical factors such as physiological hypoxia and other molecules (e.g. soluble molecules or circulating vesicles (EVs)) have been put to the test, and their role in tumor or immune activation or monitoring of various events could contribute to further understanding of such processes [10].

Extracellular vesicles (EVs)

Extracellular vesicles (EVs) are membranous vesicles produced by all cells under physiological and pathological conditions [11]. EV mediated information transfer allows a crosstalk between cells of the hematopoietic system and interactions between hematopoietic cells and local or distant tissue cells [12]. Emerging evidence suggests that EVs play a key role in the regulation of the entire physiology, including tissue differentiation and repair, hematopoietic stem cell development, coagulation, pregnancy or immune surveillance [13–15]. Due to the heterogeneity, the small size of EVs and the lack of standardization and, in particular, a qualified method to analyze multiple parameters of single EVs, a qualitative and quantitative detection and evaluation of EVs is challenging. As of now, only a few EV surface markers have been reliably linked to specific cell sources [7, 16].

Methods to isolate and characterize EVs

EVs can be isolated by various methods, e.g. ultracentrifugation, precipitation, size exclusion chromatography, ultrafiltration, and other immunoaffinity-based binding strategies [17]. A combination of isolation methods will increase the EV purity. Isolated pure EVs can be characterized by multiple methods to prove their specific properties. For example, EV morphology and size can be assessed by transmission electron microscopy (TEM) [18]. Moreover, (fluorescence) nanoparticle tracking analysis (fNTA) [15] allows to determine particle size and concentration of vesicle preparations. EV specific (surface) protein markers (e.g. CD9, CD63, CD81) can be detected using Western blot and different flow cytometry-based methods.

While high sensitivity and high-resolution methods like Imaging Flow Cytometry (IFCM) are very promising to unravel EV heterogeneity and to quantify subsets more accurately, such methods also require further benchmarking against other methods and standardization before they can be used widely and ultimately in a clinical context [15, 19]. Here, we instead have further explored the use of multiplex bead-based flow cytometry (MBFCM) for analysis of EVs in human serum samples. MBFCM does not provide information about single EVs, however, it is a robust method to assess the overall EV surface protein signature in isolated EV samples including human body fluids such as blood serum/plasma [14, 15]. We previously have optimized this multiplex bead-based method with a focus on cell culture-derived EVs [20], and here we aim to further explore MBFCM to characterize EVs from patients suffering from various hematological malignancies and relate results to clinical data in a first proof-of-concept study.

The aim of this study was to (1) prepare EVs from standard serum samples of leukemia patients and healthy donors; (2) characterize resulting EV preparations by standard methods, i.e., TEM and fNTA; (3) evaluate the use of MBFCM for comparing the overall EV surface protein composition on EVs in minimally processed samples from leukemia patients versus healthy donors; (4) evaluate resulting data for potential correlation with patient's cellular or clinical data. (5) Proof of concept deduction of EV-associated prognostic and diagnostic classification strategy for leukemia patients.

MATERIALS AND METHODS

Patients' characteristics and diagnostics

Samples from patients with AML ($n = 4$), ALL ($n = 3$), CLL ($n = 2$), and healthy donors ($n = 4$), provided by the University Hospitals of Munich, Stuttgart, Oldenburg, and Augsburg, were collected after obtaining patients' written informed consent in accordance with the Helsinki protocol and the local Ethic Committee (Pettenkoferstr. 8a, 80336 Munich, Ludwigs-Maximilians-University Hospital in Munich; Vote-No 339-05). The mean age of AML patients was 60.75 (range: 38–81) years, of ALL patients 59 (range: 57–62) years, of CLL patients 80 (range: 76–84) years and of healthy controls 36 (range: 29–56) years (Table 1).

AML patients presented with primary (p) ($n = 2$) or secondary (s) AML ($n = 2$) disease, three patients were analyzed at first diagnosis, one at relapse. According to National Comprehensive Cancer Network (NCCN) guideline, all four AML patients were risk categorized as Adverse. All three ALL patients were classified as c-B/ALL according to the European Group of Immunophenotyping of Leukemias classification and were risk-categorized as "standard" ($n = 1$), "high" ($n = 1$) or "highest risk" ($n = 1$) based on the Study Group for Adult Acute Lymphoblastic Leukemia (GMALL). The two CLL patients were classified as p/B-CLL and risk-categorized as Binet A (Table 1). Leukemia samples contained between 18 and 82% of immune cytologically detected leukemic cells (IC leukemic cells) and decreased sequence of monocytes, T-, B-, and NK-cells compared to healthy samples (Table 2).

Preparation of serum samples

Around 10 ml serum were taken from patients with AML, ALL, CLL, and healthy donors. Cells were sedimented and serum retained by centrifugation at room temperature for 10 min at 2000x *g*. The resulting supernatants (containing EVs) were aliquoted in 0.5 ml tubes and stored at -80 °C until further processing.

Enrichment of EVs from serum samples by immunoaffinity

As recommended by MISEV2018 guidelines, EVs were characterized by TEM and fNTA [20]. For this purpose, EVs were enriched from 1.5 ml serum, respectively, by immunoaffinity applying the Exosome isolation kit pan, human (Miltenyi Biotec, Germany) as recommended by the manufacturer including a one-by-one dilution with 1x PBS prior to an additional centrifugation at 10,000x *g* for 45 min. After elution in 100 µl isolation buffer, EV preparations were vacuum evaporated to a final volume of around 20 µl, recording the exact volumes for later re-calculations.

Transmission electron microscopy (TEM)

TEM was performed to evaluate EV morphology and size, and to assess the purity of enriched EV fractions. Therefore, five µl of freshly isolated EV preparations were loaded onto formvar carbon-coated grids (Nickel Grid 200 mesh; Electron Microscopy Sciences, USA) and left there to adhere for five minutes prior to five minutes of negative staining with 2% aqueous uranyl acetate at room temperature in the dark. Surplus liquids were removed. Images were acquired of air-dried grids on the same day at 80 kV using the Zeiss EM 900 instrument (Zeiss, Germany) equipped with a wide-angle dual-speed 2KCCD camera.

Fluorescence Nanoparticle Tracking Analysis (fNTA)

Particle diameter/size distribution and concentration in resulting EV preparations were analyzed by fNTA. For discrimination between biological and non-biological particles a fluorescent membrane dye was used. Analyses were performed on a ZetaView PMX110 instrument (Particle

Table 1. Characteristics of WB-samples from healthy donors and AML, ALL, CLL-patients.

	Pat.Nr	Age/Sex	Dgn. subtypes	stage	ML LC (%)	IC LC (%)	blast phenotype (CD)	Risk Stratification	WBC [G/l]	Hb [g/dl]	PLT [G/L]	Response to (induction)-chemotherapy
AML	1562	38/M	p/nd	Dgn	12	23	**34**,**117**,13,56, HLA-DR	Adverse[1]	1.79	9.9	190	CR
	1564	68/M	p/M4	Rel.	72	70	**34**,**117**,13,33, 65,56, HLA-DR	Adverse[1]	21.7	8.8	65	NCR
	1574	56/M	s/nd	Dgn	77	60	**34**, **117**,15,19, HLA-DR	Adverse[1]	4.5	9.4	58	NCR
	1584	81/M	s/nd	Dgn	40	82	**15**,**65**,**56**,33, HLA-DR	Adverse[1]	4.92	8.9	77	NCR
ALL	1587	58/M	c/B-ALL	Dgn	34	40	**34**,**19**,15,10,56, HLA-DR	High[2]	4.83	8.6	478	NCR
	1588	62/M	c/B-ALL	Dgn	1	18	**34**,**19**,56,20, HLA-DR	Standard[2]	1.74	11.9	186	NCR
	1605	57/F	c/B-ALL	Dgn	80	78	**34**,**19**,20,22,10,56, HLA-DR	Highest[2]	133	12.1	76	NCR
CLL	1589	84/M	p/B-CLL	Dgn	30	71	**5**,**19**,20,15,23,56, HLA-DR	A[3]	10.15	15.3	142	nd
	1591	76/F	p/B-CLL	Dgn	52	30	**5**,**19**,15,20, HLA-DR	A[3]	23.46	13.5	203	nd
H	1561	30/F	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	1566	29/F	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	1576	56/M	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	1582	29/M	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

AML acute myeloid leukemia; ALL acute lymphoid leukemia; CLL chronic lymphoid leukemia; H healthy donors; Pat. Nr. Patient's number; F female; M male; p primary; s secondary; c: common; CD Cluster of differentiation; dgn first diagnosis; rel relapse; CR Complete remission; NCR no complete remission; pers. persisting disease; PLT platelets; WBC white blood cells; IC LC immune cytologically detected leukemic cells; ML LC morphologically detected leukemic cells in peripheral blood; nd no data.

[1]AML patients were prognostically classified based on the National Comprehensive Cancer Network (NCCN) guidelines as "favorable", "intermediate" or "adverse risk".

[2]Risk stratification for adult ALL was based on the Study Group for Adult Acute Lymphoblastic Leukemia (GMALL) as "standard", "high" or "highest risk".

[3]According to Binet-classification, CLL patients were classified by Binet A, Binet B, Binet C. Bold blast markers were used for (co)expression analyses.

Table 2. Cellular composition of AML, ALL, CLL, and healthy samples.

	Pat.Nr	CD14+ expressing cell	CD19+ expressing cell	CD3+ expressing cell	CD56+ expressing cell	CD56+/CD3- expressing cell
AML	P1562	0.12	2.88	64.96	9.52*	2.96*
	P1564	0.25	3.17	10.27	5.24*	3.34*
	P1574	3.87	39.5*	11.93	1.02	0.32
	P1584	8.15	0.77	8.55	26.9*	17.35*
ALL	P1587	9.53	35.04*	32.87	31.72*	21.17*
	P1588	1.43	14*	32.73	5.08*	3.82*
	P1605	5.75	68*	10.78	8.23*	5.52*
CLL	P1589	5.03	71*	11.38	9.4*	4.42*
	P1591	1.08	45.92*	11.9	1.78	1.77
H	1561	5.46	2.39	20.75	9.92	6.67
	1566	3.15	0.74	11.83	4.67	3.16
	1576	9.54	2.14	8.96	12.28	4.78
	1582	1.59	0.65	nd	5.16	2.83

* (aberrant) expression of these markers on leukemic cells.

Metrix, Germany), and the corresponding software version 8.05.12 SP1 was used as described before [21]. In brief, EV preparations were stained with 5 µg/ml CellMask Orange Plasma Membrane Stain (Invitrogen, USA) and incubated for 30 minutes at 37 °C prior to appropriate sample dilution in 1x PBS. After instrument calibration, the temperature was clamped at 23 °C, and the pre-acquisition parameters to measure in the fluorescence mode were set to a shutter of 70, frame rate of 30 and a sensitivity of 95 % at high resolution. Post-acquisition parameters were as follows: minimum brightness of 25, size range of 5–1000 nm and a trace length of 15. Two cycles of measurement at eleven positions were conducted. To obtain EV concentrations of initial serum samples, a recalculation according to Eitan et al. [22] was applied accounting for sample dilution and EV sample and serum volume. Additionally, thereby obtained EV concentrations were normalized to the number of one million white blood cells.

Multiplex Bead-Based Flow Cytometry (MBFCM)

Serum samples were subjected to multiplex bead-based EV flow cytometry analysis (MBFCM; MACSPlex Exosome Kit, human, Miltenyi Biotec) as described previously [15]. In brief, EV-containing serum samples were thawed and (without further purification) subjected to centrifugation at 2,500 x g for 15 minutes before supernatants were processed as follows: Unless indicated otherwise, 30 µL of sample was diluted 1:1 with MACSPlex buffer (MPB) to a total volume of 60 µL and loaded onto wells of a pre-wet and drained MACSPlex 96-well 0.22 µm filter plate before 8 µL of MACSPlex Exosome Capture Beads (containing 39 different antibody-coated bead subsets) were added to each well and counterstained with APC-labelled pan-tetraspanin antibodies (CD9, CD63, CD81) as described previously. Unless mentioned otherwise in the results section data was analyzed as described before [15].

Statistical analysis

Data is presented as mean ± standard deviation unless otherwise stated. For the comparison of two groups a paired t-test was used, more than two groups were analyzed applying one-way analysis of variance (ANOVA) with Benjamini-Hochberg adjustments for multiple comparisons [23]. Differences were considered as 'not significant' in cases with p-values > 0.1, as 'borderline significant' (#) with p-values between 0.05 and 0.1, as 'significant' (*) with p values between 0.01 and 0.05, as 'highly significant' (**) with p values between 0.001 and 0.01, as 'very highly significant' (***) with p values between 0.0001 and 0.001 and as 'extremely significant' (****) with p-values < 0.0001. Pairwise Pearson-correlation with t-test was used for correlation analyses. Statistical analyses and creation of diagrams were performed using Microsoft Excel 2016, GraphPad Prism, version 8.4.0 and 8.4.3 and R programming language, version 4.1.0.

RESULTS

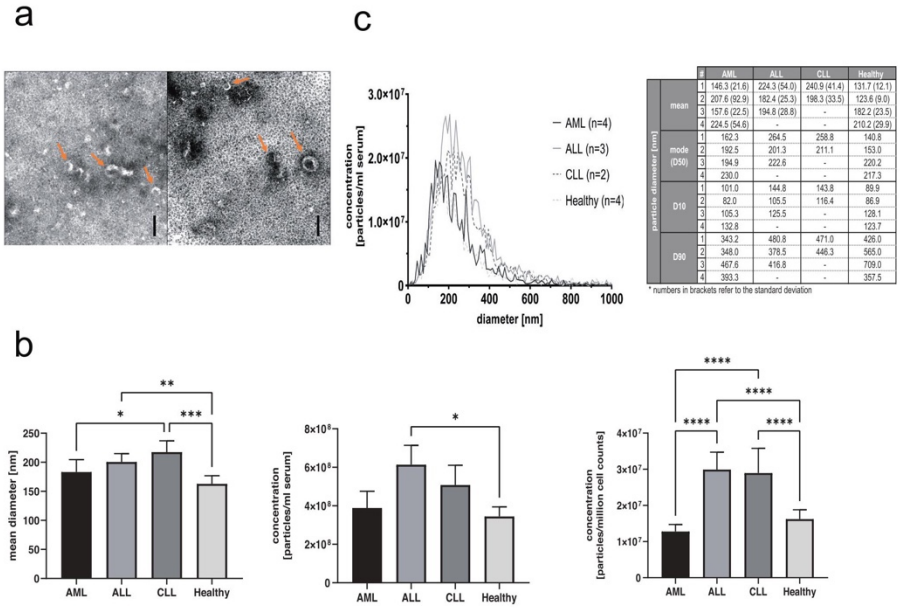
In this project, TEM, fNTA-based and MBFCM EV detection technologies were used to characterize and quantify EVs in serum of leukemia and healthy samples, aiming to establish a proof-of-concept workflow to evaluate EVs as potential diagnostic/prognostic or predictive markers for clinical entities and to identify EV protein markers potentially predicting immune reactions in the serum leukemic compared to healthy samples. Enriched EVs derived from leukemia patients and healthy donors were first characterized by TEM and fNTA, and in a next step MBFCM was adapted to analyze EV surface protein compositions in minimally processed serum samples derived from leukemia patients versus healthy donors.

Characterization of serum-derived EVs in leukemic and healthy control samples

First, we performed TEM imaging, as recommended by the MISEV2018 guidelines, to characterize morphology of freshly prepared serum EVs and confirm successful enrichment thereof by an immunoaffinity-based strategy. Indeed, we could ascertain a typical cup-shaped appearance of serum EVs with heterogeneous desiccated diameters around 100 nm in both AML and healthy samples. No differences between EVs derived from patients versus healthy donors could be detected (Fig. 1a).

Next, we performed fNTA to quantify EV concentrations (particles/ml serum and particles/million cell counts) and assess size distribution profiles of purified EV samples from the peripheral blood of healthy donors and EVs derived from AML, ALL, and CLL patients (Fig. 1b, c). We utilized a protocol we previously optimized [21] and analyzed EVs derived from four AML-patients, three ALL-patients, two CLL-patients and four healthy donors. While conventional NTA only allows detection of total particles including non-EV particles, we here applied a protocol based on utilizing a fluorescent membrane dye to stain and quantify concentrations of stained EVs and not non-EV particles which was previously optimized by Mussack et al. [21]. Overall, mean diameters of particles ranged between 163 and 218 nm, as typically observed in EVs obtained after preparation by immunomagnetic separation. EVs obtained from healthy serum appeared with a (very) highly significantly lower diameter of 163 (95% CI: 149–177) nm compared to EVs derived from ALL- or CLL-serum with 201 (95% CI: 186–215) nm and 218 (95% CI: 198–237) nm, respectively (Fig. 1b). The diameter of CLL-derived EVs was even significantly larger compared to AML-derived EVs representing a mean diameter of 183 (95% CI: 162–205) nm (Fig. 1b).

In total, fNTA revealed average concentrations of 1.63 (95% CI: 1.37–1.88) x 10^7 particles/million cell counts for healthy controls,



	#	AML	ALL	CLL	Healthy
particle diameter [nm] mean	1	146.3 (21.6)	224.3 (54.0)	240.9 (41.4)	131.7 (12.1)
	2	207.6 (92.9)	182.4 (25.3)	198.3 (33.5)	123.6 (9.0)
	3	157.6 (22.5)	194.8 (28.8)	-	182.2 (23.5)
	4	224.5 (54.6)	-	-	210.2 (29.9)
mode (D50)	1	162.3	264.5	258.8	140.8
	2	192.5	201.3	211.1	153.0
	3	194.9	222.6	-	220.2
	4	230.0	-	-	217.3
D10	1	101.0	144.8	143.8	89.9
	2	82.0	105.5	116.4	86.9
	3	105.3	125.5	-	128.1
	4	132.8	-	-	123.7
D90	1	343.2	480.8	471.0	426.0
	2	348.0	378.5	446.3	565.0
	3	467.6	416.8	-	709.0
	4	393.3	-	-	357.5

* numbers in brackets refer to the standard deviation

Fig. 1 Identification and quantification of EVs purified from serum samples using TEM and fNTA. EVs were prepared by immunoaffinity enrichment applying Exosome isolation kit pan prior to an additional centrifugation. **a** TEM identifies EVs with typical cup-shaped appearance in healthy (left side) and AML (right side) serum samples. Scale bars are the same for all images and represent 100 nm. Arrows exemplarily highlight vesicular structures. **b** fNTA enables size and concentration analyses of EVs. Results of mean diameter (nm) and concentration measurements (particles/ml serum and particles/million cell counts) of purified EVs from AML ($n = 4$), ALL ($n = 3$), CLL ($n = 2$) and Healthy ($n = 4$) samples are given. Detected particle concentrations were corrected for sample dilution and normalized to serum cell counts. Data is given as mean +/− 95% CI of values. c Size distributions of EVs measured by fNTA are given in histograms and tables. #Orders with increasing patient numbers are given. Statistical analysis was done with GraphPad Prism, version 8.4.3, by one-way analysis of variance (ANOVA) with Benjamini-Hochberg adjustments for multiple comparisons. Differences were considered as 'significant' (*) with adjusted p-values between 0.01 and 0.05, as 'highly significant' (**) with adjusted p values between 0.001 and 0.01, as 'very highly significant' (***) with adjusted p-values between 0.0001 and 0.001 and as 'extremely significant' (****) with adjusted p values < 0.0001.

1.28 (95% CI: 1.09–1.47) x 10^7 particles/million cell counts for AML patients, 2.99 (95% CI: 2.51–3.47) x 10^7 particles/million cell counts for ALL patients, and 2.89 (95% CI: 2.21–3.58) x 10^7 particles/million cell counts for CLL patients (Fig. 1b). The obtained normalized EV concentrations of ALL and CLL samples were extremely significantly higher compared to normalized EV concentrations of healthy and AML samples. Size distributions of EVs from healthy and leukemic sera appeared comparably with one peaking area around 200 nm (Fig. 1c).

Robust characterization of EV surface protein signatures with MBFCM

Next, we performed MBFCM analyses to compare the EV surface protein expression on healthy donor versus leukemia patient-derived EVs. We previously have optimized an MBFCM-based assay for analysis of cell culture-derived EV and demonstrated that this assay also facilitates the detection of EV surface markers in different biological fluid samples [15]. Of note, we also showed that freeze thaw cycles do not affect detected EV surface marker profiles notably.

The MBFCM assay used here is based on the co-detection of two EV surface markers: One marker based on specificity of one of 37 capture beads coated with specific capture antibodies included in the assay, and the other marker based on the fluorescence-labelled detection antibody added, here a mixture of pan anti-tetraspanin (CD9, CD63, CD81) antibodies aiming to detect all tetraspanin-positive EVs bound to the respective capture bead [14]. The assay principle ensures that only EVs and not free proteins are detected, thereby facilitating specific detection of EVs without further purification, in both cell culture supernatants and biological fluids [15]. Detected signal quantities directly correlate with the abundance of respective surface proteins in EV samples. Analysis of serum samples with this MBFCM assay can lead to background or unspecific signals if EVs are not further purified, e.g. by size exclusion chromatography [15], which is why we previously have purified EVs before analysis, and normalized the assay input between different donors based on measured NTA particle concentrations. Since this doesn't allow direct comparison of the abundance of respective EV surface proteins between donors and potentially can introduce a bias from purification steps, we here aimed to directly measure unprocessed serum samples instead.

In a first step, we therefore measured blood serum samples at different input doses by MBFCM to establish a simple yet robust assay workflow suitable for relating data directly to abundance per blood volume without further sample processing. Capture beads were identified as described previously [15] (Fig. 2a). We chose serum input amounts of 3, 10, 30, and 60 µL in a total volume

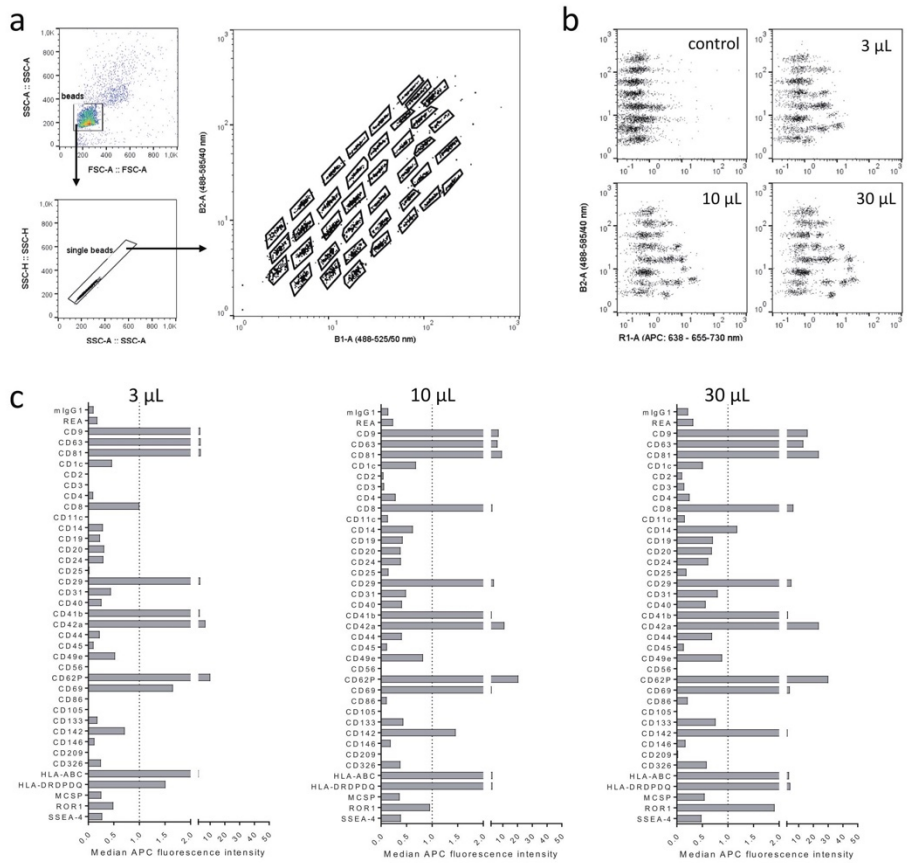


Fig. 2 Establishment of a robust workflow to directly quantify EV surface protein expression in serumsamples by MBFCM. a Gating strategy applied to identify capture bead populations. **b** Examples for signals detected when using different seruminput volumes for the MBFCM assay. Control indicates procedural control without EVs but stained with pan-tetraspanin detection antibodies, volumes given indicate seruminput volumes. Allsamples were diluted in MACSPlex buffer to a final volume of 60 µL during the initial capture step. **c** Example data showing detected background-subtracted signal intensities for each marker at different seruminput volumes.

of 60 µL during the capture step (Fig. 2b). During processing, samples with 60 µL input regularly clogged the filter plates used which resulted in low bead counts and highly variable data (not shown). 3–30 µL input resulted in low background based on internal mIgG and REA isotype control signals, with highest signals detected for positive markers at 30 µL assay input (Fig. 2c). Based on these results, we decided to use 30 µL serum as assay input for pre-cleared and otherwise unprocessed serum samples throughout this study.

MBFCM measurement of EV surface protein profiles on leukemia patient and healthy donor-derived serum samples

In this study we included samples from patients diagnosed with AML ($n = 3$), ALL ($n = 3$), CLL ($n = 2$) and Healthy donor-derived samples ($n = 4$; Tables 1, 2) and analyzed the EV surface protein profile by MBFCM. This assay comprises 39 hard-dyed capture bead populations (4 µm diameter), each of them coated with different monoclonal antibodies against 37 potential EV surface antigens or two internal isotype negative controls (details summarized in Table 3 [24–27]). Surface proteins included in the MBFCM assay comprise the tetraspanins CD9, CD63, and CD81, and other surface proteins such as various leukocyte, T cell (CD4, CD8), B cell (CD19, CD20, CD24), monocyte (CD14), thrombocyte (CD41b, CD42a, CD62Pa, CD69), integrin (CD11c (integrin αX or CR4), CD29 (integrin β1), CD41b (integrin αIIβ), CD49e (integrin α5)), endothelial (CD31, CD105, CD146 (Mel-CAM)), or MHC-associated (HLA-ABC (MHC-I), HLA-DRDPDQ (MHC-II)) associated antigens. MBFCM results obtained are given in Fig. 3. The commonly used EV markers CD9, CD63, and CD81 were detected on EVs in all measured samples, as expected (Fig. 3a). In addition, lineage-associated markers (e.g. CD8, CD42a, CD62P and HLA-DRDPDQ) were found on EVs in high expression in all sample

Table 3. List of antibodies used as capture antibodies bound to the polystyrene particles in the multiplex platform [24–27].

Capture antibody	Clone	Target
mIgG1	IS5-21F5	Isotype control
REA	REA293	Isotype control
CD9	SN4	Extracellular Vesicle marker
CD63	H5C6	Extracellular Vesicle marker
CD81	5A6	Extracellular Vesicle marker
CD1c	AD5-8E7	BDCA-1, major subpopulation of human myeloid dendritic cells
CD2	LT2.2	T cells, subset of NK cells
CD3	BW 264/56	mature human T cells, thymocytes, subset of NK cells
CD4	Vit-4.3	T helper cells, thymocytes, monocytes, dendritic cells
CD8	BW 135/80	cytotoxic T cells, thymocytes, subset of NK cells
CD11c	MJ4-27G12	integrin αX or CR4, monocytes, macrophages, NK cells, granulocytes, myeloid dendritic cells (MDCs), subsets of T and B cells
CD14	TÜK4	monocytes and macrophages, subset of neutrophils and myeloid dendritic cells
CD19	LT-19	B cells
CD20	LT20.34	B lineage cells from the pre-B cell stage to the B cell lymphoblast stage
CD24	32D12	heat-stable antigen (HSA)
CD25	3G10	activated T and B cells, macrophages, subset of non-activated CD4+ regulatory T cells
CD29	TS2/16.2.1	integrin beta 1
CD31	AC128	monocytes, platelets, and granulocytes
CD40	HB14	B cells, macrophages, dendritic cells, endothelial cells, fibroblasts, plasma cells, subset of peripheral T cells
CD41b	REA336	β chain of Integrin α-IIb, megakaryocytes, platelets
CD42a	REA209	Platelets, megakaryocytes
CD44	DB105	Cancer stem cells (CSC), hematopoietic, fibroblastic, and glial cells.
CD45	5B1	leukocyte common antigen
CD49e	NKI-SAM1	integrin α5 chain, lymphocytes, monocytes, fibroblasts, endothelial cells
CD56	REA196	neural cell adhesion molecule (NCAM), resting and activated NK cells, minor subset of CD3+ T cells
CD62P	REA389	P-selectin, vascular endothelial cells and platelets
CD69	FN50	Activated lymphocytes, monocytes, and platelets
CD86	FM95	B7-2, activated B and T cells, dendritic cells, and monocytes/macrophages
CD105	43A4E1.71	mature endothelial cells, some leukemic cells of B lymphoid and myeloid origin
CD133	AC133.1.6. 2.1.1	multipotent progenitor cells, including immature hematopoietic stem and progenitor cells, circulating endothelial progenitor cells, fetal neural stem cells, other tissue-specific stem cells, cancer stem cells
CD142	HTF-1	Tissue factor, activated endothelial cells, monocytes, macrophages, platelets, and some tumor cell types
CD146	541-10B2	MUC18, MCAM, Mel-CAM, endothelial cells, pericytes, smooth muscle cells, follicular dendritic cells, melanoma cells, sub-population of activated T lymphocytes, marrow stromal cells (MSCs)
CD209	DCN-47.5.4	DC-SIGN, dendritic cells, endothelial cells, macrophages, spleen
CD326	HEA125	EpCAM, basolateral surface of carcinoma and epithelial cells in tissues, circulating tumor cells, cancer stem cells, not on melanoma, neuroblastoma, sarcoma, lymphoma, leukemia cells, or normal fibroblasts
HLA-ABC	REA230	Nuclear cells
HLADP/DQ/D R	REA332	Antigen-presenting cells
MCSP	EP-1	melanoma-associated chondroitin sulfate proteoglycan antigen, melanoma tissues and melanoma cell lines but not carcinoma cells, fibroblastoid cells, and cells of hematopoietic origin
ROR1	2A2	receptor tyrosine kinase-like orphan receptor 1, chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (McLellan), ovarian cancer, renal cancer, melanoma, and lung adenocarcinoma, adipose tissue, at early stages of B cell development
SSEA-4		Stage-specific embryonic antigen 4, undifferentiated human embryonic stem (ES) cells, induced pluripotent (iPS) cells embryonal carcinoma (EC) cells, and embryonic germ (EG) cells, somatic stem cells

entities, whereas the markers CD2, CD3, CD25, CD56, CD142, and CD209 showed low expression on EVs as given in the heatmap analysis. A more detailed presentation of results of single cases (Fig. 3b–e) showed that the thrombocyte or myeloid blast cell-associated markers (CD42a, CD62P, and CD133) were highly expressed on AML-sample-derived EVs (Fig. 3b), whereas T cell (CD8), thrombocyte (CD42a, CD62P) or MHC associated (HLA-DRDPDQ) markers were highly expressed on lymphoid leukemia-sample-derived EVs (Fig. 3c, d).

On a first glance EVs from healthy samples also showed a high expression of CD8, CD19, CD29, CD41b, CD42a, CD62P, CD69 and ROR1 markers (Fig. 3e). This means that coexpression of several

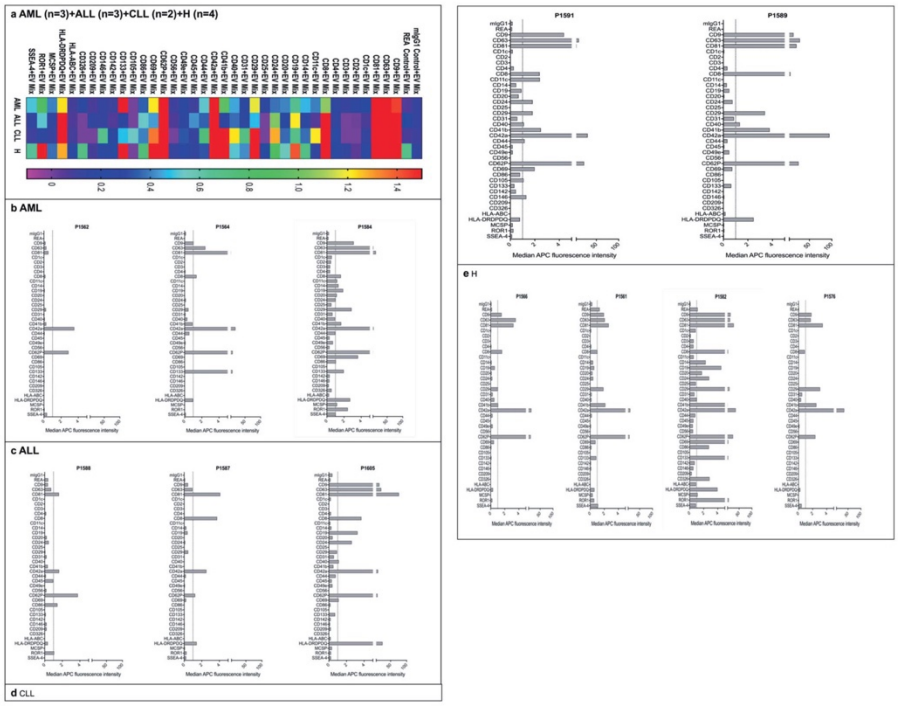


Fig. 3 Identification of serum-derived EVs from AML, ALL, CLL and healthy samples by MBFCM. MBFCM allows the detection of EVs (co-) expressing 37 different antigens in a semi-quantitative manner. Results (median fluorescence intensity) obtained with AML ($n = 3$), ALL ($n = 3$), CLL ($n = 2$) and Healthy (H) ($n = 4$) samples are given in a heatmap analysis (Fig. 3 **a**). Data from single cases referring to individual P-numbers are given (Fig. 3 **b**–**e**). Median APC fluorescence intensities are displayed resulting from MACSPlex analysis of EVs isolated from serum of all samples.

lineage-associated markers can be demonstrated in varying expressions in individual samples from leukemic and healthy sample donors.

Differential serum-derived EV surface marker detection from leukemic and healthy samples as sorted by surface markers

When sorting our results according to expressions of CD markers on EVs in different leukemic compared to healthy samples, we found remarkable differences (Fig. 4). Myeloid leukemic marker CD133 was detected with highest signal intensities in serum-derived EVs from AML compared to ALL, CLL, and Healthy samples. We further observed strong variations in signal intensities for CD209, which was not detected in CLL samples, and HLA-DRDPDQ, which showed the highest detection signals on serum-derived EVs from ALL samples. Of note, the expression of CD8, CD11c, CD31, CD40, CD41b, CD42a, and CD62P was detected at high signal intensities especially on serum-derived EVs from CLL samples. We observed a complete lack of signal detection for CD56 and CD209 on serum-derived EVs from CLL samples, while there was a clear signal on serum-derived EVs from AML, ALL, and Healthy samples (Fig. 4).

Significantly lower (*) signal intensities of CD42a positive serum-derived EVs were found in AML and ALL compared to CLL samples. Borderline significantly lower ($^{\#}$) signal intensities of CD62P positive serum-derived EVs were found in AML compared to CLL samples, while significantly lower (*) signal intensities of CD62P positive serum-derived EVs were found in ALL compared to CLL samples. Borderline significantly lower ($^{\#}$) signal intensities of CD41b positive serum-derived EVs were found in ALL compared to CLL and Healthy samples (Fig. 4).

Differential EV-marker detection in leukemic or healthy samples normalized to several subgroups

In order to evaluate differences between leukemic and healthy samples in more detail, we normalized data of leukemic samples to healthy samples (a), to leukemic cell counts (b) and to WBC counts (c).

Differential EV-marker detection in leukemic samples normalized to healthy samples. To detect and describe the differences between leukemic and healthy EV profiles in more detail we normalized results of leukemic samples to results obtained from healthy samples. As given in Fig. 5a we found higher fold-changes (> 1.5) of CD11c, CD44, CD133, and lower fold-changes (> 1) of CD49e and MCSP positive serum-derived EVs in AML than in healthy samples. We found higher fold-changes of CD81, CD45, HLA-DRDPDQ positive serum-derived EVs in ALL than in healthy samples. We found higher fold-changes of CD63, CD8, CD11c,

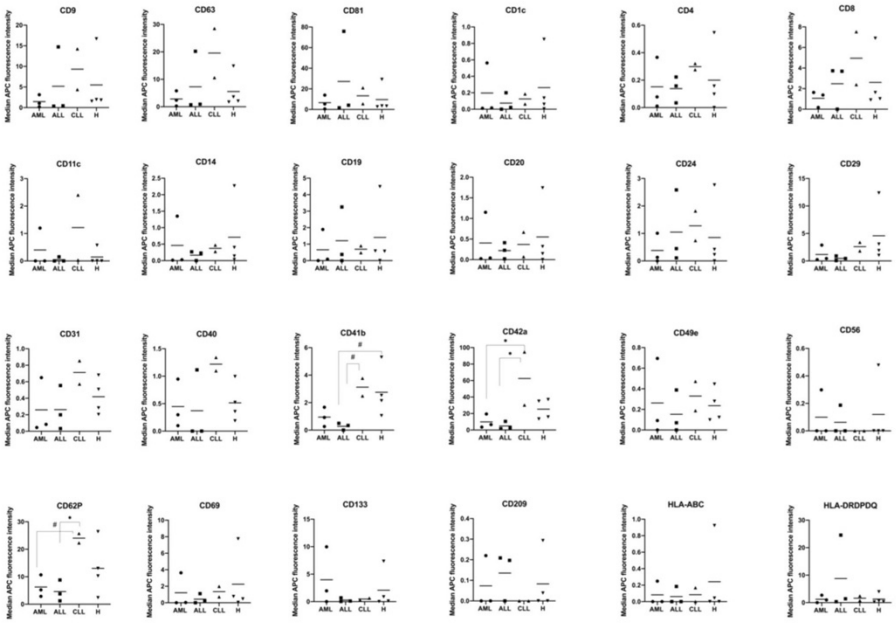


Fig. 4 Quantification and comparison of EV surface marker expression in leukemic and healthy serum samples with MBFCM. Serum samples were analyzed by MBFCM. Differences in marker expressions on serum-derived EVs from AML ($n = 3$), ALL ($n = 3$), CLL ($n = 2$) and Healthy samples ($n = 4$) are shown in mean values. For statistical comparison of more than two groups one-way analysis of variance (ANOVA) with Benjamini-Hochberg adjustments for multiple comparisons was applied. Differences were considered as 'borderline significant' (#) with p-values between 0.05 and 0.1 and as 'significant' (*) with p-values between 0.01 and 0.05.

CD40, CD42a, CD44, CD62P, and CD146 positive serum-derived EVs in CLL compared to healthy samples (Fig. 5a).

In summary, these data show that serum-derived EVs from AML, ALL, or CLL can be differentiated from healthy serum-derived EVs. Especially, AML derived EVs (positive for CD11c and CD133), ALL derived EVs (positive for CD45, HLA-DRDPDQ) and CLL derived EVs (positive for CD11c and CD146) were found in higher fold-changes compared to EVs derived from healthy samples.

Differential EV-marker detection in leukemic samples normalized to IC leukemic cells. In addition, we normalized results obtained from leukemia samples to IC leukemic cell proportions. As given in Fig. 5b, IC leukemic cell normalized signal intensities were higher for CD133 in AML than ALL and CLL. We found significantly lower signal intensities of CD81 (****) positive serum-derived EVs comparing AML with ALL derived EVs and significantly lower signal intensities of CD9 (*), of CD63 (****), of CD81 (*), of CD42a (****), of CD62P (****) positive serum-derived EVs comparing AML with CLL derived EVs with results normalized to IC leukemic cell counts.

We found significantly lower (****) signal intensities of CD63, CD42a, CD62P positive serum-derived EVs, but significantly higher (*) signal intensities of CD81 positive serum-derived EVs comparing ALL with CLL derived EVs with results normalized to IC leukemic cell counts (Fig. 5b).

Differential EV-marker detection in leukemic samples normalized to WBC counts. To detect and describe differences in more detail, we normalized MBFCM results of leukemic samples to WBC counts. As given in Fig. 5c, we found high signal intensities (positivity) of CD29, CD69, and CD133 on serum-derived EVs in AML when normalized to WBC counts.

We found high signal intensities (positivity) of CD86 and HLA-DRDPDQ on serum-derived EVs in ALL and high signal intensities of CD8, CD42a on serum-derived EVs in CLL when normalized to WBC counts. We found significantly lower signal intensities of CD63 (*), of CD42a (****) positive serum-derived EVs comparing AML with CLL derived EVs and significantly lower signal intensities of CD63 (*), of CD42a (****) positive serum-derived EVs comparing ALL with CLL derived EVs when normalized to WBC counts (Fig. 5c).

Comparisons and correlation analyses of serum-derived EV marker expression by MBFCM and cellular marker expression by flow cytometry in leukemic and healthy samples

Possible relationships between EV marker expressions (as detected by MBFCM) and cellular marker expressions (as detected by cellular flow cytometry) in leukemic and healthy samples were assessed.

Correlation analyses of serum-derived EV and cellular marker expressions (monocytes, B cells, T cells) in leukemic and healthy samples. Since leukemic cells in none of the AML-cases were positive for CD14, we compared CD14 marker expressions on EVs from all pooled AML, ALL and healthy samples. We found a significant positive correlation between serum-derived EV and cellular positive CD14 marker expressions in pooled AML and ALL samples ($r = 0.63$, $p = 0.04$, $n = 6$), while a significant negative correlation of this marker was seen in healthy samples ($r = -0.68$,

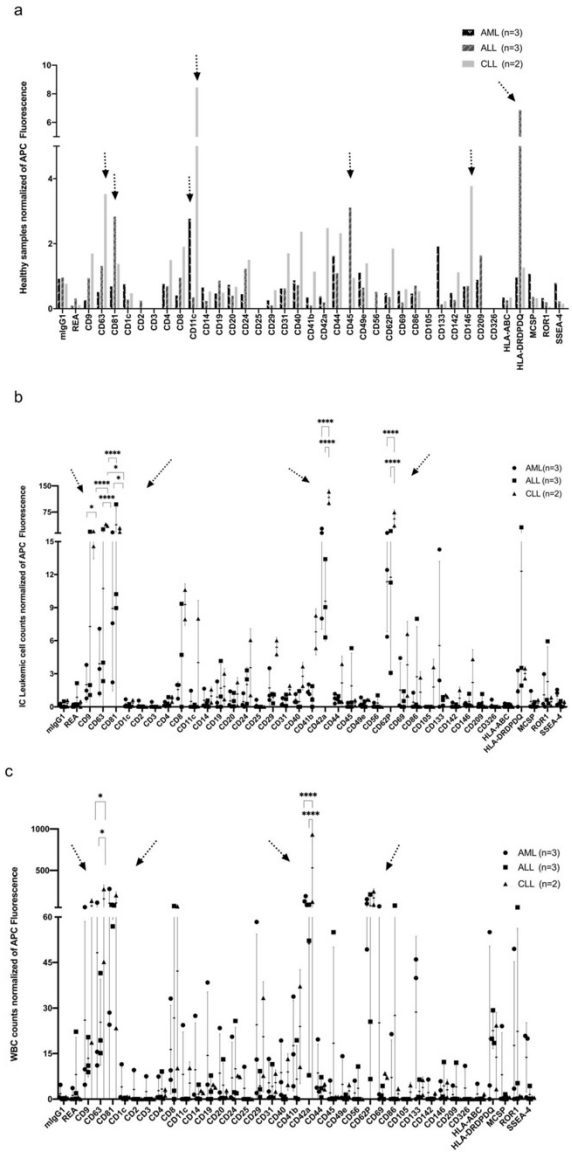


Fig. 5 Identification and comparison of serum-derived EVs from leukemic after normalization to healthy data (a) **IC leukemic cell counts** (b) **or WBC** (c)**.** MBFCM allows a semi-quantification and comparison of results in different diagnostic entities based on their (differential) expression profile of 37 different antigens. Results (median fluorescence intensities) normalized to healthy data (**a**), IC leukemic cell counts (**b**) or WBC-counts (**c**) are given. **a** Presentation of EV data normalized to healthy samples; **b** Presentation of EV data normalized to IC leukemic cell proportions; c Presentation of EV data normalized to WBC. **b**, **c** are given mean data with SD in individual dot plots. Fold changes (FC) of APC fluorescence of different marker expressions are given. Arrows point to the most abundant findings. For statistical comparison of more than two groups were analyzed applying one-way analysis of variance (ANOVA) with Benjamini-Hochberg adjustments for multiple comparisons. Differences were considered as 'significant' (*) with p-values between 0.01 and 0.05 and as 'extremely significant' (****) with p-values < 0.0001.

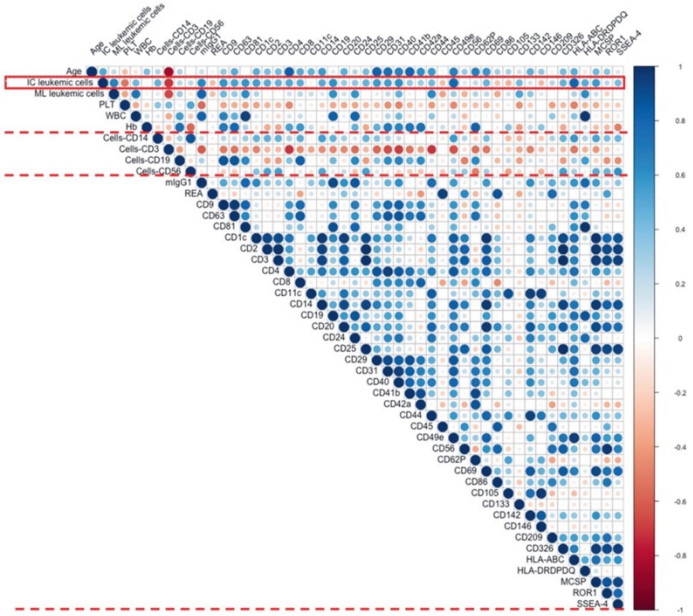


Fig. 6 Correlation analyses of serum-derived EV markers (determined by MBFCM) with IC leukemic cells, cellular platelets (PLT) and white blood cells (WBC) in all leukemic samples. Correlation analyses of serum-derived EV marker expression (detected by MBFCM) with IC leukemic cells, cellular PLT and WBC in all AML, ALL, and CLL samples. Immune cytologically detected leukemic cells (IC leukemic cells), platelet counts (PLT) and white blood cells (WBC) are evaluated and given in Table 1. Especially correlation analyses of IC leukemic cells and serum-derived leukemic EV markers (CD19, CD133, CD24, CD44, CD142, MCSP, ROR1, SSEA-4), Integrin-related EV markers (CD11c, CD29, CD41b, CD49e), MHC-related EV-markers (HLA-ABC and HLA-DRDPDQ), platelet-related EV-markers (CD42a, CD62P, and CD69) and endothelial-related EV-markers (CD31and CD146), cellular PLT, WBC and serum-derived EV markers (CD41b, CD42a, CD62P, CD69, and CD40) were also supported. Blue color shows positive correlations (positive correlation coefficient r), red color shows negative correlations (negative correlation coefficient r). Statistical analyses and creation of diagrams were performed using Microsoft Excel 2016 and R programming language. H: healthy; r: Pearson's correlation coefficient; n: numbers.

$p = 0.05$, $n = 4$). Moreover, we found a high negative correlation between serum-derived EV and cellular CD19 marker expressions in pooled AML and Healthy samples ($r = -0.70$, $p = 0.34$, $n = 7$). While a significant positive correlation between serum-derived EV and cellular CD19 marker expression was found in pooled ALL and CLL samples ($r = 0.61$, $p = 0.0026$, $n = 5$). Actually, we found a very weak negative correlation between the serum-derived EV and cellular CD3 marker expressions in pooled leukemic and healthy samples (CD3: $r = -0.23$, $p = 0.0008$, $n = 11$), and no correlation between the serum-derived EV and cellular CD56 marker expression in all leukemic and healthy samples was seen (CD56: $r = 0.06$, $p = 0.0005$, $n = 12$) (Supplementary Fig. 1).

Correlation analyses of serum-derived EV marker expressions with IC leukemic cells. We correlated leukemic cell counts (as evaluated by flow cytometry as 'IC leukemic cells' in leukemic samples) with leukemic cell-lineage-associated CD marker expressions on EVs (details are given in Tables 1, 2).

There was a positive correlation between results obtained from serum-derived EV markers and results obtained from AML, ALL and CLL samples' IC leukemic cells.

Moreover, we found a positive correlation between serum-derived EV CD24 (GPI-anchored protein) ($r = 0.36$, $p = 0.3$, $n = 8$), CD44 ($r = 0.43$, $p = 0.2$, $n = 8$), CD133 ($r = 0.42$, $p = 0.2$, $n = 8$), CD142 ($r = 0.16$, $p = 0.6$, $n = 8$), MCSP ($r = 0.56$, $p = 0.1$, $n = 8$), ROR1 ($r = 0.23$, $p = 0.5$, $n = 8$), SSEA-4 ($r = 0.42$, $p = 0.3$, $n = 8$) and IC leukemic cells in all pooled leukemic samples. A significant positive correlation between serum-derived EV leukemic cell marker CD19 ($r = 0.68$, $p = 0.004$, $n = 5$) and IC leukemic cells in ALL and CLL samples also was seen (Fig. 6).

A significant positive correlation was found between serum-derived EV CD29 (integrin β1) ($r = 0.56$, $p = 0.1$, $n = 8$), CD41b (integrin αIIβ) ($r = 0.34$, $p = 0.4$, $n = 8$), CD49e (integrin α5) ($r = 0.80$, $p = 0.01$, $n = 8$) and IC leukemic cells in AML, ALL and CLL samples. A low negative correlation between serum-derived EV CD11c (integrin αX or CR4) ($r = -0.06$, $p = 0.8$, $n = 8$) and IC leukemic cells (Fig. 6), and a significant positive correlation between serum-derived EV HLA-ABC (MHC-I) ($r = 0.80$, $p = 0.01$, $n = 8$) and HLA-DRDPDQ (MHC-II) expression ($r = 0.49$, $p = 0.2$, $n = 8$) and IC leukemic cells in AML, ALL, and CLL samples were found (Fig. 6).

Positive correlations were also observed between serum-derived platelet associated EV CD42a ($r = 0.32$, $p = 0.4$, $n = 8$) and CD62P ($r = 0.23$, $p = 0.5$, $n = 8$), CD69 ($r = 0.45$, $p = 0.2$, $n = 8$) with IC leukemic cells in AML, ALL and CLL samples (Fig. 6b), even though not significant.

There was a significant positive correlation between serum-derived endothelial associated EV CD31 ($r = 0.54$, $p = 0.1$, $n = 8$)

and IC leukemic cells, while a negative correlation was seen between serum-derived endothelial associated EV CD146 ($r = -0.29$, $p = 0.4$, $n = 8$) and IC leukemic cells (Fig. 6).

Correlation analyses of serum-derived EV markers and cellular platelets (PLT), white blood cells (WBC) in AML, ALL, and CLL. We correlated PLT counts (as evaluated in leukemia samples Table 1) with thrombocytes-lineage-associated CD marker expressions on EVs.

We found a low and non-significant negative correlation between serum-derived platelet associated EV CD41b (integrin αIIβ) ($r = -0.31$, $p = 0.4$, $n = 8$), CD42a ($r = -0,19$, $p = 0.6$, $n = 8$), CD62P ($r = -0.29$, $p = 0.4$, $n = 8$), CD69 ($r = -0.32$, $p = 0.4$, $n = 8$) and platelets counts in all leukemic samples, while a positive correlation between serum-derived EV CD19 ($r = 0.82$, $p = 0.01$, $n = 8$), CD24 ($r = 0.8$, $p = 0.01$, $n = 8$), CD40 ($r = 0.43$, $p = 0.2$, $n = 8$) and cellular WBC in all leukemic samples were seen (Fig. 6).

In summary, the results presented in this study demonstrate that MBFCM facilitates assessment of EV surface protein compositions in biological fluid samples with minimal processing. Most importantly, this study also demonstrates that there is a correlation between serum-derived EV marker expression detected by MBFCM and cellular marker expression detected by flow cytometry or blood cell counting in leukemia and healthy samples. Especially a significant correlation on monocytes, B cells, T cells, blast markers, integrin associated markers, platelet associated markers, and endothelial associated marker could be assessed.

DISCUSSION

Leukemia and prognosis

A focus of leukemia research lies on the development of new therapeutic strategies to reinduce an effective anti-leukemic immunity and the development of new diagnostic strategies to detect and monitor (risk associated) tumor-or immune-associated processes during the course of the disease [28].

Monitoring of tumor load and immune reactions

Quantification and monitoring of leukemic cells (in peripheral blood (PB)) in chronic and acute leukemia is done by morphological, immunological (immunophenotyping) [29, 30], cytogenetic and molecular methods (e.g. PCR or FISH-analysis) [31]. New strategies with higher sensitivity like next-generation sequencing or digital droplet PCR expand the armamentarium for risk stratification, treatment monitoring and for the detection of minimal residual disease [32]. The advantage of these methods is their high sensitivity enabling the detection of low amounts of (mutated) DNA, including circulating tumor DNA also in blood samples- so-called liquid biopsies.

Despite these advantages, open questions are thresholds to differentiate malignant from benign mutated DNA or whether all relevant mutated gene sequences are addressed [32]. In addition, the full implementation of these novel diagnostic tools is expensive [33].

Quantification and qualitative monitoring of immune cells (especially in PB) is possible by refined immunophenotyping like multiparameter flow cytometry [29, 34].

EVs: new particles of biologic significance

EVs are highly heterogeneous vesicles of different size originating from distinct subcellular compartments with a diverse molecular make up [35]. EVs carry a wide variety of proteins, including MHC molecules, chaperones, receptors, receptor ligands, cytokines, nucleic acids (i.e. mRNA, miRNA, DNA), and lipids [36]. The term 'EV' is used as an umbrella term for mainly microvesicles, exosomes, and apoptotic bodies. EVs are known to incorporate proteins, including cell type-specific markers from their parental cells and might therefore qualify to provide information to monitor malignant disease [37, 38]. Furthermore, it was shown that molecular profiles of secreted vesicles faithfully reproduce those of cancer cells [39]. It has been shown that EVs influence immune responses and tumor progression: on the one hand, EVs secreted by DCs have been shown to carry MHC-peptide complexes allowing efficient activation of T lymphocytes, thus displaying a potential as promoters of (adaptive) immune responses [11]. On the other hand suppressive effects, e.g. of leukemia EVs in a context of bone marrow-related stromal cells or hematopoietic progenitor cells have been demonstrated [10]. Up to now the influence of EVs produced by leukemic cell lines or prepared from plasma on immune reactive cells of several lines in ex vivo settings have been tested and 'EV-derived suppressive or stimulatory effects' to modulate immune reaction were deduced [12].

In vivo EVs delivered in experimental animals such as mice supported ex vivo data, that differentiation, expansion, migration to lymph nodes and survival of hematopoietic cells could be modulated by EVs [40].

Potential of TEM and fNTA to detect and characterize EVs in leukemic and healthy serum

In the context of this project, EVs prepared from serum from healthy donors as well as from patients with leukemia should be analyzed and compared to potentially deduce strategies to monitor tumor- or immune-related EVs or processes.

Serum is known to contain mixtures of healthy (and malignant) cells and their EV- derivatives [12]. The 'identity' of captured healthy or leukemia-derived serum EVs in our settings was confirmed by TEM, although a differentiation of malignant and non-malignant EVs was not provided by TEM, demonstrating 'cup shaped' forms in both entities [18].

Performing fNTA from leukemic and healthy serum samples, we could detect differences in EV size and concentration: the mean diameters of particles in leukemic samples were higher than in healthy samples, the EV concentrations of ALL and CLL samples were significantly higher compared to healthy samples and EV concentration in AML samples were significantly lower compared to ALL and CLL samples.

This means that we can confirm that fNTA allows a 'rough' characterization, however no refined differentiation of leukemic compared to healthy EVs and especially no subclassification of tumor or immune derived EVs [15, 21].

Multiplex bead-based flow cytometry assay for assessment of EV surface protein profiles on EVs derived from biological fluids

In general, it is meanwhile well known that the EV content, including the protein and surface marker composition, is probably strongly dependent on the cell source, the cells' activation status, and multiple other parameters. Since any EV will show similar surface profiles than the cell type releasing them, analysis of EV surface signatures in the biological fluid has the potential to reveal any changes happening in abundance, frequency, and behavior of respective cell types releasing the EVs. Thus, it is a promising approach to identify EV surface marker profiles that correspond to certain diseases such as leukemia and ultimately use EV-based liquid biopsies for diagnosis and therapy decision making. Importantly, we here demonstrated that MBFCM can be used to detect and quantify EV surface proteins on EVs in blood serum samples without the need for any processing or enrichment steps. Blood samples were pre-cleared from cells and bigger aggregates merely based on centrifugation which is typical for biobanked samples. We titrated the input dose of serum volume and ultimately used 30 µL of human serum as input for MBFCM, diluted 1:1 with assay buffer. This has not led to increased assay background but still yielded clear signals for all samples included.

Due to the specificity of this sandwich MBFCM assay and the requirement on both bead capture and binding of detection antibodies on the same EV in order to measure positive signals in this assay, non-EV contaminants like free protein, lipoproteins, and other molecules won't be detected, thereby facilitating the direct analysis of minimally processed human serum samples without any purification steps. Thus, in contrast to an approach we used previously where assay input would be dosed based on NTA particle counts following sample isolation by SEC, the approach presented in this study facilitates the relation of measured signal intensities for respective markers directly to abundance in donor's whole blood.

MBFCM data presentation and correlation

MBFCM provides data for many different EV surface proteins, often resembling classical lineage-specific blood cell surface markers. In this proof-of-concept study, we evaluated different ways how to present MBFCM data and correlate the data with clinical parameters. In heatmap analyses and bar diagrams of our study, we demonstrated that we could detect EVs positive for CD9, CD63, and CD81 by MBFCM using 37 specific markers (Fig. 3), as already shown by Koliha et al. and Wiklander et al. [14, 15]. MBFCM was moreover shown to be sensitive enough in our hands to detect different EV surface markers in serum from leukemic and healthy samples, as already detected in other tumors or multiple cell type associated with EV-subclassed analyses by other groups [14, 24, 41, 42]. As shown in all entities and given in Fig. 3, lineage-associated EV markers CD8, CD42a, CD62P, and HLA-DRDPDQ were highly expressed in all sample entities. On a first glance and presenting data of single as well as of pooled cases, we found a high expression of some markers in leukemia or healthy serum samples: thrombocyte or myeloid leukemic cell-associated markers (CD42a, CD62P, and CD133) seemed to be highly expressed on AML sample derived EVs (Fig. 3b), T cell (CD8), thrombocyte (CD42a, CD62P) or MHC associated (HLA-DRDPDQ) markers seemed to be highly expressed on lymphoid leukemia-sample-derived EVs (Fig. 3c, d) and EVs from healthy samples also showed a high expression of CD8, CD19, CD29, CD41b, CD42a, CD62P, CD69, and ROR1 markers (Fig. 3e).

Focusing on certain EV markers and comparing their expression in different leukemic and healthy samples, we demonstrated differences: As given in Fig. 4, significantly lower frequencies of platelet-derived EV CD42a marker expression was found in AML and ALL compared to CLL samples, platelet-derived EV CD62P was found with (borderline significantly) lower frequencies in AML compared to CLL samples and significantly lower frequencies in ALL compared to CLL samples. Borderline significantly lower frequencies of platelet-derived EV CD41b were found in ALL compared to healthy samples. All platelet-derived EV markers (CD41b, CD42a, and CD62P) were expressed high in CLL. This could mean that the frequencies of these platelet EV markers are altered in different leukemic patients. Platelet microparticles have been also shown to be involved in metastasis, angiogenesis, and invasiveness in lung cancer [43], breast cancer [44], and melanoma [24]. Therefore, a surplus of platelet-derived EVs might indicate tumor progression.

In addition, analyses of plasma-derived EVs from healthy male athletes during the course of physical exhaustion showed elevated frequencies of platelet-derived markers CD41b, CD42a and CD62P positive on EVs, which could indicate that a release of EVs by activated platelets might not be restricted to large EVs (>500 nm), but also be related to smaller EV populations [42]. This could mean that platelet-derived EVs may play a role in exercise-triggered processes such as immune-modulation and inflammation-associated tissue regeneration even in healthy samples.

Interestingly, we found higher frequencies of EV-associated myeloid leukemic cell marker CD133 in AML. According to Tolba et al., cellular CD133 expression correlates with poor prognosis in AML patients [45]. Our findings might point to a positive relation of cellular CD133 with EV CD133 expression, although our patient samples were not tested for cellular CD133 expression.

In a next step, we tried to find relevant differences in EV frequencies when normalizing results from leukemic to healthy samples, IC leukemic cell counts, or WBC (Fig. 5). Compared to healthy samples we found elevated fold changes of CD133 in AML derived EVs, HLA-DRDPDQ in ALL derived EVs and of CD11c and CD146 in CLL derived EVs. As we discussed before, EV CD133 might have a closer relation with AML compared to ALL and CLL. Human leukocyte antigens (HLA) can be divided into HLA-A, B, and C which are encoded by major histocompatibility complex (MHC)-I and HLA-DP, DQ, and DR which are encoded by MHC-II. Here, we found HLA-ABC antigens expressed with low signal intensities on serum EVs in all samples. This might be due to a high percentage of EVs with low MHC-I expression, such as those secreted by NK cells and platelets. According to Merkenschlager et al., cellular MHC-II expression restrained growth of murine B-cell leukemia cell lines in vitro and in *vivo*, independently of CD4+ T-cell surveillance [46]. Their results showed that MHC-II cells autonomously regulate the balance between self-renewal and differentiation of normal and malignant B cells. Our findings might point to a possible relation of MHC-II positive EVs with cellularly expressed MHC-II. Moreover, MHC-II positive EVs might also regulate differentiation of normal and malignant B cells.

An increased production of integrin CD11c (integrin αX or CR4) positive EVs was shown in melanoma patients [24]. We could also find an increased production of CD11c positive EVs in serum of CLL samples.

Umit et al. demonstrated that cellular CD11c is not only expressed on CLL including 259 CLL patients, but also on dendritic cells, macrophages and monocytes as a marker for inflammation [47]. In our context, this might reflect that released CD11c positive EVs and cellularly expressed CD11c could point to inflammation processes in CLL. Prolonged inflammation in the microenvironment of CLL cells may cause a pragmatically unfavorable susceptibility to autoimmune disorders and secondary tumors in CLL [47].

While platelet markers and lymphocytic markers are highly specific, markers like CD146 are regularly found on endothelial cells and on mesenchymal stromal cell subtypes and their derived EVs might point to an involvement of endothelial cells and mesenchymal stromal cells in these diseases [48].

Increased production and release of CD133 positive EVs to serum in AML, of HLA-DRDPDQ positive EVs to serum in ALL and of CD42a, CD62P positive EVs to serum in CLL were found after normalization of results to IC leukemic cell and WBC counts. This might reflect again that leukemic cell-derived EV marker CD133 and platelet-derived EV marker CD42a and CD62P have a clear correlation with the cellular expression of these markers [24, 45]. These studies confirm that EVs may play a role in immune modulation, and moreover, EVs liberated from AML, ALL, and CLL were hypothesized to be involved in multisystemic signaling mediating regeneration and long-term adaptive responses [14, 42].

Comparison and correlation analysis between cellular and EV associated antigen expressions by MBFCM

Serum-derived EVs might provide information about the cell–cell interactions, resulting stimulations or inhibitions of immune cells. These correlations could be detected using MBFCM to quantify EVs and their corresponding cellular markers by flow cytometry in leukemic and healthy samples.

We found a significant positive correlation between serum-derived EV and cellular CD14 marker expressions in pooled AML and ALL samples. Although the detailed characteristics are not known, microvesicles isolated from plasma of advanced

melanoma patients, but not from healthy donors were shown to address CD14+ monocytes, resulting in CD14+ suppressed T-cell functions (possible with downregulation of HLA-DR) [49]. These findings could suggest that an immunosuppressive circuit conduct (e.g. mediated by tumor cells) and the generation of suppressive myeloid cells through the release of circulating microvesicles might work without the need for cell-to-cell contact. Likewise, cellular CD14 is mainly associated with monocytes/macrophages, but also present on the surface of neutrophils, though at lower levels [50]. This could mean that CD14+ cells could produce EVs, that could mediate various reaction in leukemia and healthy samples.

We found a high negative correlation between serum-derived EV and cellular CD19 marker expression in AML and healthy samples, however, a significant positive correlation in pooled ALL and CLL samples. These findings might reflect different functions of CD19 positive EVs (in AML and healthy serum) compared to CD19 positive EVs (in ALL and CLL with CD19 being the leukemic cell marker).

According to Gutzeit et al., exosomes derived from Burkitt's lymphoma cell lines induce B cell (CD19+ or CD20+) proliferation and differentiation towards a plasma cell-like phenotype with class-switched recombination [51]. Human B cell lymphomas produce EVs which carry components of the Wnt signaling pathway, transfer them to B cells, and thus promote tumor progression and stabilize the malignant phenotype [52]. We hypothesize that, along with the previously discussed elevated frequencies of CD19 positive EVs in ALL and CLL, down regulated frequencies of CD19 positive EVs from AML and healthy serum samples might indicate tumor progression and an attenuated immune response. Our findings might suggest that CD19 positive EVs derived from leukemia in general could induce proliferation and differentiation.

Additionally, a positive correlation for CD3 positive EVs and cellularly expressed CD3 was found, implying a contribution of T cells to the mixture of vesicles in serum, but with low correlation between EVs' and cellular CD3 expression. It is known from the literature that T cell-derived EVs might transfer functional miRNAs to antigen-presenting cells (APCs) to generate an immune response [53]. This could mean that T cell-derived EVs might be able to support immune responses against tumors.

Studies of molecular EV profiles have indicated that there are significant differences in protein and nucleic acid content of EVs derived from tumor cells compared to that of EVs produced by normal cells [37].

Leukemia-derived EVs have been shown to suppress activities of various immune cells, to induce apoptosis of activated CD8 T cells, to promote the expansion of regulatory T cells (Treg), and to interfere with differentiation of DC, favoring the proliferation of myeloid-derived suppressor cells [54]. These attributes of tumor-derived EVs are a manifestation of their distinctive molecular profiles and seem to be general phenomena in cancer. Interestingly, many of the proteins (e.g. oncoproteins, oncogenes, transcripts of proteins) found in tumor-derived EVs are well-known for their role in promoting tumor progression. Many of these proteins are also involved in inflammatory reactions, chemokine receptors, immunosuppressive ligands, or soluble factors involved in regulating angiogenesis [55, 56]. This suggests that tumor-derived EVs might play a critical role in cancer development and progression, although much of the currently available evidence originates from in vitro studies. In vivo evidence for exosomes as drivers of cancer pathogenesis is still incomplete.

Clinical correlation analyses between clinical diagnosis and EV expression by MBFCM

Correlations of experimental findings with clinical subtypes of leukemia could contribute to refine the classification of the disease. We found direct correlations between immune cytologically detected leukemic cells (IC leukemic cells) with EV marker expressions (e.g. of CD24 (GPI-anchored protein), CD44, CD133, CD142, MCSP, ROR1, SSEA-4) as evaluated by MBFCM.

Detection of lymphoid-derived EV marker CD19 or myeloid-derived EV marker CD133 in leukemia could contribute to refine detection of residual disease [24, 45].

Our data moreover might point to a role of integrins: EV-associated integrinmarkers such as CD11c (integrin αX or CR4), CD29 (integrin β-1), CD41b (integrin αIIβ) and CD49e (integrin α-5) as described here could play a role for targeting special EV subtypes. CD29 is known as an interaction partner of tetraspanins on cells and is probably transferred together with tetraspanins to exosomes during their biogenesis [24]. Accordingly, CD29 had already been detected on different types of exosomes [57]. CD49e (integrin α5) was considered as a potential marker for melanoma-derived EVs because it was found to be expressed by melanoma cells and we detected CD49e signals on EVs from melanoma cell cultures [24].

Our data might point to an additional role in leukemia: we found a positive correlation of EVs positive for CD29, CD41b and CD49e with IC leukemic cells in all pooled leukemia samples. These findings might suggest that the complexity of EV signaling in leukemia and expand the spectrum of conceivable functions of EVs.

In addition, platelet-derived EV markers CD42a, CD62P and CD69 showed direct positive correlations with IC leukemic cell counts. By contrast, a negative correlation of these markers with platelet counts was shown. These findings could confirm again that platelet microparticles might be involved in metastasis, angiogenesis and invasiveness, might indicate tumor progression [24, 42, 43].

We found a direct positive correlation of EV-associated markers HLA-ABC (MHC-I) and HLA-DRDPDQ (MHC-II) with IC leukemic cells in all leukemia samples. MHC-peptide complexes on exosomes can be presented to T cells either in a direct or an indirect manner [10, 13]. Zitvogel and coworkers demonstrated that murine bone marrow-derived DCs secreted EVs carrying MHC-I, MHC-II, and T cell costimulatory molecules, leading to a priming of tumor-specific cytotoxic T lymphocytes (CTLs) and suppress tumor growth in vivo [13, 16, 58]. In our context this could mean that HLA-ABC and HLA-DRDPDQ positive EVs might directly indicate progressive in leukemia.

LAMA84 CML cell-derived EVs are able to alter functions of various tissue cells, including endothelial cells (EC), and thus exert proangiogenic effects [59]. Similar effects on angiogenesis were induced by EVs from K562 cells [60]. Here we detected a positive correlation of EV marker CD31 and a negative correlation of EV marker CD146 with IC leukemic cells. Overall, these data emphasize that exosomes released from leukemic cells could directly affect EC and modulate the process of neovascularization.

CONCLUSION

In summary, we have comprehensively evaluated and optimized MBFCM based EV detection technology: MBFCM can not only quantify robust EV surface signatures in a given sample but is also useful for comparing differentially expressed surface markers between samples. It thereby facilitates the identification of heterogeneities between different EV sources, which may lead to the identification of EV markers being specific for certain cell types.

Our own data and findings of the literatures suggest that EVs may play a role in immune modulation, inflammation-associated tissue regeneration and regulation of coagulation. Although it is not yet clear which signals trigger the release of EVs from all these cell types and the target cells.

EV profiling might qualify as a highly reliable strategy to indicate the involvement of different subtypes in leukemia or in

the mediation of antitumor reaction in leukemia compared to healthy samples. MBFCM is qualified as a suitable marker to detect heterogeneity of EV markers as well as the role of specific EVs in the classification of diseases as well as the monitoring of (disease related or unrelated) of EVs derived and released from cells.

However, the results obtained from this assay could be influenced by several factors, including cross-linking of beads by single EVs binding to more than one bead population and thus should be interpreted not as a single vesicle quantification.

In general, the combination of this rather robust and fast approach with more dedicated methods to validate candidate surface markers distinguishing EV subpopulations (i.e., single EV flow cytometric analysis cell sorting or detection) would pave the way to studying the function of EV subsets, which will be of the highest relevance to further improve our understanding of their molecular content and related functions. In addition, new applications provide a potential prognostic role or might allow to monitor the disease under the influence of new (immune) therapeutic approaches.

DATA AVAILABILITY

All data generated or analysed during this study are included in this published article [and its supplementary information files].

CODE AVAILABILITY

Not applicable.

REFERENCES

1. Siegel RL, Miller KD, Jemal A Cancer statistics, 2016. CA Cancer J Clin. 2016;66:21332.
2. Döhner H, Estey E, Grimwade D, Amadori S, Appelbaum FR, Büchner T et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. Blood. 2017;129:424–47.
3. Cancilla D, Rettig MP, DiPersio JF. Targeting CXCR4 in AML and ALL. Front Oncol. 2020;10:1672.
4. Yosifov DY, Wolf C, Stilgenbauer S, Mertens D. From biology to therapy: The CLL success story. HemaSphere. 2019;3:e175.
5. Reyes C, Engel-Nitz NM, DaCosta Byfield S, Ravelo A, Ogale S, Bancroft T et al. Cost of Disease Progression in Patients with Chronic Lymphocytic Leukemia, Acute Myeloid Leukemia, and Non-Hodgkin's Lymphoma. Oncologist. 2019;24:1219–28.
6. Boeck CL, Amberger DC, Doraneh-Gard F, Sutanto W, Guenther T, Schmohl J, et al. Significance of Frequencies, Compositions, and/or Antileukemic Activity of (DC-stimulated) Invariant NKT, NK and CIK Cells on the Outcome of Patients with AML, ALL and CLL. J Immunother. 2017;40:224–48.
7. Balan S, Arnold-Schrauf C, Abbas A, Couespel N, Savoret J, Imperatore F et al. Large-Scale Human Dendritic Cell Differentiation Revealing Notch-Dependent Lineage Bifurcation and Heterogeneity. Cell Rep. 2018;24:1902–15.e6.
8. Ansprenger C, Amberger DC, Schmetzer HM. Potential of immunotherapies in the mediation of antileukemic responses for patients with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) - With a focus on Dendritic cells of leukemic origin (DCleu). Clin Immunol. 2020;217:1–6.
9. Vogt V, Schick J, Ansprenger C, Braeu M, Kroell T, Kraemer D et al. Profiles of activation, differentiation-markers, or β-integrins on T cells contribute to predict T cells' antileukemic responses after stimulation with leukemia-derived dendritic cells. J Immunother. 2014;37:331–47.
10. Tkach M, Kowal J, Zucchetti AE, Enserink L, Jouve M, Lankar D, et al. Qualitative differences in T-cell activation by dendritic cell-derived extracellular vesicle subtypes. EMBO J. 2017;36:3012–28.
11. Boyiadzis M, Whiteside TL. The emerging roles of tumor-derived exosomes in hematological malignancies. Leukemia. 2017;31:1259–268.
12. Boyiadzis M, Whiteside TL. Information transfer by exosomes: A new frontier in hematologic malignancies. Blood Rev. 2015;29:281–90.
13. Théry C, Duban L, Segura E, Væron P, Lantz O, Amigorena S. Indirect activation of naïve CD4+ T cells by dendritic cell-derived exosomes. Nat Immunol. 2002;3:1156–62.
14. Koliha N, Wiencek Y, Heider U, Jüngst C, Kladt N, Krauthäuser S et al. A novel multiplex bead-based platform highlights the diversity of extracellular vesicles. J Extracell Vesicles. 2016;5:29975.
15. Wiklander OPB, Bostancioglu RB, Welsh JA, Zickler AM, Murke F, Corso G et al. Systematic methodological evaluation of a multiplex bead-based flow cytometry assay for detection of extracellular vesicle surface signatures. Front Immunol. 2018;9:1326.
16. Lindenbergh MFS, Stoorvogel W. Antigen Presentation by Extracellular Vesicles from Professional Antigen-Presenting Cells. Annu Rev Immunol. 2018;36:435–59.
17. Buschmann D, Kirchner B, Hermann S, Märte M, Wurmser C, Brandes F et al. Evaluation of serum extracellular vesicle isolation methods for profiling miRNAs by next-generation sequencing. J Extracell Vesicles. 2018;7:1481321.
18. Hornick NI, Huan J, Doron B, Goloviznina NA, Lapidus J, Chang BH et al. Serum Exosome MicroRNA as a minimally-invasive early biomarker of AML. Sci Rep. 2015;5:11295.
19. Görgens A, Bremer M, Ferrer-Tur R, Murke F, Tertel T, Horn PA et al. Optimisation of imaging flow cytometry for the analysis of single extracellular vesicles by using fluorescence-tagged vesicles as biological reference material. J Extracell Vesicles. 2019;8:1587567.
20. Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. J Extracell Vesicles. 2018;7:1535750.
21. Mussack V, Wittmann G, Pfaffl MW. Comparing small urinary extracellular vesicle purification methods with a view to RNA sequencing—Enabling robust and non-invasive biomarker research. Biomol Detect Quantif. 2019;17:100089.
22. Eitan E, Green J, Bodogai M, Mode NA, Bæk R, Jørgensen MM et al. Age-Related Changes in Plasma Extracellular Vesicle Characteristics and Internalization by Leukocytes. Sci Rep 2017;7:1342.
23. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. J R Stat Soc Ser B 1995;57:289–300.
24. Koliha N, Heider U, Ozimkowski T, Wiemann M, Bosio A, Wild S. Melanoma affects the composition of blood cell-derived extracellular vesicles. Front Immunol. 2016;7:282.
25. McLellan AD. Exosome release by primary B cells. Crit Rev Immunol. 2009;29:203–17.
26. Mullier F, Bailly N, Chatelain C, Chatelain B, Dogné JM. Pre-analytical issues in the measurement of circulating microparticles: Current recommendations and pending questions. J Thromb Haemost. 2013;11:693–6.
27. Witwer KW, Buzás EI, Bemis LT, Bora A, Lässer C, Lötvall J et al. Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. J Extracell Vesicles. 2013;2.
28. Blackburn LM, Bender S, Brown S. Acute Leukemia: Diagnosis and Treatment. Semin Oncol Nurs. 2019;35:150950.
29. Galera PK, Jiang C, Braylan R. Immunophenotyping of Acute Myeloid Leukemia. Methods Mol Biol. 2019;2032:281–96.
30. Finak G, Langweiler M, Jaimes M, Malek M, Taghiyar J, Korin Y et al. Standardizing Flow Cytometry Immunophenotyping Analysis from the Human ImmunoPhenotyping Consortium. Sci Rep 2016;6:20686.
31. Haferlach T, Schmidts I. The power and potential of integrated diagnostics in acute myeloid leukaemia. Br J Haematol. 2020;188:36–48.
32. Brüggemann M, Kotrová M, Knecht H, Bartram J, Boudjogrha M, Bystry V et al. Standardized next-generation sequencing of immunoglobulin and T-cell receptor gene recombinations for MRD marker identification in acute lymphoblastic leukaemia; a EuroClonality-NGS validation study. Leukemia. 2019;33:2241–53.
33. Flach J, Shumilov E, Joncourt R, Porret N, Novak U, Pabst T et al. Current concepts and future directions for hemato-oncologic diagnostics. Crit Rev Oncol Hematol. 2020;151:102977.
34. Koido S, Homma S, Takahara A, Namiki Y, Komita H, Nagasaki E et al. Immunologic monitoring of cellular responses by dendritic/tumor cell fusion vaccines. J Biomed Biotechnol. 2011;2011:910836.
35. Kim DK, Kang B, Kim OY, Choi DS, Lee J, Kim SR et al. EVpedia: An integrated database of high-throughput data for systemic analyses of extracellular vesicles. J Extracell Vesicles 2013;2.
36. Andreu Z, Yáñez-Mó M. Tetraspanins in extracellular vesicle formation and function. Front Immunol 2014;5:442.
37. Martins VR, Dias MS, Hainaut P. Tumor-cell-derived microvesicles as carriers of molecular information in cancer. Curr Opin Oncol. 2013;25:66–75.
38. Record M, Subra C, Silvente-Poirot S, Poirot M. Exosomes as intercellular signalosomes and pharmacological effectors. Biochem Pharmacol. 2011;81:1171–82.
39. Harshman SW, Canella A, Ciarlariello PD, Rocci A, Agarwal K, Smith EM et al. Characterization of multiple myeloma vesicles by label-free relative quantitation. Proteomics. 2013;13:3013–29.
40. Zhang HG, Grizzle WE. Exosomes: A novel pathway of local and distant intercellular communication that facilitates the growth and metastasis of neoplastic lesions. Am J Pathol. 2014;184:28–41.

41. Castellani C, Burrello J, Fedrigo M, Burrello A, Bolis S, Di Silvestre D et al. Circulating extracellular vesicles as non-invasive biomarker of rejection in heart transplant. J Hear Lung Transplant. 2020;39:1136–48.
42. Brahmer A, Neuberger E, Esch-Heisser L, Haller N, Jorgensen MM, Baek R et al. Platelets, endothelial cells and leukocytes contribute to the exercise-triggered release of extracellular vesicles into the circulation. J Extracell Vesicles. 2019;8:752–60.
43. Janowska-Wieczorek A, Wysoczynski M, Kijowski J, Marquez-Curtis L, Machalinski B, Ratajczak J et al. Microvesicles derived from activated platelets induce metastasis and angiogenesis in lung cancer. Int J Cancer. 2005;113:752–60.
44. Janowska-Wieczorek A, Marquez-Curtis LA, Wysoczynski M, Ratajczak MZ. Enhancing effect of platelet-derived microvesicles on the invasive potential of breast cancer cells. Transfusion. 2006;46:1199–209.
45. Tolba FM, Foda ME, Kamal HM, Elshabrawy DA. Expression of CD133 in acute leukemia. Med Oncol. 2013;30:527.
46. Merkenschlager J, Eksmond U, Danelli L, Attig J, Young GR, Nowosad C et al. MHC class II cell-autonomously regulates self-renewal and differentiation of normal and malignant B cells. Blood. 2019;133:1108–18.
47. Umit EG, Baysal M, Durmus Y, Demir AM. CD11c expression in chronic lymphocytic leukemia revisited, related with complications and survival. Int J Lab Hematol. 2017;39:552–6.
48. Börger V, Bremer M, Görgens A, Giebel B. Mesenchymal stem/stromal cell-derived extracellular vesicles as a new approach in stem cell therapy. ISBT Sci Ser. 2016;11:1450.
49. Valenti R, Huber V, Filipazzi P, Pilla L, Sovena G, Villa A, et al. Human tumor-released microvesicles promote the differentiation of myeloid cells with transforming growth factor-β-mediated suppressive activity on T lymphocytes. Cancer Res. 2006;66:9290–8.
50. Antal-Szalmas P, Van Strijp JAG, Weersink AJL, Verhoef J, Van Kessel KPM. Quantitation of surface CD14 on human monocytes and neutrophils. J Leukoc Biol. 1997;61:721–8.
51. Gutzeit C, Nagy N, Gentile M, Lyberg K, Gumz J, Vallhov H et al. Exosomes Derived from Burkitt's Lymphoma Cell Lines Induce Proliferation, Differentiation, and Class-Switch Recombination in B Cells. J Immunol. 2014;192:5852–62.
52. Koch R, Demant M, Aung T, Diering N, Cicholas A, Chapuy B et al. Populational equilibrium through exosome-mediated Wnt signaling in tumor progression of diffuse large B-cell lymphoma. Blood. 2014;123.
53. Mittelbrunn M, Gutiérrez-Vázquez C, Villarroya-Beltri C, González S, Sánchez-Cabo F, González MÁ et al. Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. Nat Commun. 2011;2:282.
54. Whiteside TL. Immune modulation of T-cell and NK (natural killer) cell activities by TEXs (tumour-derived exosomes). Biochem Soc Trans. 2013;41:245–51.
55. Rak J, Guha A. Extracellular vesicles - vehicles that spread cancer genes. BioEssays 2012;34:489–97.
56. Taylor DD, Gercel-Taylor C. Exosomes/microvesicles: mediators of cancer-associated immunosuppressive microenvironments. Semin Immunopathol. 2011;33:441–54.
57. Clayton A, Turkes A, Dewitt S, Steadman R, Mason MD, Hallett MB. Adhesion and signaling by B cell-derived exosomes: the role of integrins. FASEB J 2004;18:977–9.
58. Zitvogel L, Regnault A, Lozier A, Wolfers J, Flament C, Tenza D et al. Eradication of established murine tumors using a novel cell-free vaccine: Dendritic cell-derived exosomes. Nat Med. 1998;4:594–600.
59. Taverna S, Flugy A, Saieva L, Kohn EC, Santoro A, Meraviglia S et al. Role of exosomes released by chronic myelogenous leukemia cells in angiogenesis. Int J Cancer. 2012;130:2033–43.
60. Mineo M, Garfield SH, Taverna S, Flugy A, De Leo G, Alessandro R et al. Exosomes released by K562 chronic myeloid leukemia cells promote angiogenesis in a src-dependent fashion. Angiogenesis. 2012;15:33–45.

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AUTHOR CONTRIBUTIONS

HMS and LL designed the study and contributed to the writing, review and discussion of the manuscript. LL, EP, and ASH prepared serum supernatants and conducted cell-culture experiments and clinical reports and statistical-analysis. JS, DK, and AR provided leukemic samples and corresponding patients' diagnostic reports. AG conducted MBFCA experiments and discussion of the project and revision of the manuscript. VM conducted TEM and fNTA experiments. VM and MP discussed the results and revised the manuscript.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL

All human blood samples and medical reports were provided from the University Hospitals of Munich, Stuttgart, Oldenburg and Augsburg. All blood samples was conducted after obtaining patients' written informed consent in accordance with the World Medical Association Declaration of Helsinki and the ethic committee of the Ludwig-Maximilians-University-Hospital Munich Pettenkoferstr. 8a, 80336 Munich, Ludwigs-Maximilians-University Hospital in Munich; Vote-No 339-05.

CONSENT TO PARTICIPATE

All authors agreed to participate in this study.

CONSENT FOR PUBLICATION

All authors agreed to submit this manuscript.

ADDITIONAL INFORMATION

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6. Paper II

The potential role of serum extracellular vesicle derived small RNAs in AML research as non-invasive biomarker

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Abbreviations: AML, acute myeloid leukemia; H, healthy; MDS, myelodysplastic syndrome; EV(s), extracellular vesicle(s); WB, whole blood； TEM, transmission electron microscopy; fNTA, fluorescence nanoparticle tracking analysis; RNA-Seq, RNA sequencing; miRNA, microRNA; mRNA, messenger RNA; rRNA, ribosomal RNA; snRNA, small nuclear RNA; snoRNA, small nucleolar RNA; tRNA, transfer RNA; nm, nanometer(s); nt, nucleotide(s); RIN, RNA integrity number; AML-DS, acute myeloid leukemia-down syndrome; PB, peripheral blood; LSC, leukemic stem-like cells; RR, relapsed/refractory; CR, complete remission; WM, waldenström's macroglobulinemia; MM, multiple myeloma; FL, follicular lymphoma; DLBCL, diffuse large B-cell lymphoma; HL, Hodgkin's Lymphoma; HucMSC, human umbilical cord mesenchymal stem cells; AIP, autoimmune pancreatitis; CP, chronic pancreatitis; FC, fold change; BM, bone marrow; SHR, spontaneous hypertensive rats; MIBC, muscle-invasive bladder cancer; NMIBC, non-muscle-invasive bladder cancer; PCa, prostate cancer; HIV, human immunodeficiency virus; ARDS, acute respiratory distress syndrome; ITP, Immune thrombocytopenia; AD, alzheimer's disease; DLB, dementia with Lewy bodies; BC, breast cancer; EAE experimental autoimmune encephalomyelitis.

Abstract

Background: Extracellular vesicles (EV) are cell-derived vesicles released by all cells in health and disease. Accordingly, EVs are also released by cells in acute myeloid leukemia (AML), a hematologic malignancy characterized by uncontrolled growth of immature myeloid cells, and these EVs likely carry markers and molecular cargo reflecting the malignant transformation occurring in diseased cells. Monitoring antileukemic or proleukemic processes during disease development and treatment is essential. Therefore, EVs and EV-derived microRNA (miRNA) from AML samples were explored as biomarkers to distinguish disease-related patterns *ex vivo* or *in vivo.*

Methodology: EVs were purified from serum of healthy (H) volunteers and AML patients by immunoaffinity. EV surface protein profiles were analyzed by multiplex bead-based flow cytometry (MBFCM) and total RNA was isolated from EVs prior to miRNA profiling via small RNA sequencing.
Results: MBFCM revealed different surface protein patterns in H versus AML EVs. miRNA analysis showed individual as well as highly dysregulated patterns in H and AML samples.
Conclusions: In this study, we provide a proof-of-concept for the discriminative potential of EV derived miRNA profiles as biomarkers in H versus AML samples.

Introduction

Acute myeloid leukemia (AML)

AML is characterized by an uncontrolled and excessive proliferation and an impaired differentiation of myeloid progenitor cells, leading to an expansion of leukemic cells (blasts) in the bone marrow (BM), peripheral blood (PB) and other tissues, going along with a replacement of the physiological hematopoiesis, leading to anemia, bleeding and infections. [1,2] In order to fight AML, several (immune) therapeutic cellular, antibody based or chemotherapeutic strategies have been developed (e.g. checkpoint blockades, chimeric antigen receptor (CAR)-T cell therapies and vaccinations or immune modulating hypomethylating agents). [3,4] Currently, the major challenge is still in the development of effective (immune) therapies in AML to overcome many immunosuppressive mechanisms. [4]

Extracellular vesicles (EVs)

The term ‘EVs’ comprises a broad variety of extracellular vesicle types with overlapping chemical and biophysical properties, including exosomes (which originate from the endocytic system) and microvesicles or ectosomes (formed by budding from the plasma membrane). [5] EVs carry and deliver essential molecular information for cell physiology and metabolism. Overall, their composition, concentration and biodistribution as well as the functional role in intercellular signaling can impact the general health status, but EVs also can play a role in pathological events, including malignant transformations of cells. [6] In hematological malignancies, tumor- or immune cell-derived EVs might reprogram the bone marrow environment, suppress or activate anti-leukemic immune response, modulate drug resistance, and could therefore interfere with immunotherapies. [7,8] Composition of EVs collected

from patients' serum might correlate with disease stage, drug- and/or immunological resistance, or response to (immune) therapies. [9]

A comprehensive EV purification and characterization to provide high reproducibility and comparability of data is necessary. [10] According to MISEV guidelines, [5] EVs can be enriched by various methods, [11] e.g. ultra-centrifugation, precipitation, size exclusion chromatography, ultrafiltration, immunoaffinity-based binding strategies. Obtained EVs can be characterized by multiple methods to assess purity and specificity. [12] Vesicular surface marker expressions (e.g. CD9, CD63, CD81) can be studied using flow cytometry, size and concentration measurements by fluorescence nanoparticle tracking analysis (fNTA). [13,14] EV morphology can be investigated by Transmission Electron Microscopy (TEM). [15] Western blotting is used to assess the presence of EV specific and non-specific (surface) protein markers. Detailed phenotyping of EV subsets co-expressing specific pairs of protein surface markers can be achieved for instance through high resolution single vesicle imaging flow cytometry (IFCM) [16] or by multiplex bead-based flow cytometry (MBFCM). The MBFCM assay includes 39 hard-dyed capture bead populations, each of them coated with different monoclonal antibodies against 37 potential EV surface antigens or two internal isotype negative controls. [13]

Characteristics and potential of small RNAs

MicroRNAs (miRNA) are evolutionarily conserved small non-coding RNAs (20-24 nucleotides) and have important physiological effects through their post-transcriptional gene regulation mainly by repression of target messenger (m)RNA. [17,18] It has been discovered that miRNAs are released into various body fluids in complexes with lipoproteins, soluble proteins or encapsulated in EVs. [7] EV-associated miRNAs as well as other molecular cargo can be shuttled to recipient cells via transfer through the blood circulation, which plays an important role in long distance cell-to-cell communication. [19]

Beyond dysregulated miRNA expression profiles, it is now well accepted that selected cellular/extracellular miRNAs can function as either tumor activating (oncomiRs) or tumor suppressing miRNAs in many subtypes of tumor processes, including tumor proliferation, survival, differentiation, self-renewal, epigenetic regulation, going along *in vivo* with chemotherapy resistance and disease progression. [7,17] These observations indicate that the circulating miRNA profiles may reflect physiological and pathological processes occurring in different cells and tissues, and might qualify as valuable blood-based biomarkers for various diseases. [7]

Biomarker research has already shown, that EV marker profiles and their miRNA cargo might contribute to monitor the disease as well as the (antileukemic) immune status and to select risk-adapted therapies.[20] These findings might be used as a powerful tool to detect both novel and known (EV-derived) miRNAs that could qualify as biomarkers.

The aim of this study was to (1) prepare and characterize EVs from serum samples of healthy donors and AML patients; (2) evaluate the use of MBFCM for comparing the overall EV surface protein composition on EVs in minimally processed samples from healthy donors versus AML patients; and (3) assess EV-associated miRNA profiles that could be applied as potential biomarkers comparing healthy donors' with AML patients' serum.

Material and Methods

Sample preparation

Blood sample collection was conducted after obtaining written informed consent of the blood donor and in accordance with the World Medical Association Declaration of Helsinki and the ethic committee of the Ludwig-Maximilians-University-Hospital Munich (vote no. 33905). Serum samples were collected with 4.5 ml serum tubes (S-Monovette) and provided by the University Hospitals of Augsburg, Stuttgart and Munich. Around 3 ml of serum were isolated from each healthy or AML donors' sample. Cells and larger particles were sedimented from serum after centrifugation (2,000 x g, 10 min, room temperature). The resulting supernatant (containing EVs) was aliquoted in 0.5 ml tubes and stored at -80°C until further processing.

Serum samples were obtained from healthy (H) volunteers (n=5) with a mean age of 28.6 (range 24-31 years) and from AML patients (n=5) with a mean age of 77 years (range 61-98 years). AML patients were classified by the French-American-British-(FAB)-classification (M1-M7), the aetiology (primary or secondary AML), the stage of disease (first diagnosis, relapse) and the frequencies of blasts and blast phenotype are given in Table 1.

Flow cytometric analyses were carried out to evaluate and quantify frequencies and phenotypes of blasts, T-, B- and monocyte subsets in the WB-fractions. Panels with various monoclonal antibodies (moAbs) labeled with Fluorescein isothiocyanat (FITC), phycoerythrin (PE), tandem Cy7-PE conjugation (Cy7-PE), or allophycocyanin (APC) were used. Erythrocytes in blood samples were lysed using Lysing-Buffer (BD, Heidelberg, Germany). Cells dissolved in PBS (Biochrom, Berlin, Germany) and 10% fetal

calf-serum (FCS, Biochrome, Berlin, Germany) were performed by a 15-min incubation in the dark at room temperature. Afterwards, the cells were washed, centrifuged, resuspended in 100–200 uL PBS and measured using fluorescence-activated cell sorting Flow-Cytometer (FACSCaliburTM) and Cell-Quest-data-acquisition and analysis software (Becton Dickson, Heidelberg, Germany). Isotype controls were conducted according to manufacturer's instructions.[21] The cellular composition of AML patients presented an average of 44.4% (immune cytologically detected, IC) blasts (range 30-72), 14.74% CD3+ cells (range 7.14-27.68), 3.88% CD19+ cells (range 1.32-6.98), 27.37% CD56+ cells (range 7.18-64.58), and 10.53% CD14+ (range 3.81-28.18). H-controls presented with 10.51% CD3+ cells (range 1.27-20.69), 1.33% CD19+ cells (range 0.28-2.38), 6.38% CD56+ cells (range 1.41-11.95), and 3.15% CD14+ (range 0.47-5.43). In case of aberrant CD56 or CD14 expression on blasts, these values were excluded from NK/monocytes quantification. An overview is given in Table 2.

Extracellular vesicle preparation

1.27 (± 0.09) ml were used as input volume for EV isolation using Exosome Isolation Kit, pan, human (Miltenyi Biotec) according to the manufacturer's instructions which includes a further centrifugation step at 10,000 x g for 45 min at room temperature.

EV characterization

As recommended by MISEV2018 guidelines, [5] purified EVs were characterized by TEM and fNTA as described before. [22]

Multiplex Bead-Based Flow Cytometry (MBFCM)

Serum samples (each tube 0.5 ml) were thawed and subjected to MBFCM (MACSPlex Exosome Kit, human, Miltenyi Biotec). [13,22] EV-containing serum samples were centrifugated at 2,500 x g for 15 minutes before supernatants were processed. 30 µL of each sample was diluted with 30 µL MACSPlex buffer (MPB) and loaded onto wells of a pre-wet and drained MACSPlex 96-well 0.22 µm filter plate. Next, 8 µL of MACSPlex Exosome Capture Beads (containing 39 different antibody-coated bead subsets) were added to each well as described elsewhere. [22]

Surface proteins included in the MBFCM assay comprise the tetraspanins CD9, CD63, and CD81, and other surface proteins such as various leukocyte, T cell (CD4, CD8), B cell (CD19, CD20, CD24),

monocyte (CD14), thrombocyte (CD41b, CD42a, CD62P, CD69), integrin (CD11c (integrin αX or CR4), CD29 (integrin β1), CD41b (integrin αIIβ), CD49e (integrin α5)), endothelial (CD31, CD105, CD146 (Mel-CAM)), or MHC-associated (HLA-ABC (MHC-I), HLA-DRDPDQ (MHC-II)) antigens.

In this study, we removed values for capture beads coated with hIgG (REA) antibodies for the samples AML P1598 and H P1580 due to high background signals for internal hIgG (REA) isotype control beads. Background-subtracted median APC fluorescence values <1 were considered negative (~2 fold average mIgG/REA control bead SD).

Extracellular RNA isolation

Total RNA of a complete EV eluate was isolated using the miRNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. The obtained RNA eluate was applied twice to the membrane to obtain higher RNA yields. The resulting eluate of 30 µL was completely vacuum-evaporated and resolved in a total volume of 12 µL nuclease-free water.

Isolated RNA was quality controlled and quantified by capillary gel electrophoresis using the RNA 6000 Pico Kit (Agilent Technologies, Germany) and the Bioanalyzer 2100 (Agilent Technologies). Total RNA was stored at -80°C until small RNA-Seq library preparation.

Small RNA library preparation and sequencing reaction

Equal amounts of RNA of the biological replicates were pooled, and 6.0 ng total RNA were used as starting material for library preparation using the NEBNext Multiplex Small RNA Library Prep Set for Illumina (New England BioLabs, USA) in accordance with the manufacturer's instructions. After a pre-amplification, PCR products were purified by applying the Monarch PCR Cleanup Kit (New England Biolabs). Then, cDNA libraries were evaluated via capillary gel electrophoresis using the DNA 1000 Kit and the Bioanalyzer 2100 (Agilent Technologies) according to the manual. A miRNA-specific length of 130–150 base pairs of barcoded cDNA libraries was selected by applying and fractionating 5 ng of pooled cDNA on a 4% agarose gel (MetaPhor, USA). Clean-up of cut gel slices at the appropriate size range was performed using the Monarch Gel Extraction Kit (New England Biolabs) and correct size and molarity were analyzed via capillary gel electrophoresis using the Bioanalyzer DNA High Sensitivity Kit (Agilent Technologies). Finally, 50 cycles of single-end sequencing-by-synthesis reactions were

conducted on the HiSeq 2500 instrument (Illumina, USA) with the HiSeq Rapid SBS Kit v2 (Illumina, USA).

Small RNA-Seq data analysis

The Quality Phred Score generated by the FastQC software (Babraham Bioinformatics, UK, Version 0.11.9) was used to explore successful sequencing. [23] Next, 3' adapter sequences were trimmed from raw sequencing reads using Btrim. [24] In case no adapter could be detected or reads appeared with less than 16 nucleotides, the respective reads were discarded. Residual reads were aligned to sequences provided by RNAcentral v12. [25] Reads that mapped to ribosomal RNA (rRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), or transfer RNA (tRNA) were not considered in further processing. Remaining reads were then mapped to human precursor miRNA sequences while allowing for one mismatch (miRBase, release 22.1 and Bowtie). [26,27] Reads that still remained unmapped were named as such and not processed further. Read count tables were created by summing up all hits per specific sequence.

Statistical analysis

Data is presented as mean ± SD unless otherwise stated. For comparison of two groups a paired t-test was applied. Differences were considered as 'not significant' with (adjusted) p-values > 0.1, as 'borderline significant' (#) with (adjusted) p-values between 0.05 and 0.1, as 'significant' (*) with (adjusted) p-values between 0.01 and 0.05, as 'highly significant' (**) with (adjusted) p-values between 0.001 and 0.01, as 'very highly significant' (***) with (adjusted) p-values between 0.0001 and 0.001 and as 'extremely significant' (****) with (adjusted) p-values < 0.0001. Statistical analyses and creation of diagrams were performed using Microsoft Excel 2016, GraphPad Prism version 8.4.0 and R programming language, version 4.1.0.

Processed by the Bioconductor package DESeq2 (version 1.20.0) and pheatmap (version 1.0.12), only miRNAs with more than 20 DESeq2- normalized reads were treated as valid. Exploratory data analysis was visualized by Venn diagram, hierarchical clustering and heatmap analysis based on Euclidean distances. Differential gene expression was assessed by calculating miRNA-specific log2 fold chances (FC).

Results

We aimed to establish a proof-of-concept workflow to evaluate EV-associated miRNA profiles as potential diagnostic, prognostic or predictive markers to predict immune reaction, clinical subvision or response to treatment in AML patients compared to healthy volunteers.

Typical cup-shaped appearance of serum EVs were identified by TEM and EV concentrations and size distribution profiles of purified serum EV samples from H and AML patients by fNTA, as shown before. [22] In this manuscript, we focused on the quantitative and qualitative assessment of EV surface characteristics via MBFCM and especially the characterization of the EV-derived miRNA cargo by RNA-Seq to evaluate their potential as biomarkers in AML samples compared to healthy volunteers.

EV surface protein profiles of H and AML samples by MBFCM

The MBFCM assay is based on the co-detection of two EV surface markers: one marker (based on the fluorescence-labelled detection antibody), aims to detect all tetraspanin-positive EVs bound to the respective capture bead, which is a mixture of pan anti-tetraspanin (CD9, CD63, CD81) antibodies. Another marker (based on specificity of one of 37 capture beads) is coated with specific capture antibodies. [28] The principle of MBFCM make sure that only EVs are directly captured, not free proteins, without further purification, specific detection of EVs are identified. [13]

In this study we included serum from 5 H and 5 AML samples (Table 1) and analyzed the EV surface protein profiles by MBFCM. An overview presentation of results showed that EV markers CD81, CD63, CD9 and in addition lineage markers CD8, CD41b, CD42a, CD62P, HLA-DRDPDQ and SSEA-4 markers were both highly expressed on H and AML derived EVs (Fig. 1). Comparing individual EV markers in AML and H samples, we found differences for some markers (Fig. 2).

Characterization of extracellular vesicle associated RNA by RNA-Seq

To systematically characterize the miRNA profiles associated with serum-derived EVs, we performed small RNA-Seq analysis in 5 AML and 5 H sample pools. First, total RNA was extracted from purified EVs. Quality control using capillary gel electrophoresis revealed low average RIN values of 1.6 (±0.5) for RNA samples obtained from H derived EVs and 1.7 (±1.6) for RNA samples obtained from AML derived EVs. This is a typical range for EV-associated RNAs, as EVs are considered to have minimum

amounts of ribosomal 18S and 28S rRNA amounts, mostly bound to the EV surface. Further, total EV-associated RNA yields of 2.1 (±0.8) ng (H) and 2.5 (±1.1) ng (AML) were quantified, respectively. Furthermore, small RNA-Seq was conducted to analyze the miRNA cargo of EVs. In total, in average 8.7 x10^6 reads were detected in serum-derived EVs from H samples and 14.2 x10^6 reads in serum-derived EVs from AML samples (Fig. 3A and C).

For an overview of mapping distributions, most reads were shown to be unmapped or short. The other reads mainly mapped to rRNAs and miRNAs in both serum H and AML samples. Interestingly, more reads in total and of all types of RNA were detected in AML compared to H samples (Fig. 3A and C). Since a large proportion of reads were unmapped or shorter than 16 reads, we excluded these reads as well as *no_adapter* reads and prepared a relative mapping distribution among the remaining RNA reads. The most abundant category of transcripts was rRNA, followed by miRNA, tRNA, snRNA and snoRNA. Of note, more than 60% of retained reads mapped to rRNA, more than 20% of retained reads mapped to miRNA and smaller fractions to tRNA, snRNA and snoRNA (Fig. 3B and D). Interestingly we found that AML showed a substantially higher proportion of all categories of RNA compared to H (not including miRNA) in relative mapping distributions. Although the absolute miRNA read count was higher in AML samples, the relative mapping distribution of reads mapping to small RNA species showed a higher percentage of miRNAs in H samples.

Comparison of miRNA profiles in extracellular vesicles from H vs. AML serum samples

After filtering miRNAs with at least 20 DESeq2-normalized reads 152 miRNAs were retained in H and 159 in AML samples, 136 of which were detected in both H and AML samples (Fig. 4A, Supplementary Table). 16 miRNAs, (10.5% (16/152) of the detected miRNAs) were exclusively found in EVs purified from H samples. 23 miRNAs, (14.5% (23/159) of the detected miRNAs) were exclusively found in EVs purified from AML samples (Fig. 4A). Based on the 136 shared miRNAs heatmap analysis depicted different miRNA patterns in H or AML samples (Fig. 4B) emphasized by the high number of miRNAs (45/33.1%) that showed a log2 fold change >|1| (Fig. 4C).

To examine expression differences between H and AML samples in more detail, we selected 5 highly up regulated miRNAs (miR-10a-5p, miR-155-5p, miR-100-5p, miR-146b-5p, let-7a-5p) (Fig. 5A) and 5 highly down regulated miRNAs (miR-185-5p, miR-4433b-3p, miR-199a-5p, miR-451a, miR-151a-3p) identified by small RNA sequencing (Fig. 5B). The largest difference in AML compared to H was

detected for miR-10a-5p (Log2FC=4.89).

We summarized that EVs could be prepared from healthy and AML serum samples: some EV-derived miRNAs prepared from AML samples are up regulated and others are down regulated compared to healthy samples. These disproportions might indicate physiological changes related to the disease and that these miRNA mechanisms may be affected in AML in different ways.

Discussion

In this study, we compared serum EV-derived miRNA contents from H and AML patients to assess relevant differences that could qualify as novel non-invasive and RNA-based biomarkers. Both H and AML patient samples were further subjected to MBFCM, which is a reliable method [13,28] to detect and quantify EV surface protein signatures. By applying MBFCM, we demonstrated that serum-derived EVs of H and AML patients both showed high abundancies of the tetraspanins CD9, CD63 and CD81 on their surface (Fig. 1). Several other EV surface markers were detected in blood in accordance with previous findings: [13] T cell EV associated marker CD8, integrin associated EV marker CD29, megakaryocyte (or thrombocyte) associated EV marker CD41b (integrin αII β) and CD42a and MHC-II associated EV marker HLA-DRDPDQ could be detected in H as well as AML samples.

Previously it was shown that leukemic blasts can express CD133. [29] Moreover it was shown, that CD133 can be secreted into EVs. [22] Here, we detected elevated levels of CD133 in AML compared to healthy samples by MBFCM, suggesting that CD133+ EVs were released by AML blasts and subsequently became detectable in human blood. This could mean that these markers (in combination) could qualify as biomarkers to detect even a low tumor load.

In summary, this study presents a proof-of-concept approach to characterize EVs in the blood of AML patients versus healthy donors. The authors would like to emphasize that based on the relatively small patient cohort and other limitations (e.g. not age matched cohorts) that impact the data no ultimate conclusions can be drawn in terms of specificity for observed differences for AML. Respective differences will have to be confirmed and investigated in larger studies and more complementary upcoming EV analysis methods such as single EV Imaging Flow Cytometry[16] for additional markers and combined subpopulations of EVs, thereby leading to a higher resolution between EV subpopulations. The MBFCM data obtained from this proof-of-concept study still provides some more

insight into general expression of EV surface markers in blood of leukemia patients and provides some starting points for future studies.

MiRNA profiles might serve as highly promising biomarkers in AML for improved classification and determination of the appropriate treatment in patients initially presenting with leukemia. Interestingly, specific subtypes and mutant drivers of AML are associated with distinctive miRNA expression profiles, again suggesting that miRNAs could be useful in the initial classification of the disease. [30]

In general miRNAs are known to function as tumor activator or suppressor RNA. [10,19] To obtain a better insight into changes of molecular profiles specific for H in comparison with AML, we focused on the cellular (Table 3) and EV (Table 4) levels of individual miRNAs and observed that ten miRNAs were deregulated in AML patients' samples. In addition to AML, we also identified a large set of other tumors to support or investigate our selected ten miRNAs' function, e.g. tumor activator (oncomiR) or suppressor, based on EV level detected by the RNA-Seq.

Concerning cellular derived miRNAs (Table 3), the expression level of miR-10a-5p in AML and myelodysplastic syndrome (MDS) was shown to be significantly higher compared to controls. [7,31] MiR-10a-5p and miR-155-5p are highly expressed in FLT3-ITD associated AMLs. [32] MiR-155 was shown to control B and T-cell differentiation and the development of regulatory T-cells. [17,33,34] Inhibiting miR-155 expression in LPS activated DCs resulted in an increase in pro- inflammatory cytokines gene expression (eg. IL-1α, IL-1β IL-6, TNF-α and IL-23). [35] MiR-100 expressed highly and regulated cell differentiation and survival by targeting RBSP3 in AML. [36–38] MiR-146b-5p was shown to be significantly increased in MDS patients compared to controls in plasma. [7] In human AML cell lines OCI-AML3, cellular let-7a was shown to be downregulated by SDF-1α–mediated CXCR4 activation and increased by CXCR4 inhibition. [39] By targeting GPX1, miR-185-5p inhibited AML progression and downregulated AML cells' proliferation and invasion. [40] MiR-185-5p was shown to be associated with the negative regulation of myeloid leukocyte differentiation, negative regulation of myeloid cell differentiation, and positive regulation of hematopoiesis in the regulation of CN-AML. [41] MiR-199a-5p was shown to play an important role in regulating the sensitivity of AML cells to Adriamycin (ADM) treatment. [42]

Concerning EV derived miRNAs (Table 4), miR-10a-5p was significantly increased in MDS, prostate cancer (PCa) and human immunodeficiency virus (HIV) patients compared to controls in plasma derived EVs. [7,43,44] We found miR-155 derived from EVs upregulated in AML, chronic lymphocytic leukemia (CLL) and Waldenström's macroglobulinemia (WM) compared to controls. [33] Dunand-Sauthier et al

found that miR155-induced repression of Arg2 expression appeared critical for DCs to activate T cells by controlling arginine availability in the extracellular environment. [34] EV-associated miR-100–5p was significantly upregulated (>3-fold) in type 1 autoimmune pancreatitis (AIP) patients when compared with controls. [45] MiR-146b-5p was significantly increased in MDS patients compared to controls in EVs. [7] In urinary EVs, miR-146b-5p was exhibited significantly higher expression from patients with muscle-invasive bladder cancer (MIBC) compared with non-muscle-invasive bladder cancer (NMIBC). [46] Let-7a-5p purified from EVs was upregulated in plasma of both P. vivax-infected and P. falciparum-infected patients compared to controls. [47] EV derived let-7a-5p was found to be abnormally expressed in brain microvascular pericytes obtained from spontaneous hypertensive rats compared to normotension Wistar Kyoto rats. [48] In addition, let-7a-5p in EVs could also serve as the mechanism contributing to the reduced cell apoptosis and elevated cell autophagy in AKI. [49]

On the other hand, EVs with miR-185-5p as cargo was shown to be elevated in the setting of profound epithelial cell death. [50] Exosomal miRNAs miR-4433b-3p was found downregulated in immune thrombocytopenia (ITP) patients compared to control. [51] EV miR-199a-5p and miR-451a were reduced in higher-risk MDS compared to lower-risk disease. [52] EV miR–199a-5p was associated with three metals (barium, mercury, and thallium) in early pregnancy, and their predicted target genes were enriched in pathways important for placental development. [53] A significant increase of miRNA-451a copies (in EVs) was detected in breast cancer (BC) patients' sera compared to controls. [14] EV miR-451a levels correlated also with the severity of experimental autoimmune encephalomyelitis (EAE). [54] Plasma derived EV miR-151a-3p was shown to be significantly decreased in Alzheimer's disease (AD) compared to controls. [55]

As evidenced in differential gene expression (DGE) analyses based on small RNA-Seq, we focused on 136 shared miRNAs between H and AML (Fig. 4B) and selected ten miRNAs (miR-10a-5p, miR-155-5p, miR-100-5p, miR-146b-5p, let-7a-5p and miR-185-5p, miR-4433b-3p, miR-199a-5p, miR-451a, miR-151a-3p) expressed with Log2FC >|1| (Fig. 4C). The highest fold change (Log2FC=4.89) was detected for EV-derived miR-10a-5p, which was upregulated in AML compared to H. It is well known that, miRNAs impact disease development and progression through collaboration with known oncogenes or tumor suppressors, either by directly targeting them on the mRNA level or by working in concert with resulting proteins to promote malignancy.

In this study, we found miR-10a-5p derived from EVs (Log2FC=4.89) upregulated in AML compared to H samples, which might confirm a direct correlation between EV miR-10a-5p level with poor

prognosis in leukemic patients. [31] miR-155-5p derived from EVs was also upregulated (Log2FC=3.39) in AML compared to H samples, which might suggest that EV-associated miR-155 deficiency in these hematologic compartments may cause attenuated immune regulation. Our findings confirm that miR-100-5p, miR-146b-5p and let-7a-5p derived from EVs were upregulated in AML compared to H samples. Let-7a-5p might regulate genes involved in adherents junction or transforming growth factor-β pathways. Interestingly, our data showed that miR-185-5p (Log2FC=-2.27) from serum derived EVs was down regulated in AML compared to H samples, what might support that miR-185-5p in extracellular vesicles might be a key regulator of pathological processes. Our serum data also showed that miR-4433b-3p, miR-199a-5p, miR-451a and miR-151a-3p derived from EVs were down regulated in AML compared to H samples. These EV derived miRNAs have been shown to accumulate phosphorylation enzymes, proteasome-related proteins and genes involved in cell death among others and could therefore point to sufficiently altered metabolic processes.

Conclusions

In summary, we demonstrate that MBFCM is generally suitable for EV characterization in minimally processed AML samples. Our data indicates that EV-derived miRNA cargo changes between H and AML might not only reflect differences in levels of many hematopoiesis-related, but also general metabolic differences. In particular, miRNAs derived from EVs could be considered privileged players as biomarkers due to their impact on target cells and tissues, and we assume that EV derived miRNA dysregulation in blood circulation by tumor activators (oncomiR) or tumor suppressors could have a significant impact on multiple recipient cells, affecting their physiological features and contributing to the development of myelodysplasia.

Declarations

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Availability of data and material All human blood samples and medical reports were provided from the University Hospitals of Munich, Stuttgart and Augsburg.

Data Availability Statement All data generated or analysed during this study are included in this published article [and are given as supplementary information files] or are available from the corresponding authors on request.

Code availability Not applicable.

Authors' contributions H.M.S, L.L, V.M and M.W.P. designed the study. H.M.S, L.L. and V.M. contributed to the writing, review and discussion of the manuscript. L.L., E.P., A.S.H, H.A, E.R, prepared serum supernatants and conducted cell-culture experiments and clinical reports and statistical-analysis. A.R. and J.S. provided leukemic samples and corresponding patients' diagnostic reports. A.G. conducted MBFCA experiments, provided discussion of the project and revised the manuscript. V.M. conducted miRNA sequencing experiments and provided statistical analyses and graphical illustration thereof. V.M. and M.W.P. took part in the discussion of the results and revised the manuscript.

Ethics approval All blood sampling was conducted after obtaining patients' written informed consent in accordance with the World Medical Association Declaration of Helsinki and the ethic committee of the Ludwig-Maximilians-University-Hospital Munich, Pettenkoferstr. 8a, 80336 Munich, Ludwigs-Maximilians-University Hospital in Munich; Vote-No 339-05.

Consent to participate All authors agreed to participate in this study.

Consent for publication All authors agreed to submit this manuscript.

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References

1. Döhner H, Estey E, Grimwade D, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood*. 2017;129(4):424–447.
2. Cancilla D, Rettig MP, DiPersio JF. Targeting CXCR4 in AML and ALL. *Front Oncol*. 2020;10:.
3. Boeck CL, Amberger DC, Doraneh-Gard F, et al. Significance of Frequencies, Compositions, and/or Antileukemic Activity of (DC-stimulated) Invariant NKT, NK and CIK Cells on the Outcome of Patients with AML, ALL and CLL. *Journal of Immunotherapy*. 2017;40(6):224–248.
4. Moussa Agha D, Rouas R, Najar M, et al. Impact of Bone Marrow miR-21 Expression on Acute Myeloid Leukemia T Lymphocyte Fragility and Dysfunction. *Cells*. 2020;9(9):1–18.
5. Théry C, Witwer KW, Aikawa E, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles*. 2018;7(1):1535750.
6. Boyiadzis M, Whiteside TL. The emerging roles of tumor-derived exosomes in hematological malignancies. *Leukemia*. 2017;31(6):.
7. Hrustincova A, Krejcik Z, Kundrat D, et al. Circulating Small Noncoding RNAs Have Specific Expression Patterns in Plasma and Extracellular Vesicles in Myelodysplastic Syndromes and Are Predictive of Patient Outcome. *Cells*. 2020;9(4):.
8. Huang S, Li Y, Wu P, et al. microRNA-148a-3p in extracellular vesicles derived from bone marrow mesenchymal stem cells suppresses SMURF1 to prevent osteonecrosis of femoral head. *J Cell Mol Med*. 2020;24(19):11512–11523.
9. Hao S, Bai O, Yuan J, Qureshi M, Xiang J. Dendritic cell-derived exosomes stimulate stronger CD8+ CTL responses and antitumor immunity than tumor cell-derived exosomes. *Cell Mol Immunol*. 2006;3(3):.
10. Mussack V, Wittmann G, Pfaffl MW. Comparing small urinary extracellular vesicle purification methods with a view to RNA sequencing—Enabling robust and non-invasive biomarker research. *Biomol Detect Quantif*. 2019;17(January):
11. Buschmann D, Kirchner B, Hermann S, et al. Evaluation of serum extracellular vesicle isolation methods for profiling miRNAs by next-generation sequencing. *J Extracell Vesicles*. 2018;7(1):.
12. Buschmann D, Mussack V, Byrd JB. Separation, characterization, and standardization of extracellular vesicles for drug delivery applications. *Adv Drug Deliv Rev*. 2021;174:348–368.
13. Wiklander OPB, Bostancioglu RB, Welsh JA, et al. Systematic methodological evaluation of a multiplex bead-based flow cytometry assay for detection of extracellular vesicle surface signatures. *Front Immunol*. 2018;9(JUN):

14. Moloney BM, Gilligan KE, Joyce DP, et al. Investigating the Potential and Pitfalls of EV-Encapsulated MicroRNAs as Circulating Biomarkers of Breast Cancer. *Cells*. 2020;9(1):1–13.
15. Hornick NI, Huan J, Doron B, et al. Serum Exosome MicroRNA as a minimally-invasive early biomarker of AML. *Sci Rep*. 2015;5:.
16. Görgens A, Bremer M, Ferrer-Tur R, et al. Optimisation of imaging flow cytometry for the analysis of single extracellular vesicles by using fluorescence-tagged vesicles as biological reference material. *J Extracell Vesicles*. 2019;8(1):.
17. Wallace JA, O'Connell RM. MicroRNAs and acute myeloid leukemia: Therapeutic implications and emerging concepts. *Blood*. 2017;130(11):1290–1301.
18. Guo Q, Luan J, Li N, et al. MicroRNA-181 as a prognostic biomarker for survival in acute myeloid leukemia: A meta-analysis. *Oncotarget*. 2017;8(51):89130–89141.
19. Tadokoro H, Umezu T, Ohyashiki K, Hirano T, Ohyashiki JH. Exosomes derived from hypoxic leukemia cells enhance tube formation in endothelial cells. *Journal of Biological Chemistry*. 2013;288(48):34343–34351.
20. Mateescu B, Kowal EJK, van Balkom BWM, et al. Obstacles and opportunities in the functional analysis of extracellular vesicle RNA - An ISEV position paper. *J Extracell Vesicles*. 2017;6(1):.
21. Amberger DC, Doraneh-Gard F, Gunsilius C, et al. PGE1-containing protocols generate mature (leukemia-derived) dendritic cells directly from leukemic whole blood. *Int J Mol Sci*. 2019;20(18):.
22. Li L, Mussack V, Pepeldjiyska E, et al. Description and optimization of a multiplex bead-based fl ow cytometry method (MBFCM) to characterize extracellular vesicles in serum samples from patients with hematological malignancies. 2022;(March):
23. Andrews S. FastQC - A quality control tool for high throughput sequence data. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/. *Babraham Bioinformatics*. 2010;
24. Kong Y. Btrim: A fast, lightweight adapter and quality trimming program for next-generation sequencing technologies. *Genomics*. 2011;98(2):.
25. The RNA Central Consortium. RNAcentral: a hub of information for non-coding RNA sequences The RNAcentral Consortium 1-38. *Nucleic Acids Res*. 2018;47:.
26. Kozomara A, Griffiths-Jones S. MiRBase: Annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res*. 2014;42(D1):.
27. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol*. 2009;10(3):.
28. Koliha N, Wiencek Y, Heider U, et al. A novel multiplex bead-based platform highlights the diversity of extracellular vesicles. *J Extracell Vesicles*. 2016;5(1):.
29. Tolba FM, Foda ME, Kamal HM, Elshabrawy DA. Expression of CD133 in acute leukemia. *Medical Oncology*. 2013;30(2):.
30. Mi S, Lu J, Sun M, et al. MicroRNA expression signatures accurately discriminate acute lymphoblastic leukemia from acute myeloid leukemia. *Proc Natl Acad Sci U S A*. 2007;104(50):.
31. Zhi Y, Xie X, Wang R, et al. Serum level of miR-10-5p as a prognostic biomarker for acute myeloid leukemia. *Int J Hematol*. 2015;102(3):296–303.
32. Gerloff D, Wurm AA, Hartmann J-U, et al. Next Generation Sequencing and Functional Analysis of Mirna Expression in Acute Myeloid Leukemia Patients with Different FLT3 Mutations: Block of MiR-155 in FLT3-ITD Driven AML Leads to Downregulation of Myeloid Blasts in Vivo. *Blood*. 2015;126(23):.

33. Caivano A, La Rocca F, Simeon V, et al. MicroRNA-155 in serum-derived extracellular vesicles as a potential biomarker for hematologic malignancies - a short report. *Cellular Oncology*. 2017;40(1):97–103.
34. Dunand-Sauthier I, Irla M, Carnesecchi S, et al. Repression of Arginase-2 Expression in Dendritic Cells by MicroRNA-155 Is Critical for Promoting T Cell Proliferation. *The Journal of Immunology*. 2014;193(4):.
35. Smyth LA, Boardman DA, Tung SL, Lechler R, Lombardi G. MicroRNAs affect dendritic cell function and phenotype. *Immunology*. 2015;144(2):197–205.
36. Zheng YS, Zhang H, Zhang XJ, et al. MiR-100 regulates cell differentiation and survival by targeting RBSP3, a phosphatase-like tumor suppressor in acute myeloid leukemia. *Oncogene*. 2012;31(1):80–92.
37. Krejcik Z, Belickova M, Hrustincova A, et al. MicroRNA profiles as predictive markers of response to azacitidine therapy in myelodysplastic syndromes and acute myeloid leukemia. *Cancer Biomarkers*. 2018;22(1):101–110.
38. Garcia-Orti L, Cristobal I, Marcotegui M, et al. Integration of global snp-based mapping and expression arrays with microrna patterns reveals deregulated mirnas and their gene candidate targets in acute myeloidleukemia. *Haematologica*. 2010;95:.
39. Chen Y, Jacamo R, Konopleva M, et al. CXCR4 downregulation of let-7a drives chemoresistance in acute myeloid leukemia. *Journal of Clinical Investigation*. 2013;123(6):2395–2407.
40. Pang B, Mao H, Wang J, Yang W. MiR-185-5p suppresses acute myeloid leukemia by inhibiting GPX1. *Microvasc Res*. 2022;140:.
41. Esa E, Hashim AK, Mohamed EHM, et al. Construction of a microRNA-mRNA Regulatory Network in de Novo Cytogenetically Normal Acute Myeloid Leukemia Patients. *Genet Test Mol Biomarkers*. 2021;25(3):.
42. Li Y, Zhang G, Wu B, Yang W, Liu Z. MiR-199a-5p Represses Protective Autophagy and Overcomes Chemoresistance by Directly Targeting DRAM1 in Acute Myeloid Leukemia. *J Oncol*. 2019;2019:.
43. Worst TS, Previti C, Nitschke K, et al. Mir-10a-5p and mir-29b-3p as extracellular vesicle-associated prostate cancer detection markers. *Cancers (Basel)*. 2020;12(1):.
44. Chettimada S, Lorenz DR, Misra V, Wolinsky SM, Gabuzda D. Small RNA sequencing of extracellular vesicles identifies circulating miRNAs related to inflammation and oxidative stress in HIV patients. *BMC Immunol*. 2020;21(1):1–20.
45. Nakamaru K, Tomiyama T, Kobayashi S, et al. Extracellular vesicles microRNA analysis in type 1 autoimmune pancreatitis: Increased expression of microRNA-21: miR-21 and type 1 AIP. *Pancreatology*. 2020;20(3):318–324.
46. Baumgart S, Meschkat P, Edelmann P, et al. MicroRNAs in tumor samples and urinary extracellular vesicles as a putative diagnostic tool for muscle-invasive bladder cancer. *J Cancer Res Clin Oncol*. 2019;145(11):2725–2736.
47. Ketprasit N, Cheng IS, Deutsch F, et al. The characterization of extracellular vesicles-derived microRNAs in Thai malaria patients. *Malar J*. 2020;19(1):1–14.
48. Wu Q, Yuan X, Li B, et al. Differential miRNA expression analysis of extracellular vesicles from brain microvascular pericytes in spontaneous hypertensive rats. *Biotechnol Lett*. 2020;42(3):389–401.

570 49. Zhang C, Shang Y, Chen X, et al. Supramolecular Nanofibers Containing Arginine-Glycine-
571 Aspartate (RGD) Peptides Boost Therapeutic Efficacy of Extracellular Vesicles in Kidney
572 Repair. *ACS Nano*. 2020;14(9):12133–12147.
573 50. Carnino JM, Lee H, He X, Groot M, Jin Y. Extracellular vesicle-cargo miR-185-5p reflects type
574 II alveolar cell death after oxidative stress. *Cell Death Discov*. 2020;6(1):.
575 51. Sun Y, Hou Y, Meng G, et al. Proteomic analysis and microRNA expression profiling of plasma-
576 derived exosomes in primary immune thrombocytopenia. *Br J Haematol*. 2021;194(6):1045–
577 1052.
578 52. Dostalova Merkerova M, Hrustincova A, Krejcik Z, et al. Microarray profiling defines
579 circulating microRNAs associated with myelodysplastic syndromes. *Neoplasma*. 2017;64(4):.
580 53. Howe CG, Foley HB, Farzan SF, et al. Urinary metals and maternal circulating extracellular
581 vesicle microRNA in the MADRES pregnancy cohort. *Epigenetics*. 2021;
582 54. Nakashima M, Ishikawa K, Fugiwara A, et al. miR-451a levels rather than human papillomavirus
583 vaccine administration is associated with the severity of murine experimental autoimmune
584 encephalomyelitis. *Sci Rep*. 2021;11(1):1–13.
585 55. Gámez-Valero A, Campdelacreu J, Vilas D, et al. Exploratory study on microRNA profiles from
586 plasma-derived extracellular vesicles in Alzheimer's disease and dementia with Lewy bodies.
587 *Transl Neurodegener*. 2019;8(1):1–17.
588

Figure 1 Characterization of serum derived EVs from H and AML samples by MBFCM

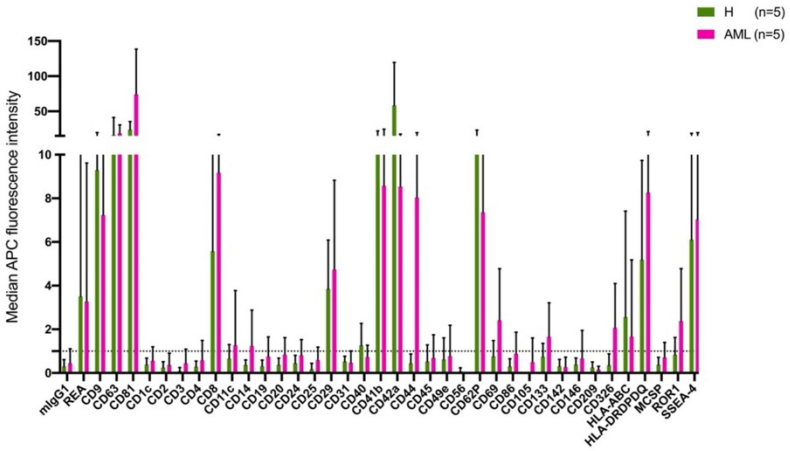


Figure 1 MBFCM allows the detection of EVs (co-) expressing 37 different antigens in a semi-quantitative manner. Results (median APC fluorescence intensities) detected by MBFCM in H (left side, A) and AML (right side, B) serum samples are displayed. Results are given as mean ± SD.

Figure 2 Quantification and comparison of EV surface marker expressions from serum derived EVs in H and AML samples with MBFCM

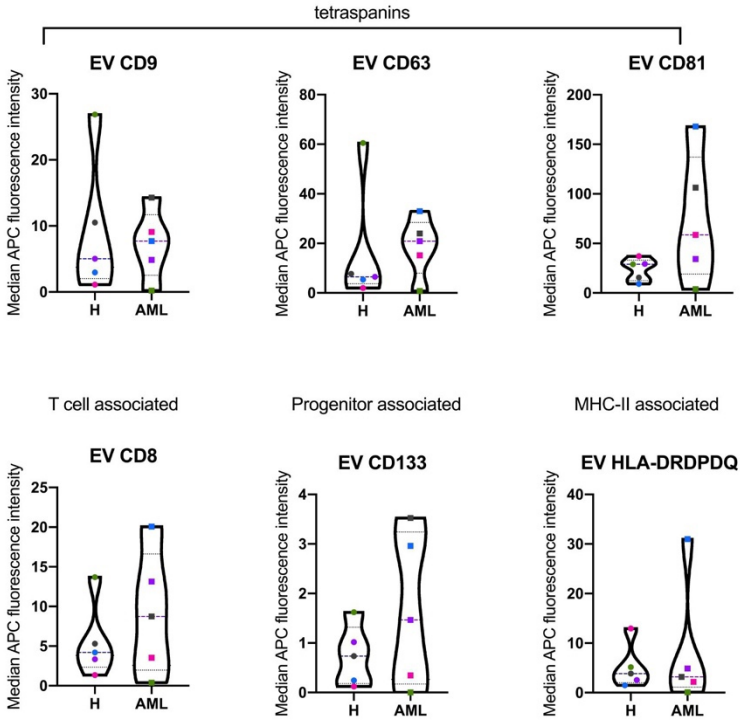
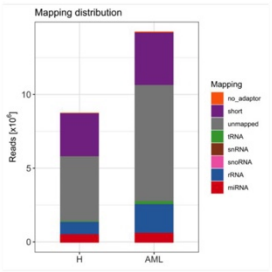


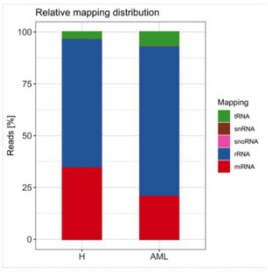
Figure 2 Serum samples were analyzed by multiplex bead-based flow cytometry (MBFCM). Selected several lineage associated EV marker expression on serum from 5 H and 5 AML samples are shown by violin plots (with all individual points) of median APC intensities. Longdash horizontal lines means 'median data', dotted horizontal line means 'quartile data'. For statistical comparison of two groups applying two-way analysis of variance (ANOVA) with Benjamini-Hochberg adjustments were analyzed.

Figure 3 Small RNA species in serum derived EVs of H and AML samples

A



B



C

Mapping distribution (RNA reads)	H	AML
no_adaptor	42,221	67,494
short	2,911,996	3,560,493
unmapped	4,407,416	7,879,508
rRNA	832,970	1,949,259
snRNA	2,136	4,556
snoRNA	1,054	2,955
tRNA	49,714	195,230
miRNA	462,566	557,484

D

Relative mapping distribution (RNA reads)	H serum	H (%)	AML serum	AML (%)
rRNA	832,970	61.77%	1,949,259	71.94%
snRNA	2,136	0.16%	4,556	0.17%
snoRNA	1,054	0.08%	2,955	0.11%
tRNA	49,714	3.69%	195,230	7.20%
miRNA	462,566	34.30%	557,484	20.58%

Figure 3 Assignment of small RNA sequencing results to different RNA species from isolated EVs in 5 H and 5 AML serum samples: A Reads mapping to miRNA, tRNA, snoRNA, snRNA and rRNA, (remained) unmapped, short (shorter than 16 nucleotides) and no-adapter (no adapter detectable). B Relative frequencies of reads that mapped to miRNA, tRNA, snoRNA, snRNA and rRNA (uncharacterized unmapped reads were excluded). C Mapping distribution of RNA reads. D Relative mapping distribution of RNA reads (uncharacterized unmapped reads were excluded).

Figure 4 Profiling and characterization of DESeq2-normalized miRNA data derived from EVs in H and AML serum samples

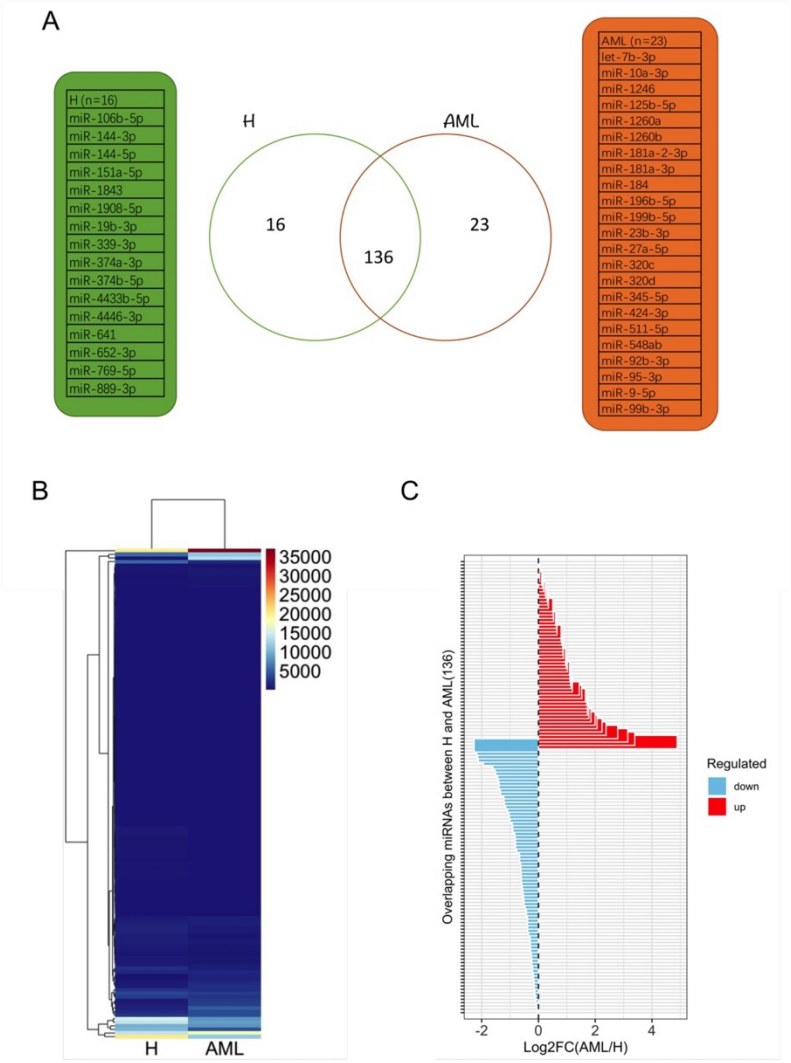


Figure 4 Various expression patterns of miRNAs with more than 20 DESeq2-normalized read counts derived from EVs in H and AML serum samples: A Venn diagram of distinct miRNA patterns detected in H and AML samples. B Heatmap and hierarchical cluster analysis of H from AML samples. C Log2 fold change (FC) of overlapping miRNAs (136) between H and AML samples.

Figure 5 Selected 10 DESeq2-normalized miRNAs derived from EVs in H and AML serum samples

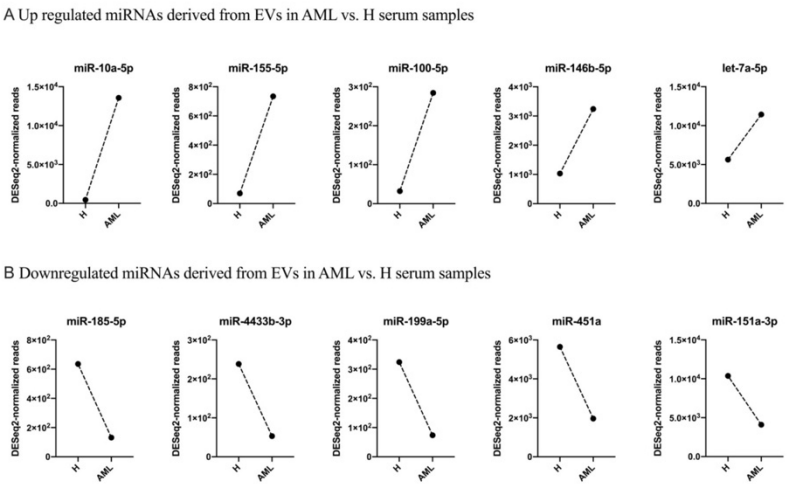


Figure 5 Given are up or down regulated miRNAs derived from EVs in AML vs. H serum samples: A 5 miRNAs (miR-10a-5p, miR-155-5p, miR-100-5p, miR-146b-5p, let-7a-5p) were up regulated. B 5 miRNAs (miR-185-5p, miR-4433b-3p, miR-199a-5p, miR-451a, miR-151a-3p) were down regulated.

Table 1 Patients' and healthy individuals' characteristics

	Pat.No.	Age/Sex	Dgn. subtypes	stage	IC Blasts (%)	blast phenotype (CD)	Risk Stratification[1]	WBC [G/l]	Hb [g/dl]	PLT [G/L]	Response to (induction)-chemotherapy
AML	1567	98/F	pAML	Dgn	16	**34**,**117**,**56**,**14**,15,65	Adverse	7.96	8.3	12	NCR
	1597	84/F	sAML	Dgn	72	**117**,**34**,**56**,65,15,33,13	Intermediate	88.6	11.3	41	NCR
	1609	72/M	sAML	Dgn	59	**117**,**34**,**56**,65,33	Favourable	10.04	9.5	114	CR
	1594	70/F	pAML/M4	Pers.	30	**34**,**117**,13,33,65	Favourable	1.32	6.8	125	NCR
	1598	61/F	sAML	Rel.	45	**117**,33,13	Adverse	19.6	8.3	15	NCR
H	1579	31/M	nd	nd	nd	nd	nd	nd	nd	nd	nd
	1580	24/F	nd	nd	nd	nd	nd	nd	nd	nd	nd
	1583	29/M	nd	nd	nd	nd	nd	nd	nd	nd	nd
	1585	30/M	nd	nd	nd	nd	nd	nd	nd	nd	nd
	1586	29/M	nd	nd	nd	nd	nd	nd	nd	nd	nd

Table 1 AML acute myeloid leukemia; H healthy; Pat. No. Patient's number; F female; M male; p primary AML; s secondary AML; CD Cluster of differentiation; dgn first diagnosis; Hb hemoglobin; IC Blast immune cytologically detected blasts; Bold markers used to quantify blasts; rel relapse; CR Complete remission; NCR no complete remission; pers. persisting disease; PLT platelets; WBC white blood cells; nd, no data. [1]AML patients were classified based on the National Comprehensive Cancer Network (NCCN) guidelines as "favorable", "intermediate", or "adverse risk".

Table 2 Cellular composition of H and AML peripheral blood samples

	Pat. No.	CD14+ expressing cells (%)	CD19+ expressing cells (%)	CD3+ expressing cells (%)	CD56+ expressing cells (%)
AML	P1567	28.18*	2.74	7.14	44.9*
	P1594	9.42	2.56	16.91	8.18
	P1597	6.02	1.32	9.29	64.58*
	P1598	3.81	5.81	12.68	7.18
	P1609	5.22	6.98	27.68	12*
H	1579	2.94	2.38	12.73	3.21
	1580	5.43	1.14	8.71	11.95
	1583	3.12	0.72	9.13	4.38
	1585	0.47	0.28	1.27	1.41
	1586	3.8	2.12	20.69	10.94

*(aberrant) expression of these markers on leukemic cells; AML acute myeloid leukemia; H healthy donors; Pat. No. Patient's number;

Table 3 Selected miRNAs involved in AML pathogenesis

	Cellular sources	Compared to Healthy (H)	Confirmed Targets	Targeted cells	Functional effect of expression		References
					OncomiR	Tumor suppressor	
miR-10a-5p	serum,BM,plasma	↑ in AML vs.H (FC=4.8); ↑MDS (plasma) vs.H;	FLT3-ITDNPM1, CEBPA, C-KIT, MDM4	nd	↑ cell survival,↑ cellular growth, and induce angiogenesis, protease inhibitors ↑ chemotherapy drug sensitivity of AML, ↑ pathogenesis of NPM1 mutated (NPM1mut), ↓ MDM4 downregulation	nd	7, 31, 32
miR-155-(5p)	BM, plasma, cell lines (KG1 and NB4)	↑CN-AML vs.H (normal haematopoietic cell);	FLT3-ITD, CEBPB, SHIP1, PU.1	T cells	↑ Proliferation, ↑ survival, overexpression leads to myeloproliferative neoplasm in mice, confers negative prognosis in CN-AML, no effect in MLL-AF9 mouse model of leukemia	nd	17, 32, 33, 34, 35
miR-100-(5p)	BM, plasma, cell lines	nd	RBSP3	nd	↑ Proliferation, ↑ survival, ↓ differentiation	↓ human granulocyte and monocyte differentiation, ↑ cell survival	36, 37, 38
miR-146b-5p	plasma	↑MDS (plasma) vs.H	SF3B1, DNMT3A	nd	nd	↓ Proliferation, ↓ survival, ↓ NF-kB activation, deletion leads to myeloproliferation	7
let-7a-5p	cell lines	nd	CXCR4	nd	nd	↓SDF-1α–mediated CXCR4 activation and ↑ by CXCR4 inhibition, ↓ c-Myc and BCL-XL protein, ↑ chemosensitivity	39
miR-185-5p	cell lines	nd	GPX1	nd	nd	↓ Proliferation, ↓ invasion, ↑ diffenrentiation and apoptosis	40, 41
miR-4433b-3p	nd	nd	nd	nd	nd	nd	nd
miR-199a-5p	BM, cell lines (KG1 and NB4)	↓ RR(BM) vs. CR	DRAM1	nd	nd	↑ inducible autophagy, ↑prosurvival, ↓drug sensitivity, ↑chemoresistance upon ADM	42
miR-451a	nd	nd	nd	nd	nd	nd	nd
miR-151a-3p	plasma	nd	SF3B1, DNMT3A	nd	nd	nd	7

Table 3 AML acute myeloid leukemia; H healthy donors; FC fold change; BM: bone marrow; AML-DS Down syndrome; PB peripheral blood; MDS myelodysplastic syndromes; LSC: leukemic stem-like cells; RR relapsed/refractory; CR complete remission; ↑ up regulated; ↓ down regulated; nd: no data;

Table 4 Selected extracellular miRNAs involved in tumor pathogenesis

EV-miRNA	Cellular sources	Compared to Healthy (H)	Confirmed Targets	Targeted cells	Functional effect of expression		References	Predicted change (serum)
					OncomiR	Tumor suppressor		
miR-10a-5p	MDS, plasma; PCa, cell lines, plasma; HIV, plasma; breast cancer, hepatocellular carcinoma	↑MDS (plasma derived EV) vs.H; ↑(p = 0.018) PCa (plasma derived EV) vs. H; ↑ (plasma derived EVs) HIV-positive vs. HIV-negative subjects	SF3B1, DNMT3A (MDS)	nd	↓cell apoptosis ↑ viability of AML cells in vivo and vitro, oncogene in leukemogenesis	involved in angiogenesis, transcription actin cytoskeleton, and ephrin receptor signaling	7, 43, 44	↑
miR-155-5p	HMs (AML, CLL,WM, MDS, MM, FL, DLBCL, HL), serum	↑AML, CLL, WM (serum derived EV) vs.H	nd	nd	nd	nd	33	↑
miR-100-5p	HucMSC; type 1 AIP, CP, serum	↑(p = 3.23) AIP (serum derived EV) vs. H	NOX4	nd	nd	↓ DOX-induced ROS, LDH, and MDA, ↓ SOD, ↑apoptotic cells, ↑ NOX4 and cleaved-caspase-3 protein expression	45	↑
miR-146b-5p	MDS,plasma; MIBC, tumor tissues and urinary EV;HIV, plasma	↑(FC = 7.84;P = 0.004) (urinary EVs) MIBC VS NMIBC patients; ↑(plasma derived EVs) HIV-positive vs. HIV-negative	SF3B1, DNMT3A (MDS)	nd	↓cell apoptosis ↑ viability of AML cells in vivo and vitro, oncogene in leukemogenesis	nd	7, 44, 46	↑
let-7a-5p	Brain microvascular pericytes from SHR; plasma of Thai P. vivax-infected patients	abnormally expressed in brain microvascular pericyte EVs of SHR vs. WKY rats.	TGF- β pathway	nd	nd	brain microvascular pericytes and the pathogenesis of hypertension	47, 48	↑
miR-185-5p	murine model (mimics human ARDS), cell lines	↑Hyperoxia vs. Room air, ↑ Mimics vs. Control	FADD/ caspase-8	nd	nd	↓FADD and caspase-8, ↑ necroptosis	50	↓
miR-4433b-3p	ITP, plasma	↓ ITP (plasma derived EV) vs. H	nd	nd	nd	nd	51	↓
miR-199a-5p	MDS, plasma; pregnancy	↓MDS (plasma derived EV) (p<0.05) vs. H	nd	nd	nd	nd	7, 52, 53	↓
miR-451a	MDS, plasma; BC, AD, DLB, murine EAE	↓MDS (plasma derived EV) (p<0.05) vs. H; ↑BC (serum derived EV) vs. H;↓ AD (plasma derived EV) vs. H	AKT1, CAB39 (BC)	nd	nd	involved in neuron differentiation-proliferation and death, genes related to SMAD	14, 52, 54	↓
miR-151-3p	MDS plasma; AD, plasma	↓ AD (plasma derived EV) vs. H	SF3B1, DNMT3A (MDS)	nd	↓ cell apoptosis ↑ viability of AML cells in vivo and vitro, oncogene in leukemogenesis	nd	7, 55	↓

Table 4 AML acute myeloid leukemia; CLL chronic lymphocytic leukemia; WM Waldenström's macroglobulinemia; MDS myelodysplastic syndrome; MM multiple myeloma, FL follicular lymphoma, DLBCL diffuse large B-cell lymphoma; HL Hodgkin's Lymphoma, H healthy donors; HucMSC human umbilical cord mesenchymal stem cells; AIP autoimmune pancreatitis; CP chronic pancreatitis; FC fold change; BM bone marrow; MIBC muscle-invasive bladder cancer; SHR spontaneous hypertensive rats; PCa prostate cancer; HIV human immunodeficiency virus; ARDS acute respiratory distress syndrome; ITP Immune thrombocytopenia; AD Alzheimer's disease; DLB dementia with Lewy bodies; BC breast cancer; EAE experimental autoimmune encephalomyelitis; ↑ up regulated;↓down regulated; nd no data;

Supplementary Table 136 DESeq2-normalized miRNAs detected in H and AML samples

	H	AML	Fold change (FC)	Log2FC
miR-10a-5p	458.8083	13568.8	29.57401462	4.886258195
miR-155-5p	69.89218	734.3148	10.50639422	3.393195718
miR-100-5p	32.12786	284.4989	8.855210192	3.146526552
miR-181b-5p	43.96444	305.0034	6.937501817	2.794416244
miR-139-5p	243.4953	1269.352	5.213045177	2.382126363
miR-10b-5p	643.6844	3041.704	4.725457784	2.240454099
miR-125a-5p	117.8021	493.3878	4.188275091	2.066356203
let-7b-5p	1238.895	4935.8	3.984033391	1.994229739
miR-378a-3p	46.21902	166.5985	3.604543371	1.849816508
let-7c-5p	82.29241	281.9359	3.426025417	1.776535854
miR-181a-5p	395.6799	1307.798	3.305191692	1.724733947
miR-99a-5p	629.5933	2078.636	3.301554208	1.723145334
miR-30a-3p	46.21902	152.5017	3.299543547	1.722266459
miR-941	55.23737	179.4137	3.248050071	1.699573873
miR-146b-5p	1030.346	3241.622	3.146148719	1.653586869
miR-222-3p	155.0028	446.6121	2.88131569	1.526727737
miR-150-5p	108.7838	294.7511	2.709513271	1.438033714
miR-30a-5p	199.5309	456.8643	2.289691794	1.195153416
miR-27a-3p	851.1064	1890.252	2.220934899	1.151167105
let-7d-3p	49.03726	107.6482	2.195233841	1.134374627
miR-197-3p	22.54586	48.69801	2.159953297	1.111000118
miR-122-5p	1869.616	4035.528	2.158479581	1.110015445
let-7e-5p	136.9661	292.1881	2.133287207	1.09307821
let-7a-5p	5637.03	11428.66	2.027424995	1.019648542
miR-29a-3p	69.89218	137.7641	1.971094902	0.978997239
miR-320b	51.29184	99.31832	1.936337542	0.953330465
miR-99b-5p	387.2252	749.6931	1.93606478	0.953127225
miR-126-3p	20127.26	36998.32	1.838219516	0.87830906
miR-181d-5p	21.41857	39.08656	1.82489129	0.867810524
miR-342-5p	25.3641	46.13496	1.81890804	0.863072605
miR-139-3p	31.56421	57.02794	1.806727852	0.853379209
miR-1-3p	47.90996	84.58076	1.765410744	0.820003883
miR-361-3p	101.4564	176.2099	1.736804552	0.796435412
miR-223-5p	321.2786	557.4641	1.735142538	0.795054182
miR-629-5p	43.96444	69.20244	1.574055034	0.654485983
miR-30c-5p	433.4442	676.6461	1.561091425	0.642555031
miR-335-3p	80.03782	124.3081	1.5531169	0.635166423
miR-543	73.83771	112.1336	1.518649365	0.60278881
miR-342-3p	27.05504	39.08656	1.444705604	0.530775537
miR-92a-3p	2735.377	3919.549	1.432910117	0.518948116
miR-532-5p	98.63816	140.9679	1.429142031	0.515149302
miR-192-5p	132.457	187.7437	1.417393765	0.503240608
miR-148a-3p	13972.8	17839.49	1.27673	0.35245346
miR-16-2-3p	117.8021	148.0163	1.256482527	0.329390609
miR-320a-3p	941.2898	1162.345	1.234842509	0.304327053
miR-25-3p	865.7612	1031.629	1.191586077	0.252883172
miR-23a-3p	129.0751	152.5017	1.181495943	0.240614675
miR-625-3p	80.03782	90.9884	1.136817525	0.1850007
miR-486-3p	20.85492	23.06748	1.106092727	0.145472336
miR-140-3p	217.5676	235.1601	1.080860185	0.112179915
miR-194-5p	23.67316	24.34901	1.028549189	0.040610791
miR-203a-3p	105.4019	108.289	1.027391239	0.038985676
miR-20a-5p	157.2574	160.1908	1.018653696	0.026663673
miR-1301-3p	29.30962	29.47511	1.005646272	0.008122938
miR-32-5p	33.8188	33.96046	1.004188813	0.006030559
miR-24-2-5p	31.00056	30.75664	0.992131658	-0.011396513
miR-7-5p	236.7316	231.9563	0.979828438	-0.029398931
miR-382-5p	47.90996	46.77572	0.976325639	-0.034565677
miR-142-5p	645.3754	627.9481	0.972996659	-0.039493244
miR-19a-3p	21.41857	20.50443	0.957320021	-0.062926814
miR-3615	46.78267	44.21267	0.945065171	-0.081514275
miR-340-3p	49.6009	46.13496	0.930123429	-0.104505917
miR-381-3p	151.0573	139.0456	0.920482846	-0.119537258
miR-363-3p	145.4208	133.2788	0.916504051	-0.125786837
miR-3168	82.85605	74.96931	0.904813948	-0.144306925
miR-22-5p	38.32797	34.60122	0.902766858	-0.147574639
miR-423-5p	2535.846	2281.118	0.899548875	-0.152726426
miR-22-3p	313.9512	277.4505	0.88373786	-0.178309603

miR-140-5p	37.76432	33.31969	0.882306139	-0.180648772
miR-409-3p	209.1129	181.336	0.867168085	-0.205616434
miR-191-5p	1732.65	1500.027	0.865741648	-0.207991531
miR-30d-5p	3055.528	2598.936	0.850568508	-0.233500655
miR-423-3p	1189.858	1011.765	0.85032443	-0.233914708
miR-183-5p	30.43692	25.63053	0.842087055	-0.247958707
miR-143-3p	564.2103	473.5241	0.839268882	-0.252795005
miR-495-3p	31.00056	25.63053	0.826776382	-0.274430919
let-7f-5p	9902.707	8147.306	0.822735206	-0.281499916
let-7g-5p	3569.01	2923.803	0.819219578	-0.287677902
miR-186-5p	206.2947	168.5208	0.816893467	-0.291780149
miR-224-5p	77.78323	63.43557	0.815543007	-0.294167137
miR-30e-3p	379.8978	299.2365	0.787676237	-0.344325342
let-7d-5p	640.3026	494.0285	0.771554852	-0.37415937
miR-27b-3p	810.5238	624.1035	0.770000187	-0.377069299
miR-128-3p	772.1959	592.0653	0.766729484	-0.383210437
miR-28-3p	214.1857	164.0354	0.765856017	-0.384854908
miR-330-3p	29.30962	22.42672	0.765165642	-0.386156001
miR-223-3p	355.661	265.9168	0.747669212	-0.419527969
miR-101-3p	915.3621	683.0537	0.746211503	-0.422343495
miR-17-5p	91.8744	66.63939	0.725331427	-0.463287736
miR-126-5p	195.0217	137.1234	0.703118353	-0.508160541
miR-379-5p	135.8388	95.47374	0.70284569	-0.508720116
miR-134-5p	52.41913	36.52351	0.696759128	-0.521268097
miR-15b-5p	45.65538	31.3974	0.687704429	-0.540139459
miR-28-5p	53.54643	36.52351	0.682090515	-0.551964894
miR-654-3p	44.52808	30.11588	0.676334477	-0.564191197
miR-127-3p	64.25571	42.93114	0.668129598	-0.581800124
miR-26b-5p	1087.274	718.9365	0.661228234	-0.596779768
miR-493-3p	74.965	49.33878	0.658157514	-0.603495195
miR-24-3p	1299.205	851.5745	0.655457913	-0.609424946
miR-411-5p	45.09173	29.47511	0.653670077	-0.613365439
miR-425-5p	293.6599	191.5882	0.652415432	-0.616137188
miR-484	72.14677	46.77572	0.648341245	-0.625174741
miR-1307-3p	327.4787	206.9666	0.632000104	-0.662003299
miR-21-5p	13918.69	8789.351	0.631478334	-0.663194858
miR-30b-5p	114.9839	72.40626	0.629707747	-0.66724568
miR-221-3p	603.6655	355.6237	0.589107121	-0.763398104
miR-26a-5p	20441.77	11699.7	0.572342658	-0.805048956
miR-486-5p	1334.152	762.5084	0.571530568	-0.807097433
miR-30e-5p	459.9356	262.0722	0.569801921	-0.811467609
miR-182-5p	95.81992	54.46488	0.568408762	-0.8149993
miR-432-5p	44.52808	24.98977	0.561213715	-0.833377829
miR-142-3p	361.2975	194.1513	0.537372402	-0.896005863
let-7i-5p	15391.5	8190.878	0.532168983	-0.910043668
miR-328-3p	139.7844	73.68778	0.527153288	-0.92370556
miR-98-5p	353.9701	177.4914	0.50143066	-0.995877883
miR-370-3p	217.5676	107.0075	0.491835561	-1.023752045
miR-374a-5p	82.85605	40.36809	0.487207511	-1.037391721
miR-106b-3p	168.5303	79.45465	0.471456097	-1.084804664
miR-146a-5p	2130.021	958.582	0.450034141	-1.151893644
miR-199a-3p=miR-199b-3p	1030.346	449.8159	0.43656778	-1.195722435
miR-16-5p	200.0945	86.50305	0.43231089	-1.209858917
miR-93-5p	135.8388	58.30946	0.42925475	-1.220093996
miR-340-5p	371.4431	147.3756	0.396764842	-1.333643904
miR-151a-3p	10364.9	4091.915	0.394785835	-1.34085787
miR-152-3p	107.0929	41.00885	0.382928008	-1.384854908
miR-339-5p	56.92831	21.78595	0.382691048	-1.385747942
miR-493-5p	100.3291	37.80504	0.376810303	-1.408089682
miR-744-5p	564.2103	208.2481	0.369096599	-1.437929651
miR-11400	121.7477	42.93114	0.352623954	-1.503797612
miR-451a	5646.612	1961.377	0.347354606	-1.525518867
miR-103a-3p	632.4115	207.6073	0.328278857	-1.607006258
miR-584-5p	302.6782	78.81389	0.260388372	-1.941263073
miR-148b-3p	624.5204	144.1718	0.230851934	-2.114960275
miR-199a-5p	324.0968	73.68778	0.227363505	-2.136927395
miR-4433b-3p	238.4225	53.18336	0.223063486	-2.164473722
miR-185-5p	635.7934	131.9972	0.207610293	-2.268050125

7. Literaturverzeichnis

1. Dong, Y. *et al.* Leukemia incidence trends at the global, regional, and national level between 1990 and 2017. *Exp Hematol Oncol* **9**, (2020).
2. Siegel, R. L., Miller, K. D. & Jemal, A. Cancer statistics, 2016. *CA Cancer J Clin* **66**, (2016).
3. Yosifov, D. Y., Wolf, C., Stilgenbauer, S. & Mertens, D. From biology to therapy: The CLL success story. *Hemasphere* **3**, e175 (2019).
4. Hwang, S. M. Classification of acute myeloid leukemia. *Blood Research* vol. 55 Preprint at https://doi.org/10.5045/br.2020.S001 (2020).
5. Kolitz, E. Overview of acute myeloid leukemia in adults. *UptoDate* **10**, (2019).
6. Aldoss, I., Forman, S. J. & Pullarkat, V. Acute lymphoblastic leukemia in the older adult. *Journal of Oncology Practice* vol. 15 Preprint at https://doi.org/10.1200/JOP.18.00271 (2019).
7. Inaba, H. & Pui, C. H. Advances in the diagnosis and treatment of pediatric acute lymphoblastic leukemia. *J Clin Med* **10**, (2021).
8. Crompot, E. *et al.* Extracellular vesicles of bone marrow stromal cells rescue chronic lymphocytic leukemia B cells from apoptosis, enhance their migration and induce gene expression modifications. *Haematologica* **102**, 1594–1604 (2017).
9. Liu, D. & Zhao, J. Frontline therapies for untreated chronic lymphoid leukemia. *Experimental Hematology and Oncology* vol. 8 Preprint at https://doi.org/10.1186/s40164-019-0139-8 (2019).
10. Moussa Agha, D. *et al.* Impact of Bone Marrow miR-21 Expression on Acute Myeloid Leukemia T Lymphocyte Fragility and Dysfunction. *Cells* **9**, 1–18 (2020).
11. Davidson-Moncada, J., Viboch, E., Church, S. E., Warren, S. E. & Rutella, S. Dissecting the immune landscape of acute myeloid leukemia. *Biomedicines* vol. 6 Preprint at https://doi.org/10.3390/biomedicines6040110 (2018).
12. Vago, L. & Gojo, I. Immune escape and immunotherapy of acute myeloid leukemia. *Journal of Clinical Investigation* **130**, (2020).
13. Tanchot, C. *et al.* Tumor-infiltrating regulatory T cells: Phenotype, role, mechanism of expansion in situ and clinical significance. *Cancer Microenvironment* **6**, (2013).
14. Toffalori, C. *et al.* Immune signature drives leukemia escape and relapse after hematopoietic cell transplantation. *Nat Med* **25**, (2019).
15. Dougé, A., Bay, J. O., Ravinet, A. & Scanzi, J. Intestinal microbiota and allogeneic stem cell transplantation. *Bulletin du Cancer* vol. 107 Preprint at https://doi.org/10.1016/j.bulcan.2019.08.014 (2020).
16. Greil, C., Engelhardt, M., Finke, J. & Wäsch, R. Allogeneic stem cell transplantation in multiple myeloma. *Cancers* vol. 14 Preprint at https://doi.org/10.3390/cancers14010055 (2022).
17. Müller, K. *et al.* Chimeric antigen receptor T-cell therapy. *Ugeskrift for laeger* vol. 181 Preprint at https://doi.org/10.3904/kjm.2022.97.4.229 (2019).

18. Li, L. *et al.* Chimeric Antigen Receptor T-Cell Therapy in Glioblastoma: Current and Future. *Frontiers in Immunology* vol. 11 Preprint at https://doi.org/10.3389/fimmu.2020.594271 (2020).

19. Reyes, C. *et al.* Cost of Disease Progression in Patients with Chronic Lymphocytic Leukemia, Acute Myeloid Leukemia, and Non-Hodgkin's Lymphoma. *Oncologist* **24**, (2019).

20. Kremser, A. *et al.* Dendritic cells (DCs) can be successfully generated from leukemic blasts in individual patients with AML or MDS: An evaluation of different methods. *Journal of Immunotherapy* **33**, 185–199 (2010).

21. Schmetzer, H. M., Kremser, A., Loibl, J., Kroell, T. & Kolb, H. J. Quantification of ex vivo generated dendritic cells (DC) and leukemia-derived DC contributes to estimate the quality of DC, to detect optimal DC-generating methods or to optimize DC-mediated T-cell-activation-procedures ex vivo or in vivo [16]. *Leukemia* vol. 21 Preprint at https://doi.org/10.1038/sj.leu.2404639 (2007).

22. Pepeldjiyska, E. *et al.* Leukemia derived dendritic cell (DCleu) mediated immune response goes along with reduced (leukemia-specific) regulatory T-cells. *Immunobiology* **227**, (2022).

23. Klauer, L. K. *et al.* Interferon Gamma Secretion of Adaptive and Innate Immune Cells as a Parameter to Describe Leukaemia-Derived Dendritic-Cell-Mediated Immune Responses in Acute Myeloid Leukaemia in vitro. *Transfusion Medicine and Hemotherapy* **49**, (2022).

24. Plett, C. *et al.* Immunomodulatory kits generating leukaemia derived dendritic cells do not induce blast proliferation ex vivo: IPO-38 as a novel marker to quantify proliferating blasts in acute myeloid leukaemia. *Clinical Immunology* **242**, 109083 (2022).

25. Ansprenger, C., Amberger, D. C. & Schmetzer, H. M. Potential of immunotherapies in the mediation of antileukemic responses for patients with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) - With a focus on Dendritic cells of leukemic origin (DCleu). *Clinical Immunology* **217**, 1–6 (2020).

26. Freudenreich, M. *et al. In Vitro Generated Dendritic Cells of Leukemic Origin Predict Response to Allogeneic Stem Cell Transplantation in Patients With AML and MDS*. http://links.lww.com/JIT/A651 (2021).

27. Vogt, V. *et al.* Profiles of activation, differentiation-markers, or β-integrins on T cells contribute to predict T cells' antileukemic responses after stimulation with leukemia-derived dendritic cells. *Journal of Immunotherapy* **37**, 331–347 (2014).

28. Boeck, C. L. *et al.* Significance of Frequencies, Compositions, and/or Antileukemic Activity of (DC-stimulated) Invariant NKT, NK and CIK Cells on the Outcome of Patients with AML, ALL and CLL. *Journal of Immunotherapy* **40**, 224–248 (2017).

29. Amberger, D. C. *et al.* PGE1-containing protocols generate mature (leukemia-derived) dendritic cells directly from leukemic whole blood. *Int J Mol Sci* **20**, (2019).

30. Skotland, T., Sagini, K., Sandvig, K. & Llorente, A. An emerging focus on lipids in extracellular vesicles. *Advanced Drug Delivery Reviews* vol. 159 Preprint at https://doi.org/10.1016/j.addr.2020.03.002 (2020).

31. Visentin, E. C., Pogue, R. E., Rosa, T. & Marra, B. Extracellular Vesicle Influence on Wound Healing. *J Endocr Soc* **5**, (2021).

32. Lindenbergh, M. F. S. & Stoorvogel, W. Antigen Presentation by Extracellular Vesicles from Professional Antigen-Presenting Cells. *Annual Review of Immunology* vol. 36 Preprint at https://doi.org/10.1146/annurev-immunol-041015-055700 (2018).

33. Raju, D. *et al.* Detection of Cancer Exosomes By a Localized Surface Plasmon Resonance Method Using Gold Nano-Islands. *ECS Meeting Abstracts* **MA2020-01**, (2020).

34. Buzás, E. I., Tóth, E., Sódar, B. W. & Szabó-Taylor, K. Molecular interactions at the surface of extracellular vesicles. *Seminars in Immunopathology* vol. 40 Preprint at https://doi.org/10.1007/s00281-018-0682-0 (2018).

35. Gargiulo, E., Morande, P. E., Largeot, A., Moussay, E. & Paggetti, J. Diagnostic and Therapeutic Potential of Extracellular Vesicles in B-Cell Malignancies. *Frontiers in Oncology* vol. 10 Preprint at https://doi.org/10.3389/fonc.2020.580874 (2020).

36. Nehrbas, J., Butler, J. T., Chen, D. W. & Kurre, P. Extracellular Vesicles and Chemotherapy Resistance in the AML Microenvironment. *Frontiers in Oncology* vol. 10 Preprint at https://doi.org/10.3389/fonc.2020.00090 (2020).

37. Tkach, M. *et al.* Qualitative differences in T-cell activation by dendritic cell-derived extracellular vesicle subtypes. *EMBO J* **36**, 3012–3028 (2017).

38. Shao, H. *et al.* New Technologies for Analysis of Extracellular Vesicles. *Chemical Reviews* vol. 118 Preprint at https://doi.org/10.1021/acs.chemrev.7b00534 (2018).

39. Hao, S., Bai, O., Yuan, J., Qureshi, M. & Xiang, J. Dendritic cell-derived exosomes stimulate stronger CD8+ CTL responses and antitumor immunity than tumor cell-derived exosomes. *Cell Mol Immunol* **3**, (2006).

40. Kleindienst, P. & Brocker, T. Endogenous Dendritic Cells Are Required for Amplification of T Cell Responses Induced by Dendritic Cell Vaccines In Vivo. *The Journal of Immunology* **170**, (2003).

41. Divito, S. J. *et al.* Endogenous dendritic cells mediate the effects of intravenously injected therapeutic immunosuppressive dendritic cells in transplantation. *Blood* **116**, (2010).

42. Morelli, A. E. & Thomson, A. W. Tolerogenic dendritic cells and the quest for transplant tolerance. *Nature Reviews Immunology* vol. 7 Preprint at https://doi.org/10.1038/nri2132 (2007).

43. Albert, M. L., Sauter, B. & Bhardwaj, N. Dendritic cells acquire antigen from apoptotic cells and induce class I- restricted CTLS. *Nature* **392**, (1998).

44. Théry, C. *et al.* Indirect activation of naïve CD4+ T cells by dendritic cell-derived exosomes. *Nat Immunol* **3**, 1156–1162 (2002).

45. Morelli, A. E. *et al.* Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells. *Blood* **104**, (2004).

46. Montecalvo, A. *et al.* Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. *Blood* **119**, 756–766 (2012).

47. Théry, C. *et al.* Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles* **7**, 1535750 (2018).

48. Buschmann, D. *et al.* Evaluation of serum extracellular vesicle isolation methods for profiling miRNAs by next-generation sequencing. *J Extracell Vesicles* **7**, (2018).

49. Mussack, V., Wittmann, G. & Pfaffl, M. W. Comparing small urinary extracellular vesicle purification methods with a view to RNA sequencing—Enabling robust and non-invasive biomarker research. *Biomol Detect Quantif* **17**, (2019).

50. Hornick, N. I. *et al.* Serum Exosome MicroRNA as a minimally-invasive early biomarker of AML. *Sci Rep* **5**, (2015).

51. Wiklander, O. P. B. *et al.* Systematic methodological evaluation of a multiplex bead-based flow cytometry assay for detection of extracellular vesicle surface signatures. *Front Immunol* **9**, (2018).

52. Görgens, A. *et al.* Optimisation of imaging flow cytometry for the analysis of single extracellular vesicles by using fluorescence-tagged vesicles as biological reference material. *J Extracell Vesicles* **8**, (2019).

53. Riolo, G., Cantara, S., Marzocchi, C. & Ricci, C. miRNA targets: From prediction tools to experimental validation. *Methods and Protocols* vol. 4 Preprint at https://doi.org/10.3390/mps4010001 (2021).

54. Hrustincova, A. *et al.* Circulating Small Noncoding RNAs Have Specific Expression Patterns in Plasma and Extracellular Vesicles in Myelodysplastic Syndromes and Are Predictive of Patient Outcome. *Cells* **9**, (2020).

55. Wallace, J. A. & O'Connell, R. M. MicroRNAs and acute myeloid leukemia: Therapeutic implications and emerging concepts. *Blood* **130**, 1290–1301 (2017).

56. Huan, J. *et al.* RNA trafficking by acute myelogenous leukemia exosomes. *Cancer Res* **73**, 918–929 (2013).

57. Umezu, T., Ohyashiki, K., Kuroda, M. & Ohyashiki, J. H. Leukemia cell to endothelial cell communication via exosomal miRNAs. *Oncogene* **32**, (2013).

58. Boyiadzis, M. & Whiteside, T. L. The emerging roles of tumor-derived exosomes in hematological malignancies. *Leukemia* vol. 31 Preprint at https://doi.org/10.1038/leu.2017.91 (2017).

59. Peng, D. *et al.* MiR-34c-5p promotes eradication of acute myeloid leukemia stem cells by inducing senescence through selective RAB27B targeting to inhibit exosome shedding. *Leukemia* **32**, 1180–1188 (2018).

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