The diagnostic potentials of extracellular vesicles in pediatric acute myeloid leukemia

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ABSTRACT

Pediatric Acute Myeloid Leukemia (AML) has a high relapse rate of >30%. Therefore, new methods are needed to precisely measure the mutational changes between the different stages of the disease, which could provide useful information regarding the disease's progress. Extracellular vesicles (EVs) are released by both healthy and malignant cells. Due to their abilities to carry and transfer information of the parental cells, as well as to mediate changes in the microenvironment, their mutational profile can serve as a valuable diagnostic tool.

In this thesis, we aimed to establish a new detection method for AML-specific mutations in plasma-derived EV-RNA and EV-double stranded DNA (dsDNA) from primary pediatric AML samples. For this purpose, ultracentrifugation was performed to obtain EVs from the plasma of 29 pediatric AML patients at different time points of the disease, starting at the stage of diagnosis and during therapy. Afterwards, mutational analysis for the AML-specific mutations was performed using next generation sequencing (NGS) and GeneScan-based fragment-length analysis. NPM1 and FLT3-ITD mutations were detectable in the EV-RNAs of before-treatment samples, where RT-PCR and GeneScan-based fragment-length analysis were performed, respectively. The outcome was similar to the results of the mutational analysis we obtained from the genomic DNA (gDNA) of the same samples, supporting a potential use of EV-RNAs in pediatric AML diagnostics. However, the same results were not observed in the analysis of the after-treatment samples, implying an issue of reduced sensitivity. The mutational analysis of the EV-dsDNA from the initial samples mirrored the same AML-specific mutations found in the gDNA. Nonetheless, the mutations in the majority of the after-treatment samples were undetectable, suggesting again the limitation of low sensitivity. Finally, the highlight of the study was that the mutational background appeared to play an important role in the levels of the EV-RNA and EV-dsDNA as well as in the number of EVs. In conclusion, our findings support the potential of using EV-RNA and EV-dsDNA as diagnostic tools in complementation with the already existing clinical methods, leading to a more comprehensive analysis and monitoring of pediatric AML.

ZUSAMMENFASSUNG

Die akute myeloische Leukämie (AML) im Kindesalter hat mit >30 % eine hohe Rezidivrate. Deshalb sind neue Methoden notwendig, die die mutationsbedingten Änderungen zwischen den verschiedenen Stadien der Erkrankungen präzise messen und so nützliche Aussagen über den Progress der Erkrankung bereitstellen können. Extrazelluläre Vesikel (EVs) werden sowohl von gesunden als auch von malignen Zellen freigesetzt. Aufgrund ihrer Fähigkeit, Informationen ihrer Ursprungszellen zu tragen und zu übertragen sowie Veränderungen in der Mikroumgebung zu vermitteln, könnte ihr Mutationsprofil als wertvolles diagnostisches Hilfsmittel dienen.

In dieser Arbeit sollte eine neue Nachweismethode für AML-spezifische Mutationen in aus Plasma gewonnener EV-RNA und doppelsträngiger EV-DNA (dsDNA) aus initialen pädiatrischen AML Proben zu etablieren. Zu diesem Zweck, führten wir zur Gewinnung der EVs eine Ultrazentrifugation von Plasmaproben durch, die von 29 pädiatrischen AML Patienten zu unterschiedlichen Erkrankungszeitpunkten, beginnend im Stadium der Diagnose und während der Therapie, stammten. Im Anschluss wurde die Analyse auf AML-spezifische Mutationen mittels Next Generation Sequencing (NGS) und der GeneScan-basierten Fragmentlängen Analyse durchgeführt. NPM1 und FLT3-ITD Mutationen ließen sich in der EV-RNA der initialen Proben detektieren, wofür wir die Verfahren der RT-PCR und der GeneScan-basierten Fragmentlängen Analyse nutzten. Diese Ergebnisse glichen denen der Mutationsanalysen, welche wir zuvor mit der genomischen DNA (gDNA) derselben Proben durchgeführt hatten, was den potenziellen Nutzen der EV-RNA in der pädiatrischen AML-Diagnostik unterstützt. Allerdings konnten wir diese Ergebnisse in der Analyse der Proben nach Therapiebeginn nicht beobachten, was auf eine mangelnde Sensitivität hindeutet. Die Mutationsanalyse der EV-dsDNA der initialen Proben wies ebenso die gleichen AML-spezifischen Mutationen auf, welche zuvor in der gDNA gefunden wurden. Nichtsdestotrotz waren die Mutationen in dem Großteil der Proben nach Therapiebeginn nicht detektierbar, was erneut darauf hinweist, dass diese Methode aufgrund der geringen Sensitivität begrenzt ist. Allerdings spielt der Mutationshintergrund offenbar eine wichtige Rolle bei den Niveaus der EV-RNA und EV-dsDNA sowie bei der Anzahl der EVs. Zusammenfassend lässt sich sagen, dass unsere Ergebnisse das Potenzial der Verwendung von EV-RNA und EV-dsDNA als diagnostische Hilfsmittel in Ergänzung zu den bereits bestehenden klinischen Methoden unterstützen, was zu einer umfassenderen Analyse und Überwachung der pädiatrischen AML führt.

1. INTRODUCTION

1.1 Blood Cells and Stem Cells

Every blood cell that establishes the premise of the life form and homeostasis is derived from the hematopoietic compartment *(Figure 1).* The primary classifications of these cells are red blood cells, white blood cells, and platelets (Orkin and Zon 2008). Hematopoietic Stem Cells (HSCs), which are a definitive source of all types of blood cells, can be found in the adult bone marrow (Orkin and Zon 2008), activated fringe blood (Korbling and Anderlini 2001), the umbilical cord (Newcomb, Willing, and Sanberg 2009) and the fetal liver (Manesia et al. 2015). The life expectancy of blood cells is limited and they should be renewed consistently; their steady replenishment relies upon the self-renewal, separation and development capability of HSCs.

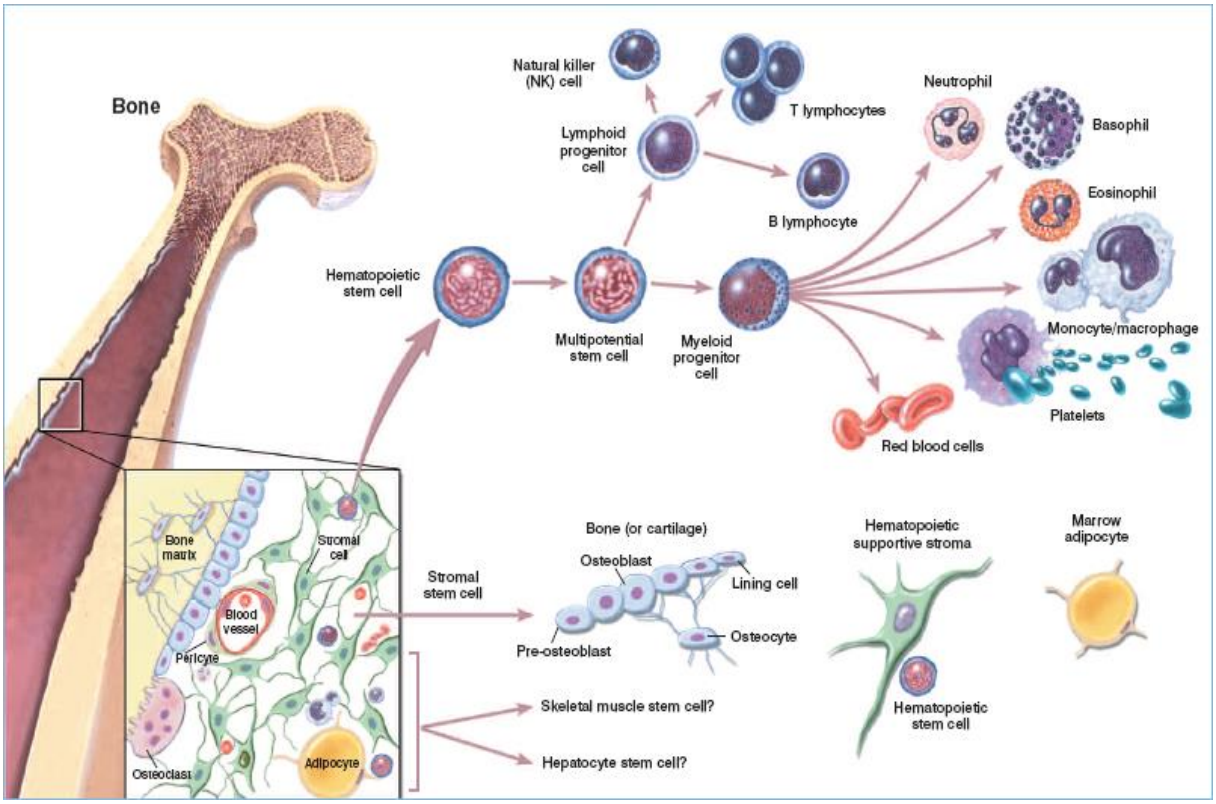

Figure 1: Differentiation of hematopoietic and stromal stem cells. © 2001 Terese Winslow (assisted by Lydia Kibiuk)

No other cell type displays the unique properties of HSCs (Domen and Weissman 1999). The generation of multi-lineage and uni-lineage progenitor cells during hematopoiesis is controlled by an ancestral HSC. In the last strides of this procedure, there are increasingly mature cells, which are progressively confined with respect to their differentiation ability.

By the end of the procedure, these matured cells are completely differentiated functional blood cells. The capacity of HSCs to experience self-renewal is the key property that makes them exceptional and effectively recognizable from their downstream progenitors (Dick 2003). That capacity is reflected in the daughter cells which possess the very similar stem cell properties of the parent cell. Contingent upon the prerequisite of the blood cells that ought to be delivered, HSC self-renewal is kept up by either symmetrical or asymmetrical division. Symmetrical self-renewal produces two indistinguishable daughter HSCs; while in asymmetrical self-renewal, one daughter cell is indistinguishable from the HSC and the one is constrained with respect to self-renewal (Greenwood and Lansdorp 2003).

A complicated system of growth factors and cytokines drives and guides the differentiation of HSCs into progenitors and mature cells (Zhang and Lodish 2008). After separation, mature cells acquire unique attributes, and as an outcome, the whole plethora of cells that are significant for the support of consistent blood production are delivered, which prompts the foundation of clonal hierarchy (Dick 2003).

The important mechanism of apoptosis regulates proliferation and differentiation, which is a way for cells to control their number without any other obliteration. It has been demonstrated that HSCs could avoid apoptosis under the bearing of two signals (Domen and Weissman 2000). Deregulation of this mechanism brings about malignancies like lymphoma or the inception and advancement of primary AML (Smith 1990), because of the proliferative ability of these cells (Greim et al. 2014).

1.2 Pediatric Acute Myeloid Leukemia

High tumor cell proliferation and a predominance of blast cells characterize most acute pediatric leukemia cases (Creutzig et al. 2012). Over 90% of pediatric leukemia is acute, mostly acute lymphoblastic leukemia. The remaining 10% includes chronic myelogenous leukemia (CML) and juvenile myelomonocytic leukemia (JMML), which belong to the category of chronic and/or subacute myeloproliferative disorders. Myelodysplastic disorders (MDS) in children represent under 5% of myeloid malignancies (Szalontay and Shad 2014). Pediatric acute myeloid leukemia

represents ~20% and is generally heterogeneous (Ries et al. 1999). Because of a superior comprehension of etiology and risk factors, the advancement of novel therapeutic strategies, classification of prognostic markers and accentuation on the significance of close follow-ups, the outcome of pediatric AML has been improved fundamentally over the last decades (Szalontay and Shad 2014).

1.3 Etiology of pediatric AML

In the majority of pediatric AML the cause of leukemogenesis is unknown. In about 7% an individual can be predisposed to pediatric AML by a number of risk factors, which include congenital abnormalities, environmental factors, cytogenetics, and gene mutations.

Congenital Abnormalities

Inherited diseases and syndromes, which have been proved to be related to AML are: Kleinefelter syndrome, Li-Fraumeni syndrome, Fanconi anemia, and neurofibromatosis (Potzsch, Voigtlander, and Lubbert 2002). A 10 to 20 fold increased likelihood of developing acute leukemia has been shown in children with Down syndrome (Fong and Brodeur 1987).

Environmental Factors

Exposure to ionizing radiation in utero has been associated with pediatric AML (Doll and Wakeford 1997). An increased risk for the development of AML is shown in chronic exposure to certain chemicals (Savitz and Andrews 1997). Parents exposed to benzene (Magnani et al. 1990), parental smoking (Rudant et al. 2008), use of antibiotics during pregnancy (Kaatsch, Scheidemann-Wesp, and Schuz 2010) and consumption of DNA topoisomerase II inhibitors in maternal diet (Spector et al. 2005) increase the risk of developing AML in the child.

Cytogenetics

One of the important prognostic factors for AML is the cytogenetics. Several identified chromosomal aberrations have been proven not only as important mutational drivers of AML development, but also as prognostic markers for the disease.

Cytogenetic abnormalities including t(8;21)(q22;q22) which disrupts AML1 gene; inv(16)(p13.1;q22), which creates two different fusion genes affecting the beta subunit quantity (de Bruijn and Speck 2004; Strout et al. 1999); and t(15;17)(q22;q12), in which the retinoic acid receptor-.α is fused to the PML gene, are AML favorable *(Figure 2A)* (de The et al. 1990).

Cytogenetics associated with poor outcome were monosomy 7, monosomy 5, del (5q), and abnormal chromosome 3. Chromosome 5q and 7 contain tumor suppressor genes which regulate the myeloid growth and differentiation (Luna-Fineman, Shannon, and Lange 1995; Giagounidis, Germing, and Aul 2006; Medeiros et al. 2010; Voutiadou et al. 2013). Trisomies 8 and 21 have also been proven to be related to AML (Khan, Malinge, and Crispino 2011).

Gene Mutations

Technological advances such as NGS have helped us to better understand and characterize the genetic background of myeloid neoplasms. The three most common mutations that have been related with a significant influence of the disease are:

i. Nucleophosmin 1 (*NPM1*): The most common mutation is found in *NPM1* gene (Kuhnl and Grimwade 2012). It can be found in about one-third of AML cases *(Figure 2B)* (Falini et al. 2005; Albiero et al. 2007). This gene encodes a nuclear protein and one of its main roles is to regulate centrosome duplication. NPM1 mutations are related to patients with improved survival rate (Hollink et al. 2009).

ii. CCAAT Enhancer Binding Protein α (*CEBPA*): The *CEBPA* gene is mutated in approximately 5–10% of AML patients *(Figure 2B)* (Leroy et al. 2005). *CEBPA* mutations promote proliferation of myeloid lineage on one hand, while on the other hand they inhibit myeloid differentiation (Pabst et al. 2001; Bereshchenko et al. 2009). Interestingly, the majority of patients harboring *CEBPA* mutations display longer event-free survival and decreased incidence of relapse (Frohling et al. 2004).

iii. Fms-Like Tyrosine Kinase 3 (*FLT3*): Mutations in FLT3 are related to an increase risk of relapse (Meshinchi et al. 2006). Mutated *FLT3* gene is found in 15–35% of AML patients *(Figure 2B)* (Yokota et al. 1997). The most frequently occurring *FLT3* mutation in AML is an internal tandem duplication located within the intracellular domain of this receptor tyrosine kinase (FLT3-ITD) (Nakao et al. 1996). Additionally, missense mutations have been described in the activation loop domain of the tyrosine kinase of *FLT3* (FLT3-TKD). These mutations are found in 5-10% of AML and rarely coexist with FLT3-ITD (Thiede et al. 2002).

Other important mutations are:

RAS: The isoforms *N-RAS* and *K-RAS* are mutated in up to 25% of de novo AML patients *(Figure 2)* (Bos 1989). In RAS proto-oncogenes, activating mutations can be defined in codons 12, 13, and 61 (Ritter et al. 2004).

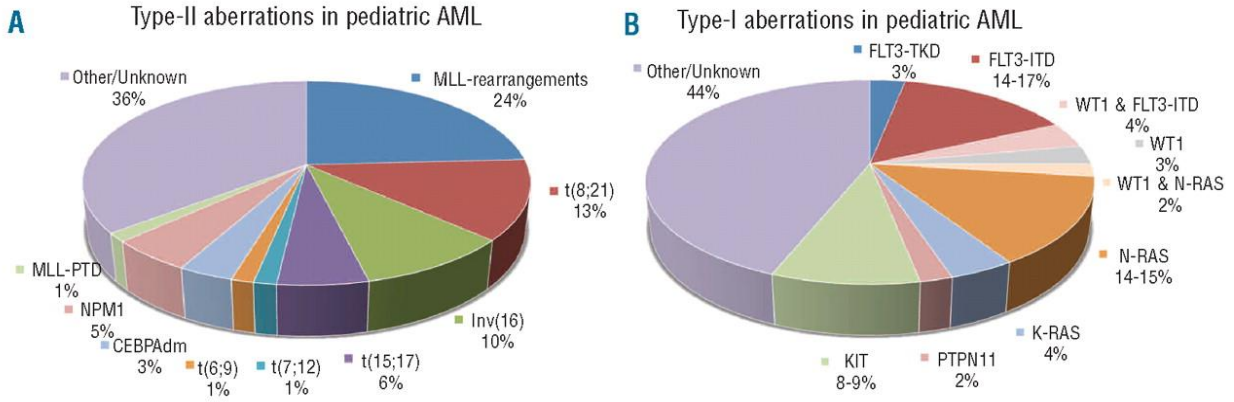

Figure 2: *Representation of the different type-I and type-II aberrations in pediatric AML. These different types of genetic aberrations reflect the heterogeneity of pediatric AML, although there is a lot of cases in which they have not yet been identified (Balgobind et al. 2011).*

Wilms tumor 1 (WT1) Overexpression: The gene, WT1, although expressed in about 90% of AMLs, is often overexpressed to a level that it can be used as a minimal residual disease (MRD) test in about 50% of cases *(Figure 2)* (Cilloni et al. 2009). However, the limited specificity and sensitivity of the use of WT1 for the significance of MRD detection is currently under debate.

c-KIT: c-KIT mutations have been reported in pediatric CBF-rearranged AML at frequencies ranging from 15 to 54.5% *(Figure 2)*; however, their prognostic significance is still controversial (Pollard et al. 2010; Goemans et al. 2005).

1.4 Microenvironmental interactions in AML

The anatomy and function of the HSC niche have been defined. It presents, within the bone marrow, one endosteal (Zhang et al. 2003; Mayack and Wagers 2008) and one perivascular compartment (Kiel et al. 2005). Important bi-directional signals inside the niche regulate the normal HSC numbers (Calvi et al. 2003) and maintain the long-term quiescence of the HSC pool (Fleming et al. 2008). In addition, perivascular structures, as well as supportive structures such as mesenchymal stem cells, also play an important role in the bone marrow niche (Kiel et al. 2005).

Apart from the role of the healthy stem cells, leukemic stem cells (LSCs) are also important by exchanging signals with the microenvironment, which play a crucial role in the regulation of cell self-renewal, by exploiting the normal homeostatic mechanisms (Lane, Scadden, and Gilliland 2009). The interaction between the microenvironment and specific cell compartments contributes to pathogenesis and is connected to the clinical outcome *(Figure 3)* (Ishikawa et al. 2007).

Interestingly, it has been shown in AML that the survival rate of LSCs is increased, while that of normal HSCs is decreased (Lane, Scadden, and Gilliland 2009). Another study has shown that the transformed niche cannot support any more normal hematopoiesis and provides survival advantage to the LSCs, as the levels of many factors have been changed. This suggests that it is very important to maintain the mesenchymal stem cell (MSC) population, especially the population of the normal osteoblasts for the prevention of the disease progression (Schepers et al. 2013). Such evidence proves that the niche is changing during the disease progression, and supports the survival of leukemic cells. Therefore, understanding the role of the microenvironment is important for constructing effective strategies for treatment regimes.

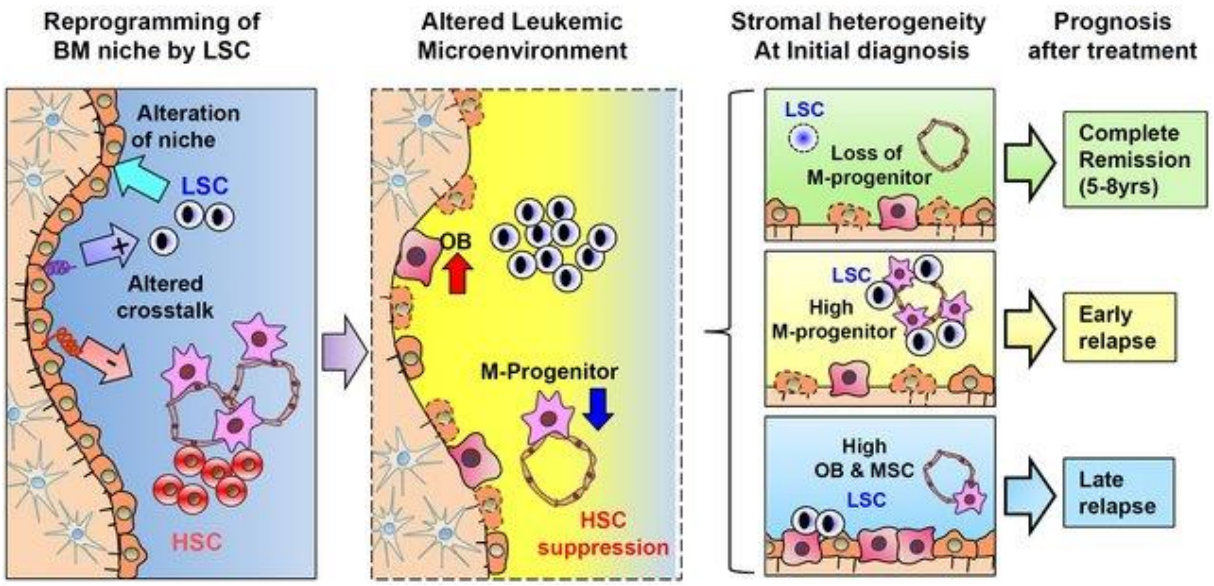

Figure 3: *Schematic representation of leukemia-induced changes in the niches and their importance in clinical outcomes. Transcriptional reprogramming of MSCs can be incited by LSC clones developed in bone marrow. In addition, they induce the niche remodeling leading to loss of M-progenitor, as well as maturation into osteoblastic cells. The altered microenvironment allows a communication between normal HSCs and LSCs in a way that controls a suppression of normal HSCs (−) and maintenance of LSCs (+), leading to the dominance of LSCs. The heterogeneity in prognosis following the treatment is related with the stromal remodeling at the stage of diagnosis. In this way, stromal remodeling can be used as a prognostic marker in AML due to its impact to leukemogenic action and clinical course (Lee, Kim, and Oh 2015).*

In combination, all these studies prove that the bone marrow niche can be transformed by leukemia cells into a microenvironment that promotes the survival of leukemic cells instead of normal HSCs, through the regulatory role of cytokines and growth factors. Besides cytokines and growth factors, the role of EVs has emerged to be critical in cell to cell communication. Therefore, in this model it is expected that EVs play an important role as mediators of the intercellular communication that contribute in the transformation of the bone marrow niche, by transferring information from the cancer stem cells to their microenvironment and vice versa.

1.5 Extracellular vesicles and the microenvironment

Extracellular vesicles (EVs) have attracted the interest of the scientific community in the last 50 years (Anderson 1969; Crawford 1971; Stegmayr and Ronquist 1982). They are small, membrane particles secreted by both healthy and non-healthy cells into the extracellular space. Large EVs (lEVs referring to microvesicles (MVs)), small EVs (sEVs referring to exosomes) and apoptotic bodies are three main categories of EVs, and they can be further categorized according to size, content and function (Borges, Reis, and Schor 2013).

sEVs have a size of ~100 nm and they are formed by the multivesicular bodies (MVB) (Dragovic et al. 2011; Sokolova et al. 2011). When the plasma membrane turns inside out or folds back on itself, small vesicles are formed. These vesicles fuse together and form the endosomes. During endosomes' maturing process, MVBs are formed (Kowal, Tkach, and Thery 2014). When these MVBs are fused with the plasma membrane, all the sEVs that they contain are released from the cell into the microenvironment (*Figure 4*) (Johnstone et al. 1987).

On the other hand, lEVs size ranges from 100 to 1000 nm and they are released from the cells by budding off of the plasma membrane (Ludwig and Giebel 2012; Raposo and Stoorvogel 2013; Kim et al. 2015). For this reason, MVs expose residuals of inner cell membrane-associated phosphatidylserine on their surface (Al-Nedawi, Meehan, and Rak 2009).

EVs are well-known for their important roles in intercellular communication. When they leave the cells, EVs travel through extracellular matrices and reach various biofluids. They can therefore be isolated from biological fluids such as breast milk, plasma, and blood (Borges, Reis, and Schor 2013; Kim et al. 2015), and can be analyzed for molecular signatures of the cell of origin as they contain and transfer many important biomolecules such as nucleic acids (DNA, messenger RNA (mRNA), and microRNA (miRNA)), proteins and lipids (Ratajczak et al. 2006; Deregibus et al. 2007; Valadi et al. 2007). EVs are markedly heterogeneous in terms of cell of origin, in which a mixture of different EV subpopulations that show a wide range of size, content and function are found (Yekula et al. 2019). They regulate many biological processes and influence the immune system by transmitting biological signals, while they also have some physiopathological roles in neurodegenerative diseases, cancer and various infections (Li et al. 2006; S et al. 2013; Yanez-Mo et al. 2015).

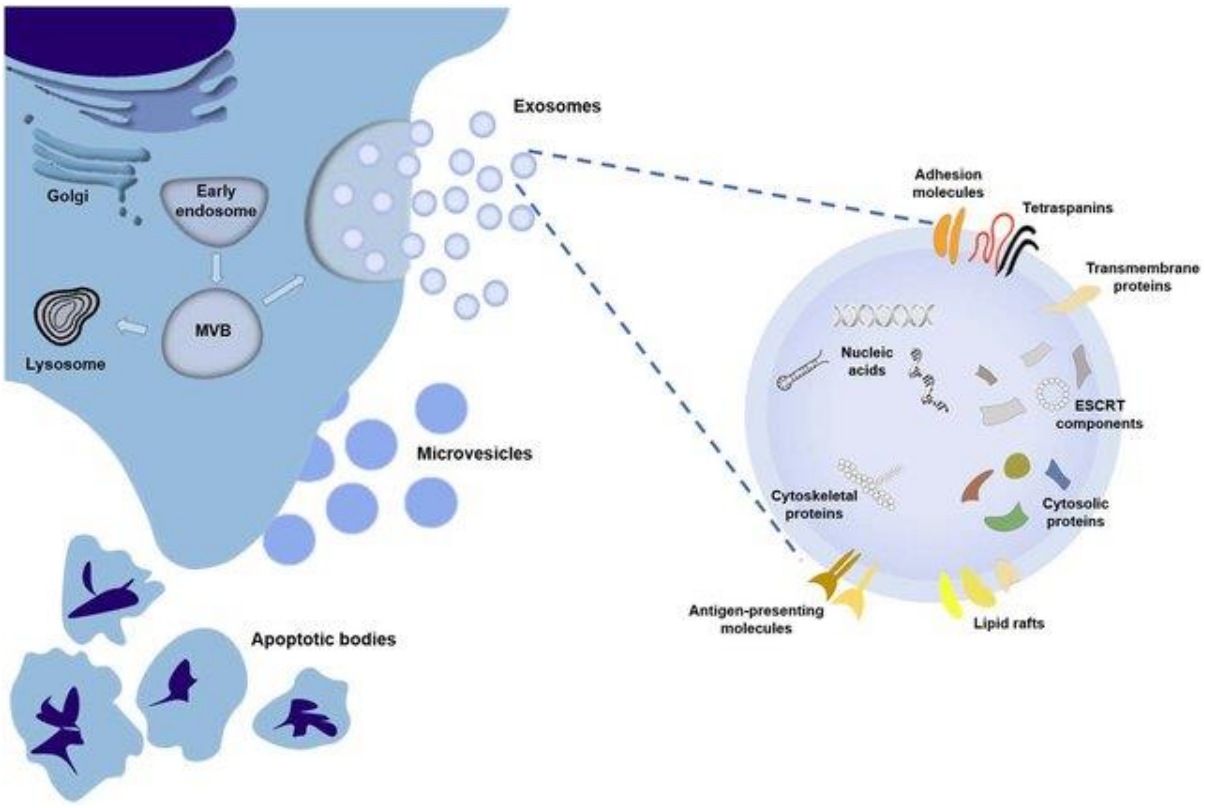

Figure 4: Biogenesis of extracellular vesicles. When the plasma membrane is budded, the early endosomes are formed. During the maturation process of these endosomes takes place the formation of MVB which contain exosomes and release them when they are fused with the plasma membrane. Microvesicles are released from the cells when they are budded off the plasma membrane. Arrows represent proposed directions (Rufino-Ramos et al. 2017).

Another important role of EVs is their potential intermediate action in communication and influence on cancer cells and their microenvironment by promoting tumor progression and dissemination. They achieve this through promotion of angiogenesis, matrix remodeling, increase of cell proliferation, alteration of immune responses, and, at last, metastasis. (*Figure 5*) (Wang and Bettegowda 2017; Zhang and Grizzle 2014; Shankar et al. 2017).

However, the mechanisms that contribute to this communication and how the EVs interact and fuse with the recipient cells remain unidentified. It is believed that target cells uptake EVs in three different ways: 1) simple membrane fusion, 2) endocytosis and 3) presence of distinct receptors (Pigati et al. 2010).

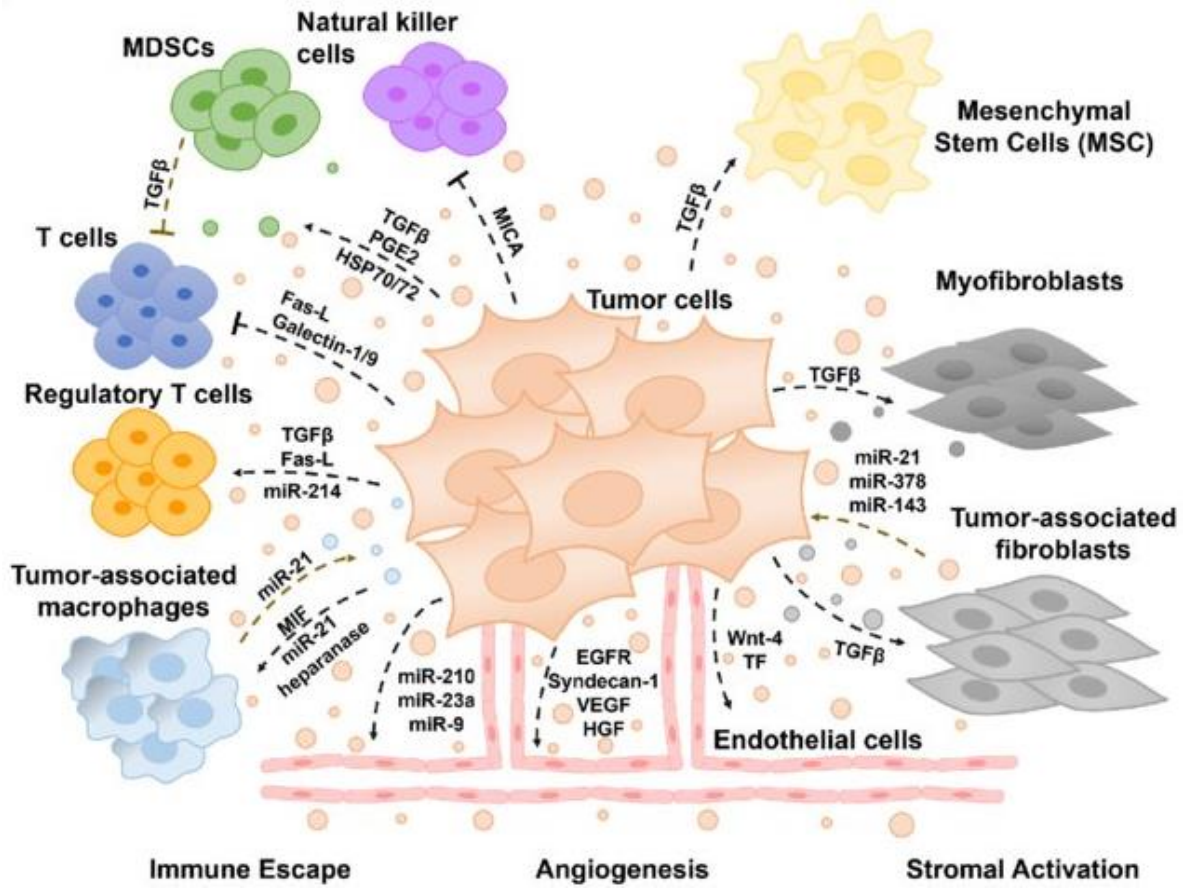

Figure 5: Extracellular vesicles derived from cancer cells affect tumor development and progression of the microenvironment. (Maacha et al. 2019).

In the case of cancer development, the malignant progression is supported by alterations in the extracellular matrix, while there is evolution in the tumor microenvironment. The genetic instability of the cancerous cells plays an important role in tumor progression and the evolution of the tumor microenvironment. During that phase, they release EVs that are multi-functional, in high amounts and continuously (Batista et al. 2011). Through these functions cancer and stromal cells communicate, thus leading to growth and proliferation of the tumor, as well as the maturation of the microenvironment (Batista et al. 2011). The exact way by which EVs contribute to the intercellular communication between cancer cells and the microenvironmental cells of the niche and how this affects the status of the microenvironment needs to be further examined.

1.6 Aim of the study

The role of EVs in hematological malignancies has not been explored thoroughly. A small number of studies suggest the potential of EVs as leukemia biomarkers (Hong et al. 2014), albeit studies addressing the mechanisms through which EVs contribute to the initiation, maintenance, progress and relapse in leukemia, are still scarce. The development of a novel and sensitive diagnostic tool based on analysis of EVs derived from the blood plasma of AML patients will help in understanding the status of the dominating leukemia cells at initial diagnostics, during treatment and relapse. Understanding this novel EV-based communication between leukemia cells and cells in the microenvironment will lead to the development of novel therapeutic approaches to specifically target the progression of leukemia.

2. RESULTS

2.1 Publications

Detection of AML-specific mutations in pediatric patient plasma using extracellular vesicle–derived RNA

Fabienne Kunz*, Evangelia Kontopoulou*, Katarina Reinhardt, Maren Soldierer, Sarah Strachan, Dirk Reinhardt, Basant Kumar Thakur

*Both authors contributed equally

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https://doi.org/10.1007/s00277-019-03608-y

Evaluation of dsDNA from extracellular vesicles (EVs) in pediatric AML diagnostics

Evangelia Kontopoulou, Sarah Strachan, Katarina Reinhardt, Fabienne Kunz, Christiane Walter, Bernd Walkenfort, Holger Jastrow, Mike Hasenberg, Bernd Giebel, Nils von Neuhoff, Dirk Reinhardt, Basant Kumar Thakur

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https://doi.org/10.1007/s00277-019-03866-w

AML is a highly heterogeneous disease and the second most common form of pediatric leukemia. Due to the high mortality rate, improved therapies to prevent relapse are of utmost importance. In this thesis, we aimed to evaluate and establish EV-dsDNA as a potential tool in AML pediatric diagnostics. A cohort of 29 pediatric AML patients (<18 years and in different disease stages) treated in Germany in the AML-BFM studies since 2004 were used for this analysis. EVs from their plasma were isolated by ultracentrifugation and were then characterized. dsDNA was extracted from those EVs and mutational analysis with NGS and GeneScan-based fragment-length analysis was performed. Importantly, this work demonstrated that the amount of EVs was related to disease progression, implying a prognostic and diagnostic potential of our method. In addition, our findings from the mutational analysis provided us with a better insight into the connection between the mutational background and the number of EVs as well as the dsDNA concentration. In combination, our results show that our method could be proven to be very important in the field of personalized treatment, as the most effective therapy could be chosen based on the mutational status of each patient which would provide the best possible outcome. In conclusion, the method is very sensitive, although further analysis is needed in order to further investigate and validate this data.

In the second study the workflow was similar to the previous one. EVs from the plasma of 16 AML pediatric patients (the same samples were included also in the first study) were isolated by ultracentrifugation, obtained EVs were characterized, EV-RNA isolated from those EVs and the mutational status was confirmed by GeneScan-based fragment length analysis and real-time PCR assays. The outcome was similar to our EV-dsDNA study and supported the potential of using EV-RNA and EV-dsDNA as diagnostic tools in pediatric AML. The high heterogeneity of EV populations and the low amount of the obtained tumor-derived EVs in combination with the small size of the EVs dampened the outcome of our study. A more sensitive EV-isolation method that could provide us with higher numbers of disease-specific EVs could help to achieve a more sensitive and reliable outcome.

2.2 Detection of AML-specific mutations in pediatric patient plasma using extracellular vesicle–derived RNA

Contribution to present publication:

- Conception: 50%
- Experimental work: 40%
  - Cell culture of AML cell lines
  - Establishment of EV isolation from AML cell lines
  - EV isolation from pediatric AML patients' samples
  - Characterization of all EVs (NTA)
  - RNA extraction and quantification
- Data analysis: 60%
- Writing the manuscript: 40%
- Revising the manuscript: 40%

The above listed contributions of Evangelia Kontopoulou to the publication are correct.

Essen, den _____ _____

Unterschrift der Doktoradin

Essen, den _____ _____

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ORIGINAL ARTICLE

Detection of AML-specific mutations in pediatric patient plasma using extracellular vesicle–derived RNA

Fabienne Kunz[1] · Evangelia Kontopoulou[1] · Katarina Reinhardt[1] · Maren Soldierer[1] · Sarah Strachan[1] · Dirk Reinhardt[1] · Basant Kumar Thakur[1]

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Abstract
Despite high remission rates, almost 25% of patients with AML will suffer relapse 3–5 years after diagnosis. Therefore, in addition to existing diagnostic and MRD detection tools, there is still a need for the development of novel approaches that can provide information on the state of the disease. Extracellular vesicles (EVs), containing genetic material reflecting the status of the parental cell, have gained interest in recent years as potential diagnostic biomarkers in cancer. Therefore, isolation and characterization of blood and bone marrow plasma-derived EVs from pediatric AML patients could be an additional approach in AML diagnostics and disease monitoring. In this study, we attempt to establish a plasma EV-RNA-based method to detect leukemia-specific FLT3-ITD and NPM1 mutations using established leukemia cell lines and primary pediatric AML plasma samples. We were successfully able to detect FLT3-ITD and NPM1 mutations in the EV-RNA using GeneScan-based fragment-length analysis and real-time PCR assays, respectively, in samples before therapy. This was corresponding to the gDNA mutational analysis from leukemic blasts, and supports the potential of using EV-RNA as a diagnostic biomarker in pediatric AML.

Keywords Extracellular vesicles · RNA · Pediatric AML · Biomarker

Introduction

Acute myeloid leukemia (AML) is a very heterogeneous hematological cancer and is the second most common form of leukemia in children [1]. Although there is a high remission rate for AML patients (up to 80%), relapse continues to be the most common cause of death in AML [2, 3]. One of the major challenges in predicting relapse in AML lies in the acquisition of novel secondary mutations in the primary leukemic cells during therapy [1, 4]. Additionally, there is lack of an effective uniform molecular biomarker which can monitor clonal evolution to predict minimal residual disease and relapse [2, 3, 5]. Although cellular analysis of blasts can be effective for diagnosis, the presence of them in the blood can reflect that the disease has already reached an advanced level; therefore, there is a strong need for an alternative diagnostic tool which can predict relapse at an earlier time point.

Extracellular vesicles (EVs) have recently gained interest in the field of cancer due to their novel roles as biomarkers and cell to cell mediators in metastasis and relapse [6, 7]. EVs are a mixed group of membrane-bound vesicles, with exosomes and microvesicles being the two main subtypes of interest in research [8–10]. They are produced by both healthy and cancerous cells and can be found in several body fluids, like blood, urine, or saliva, from where they can be easily isolated and analyzed using a simple liquid biopsy approach [6]. Additionally, it has been shown that the nucleic acids found within EVs can potentially mirror the mutational status of the parental cell from which the EVs originate [6, 11], making them potential candidates for the development of diagnostic tools that could be utilized in a clinical setting.

Furthermore, several other working groups have previously demonstrated that EVs and the RNA they contain have

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several characteristics, which could make them ideal biomarkers for the AML disease state. For example, earlier studies have claimed that cancerous cells produce more EVs than healthy cells [6, 12], which has also been observed in the AML disease state, with AML patients having higher numbers of EVs in circulation than healthy controls [13–15]. It has also been shown that the contents of EVs from AML cells can change depending on the disease stage [13], including the upregulation of several miRNAs that are relevant to AML prognosis and relapse [13, 16]. This is further supported by the previous work of Hornick et al. (2015) who showed that EV miRNA could be used to detect leukemic relapse in a mouse model [2, 5]. Furthermore, Huan et al. (2013) have previously shown that it is possible to detect AML-specific mRNA transcripts in EVs from conditioned media of cultured primary leukemia cells and AML cell lines [17] and, in addition, Hong et al. (2014) have previously shown that the level of TGF-β1 protein in EVs can be correlated to the status of therapy in AML [18]. All of these findings further support the idea of using EVs and their RNA content for the detection and monitoring of AML [2, 5, 17, 18].

In this study, for the first time, EV-RNA from primary pediatric patient plasma was investigated for its diagnostic value by attempting to detect AML-specific mutations in the EV-RNA at time points of diagnosis and during treatment. The plasma EV-based method to detect leukemia cell–specific information could potentially provide complimentary data on the stage of leukemia, disease progression, and response to therapy. In future, this could offer an alternative or additional tool to the classical bone marrow puncture for the detection and prediction of pediatric AML.

Material and methods

Ultracentrifugation of patient samples

Seventy-three plasma samples from 16 patients with childhood AML were obtained from the biobank of the Children's Hospital of Essen. Plasma from patients was obtained in varying volumes; therefore, before performing differential centrifugation and ultracentrifugation steps for EV isolation, the sample volume was adjusted to 2 ml by adding PBS, as required. A normal centrifugation for 20 min at 3000×*g* at 10 °C had been previously performed. The supernatant was collected in new tubes and centrifuged at 12,000×*g* for 20 min at 10 °C. The supernatant was transferred into 4-ml ultracentrifuge tubes (Beckman Coulter No. 355645) and samples were balanced with a maximum difference of 0.01 g before performing ultracentrifugation. Samples were ultracentrifuged using a fixed angle rotor Ti 50.4 (Beckman Coulter) at 100,000×*g* for 70 min at 10 °C. The supernatants were discarded and the pellet containing EVs was washed by resuspending in 2 ml of PBS and ultracentrifuged at 100,000×*g* for 70 min at 10 °C. After discarding the supernatant, the final EV pellet was resuspended in 250 μl of PBS. From the obtained EV pellet, 20 μl was used as aliquots for further analysis.

The same protocol was used for the isolation of EVs from conditioned media of leukemia cell lines, with the only differences being the volume and the rotor (Ti 45, Beckman Coulter) which were used. The conditioned media from each flask of each leukemia cell line were transferred into one 94-ml ultracentrifuge tube (Beckman Coulter No. 355628) and was ultracentrifuged to obtain purified EVs. The final EV pellets were resuspended in 250 μl of PBS, transferred into microcentrifuge tubes, and frozen at − 80 °C.

Cell culture

Leukemia cell line MV4-11 (acute monocytic leukemia) was cultured in RPMI-1640 media (Gibco No. 21875-034) with 10% FBS (Biowest, No. S1860-500) and 1% Pen-Strep (Gibco No. 15140-122), and OCI-AML3 (acute myeloid leukemia) was cultured in MEM Alpha media (Gibco No. 12561056) with 20% FBS and 1% Pen-Strep . When enough cells were obtained, the cells from each flask were centrifuged at 300×*g* for 5 min and the pellet was resuspended in 1 ml of RPMI-1640 media or MEM Alpha media with 10% or 20% EV-depleted FBS and 1% Pen-Strep. Next, 3.5×10^6 cells were then transferred into 12 T175 flasks (Cell star No. 660175) with 24 ml of EV-depleted media. This assured that the supernatant would contain only EVs released from the cells. After 48 h, an additional 25 ml of this media was added to the flasks and left for an additional 24 h. This supernatant was collected and used in ultracentrifugation to obtain EVs for further analysis.

ZetaView analysis—nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA-ZetaView, Particle Metrix GmbH) was performed to characterize EVs according to the recommendations of the International Society of Extracellular Vesicles (ISEV). Fresh standards were prepared using "100 nm standard beads." First, 10 μl of standard was added to 10 ml of H_20 (1:1000). Then, 188 μl of the 1:1000 solution was transferred into another falcon which contained 50 ml H_20 (1:266,000). All the settings were adjusted using the standard beads, and the concentration of the samples was adjusted using DPBS in order to obtain the number and the size of the particles that did not exert the minimum and maximum values of the settings. The final volume of diluted samples loaded onto the ZetaView was 1 ml. At least two washes of 10 ml DPBS were performed to clean the sample loading platform of the ZetaView between the measurements of each sample. The zeta potential, number of EVs, and size histograms were generated by built-in software of the ZetaView instrument.

RNA isolation and quantification

The RNA isolation was performed using the NucleoSpin® RNA XS (Macherey-Nagel No. 740902.50) according to the manufacturer's instructions. Briefly, 200 μl of Buffer RA1 and 4 μl of TCEP were added to each 50 μl isolated EV sample and vortexed vigorously. Next, 300 μl of 70% ethanol was added to the homogenized lysate and mixed by pipetting. Each sample was loaded into a Nucleospin RNA XS column and centrifuged at 11,000×*g* for 30 s. Then, 100 μl MDB (Membrane Desalting Buffer) was added and centrifuged at 11,000×*g* for 30 s. Next, 25 μl of rDNase reaction mixture was applied directly onto the center of the column and incubated at RT for 15 min. A wash step was performed by adding 100 μl of Buffer RA2 to the column, followed by a centrifugation of 11,000×*g* for 30 s. Two extra washing steps took place by applying 400 μl and 200 μl of Buffer RA3 and centrifuged at 11,000×*g* for 30 s and 2 min, respectively. RNA was eluted in 45 μl of RNase-free H_2O. The RNA concentration was quantified using the RNA Quantifluor (Promega No. E3310) system according to the instructions of the manufacturer.

cDNA synthesis

The isolated RNA was transcribed into cDNA using a Transcriptor First Strand DNA Synthesis Kit (Roche No. 04897030001). Briefly, 2 μl of the provided random hexamer primer and 11 μl of RNA were transferred into a 0.2-ml Eppendorf tube. The samples were heated for 10 min at 65 °C and then cooled on ice. Next, 7 μl of a master mix consisting of the provided reverse transcriptase reaction buffer, protector RNase inhibitor, deoxynucleotide mix, and reverse transcriptase was added to the RNA/oligonucleotide primer solution, to a final volume of 20 μl. For the cDNA synthesis, samples were heated at 25 °C for 10 min, followed by 55 °C for 30 min and 85 °C for 5 min in a C1000 Thermal Cycler (Bio-Rad).

GeneScan-based fragment-length analysis

For the detection of the FLT3-ITD mutation, GeneScan-based fragment-length analysis was performed. First, PCR was performed with the following primers: FLT3-ITD forward 5′-GTAAAACGACGGCCAGGCAATTTAGGTATGAAAGCCAGC-3′ and reverse 5′-CAGGAAACAGCTATGACCTTTCAGCATTTTGACGGCAACC-3′ (Eurofins). Next, 20.5 μl of the Master Mix (which contained 12.5 μl ALL in Hot Start Taq 2× MM (HighQu), 6 μl H_2O, and 1 μl of the forward and reverse primers with a concentration of 10 pmol/μl) was added together with 4.5 μl of the sample into a 0.5-ml Eppendorf tube. The same mixture without the sample was prepared as control. For amplification, samples were heated at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 sec, 59 °C for 15 sec, and 72 °C for 15 sec. PCR products were diluted (1:80) in H_2O. Next, 1 μl of diluted PCR products was mixed with 10 μl HiDi Formamid (Thermo Fisher) and 0.3 μl GeneScan-based fragment-length analysis 600 LIZ Size Standard v 2.0 (Thermo Fisher). PCR products were denatured for 5 min at 95 °C. GeneScan-based fragment-length analysis was performed using the 3500 genetic analyzer and data were analyzed with GeneMapper Software 5 (Thermo Fisher).

RT-PCR

Qualitative and quantitative detection of the mutated NPM1 gene (OMIM No. 1640401) was done by RT-PCR. The protocol has been optimized from the standard operational procedure (SOP), which was developed by the European molecular net. The following primers were used: NPM1_forward: 5′-CAAAGTGGAAGCCAAATTCATC-3′; NPM1_reverse: 5′-CCTCCACTGCCAGACAGA-3′; probe: 5′-TAGCCTCTTGGTCAG-TCATCCGGAAGCA [BHQ1]-3′ (Eurofins). The ABL2 gene (OMIM 164690) was used as a housekeeping gene: ABL_forward: 5′-GGGTCCACACTGCAATGTTT-3′; ABL_reverse: 5′-CCAACGAGCGGCTTCAC-3′; probe: 5′-TCAGATGCTACTGGCCGCTGAAGG [BHQ1]-3′. Probes were synthesized by Eurofins (Ebersberg). All samples were performed in triplicate, including controls (ABL2 and H_2O). In each well of a 96-well plate, 17 μl of the Master Mix, 12.5 μl of TaqMan Universal Master Mix (Thermo Fisher), 2 μl of H_2O, and 2.5 μl of 10× primer mix were added. This primer mix contained 3 μM of the primer (forward/reverse) and 2 μM of the probe (FAM and H_2O). Next, 3 μl of cDNA was added to each well, which was then sealed with optical caps. The plate was briefly spun down and loaded onto the StepOne™ Real-Time PCR System (Thermo Fisher).

Transmission electron microscopy

Transmission electron microscopy (TEM) was performed at the Imaging Center Essen (IMCES) for visualization of the EVs. Firstly, mesh copper grids coated with formvar (PLANO No. SF162) were made hydrophilic by exposing them to glow discharging for 1.5 min (PELCO easiGlow™). Afterwards, 3 μl of EV sample was added to the grids, which were then negatively stained for 1 min with 3 μl of 1% *v*/*v* uranyl acetate. The excess liquid was removed and the grids were dried for 15 min and finally observed under a JEOL 1400+ TEM Crossbeam at 120 kV (JEOL).

Results

EV isolation from leukemia cell lines

Two different cell lines, OCI-AML3 carrying a NPM1 mutation and MV4-11 carrying a FLT3-ITD mutation, were cultured for 72 h in EV-depleted growth medium. EVs were extracted from conditioned medium using differential centrifugation followed by ultracentrifugation steps. The characterization of the EVs (Supplementary Table S1), to estimate their number and their average diameter, was performed by NTA. Average particle size of both cell lines was in the range of 30–160 nm (Fig. 1a, b, Supplementary Fig. 1a). Transmission electron microscopy was performed to visualize EVs in order to further confirm their presence and size (Fig. 1b–e). These results confirmed the successful isolation of EVs based on the expected EV size range (30–150 nm), allowing the continuation of further molecular analyses.

EV-derived RNA from leukemia cell lines reflects the genomic mutational status

To firstly check whether it was possible to detect the mutations in each cell line and to compare the mutational status of the cell lines with their EVs, RNA was extracted from the EVs (Fig. 2a) and cells of both cell lines. The RNA was transcribed into cDNA and the mutational detection was performed by two different assays. RT-PCR was used for the detection of the NPM1 mutation, while GeneScan-based fragment-length analysis was performed for the FLT3-ITD mutation. The NPM1 mutation was detectable in RNA extracted from both cells and EVs (Fig. 2b, Supplementary Fig. 1b and c). The FLT3-ITD mutation was also

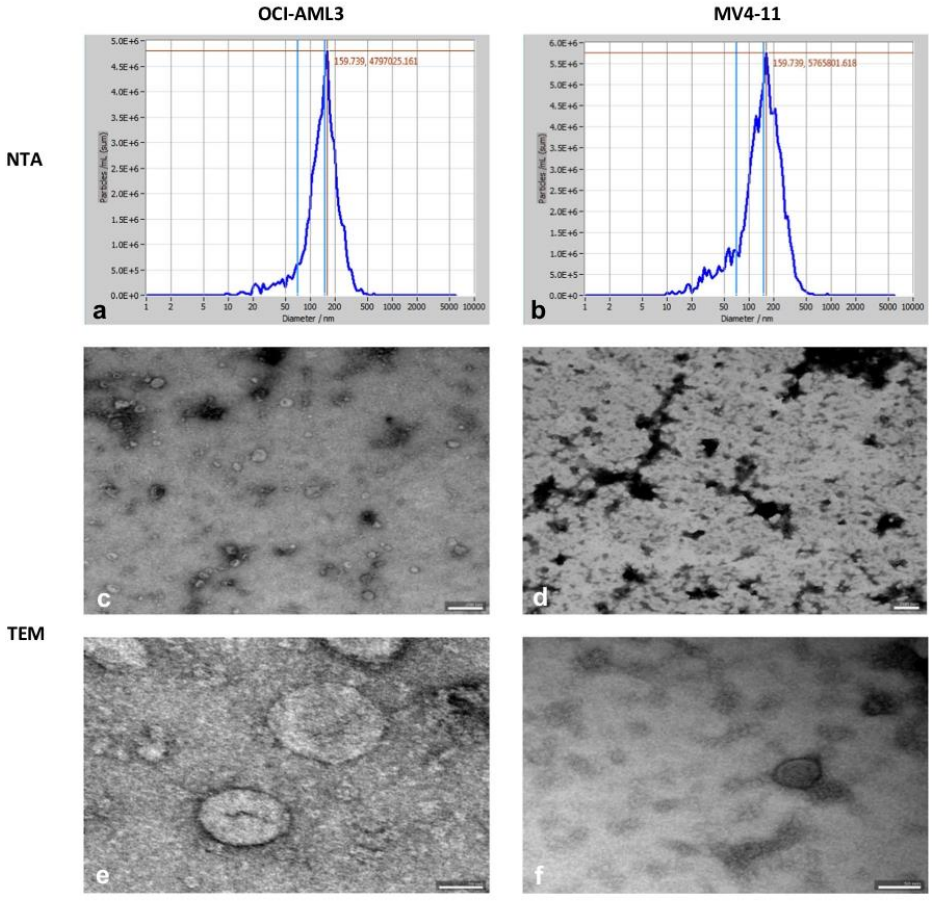
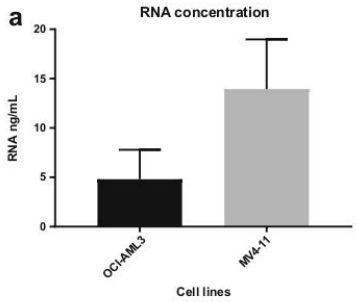


Fig. 1 **a**, **b** Particle diameter size and number of particles in leukemia cell line supernatant, as measured by nanoparticle tracking analysis (NTA). The size distribution is in the range of extracellular vesicles (30–160 nm). **c**–**f** Transmission electron microscopy of uranyl acetate–stained EVs from leukemia cell line supernatant. Scale bar 200 nm



b

Cell line	NPM1	FLT3-ITD
OCI-AML3 EVs	+	-
OCI-AML3 cells	+	-
MV4-11 EVs	-	+
MV4-11 cells	-	+

Fig. 2 **a** Comparison of RNA concentration, extracted from EVs of two leukemia cell lines. **b** Results of RNA mutational analyses. RT-PCR analyses for NPM1 mutation were performed to compare the sensitivity of mutational detection in RNA that was extracted from EVs of one cell line (OCI-AML3) with the RNA extracted from the cells of both cell lines. For the assay, maximum volume of 3 μl was used from each sample. The NPM1 AML-specific mutations were not present in negative control cell line (MV4-11), however, the OCI-AML3 cell RNA and EV-RNA were both positive for the NPM1 mutation. GeneScan-based fragment-length analyses for detection of FLT3-ITD mutation in MV4-11 cell RNA and EV RNA were also performed. Negative control OCI-AML3 cell RNA was negative for the FLT3-ITD mutation and MV4-11 cell RNA and EV-RNA were positive for the FLT3-ITD mutation

detectable in both cases and, interestingly, was higher expressed in EVs in comparison with cell-derived RNA (Fig. 2b).

EV isolation from pediatric AML patient samples

To further investigate if the mutational status of a patient can be detected from plasma-derived EVs, EVs were isolated from AML patient samples by performing differential centrifugation followed by ultracentrifugation steps. The plasma-derived EVs were analyzed for their number and size by NTA, and the number of EVs obtained from samples before and after treatment was compared (Supplementary Tables S2 and S3). The average particle size was in the range of 30–160 nm (Fig. 3a–d) and TEM was performed to confirm the NTA results (Fig. 3e–h). In patients carrying the NPM1 mutation, as well as in patients with a combined mutation (NPM1 and FLT3-ITD), the number of particles was always lower in the after-treatment EV samples than in the before-treatment EV samples (Fig. 3i). In contrast, the patient group with the FLT3-ITD mutation only revealed a higher particle concentration in the after-treatment samples compared with the before-treatment samples (Fig. 3i). A decrease in the number of EVs after treatment would be expected as this would correlate with the decrease in cancerous cells, as well as with the reduced leukemic blast cells after treatment (Supplementary Table S2). The number of the EVs in the before-treatment samples between the different mutational groups is statistically significantly different revealing a correlation between the number of the released EVs and the mutational group that they belong to.

EV samples from pediatric AML patients contain RNA

To establish the EV-RNA as a potential tool for the detection of AML mutations in patient samples, RNA was extracted from the plasma-derived EVs of 16 AML patients. The EV-RNA concentration was measured by RNA QuantiFluor (Supplementary Table S3). The amount of obtained EV-derived RNA was higher in the before-treatment samples than in the after-treatment samples in AML patients with the NPM1 mutation only or with a combined mutation (Fig. 4a). In contrast, the EV-derived RNA from patients with the FLT3-ITD mutation only showed higher concentrations after treatment in comparison with the before-treatment samples (Fig. 4a). This mirrors the results of the NTA, which indicate the same trend in EV number.

EV-derived RNA reflects the patient mutational status

Next, we investigated whether the EV-RNA of the AML patients reflected their mutational status. EV-RNA from patients carrying a NPM1 mutation was obtained, transcribed into cDNA, and analyzed for the mutational status by RT-PCR. In seven AML patients, identical mutations were detected in the EV-RNA of the initial samples before therapy and in the primary leukemic blasts. In two patients, it was not possible to detect the mutation in the initial sample at all (Fig. 4b, Supplementary Table S4). In all patient samples after therapy, the initial AML-specific mutation was no longer detectable (Fig. 4b, Supplementary Table S4). GeneScan-based fragment-length analysis was performed for the mutational analysis of nine AML patients with a FLT3-ITD mutation. For this purpose, EV-derived RNA was extracted and transcribed into cDNA and GeneScan-based fragment-length analysis was performed. The mutation was detectable in all of the initial samples before treatment (Fig. 5a–d), but it was not possible to detect it in the after-treatment samples of each patient (Supplementary Table 4). This lack of mutational detection for both mutations in all after-treatment patient samples was not always in correlation to the results of the gDNA analysis that is routinely performed in our diagnostic AML lab after every treatment.

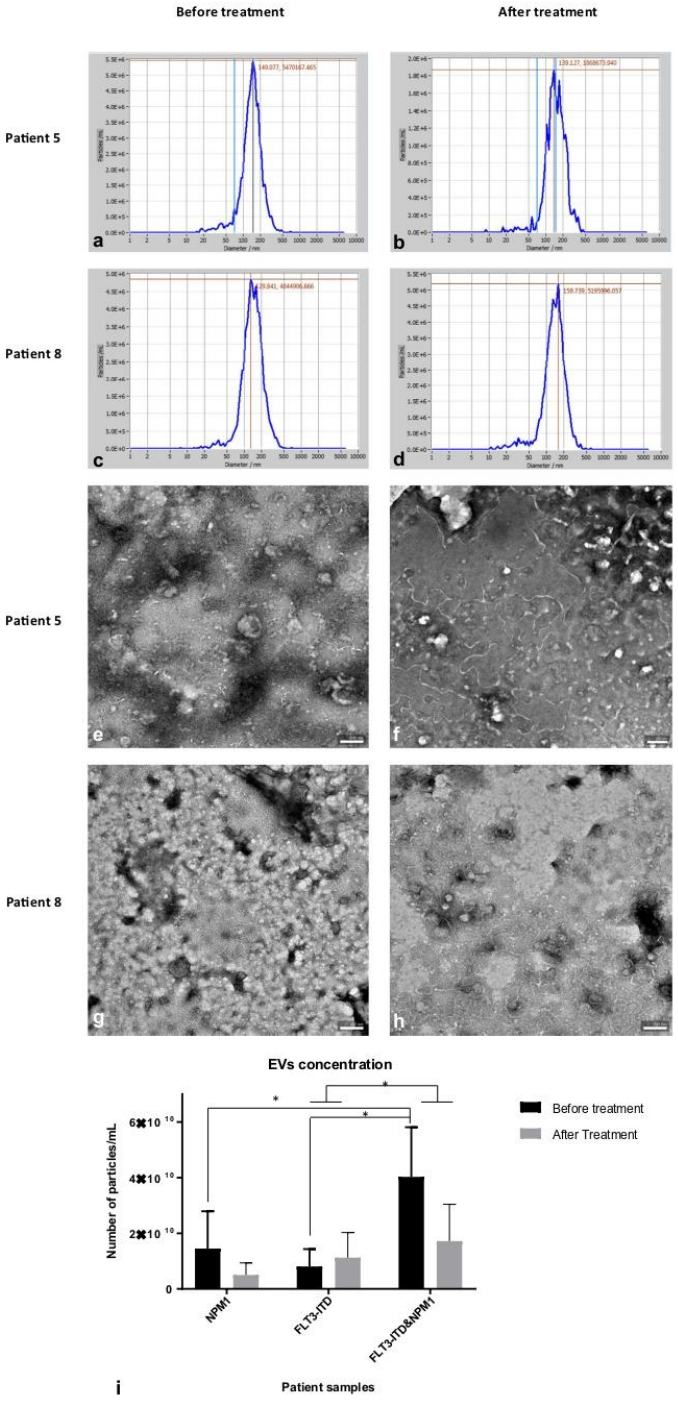
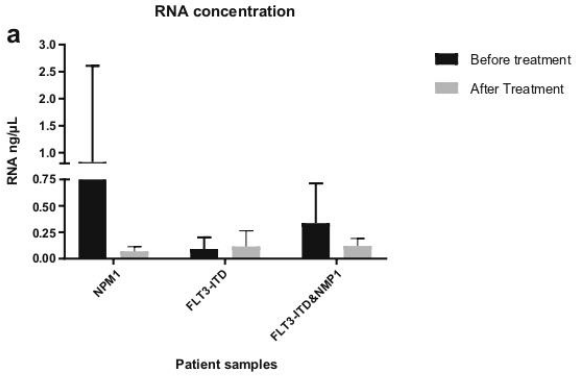


Fig. 3 **a**–**d** Particle diameter size and number of particles per mL of plasma from two AML patients before and after treatment, as measured by nanoparticle tracking analysis (NTA). The size distribution is in the range of extracellular vesicles (30–160 nm). **e**–**h** Transmission electron microscopy of uranyl acetate–stained EVs from patient plasma samples before and after treatment. Scale bar 200 nm. **i** Comparison of EVs concentration in before- and after-treatment samples from 16 AML patients, as measured by NTA analysis. Higher concentration of EVs in before-treatment samples was observed in patients with NPM1 mutation only and with combined mutations than those with a FLT3-ITD mutation only. The difference in EV concentration between patients with a FLT3-ITD mutation only and patients with FLT3-ITD and NPM1 combined mutations was statistically significant ($p = 0.0145$). In addition, EV concentration in before-treatment samples of patients carrying both mutations was statistically significantly higher than in the NPM1 only group ($p = 0.0468$) and the FLT3-ITD only group ($p = 0.0168$)

Discussion

Despite the recent advances in the treatment and diagnosis of AML, which is reflected by high remission rates, many patients are still suffering from relapse—the biggest cause of death in AML [2, 3]. This highlights the ongoing need for the development of a more sensitive approach with the ability to detect and monitor this disease more effectively. Simultaneously, this approach should encompass the advantage of reducing pain and inconvenience for the patients caused by the current standard bone marrow puncture–based diagnostic methods. In the current study, we attempted to take the first steps towards the development of a tool that would fulfill these criteria by evaluating the diagnostic potential of plasma-derived EV-RNA in pediatric AML.

Here, using both established leukemia cell lines and AML pediatric patient samples ($n = 16$), we have demonstrated the biomarker potential of EV-derived RNA in AML. Using cell line–conditioned media, we successfully established that it was possible to isolate EVs, extract RNA, and detect AML-specific mutations, NPM1 and FLT3-ITD, using RT-PCR and GeneScan-based fragment-length analysis methods, respectfully. These positive results reflected that our proposed isolation and detection methods were valid and functional at the cell line level. Subsequently, using the same methods, RNA was isolated from EVs of pediatric patient plasma ($n = 16$), before and after treatment, and analyzed for its biomarker potential. In 14 out of the 16 AML patient samples at the stage of primary diagnosis, it was possible to detect NPM1 or FLT3-ITD mutations which reflected the known gDNA information of the samples. These initial results showed that our approach was sensitive enough to detect AML-specific mutations from a source of plasma containing heterogeneous populations of EVs, highlighting that the approach does indeed have



b

Patient	Mutation	Primary leukemia blasts	EV RNA Before treatment	EV RNA After treatment in different blocks
P3	NPM1	+	+	-* -* -* -*
P4	NPM1	+	+	- - - - -
P5	NPM1	+	+	- -
P7	NPM1	+	+	-* -* -* -* -* - -
P9	NPM1	+	+	n.a
P10	NPM1	+	+	-* -* -* -*
P14	NPM1	+	+	- -*
P15	NPM1	+	n.a	-* -*
P16	NPM1	+	n.a	-* -*

Fig. 4 **a** Comparison of RNA concentration, extracted from EV fractions of 16 AML patients, prior and post treatment. **b** RT-PCR for NPM1 mutation was performed to establish the sensitivity of mutational detection in RNA that was extracted from EVs of patients, and compared with the already existing patient mutational status information of the primary leukemia database in the AML-BFM lab. Sixteen patient samples before and after treatment were used for analysis. A maximum volume of 3 µl from each sample was used. AML-specific mutations were not present in post-treatment samples. *Low-quality sequencing data. -*No mutation detected but low-quality sequencing data

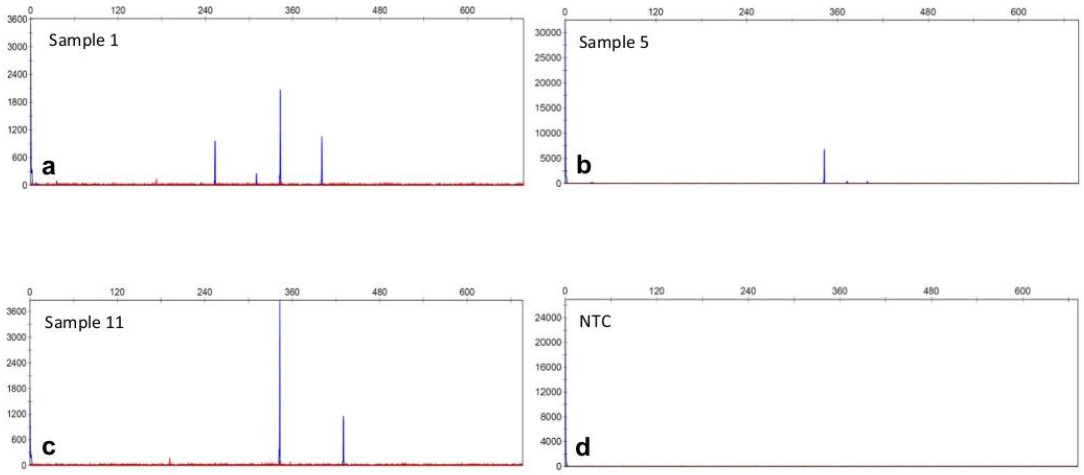


Fig. 5 **a**–**d** GeneScan-based fragment-length analysis of FLT3-ITD mutation was performed to establish the sensitivity of mutational detection in RNA that was extracted from EVs of patient samples, and compared with the already existing patient mutational status information of the primary leukemia database in the AML-BFM lab. Sixteen patient samples before and after treatment were used for analysis. 50 ng/μl or a maximum volume of 10.5 μl from each sample was used. Samples **a**, **b**, and **c** are before-treatment samples that show the FLT3-ITD mutation (size ~ 372). Sample **d** is the negative control. AML-specific mutations were not present in post-treatment samples (data not shown)

diagnostic biomarker potential. In addition, the number of EVs and the consequent amounts of RNA appeared to be influenced by the mutational background of the patients. As previous studies have shown that AML patients have more EVs than healthy controls [13–15], this was an interesting observation, which once established in a larger patient cohort study may have additional diagnostic utility.

The proposed approach, with the current sensitivity, does in fact have its limitations. Although it was successful at detecting mutations in almost all patients at initial diagnosis, it was not always possible to detect mutations after treatment when using this method. These are issues that must be addressed, as sensitivity is of utmost importance when designing diagnostic detection methods. These results could be attributed to the lower amount of RNA that was recovered from these samples; perhaps due to a reduction in EV production by mutation-containing cells or a reduction of the cells themselves. Alternatively, they could also be related to the EV heterogeneity of the plasma. Like all new approaches, this method also requires a period of optimization before its implementation as a clinically routine method can be considered. Therefore, in future, this study should be repeated using a larger cohort of patients and with an enhanced EV isolation method, capable of specifically sorting for AML-derived EVs.

Despite the discussed points of needed improvements in sensitivity, the advantage of this method over current diagnostic methods is clear, in terms of patient welfare. A liquid biopsy approach could indeed be a valuable diagnostic tool, offering a fast, pain-free, and hassle-free alternative to current painful bone marrow biopsies. Being able to diagnose or monitor a leukemic disease simply by drawing blood and isolating EVs instead of having to undergo a bone marrow puncture procedure would be much more convenient and less stressful for pediatric patients. In conclusion, our approach to establish an EV-RNA-based diagnostic platform provides valuable information that could be potentially useful in the future diagnosis and treatment of AML. This preliminary study definitely provides a starting point for the use of EV-RNA as a disease biomarker in AML and, once the sensitivity is optimized and the study is recapitulated in larger cohorts of patients, will open the door to many possible avenues of future research on this topic.

Authors' contributions Conception and design: BKT, FK, EK; collection and assembly of data: FK, EK, KR, MS; data analysis and interpretation: BKT, FK, EK; drafting of manuscript: BKT, FK, EK, SS; manuscript writing: BKT, FK, EK, SS; final approval of manuscript: all co-authors.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent Informed consent was obtained from all individual participants (or their parents) included in the study. Each patient consented following institutional review board approval AML-BFM 2004 (3VCreutzig1).

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Statement on the welfare of animals This article does not contain any studies with animals performed by any of the authors.

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2.3 Evaluation of dsDNA from extracellular vesicles (EVs) in pediatric AML diagnostics

Contribution to present publication:

- Conception: 40%
- Experimental work: 70%
  - EV isolation from pediatric AML patients' samples
  - Characterization of all EVs (NTA, Protein concentration analysis, WB)
  - DNA extraction
  - Quantitative and Qualitative DNA analysis
  - GeneScan-based fragment-length analysis
- Data analysis: 80%
- Writing the manuscript: 60%
- Revising the manuscript: 60%

The above listed contributions of Evangelia Kontopoulou to the publication are correct.

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ORIGINAL ARTICLE

Evaluation of dsDNA from extracellular vesicles (EVs) in pediatric AML diagnostics

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Abstract
Acute myeloid leukemia (AML) is a heterogeneous malignant disease characterized by a collection of genetic and epigenetic changes. As a consequence, AML can evolve towards more aggressive subtypes during treatment, which require additional therapies to prevent future relapse. As we have previously detected double-stranded DNA (dsDNA) in tumor-derived extracellular vesicles (EVs), in this current study we attempted to evaluate the potential diagnostic applications of AML EV-dsDNA derived from primary bone marrow and peripheral blood plasma samples. EVs from plasma of 29 pediatric AML patients (at initial diagnosis or during treatment) were isolated by ultracentrifugation, after which dsDNA was extracted from obtained EVs and analyzed for leukemia-specific mutations using next generation sequencing (NGS) and GeneScan-based fragment-length analysis. In 18 out of 20 patients, dsDNA harvested from EVs mirrored the (leukemia-specific) mutations found in the genomic DNA obtained from primary leukemia cells. In the nanoparticle tracking analysis (NTA), a decrease in EV numbers was observed in patients after treatment compared with initial diagnosis. Following treatment, in 75 samples out of the 79, these mutations were no longer detectable in EV-dsDNA. In light of our results, we propose the use of leukemia-derived EV-dsDNA as an additional measure for mutational status and, potentially, treatment response in pediatric AML.

Keywords Acute myeloid leukemia · EVs · dsDNA · Mutational detection · Pediatrics

Introduction

Acute myeloid leukemia (AML) is the second most common form of pediatric leukemia, with relapse rates of more than 30% in all patients. Clonal evolution of rare primary leukemic cells, which survived initial therapy or gained additional mutations, is considered as a potential cause of relapse in pediatric AML [1, 2]. With the recent advancements in the field of cancer genomics (e.g., whole genome deep sequencing at the single cell level), it has become clear that genomes acquire AML mutations in a stepwise manner, leading to the presence of genetically heterogeneous populations of leukemic cells in the hematopoietic compartment [2]. Due to this biological heterogeneity at the genomic DNA level, it is a great challenge to clinically target AML and to achieve a disease-free state in patients. In recent years, research has been intensively focused on understanding the process of clonal evolution in leukemia progression, both during therapy and relapse, as relapse remains the major cause of lethality in pediatric AML [3].

Classically, the role of extracellular signalling molecules, such as cytokines and growth factors, released by leukemic cells, has been discussed to modulate the microenvironment towards leukemogenesis [4, 5]. In the last decade, extracellular vesicles (EVs), especially exosomes and microvesicles, have been shown to mediate complex intercellular interactions

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at local and distant sites, in normal and pathophysiological conditions [6]. EV preparations contain many important biomolecules including nucleic acids (DNA, mRNA, and microRNA), proteins, and lipids [7–10], which can be transferred from EV releasing cells to their specific target cells [11]. Containing cell type–specific signatures, EVs and their cargo molecules have a huge potential to serve as novel biomarkers for a variety of different diseases [12]. Indeed, we and others previously demonstrated that EV preparations of various tumor patients contained dsDNA which mirrored the mutational information of the tumor cells from which they were produced [13–19].

The aim of the current study was to evaluate the potential diagnostic role of leukemia-derived EVs in pediatric AML. Accordingly, we harvested EVs from the plasma of AML patients, extracted their dsDNA, and analyzed it for AML-specific mutations using AML-specific next-generation sequencing (NGS) as well as GeneScan-based fragment-length analysis. Although it has been previously shown that in AML plasma EV number correlates with leukemic blood cell counts, and that miRNA from EVs has potential as a biomarker for minimal residual disease (MRD) and patient prognosis [20–22], the data that we have been able to generate in this study is the first to show the potential diagnostic applications of EV-dsDNA in primary AML pediatric patient samples.

Materials and methods

Twenty-nine patients with AML (Supplementary Table S1) were included in our study. Genetic information and the levels of hemoglobin, leukocytes, and myeloblasts of each patient are provided (Table 1 and Supplementary Table S1). Each patient was consented following institutional review board approval AML-BFM 2004 (3VCreutzig1). The age of the patients ranged from less than 1 up to 18 years. As a reference point, the plasma from 5 healthy donors between 25 and 40 years old were also collected. Plasma (1–2 mL) from bone marrow (BM) or peripheral blood (PB) was collected from each patient prior to the start of chemotherapy (twenty-nine patients) as well as after different stages of chemotherapy (twenty patients) (Table 1), and 7 mL peripheral blood from each healthy donor were collected at one time point. EVs were isolated using a differential ultracentrifugation protocol (Supplementary Figure S1). The number of EVs and their diameter were quantified by nanoparticle tracking analysis (NTA) using ZetaView (Particle Metrix GmbH). The dsDNA was isolated to allow genomic and EV-DNA sequencing for tumor profiling using MiSeqDx (Illumina Inc.) with the TruSight Myeloid (TSM) panel (Illumina, FC-130-1010) using the MiSeq V2 Reagent kit (2 × 150 cycles, paired end run) and a total of 141 kb per sample, as well as with GeneScan-based fragment-length analysis (Supplementary Figure S1). All statistical analyses were performed using Prism 7 (GraphPad, San Diego). Data were analyzed using unpaired and paired Student's *t* test. The majority of results are expressed as means including the standard deviation with $P < 0.05$ being considered statistically significant.

Ultracentrifugation of patient samples and healthy donor samples

Healthy donor blood (7 mL) was collected in red-top EDTA (ethylenediamine tetraacetic acid) tubes (#01.1605.001, SARSTEDT AG & Co. KG). Plasma samples from patients with childhood AML were obtained from the Biobank. The EVs from the healthy donors and the patient samples were isolated using 3 steps of differential centrifugation followed by ultracentrifugation according to the protocol from Kunz et al 2019 [23].

From each plasma sample, 200 μL were transferred into new microcentrifuge tubes and stored at − 80 °C as a control before EV depletion (unfractionated plasma). The supernatants after the first spin of 100,000×*g* were collected in fresh microcentrifuge tubes and stored at − 80 °C for future analysis of DNA in the plasma after EV depletion (fractionated plasma). The final EV pellet was resuspended in 200 μL of PBS. From the obtained EV fraction, 20 μL were used for the Transmission Electron Miroscopy (TEM), BCA assay, and ZetaView analysis. The rest of the samples were stored at − 80 °C for later Western blot analysis as well as DNA isolation and sequencing (Supplementary Figure S1).

ZetaView analysis-nanoparticle tracking analysis

EV size and concentration were analyzed by nanoparticle tracking analysis using the ZetaView device of Particle Metrix. Firstly, 100 nm "Standard beads" (Particle Metrix GmbH) were used to calibrate the machine. The following settings were used for all measurements: 11 positions, 5 cycles, medium quality, minimal brightness of 20, minimal size of 5 nm, maximal size of 200 nm, tracelength of 15 s, sensitivity of 75%, shutter speed of 75 ms, and a frame rate of 30. Sample dilutions were adjusted using DPBS (#14200075, ThermoFischer Scientific) to a final volume of 1 mL. For analysis, size and concentration of particles were determined using Software provided by Zetaview (version 2.3) (Particle Metrix GmbH).

Transmission electron microscopy

TEM was performed at the Electron Microscopy Unit (EMU) of the Imaging Center Essen (IMCES) to visualize the EVs. First of all, 3 μL of isolated EV fractions were added on to a Formvar-coated 200 mesh copper grid (#SF162, PLANO GmbH). The grid had been previously prepared by making

Table 1 AML patients' samples overview and mutational analysis

Patient number	Age at diagnosis (years)	FAB	% blasts	Leukocytes (103)	Hb (g/dl)	Date	Material	Remark treatment block	Mutations gDNA	Mutations EV-DNA
1	16	M1	BM: 90% PB: 90%	67.5	10.2	09.05.2016	PB	Initial	WT1 NPM1 FLT3-TKD GATA2 ETV6(SNP) ZRSR2(SNP)	WT1 NPM1 FLT3-TKD GATA2 ETV6(SNP) ZRSR2(SNP)
1a			BM: 79% PB: n.a	0.1	6.9		BM	After ADxE	n.a n.a n.a n.a n.a n.a	* * * * * *
1b			BM: 81% PB: n.a	0.1	7.8		BM	After ADxE	n.a n.a n.a n.a n.a n.a	n.d * n.d * n.d * n.d * n.d * n.d *
1c			BM: 0% PB: 0%	2.2	8.2		BM	After HAM	n.a n.a n.a n.a n.a n.a	n.d n.d n.d n.d ETV6(SNP) ZRSR2(SNP)
1d			BM: 0% PB: 0%	1.9	9.5		BM	After AI	n.a n.a n.a n.a n.a n.a	n.d n.d n.d n.d ETV6(SNP) ZRSR2(SNP)
1e			BM: 0% PB: 0%	3.7	10.2		BM	After hAM	n.a n.a n.a n.a n.a n.a	n.a n.a n.a n.a n.a n.a
2	5	M2	BM: 30% PB: 23%	7.3	7.9	20.12.2016	BM	Initial	t(8;21), FLT3-ITD, RAD21, KIT, EZH2	n.d n.d n.d n.d
2a			BM: 0% PB: 0%	0.6	7.5		BM	After ADxE	t(8;21), FLT3-ITD, RAD21, KIT, EZH2	n.d * n.d * n.d * n.d *
2b			BM: 0% PB: 0%	2.8	9.5		BM	After AI	n.d n.d n.d n.d n.d	n.a n.a n.a n.a n.a
2c			BM: 0% PB: 0%	2.3	8.1		BM	After hAM	n.d n.d n.d n.d	* * * *
3	18	M3	BM: 71% PB: 3%	0.9	7.5	28.06.2016	PB	Initial	t(15;17) GATA2(SNP) NOTCH1(SNP)	t(15;17) GATA2(SNP) NOTCH1(SNP)
3a			BM: 0% PB: 0%	48	8.6		BM	After block 2	t(15;17) GATA2(SNP) NOTCH1(SNP)	n.d GATA2(SNP) NOTCH1(SNP)
3b			BM: 0% PB: 0%	5.8	12.4		BM	After ATRA	n.d n.d n.d	n.d GATA2(SNP) NOTCH1(SNP)
3c			BM: 0%	3.6	11.8		BM	After block 4	n.d	n.d

Table 1 (continued)

Patient number	Age at diagnosis (years)	FAB	% blasts	Leukocytes (103)	Hb (g/dl)	Date	Material	Remark treatment block	Mutations gDNA	Mutations EV-DNA
			PB: 0%						n.d	GATA2(SNP)
									n.d	NOTCH1(SNP)
3d			BM: 0%	No data	No data		BM	After block 5 ATO	n.d	n.d
			PB: 0%						n.d	GATA2(SNP)
									n.d	NOTCH1(SNP)
4	1	M4	BM: 83%	11	8.6	29.11.2016	BM	Initial	t(1;11)	t(1;11)
			PB: 25%						NRAS	NRAS
									KIT (SNP)	KIT (SNP)
									PHF6 (SNP)	PHF6 (SNP)
4a			BM: 5%	0.2	6.1		BM	After d26	t(1;11)	n.d
			PB: n.a						NRAS	n.d
									KIT (SNP)	KIT (SNP)
									PHF6 (SNP)	PHF6 (SNP)
4b			BM: 0%	1.7	9.1		BM	After AI	n.d	n.d
			PB: 0%						n.d	n.d
									n.d	KIT (SNP)
									n.d	PHF6 (SNP)
14	9	M1	BM: 78%	51.75	7.8	26.09.2017	BM	Initial	FLT3-ITD	FLT3-ITD
			PB: 88%							
14a			BM: 0%	0.1	7.1	23.10.2017	BM	After 1 induction	n.a	n.d
			PB: n.a							
14b			BM: 0%	1.89	7.9	30.10.2017	BM	After CDxA	FLT3-WT	n.d
			PB: 0%							
14c			BM: 0%	n.a	n.a	21.12.2017	BM	After HAM	FLT3-WT	n.d
			PB: n.a							
15	4	M2	BM: 45%	9.86	7.3	13.01.2017	BM	Initial left	FLT3-ITD	n.d
			PB: 0%							
15a			BM: 34%	9.86	7.3	13.01.2017	BM	Initial right	FLT3-ITD	n.d
			PB: n.a							
15b			BM: 0%	3.15	9.6	21.03.2017	BM	After AI	FLT3-WT	n.d
			PB: 0%							
15c			BM: 0%	3.3	11.5	19.04.2017	BM	After HAM	FLT3-WT	n.d
			PB: n.a							
15d			BM: 0%	3.5	9.5	18.05.2017	BM	After HAM	FLT3-WT	n.d
			PB: 0%							
15e			BM: 0%	5.36	11.0	06.07.2017	BM	After HAE	FLT3-WT	n.d
			PB: n.a							
16	4	M1	BM: 93%	16.1	9.5	28.08.2017	BM	Initial	FLT3-ITD	FLT3-ITD
			PB: 50%						NPM1	NPM1
16a			BM: 2%	0.6	10.1	21.09.2017	BM	After ADxE	FLT3-ITD	n.d
			PB: n.a						NPM1	n.d
16b			BM: 0%	3.0	9.0	04.10.2017	BM	After ADxE 1st total remission	FLT3-WT	n.d
			PB: 0%						NPM1-WT	n.d
16c			BM: 0%	3.1	9.6	30.10.2017	BM	After HAM Remission	FLT3-WT	n.d
			PB: 0%						NPM1-WT	n.d
16d			BM: 0%	2.7	9.1	02.01.2018	BM	After AI Remission	FLT3-WT	n.d
			PB: 0%						NPM1-WT	n.d
17	2	M1	BM: 94%	9.7	8.4	22.08.2016	BM	Initial	NPM1	NPM1
			PB: n.a							
17a			PB: 56%	n.a	n.a	22.08.2016	PB	Initial	NPM1	NPM1
			PB: n.a							
17b			BM: 19%	0.2	10.0	13.09.2016	BM	After ADxE	NPM1	n.d
			PB: n.a							
17c			BM: 0%	2.3	9.7	21.09.2016	BM	After CDxA	NPM1-WT	n.d
			PB: n.a							
17d			BM: 0%	4.9	13.4	01.03.2017	BM	After HAE	NPM1-WT	n.d
			PB: n.a							
17e			BM: 0%	3.2	12.2	27.11.2017	BM	Suspected relapse	NPM1-WT	n.d
			PB: n.a							
18	5	M2	BM: 33%	28.7	5.7	12.12.2017	BM	Initial	NPM1	NPM1
			PB: 8%							
18a			BM: 16%	0.6	11.6	02.01.2018	BM	After AIE	NPM1	n.d
			PB: 0%							

Table 1 (continued)

Patient number	Age at diagnosis (years)	FAB	% blasts	Leukocytes (103)	Hb (g/dl)	Date	Material	Remark treatment block	Mutations gDNA	Mutations EV-DNA
18b			BM: 0% PB: 0%	2.4	13.2	25.01.2018	BM	After HAM	NPM1-WT	n.d
19	15	M5	BM: 79% PB: 11%	6.55	7.0	26.01.2017	BM	Initial	FLT3-ITD	FLT3-ITD
19a			n.a	0.01	7.4	30.03.2017	BM	After HAM	FLT3-ITD	n.d
19b			BM: 0% PB: n.a	1.13	8.5	02.05.2017	BM	After HAM	FLT3-WT	n.d
19c			BM: 0% PB: 0%	1.05	9.0	05.07.2017	BM	After HAM	FLT3-WT	n.d
19d			BM: 0% PB: n.a	0.73	8.0	29.08.2017	BM	After HAE	FLT3-WT	n.d
19e			BM: 0% PB: 0%	0.29	8.0	05.10.2017	BM	After HAE	FLT3-WT	n.d
19f			BM: 0% PB: n.a	3.33	12.2	02.11.2017	BM	After DT	FLT3-WT	n.d
19g			BM: 0% PB: 0%	0.45	10.0	21.12.2017	BM	After DT	FLT3-WT	n.d
20	7	M2	BM: 81% PB: 47%	37.24	61.9	11.09.2017	BM	Initial	NPM1	NPM1
20a			BM: 0% PB: n.a	0.37	8.6	21.09.2017	BM	After ADxE	NPM1	NPM1
20b			BM: 0% PB: n.a	0.35	10.2	05.10.2017	BM	After ADxE	NPM1	n.d
20c			BM: n.a PB: n.a	3.82	9.8	16.10.2017	BM	After ADxE 1st remission	NPM1	n.d
20d			BM: 0% PB: n.a	2.54	11.9	14.11.2017	BM	After HAM	NPM1	n.d
20e			BM: 0% PB: 0%	2.86	11.3	12.12.2017	BM	n.a	NPM1	n.d
20f			BM: 0% PB: 0%	2.81	8.8	11.01.2018	BM	Still in remission After HA(E)	NPM1	n.d
20g			n.a	n.a	n.a	13.02.2018	BM	n.a	NPM1-WT	n.d
21	18	M2	BM: 58% PB: 62%	21.0	8.0	04.08.2017	BM	Initial	FLT3-ITD	FLT3-ITD
21a			BM: 14% PB: n.a	1.0	8.3	28.08.2017	BM	After Dag-21	n.a	FLT3-ITD
21b			BM: 0% PB: 0%	1.12	7.5	04.10.2017	BM	After HAM	n.a	n.d
21c			BM: 0% PB: n.a	1.99	9.3	09.10.2017	BM	After AI	n.a	n.d
21d			BM: 0% PB: n.a	1.98	11.5	23.11.2017	BM	After AI	FLT3-WT	n.d
22	14	M6	BM: 20% PB: 5%	8.4	6.1	28.03.2017	BM	Initial	NPM1	NPM1
22a			BM: 14% PB: n.a	6.81	6.2	03.04.2017	BM	Initial	NPM1	n.a
23	17	M5	BM: 91% PB: 35%	32.86	9.7	25.07.2017	BM	Initial	FLT3-ITD, NPM1	FLT3-ITD NPM1
23a			BM: 0% PB: n.a	0.69	9.2	16.08.2017	BM	After ADxE	FLT3-WT, NPM1	n.d n.d
23b			BM: 0% PB: n.a	2.05	7.3	28.08.2017	BM	After ADxE	FLT3-WT, NPM1	n.d n.d
23c			BM: 0% PB: n.a	2.37	8.7	31.08.2017	BM	After ADxE	FLT3-WT, NPM1	n.d n.d
23d			BM: 0% PB: n.a	3.44	9.5	13.11.2017	BM	After AI	FLT3-WT, NPM1-WT	n.d n.d
24	14	M4	BM: 66% PB: 40%	9.1	6.0	08.09.2017	BM	Initial	FLT3-ITD	FLT3-ITD
24a			BM: 0% PB: n.a	0.5	9.0	28.09.2017	BM	After CDxA	FLT3-WT	n.d
24b			BM: 0% PB: 0%	1.9	9.0	11.10.2017	BM	After CDxA	FLT3-WT	n.d
24c			BM: 0%	0.7	9.0	11.01.2018	BM		FLT3-WT	n.d

Table 1 (continued)

Patient number	Age at diagnosis (years)	FAB	% blasts	Leukocytes (103)	Hb (g/dl)	Date	Material	Remark treatment block	Mutations gDNA	Mutations EV-DNA
			PB: 0%					Day 28 after BM transplantation		
24d			BM: 0% PB: 0%	2.8	7.6	24.01.2018	BM	Day 40 after BM transplantation	FLT3-WT	n.d
25	14	M4	BM: 56% PB: 31%	23.18	5.1	15.09.2017	BM	Initial	FLT3-ITD	FLT3-ITD
25a			BM: 0% PB: 0%	1.25	9.6	23.10.2017	BM	After ADxE	FLT3-WT	n.d
25b			BM: 0% PB: 0%	1.57	8.3	n.a	BM	Before AI	FLT3-WT	n.d
26	16	M4	BM: 36% PB: 14%	8.0	13.4	04.08.2017	BM	Initial	FLT3-ITD	FLT3-ITD
26a			BM: 0% PB: 0%	0.8	12.0	04.09.2017	BM	After DNX-FLA	FLT3-WT	n.d
26b			BM: 0% PB: n.a	3.1	15.3	11.09.2017	BM	After DNX-FLA	FLT3-WT	n.d
26c			BM: 0% PB: 0%	1.19	7.8	06.02.2018	BM	Month 4 after BM transplantation	FLT3-WT	n.d
27	8	M2	BM: 45% PB: 19%	3.47	7.8	01.02.2018	BM	Initial	NPM1	NPM1
27a			BM: 0% PB: n.a	0.61	10.0	01.03.2018	BM	After AIE	NPM1-WT	n.d
27b			BM: 0% PB: n.a	1.33	11.2	09.04.2018	BM	After HAM	NPM1-WT	n.d
28	7	M1	BM: 87% PB: 91%	37.8	7.7	02.03.2018	BM	Initial relapse	NPM1	NPM1
28a			BM: 96% PB: n.a	0.47	8.2	03.04.2018	BM	Day 28 relapse	NPM1	n.d
28b			BM: 0% PB: n.a	0.45	10.0	26.04.2018	BM	After Clofarabine	NPM1	n.d
29	15	M2	BM: 84% PB: 80%	24.53	15.8	22.11.2017	BM	Initial	NPM1	NPM1
29a			BM: 0% PB: n.a	0.67	10.0	13.12.2017	BM	Day 21	NPM1	n.d
29b			BM: 0% PB: n.a	1.93	9.3	19.02.2018	BM	After AI	NPM1-WT	NPM1*

Information of all the collected samples. Next-generation sequencing analysis of known mutations and GeneScan-based fragment-length analysis of FLT3-ITD and NPM1 (insertion) mutations were performed to investigate the diagnostic potential of DNA that was extracted from EVs. The results were compared with the actual mutational status of the patients that was obtained from the already existing primary leukemia database in the AML-BFM lab. Four patient samples (P1–P4) from before and after treatment were used for NGS analysis. For the sequencing, 50 ng or a maximum volume of 15 μL was used from each sample depending on their concentration. AML-specific mutations were not present in post-treatment samples. SNPs were present in all analyzed samples

Sixteen patient samples (P14–P29) from before and after treatment were used for GeneScan-based fragment-length analysis. 50 ng/μL or a maximum volume of 10.5 μL from each sample was used depending on their concentration. AML-specific mutations were not present in post-treatment samples. Any "positive" or "negative" entries are mentioned by the name of the mutations or a note that the mutations were not detectable (n.d) or not applicable (n.a). *= Low quality sequencing data

its surface hydrophilic using glow discharging for 1.5 min (easiGlow™, PELCO). Samples were then negatively stained with 10 μL of 1.5% v/v Phosphotungstic acid (PTA) for 2 min, after which excess liquid was removed. The grids were allowed to dry for at least 2 min and then observed with a JEOL JEM-1400 Plus TEM (JEOL) at 120 kV. Additional positive staining was performed for extra validation of our results. For this, 7.5 μL of isolated EV fractions were mixed and positively stained with 1.5 μL of 1% Methyl-cellulose and 1 μL of 1% Uranyl-acetate and incubated at room temperature for 30 min. From this mixture, 1.5 μL were added on to a Formvar-coated 200 mesh copper grid (#SF162, PLANO GmbH) which had been previously prepared by making its surface hydrophilic using glow discharging for 1.5 min (easiGlow™, PELCO). Samples were dried on the grid for 2 min and then observed with a JEOL JEM-1400 Plus TEM (JEOL) at 120 kV. 4K images were acquired with TemCam-F416 and EM-Menu 4 software (TVIPS GmbH).

Protein concentration analysis

A bicinchoninic acid assay (BCA) was performed using a BCA protein assay kit (#23225, ThermoFischer Scientific) according to the manufacturer's instructions. Protein concentrations were determined using the modulus microplate reader (Turner Biosystems) at a wavelength of 562 nm.

Western blots

Equal volumes of EV fractions from leukemia cell lines (MV4-11 and OCI-AML3 served as controls), healthy donors, and patient samples were treated 1:1 with RIPA buffer containing protease (cOmplete, #11697498001, Hoffman-La Roche) and phosphatase (PhosphoSTOP, #04906837001, Hoffman-La Roche) inhibitors. Next, 25 μl of the EV fractions were added to a master mix of β-mercaptoethanol (M-7522, Sigma-Aldrich) and 6× SDS Protein Loading Buffer pH 6.8 (#LB0100, Morganville Scientific), heated at 95 °C for 10 min, then loaded on to NuPAGE 4–12% Gels (#NPO321Box, Invitrogen, ThermoFischer Scientific) with the PageRuler Prestained protein ladder (#26616, ThermoFischer Scientific) for separation. THP-1 Cell Lysate (#sc-2238, Santa Cruz) was used as a control to verify the leukemia origin of the patient plasma-derived EVs and 20 μl were loaded directly to the gel after heaingt at 95 °C for 10 min. Afterwards, they were transferred on to a nitrocellulose blotting membrane (#10600001, GE Healthcare). After transfer, membranes were washed with TBS-T and blocked in 5% milk blocking solution with 0.05% Tween-20 for 30 min. The membranes were then incubated overnight at 4 °C with primary antibodies in 5% milk blocking solution (1:1000): Anti-CD63 (#EXOAB-KIT-1, System Biosciences), Anti-HSP70 (#EXOAB-KIT-1, System Biosciences), Anti-TSG101 (#HPA006161, Sigma-Aldrich), Anti-Syntenin (#ab133267, Abcam), Anti-CD33 (ab134115, Abcam), and Anti-CD13 (ab108310, Abcam). Next day, membranes were washed with TBS-T and incubated at RT for 90 min with the relevant secondary antibody (1:10,000 for the Anti-Rabbit IgG, HRP-linked Antibody, #7074S, Cell Signalling) or (1:20,000 for the Goat Anti-rabbit HRP, #EXOAB-KIT-1, System Biosciences). After washing, blots were developed with ECL Prime Western Blotting Detection reagents (#2232, GE Healthcare) and detected with a Fusion FX Machine (Vilber Lourmat Deutschland GmbH).

QIAamp DNA Micro Kit

DNA was extracted from plasma samples, EVs, and EV supernatants using the QIAamp DNA Micro Kit (#56304, Qiagen) according to the manufacturer's instructions. Briefly, 100 μL from each sample was pipetted into a 1.5-mL microcentrifuge tube with 4 μL of RNase (#56304, Qiagen) and incubated at RT for 15 min. Next, 100 μL of Buffer AL (#56304, Qiagen) was added, followed by vortexing for 15 s. Then, 10 μL proteinase K (#56304, Qiagen) was added and the samples were incubated at 56 °C for 10 min. Afterwards, 100 μL of ethanol 100% (#603-002-00-5, Honeywell Riedel-de Haën AG) were added followed by vortexing for 15 s. The samples were transferred into a QIAamp MinElute (#56304, Qiagen) column after 3 min incubation at RT. Centrifugation steps of 6000×*g* for 1 min were performed in between the washing steps with 500 μl of each washing buffer: AW1 (#56304, Qiagen) and AW2 (#56304, Qiagen). The columns were transferred into clean 1.5-mL microcentrifuge tubes and 50 μL of distilled water were applied to the center of each membrane, followed by centrifugation at full speed (20,000×*g*) for 1 min, to elute the DNA. From each eluted sample, 1 μL was used to perform Qubit or dsDNA quantifluor for the measurement of concentration. The samples were stored at − 80 °C.

DNA quantification

The dsDNA from 4 patients, unfractionated plasma, and EVs were measured using Qubit 3.0 Fluorometer (#Q33226, ThermoFischer Scientific) according to the manufacturer's instructions. Briefly, Qubit® working solution was prepared by diluting the Qubit® reagent (#Q33227, ThermoFischer Scientific) 1:200 in Qubit® buffer (#Q33227, ThermoFischer Scientific). Two Assay tubes (#Q32856, ThermoFischer Scientific) for the standards (190 μL working solution + 10 μL standards) and one for each sample (199 μL working solution + 1 μL sample) were prepared. Tubes were inserted into a Qubit® fluorometer for analysis. The dsDNA from all fractions of the remaining 25 patient samples were quantified using a dsDNA quantifluor kit (#ab27156, Promega) according to manufacturer's instructions. Briefly, 1× TE buffer, dsDNA dye working solution and standards were prepared. Next, 200 μL of dsDNA dye working solution was applied to each well (standard, blank and sample). Then, 10 μL of the standards, 10 μL 1× TE buffer for the blank or 1 μL of each sample was added. The plate was mixed thoroughly and incubated for 5 min at RT. Plate was analyzed using fluorescence (504 nm_{Ex}/531 nm_{Em}) by the modulus microplate reader (Turner Biosystems).

DNA bioanalysis

The EV-DNA fragment sizes from patient samples and healthy donor samples were analyzed using an Agilent High Sensitivity D1000 ScreenTape Assay. Briefly, a ladder was prepared by adding 2 μL High Sensitivity D1000 sample buffer and 2 μL High Sensitivity D1000 ladder (#0006371807, Agilent Technologies) in a tube strip (#0200794-260, Agilent Technologies). Samples were then

prepared by adding 2 μL High Sensitivity D1000 sample buffer and 2 μL of each sample in a tube strip. The tube strips were then covered by caps (#401425, Agilent Technologies) and mixed by vortexing for 1 min and then briefly spun down. The tube strips were then loaded in to the Agilent 4200 TapeStation instrument (4200 TapeStation, Agilent Technologies) and the caps removed. Results were then generated using the Agilent Tapestation Analysis Software.

Sequencing of patient samples

The DNA samples that were measured using the Qubit 3.0 Fluorometer (ThermoFischer Scientific) were sequenced using Next Generation Sequencing-Illumina MiSeqDx (Illumina Inc.). For targeted sequencing with the TruSight Myeloid (TSM) panel (FC-130-1010, Illumina Inc.) (Table 2), 50 ng of DNA derived from bulk bone marrow or peripheral blood cells at diagnosis were used by the AML-BFM central core facility. The plasma and EV-DNA sequencing was performed using either 50 ng of DNA or, for the samples with low concentration, a maximum volume of up to 15 μL. The libraries were prepared according to the manufacturer's protocol and sequenced with Illumina MiSeqDx using the MiSeq V2 Reagent kit (2 × 150 cycles, paired end run) (MS-102-2002, Illumina Inc.), with a total of 141 kb per sample.

GeneScan-based fragment-length analysis

The DNA from the last sixteen patients was measured using a dsDNA quantifluor kit (#ab27156, Promega) and underwent mutational detection with GeneScan-based fragment-length analysis. A master mix of reagents was prepared: Hot Start Taq 2x MM 12.5 μL, DEPC H_2O 8.5 μL, Primer F (10 pmol/μL) 1 μL, Primer R (10 pmol/μL) 1 μL. A DNA concentration of 50 ng/μL or a maximum volume of 10.5 μL of sample was used. Water can be avoided in case of 10.5 μL of DNA. For amplification, samples were heated at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 59 °C for 15 s and 72 °C for 15 s. GeneScan-based fragment-length analysis was performed for the FLT3-Internal Tandem Repeat (FLT3) and Nucleophosphomin (NPM1) mutations by using the following primers:

FLT3-ITD:

Primer fw: 5′-GTAAAACGACGGCCAGGCAA TTTAGGTATGAAAGCCAGC-3′
Primer rev: 5′-FAM-CTT TCA GCA TTT TGA CGG CAA CC-3′

NPM1:

Primer fw: 5′-GTAAAACGACGGCCAGGATG TCTATGAAGTGTTGTGGTTCC-3′
Primer rev: 5′-VIC-ATC AAA CAC GGT AGG GAA AGT TC-3′

PCR products were diluted (1:70) in H_2O. One microliter of diluted PCR products was mixed with 10 μL HiDi Formamid (Applied Biosystems) and 0.3 μL GeneScan 600 LIZ Size Standard v 2.0 (ThermoFischer Scientific). PCR products were denaturated for 5 min at 95 °C and the GeneScan-based fragment-length analysis was performed using the 3500 genetic analyzer (Applied Biosystems).

Results

Characterization of EV preparations

To characterize the EV content of plasma from pediatric AML samples, EVs were harvested by differential centrifugation/ultracentrifugation steps. Obtained EV fractions were characterized by nanoparticle tracking analysis (NTA) for the number and average diameter of obtained particles, while BCA assay was also performed to support the NTA quantification with additional protein quantification data, with a strong correlation between the two parameters being observed (Supplementary Figure 2; Supplementary Table S2). Average particle sizes obtained from AML patient plasma samples were in the range of 30–150 nm, the expected size of EVs (Figure 1a, b). To confirm the presence of EVs, transmission electron microscopy was performed. Bona fide EVs were indeed observed in all preparations studied (Fig. 1c–f). The presence of EVs in our preparations was further proven by Western blotting using EV-specific antibodies (Fig. 2a). EVs from leukemia cell lines which served as positive controls showed clear bands with the EV-specific antibodies. Furthermore, the origin of the EVs was confirmed by additional Western blots using myeloid-specific antibodies, which were present in the positive control as well as in the leukemia patient samples (Fig. 2b). For reference, EVs from healthy donors were also probed with the same myeloid-specific antibodies, which gave negative results (Fig. 2b). Healthy donor samples were also probed with EV-specific antibodies, but it was not possible to detect all tested antibodies. However, this could be explained by the lower number of EVs present in healthy donor samples in comparison to patient samples as the number of particles measured by NTA appeared to be statistically significantly higher in patient plasma samples than in healthy donor samples ["p" = 0.0057] (Supplementary Figure S3), and the same volume of EV fractions and not equal protein amount was loaded for Western blot analysis. As the healthy donor samples were from adults, this significant difference in EV

Table 2 Target regions of the TruSight Myeloid panel (Illumina)

Gene	Target region (exon)	Gene	Target region (exon)	Gene	Target region (exon)	Gene	Target region (exon)
ABL	4–6	DNMT3A	Full	KDM6A	Full	RAD21	Full
ASXL1	12	ETV6/Tel	Full	KIT	2, 8–11, 13+17	RUNX1	Full
ATRX	8–10 and 17–31	EZH2	Full	KRAS	2+3	SETBP1	4 (partial)
BCOR	Full	FBXW7	9+10+11	MLL	5–8	SF3B1	13–16
BCORL	Full	FLT3	14+15+20	MPL	10	SMC1A	2, 11, 16+17
BRAF	15	GATA1	2	MYD88	3–5	SMC3	10, 13, 19, 23, 25+28
CALR	9	GATA2	2–6	NOTCH1	26–29+34	SRSF2	1
CBL	8+9	GNAS	8+9	NPM1	12	STAG2	Full
CBLB	9, 10	HRAS	2+3	NRAS	2+3	TET2	3–11
CBLC	9, 10	IDH1	4	PDGFRA	12, 14, 18	TP53	2–11
CDKN2A	Full	IDH2	4	PHF6	Full	U2AF1	2+6
CEBPA	Full	IKZF1	Full	PTEN	5+7	WT1	7+9
CSF3R	14–17	JAK2	5+7	PTPN11	3+13	ZRSR2	Full
CUX1	Full	JAK3	3+13				

number cannot be fully concluded to be disease-related. Additionally, as normal myelopoiesis may be a rich source of EVs depending on regenerative activity and/or phases of cell destruction, our finding that steady-state PB of adults does not contain detectable amounts of myeloid EVs in Western blot experiments could be a matter of sensitivity and does not exclude that they could be detectable in other/better selected controls.

Analysis of EV numbers in pediatric AML samples at different stages of treatment

Next, we investigated whether EVs present in the plasma samples reflect the status of the leukemia blast burden in pediatric AML patients undergoing therapy. As proof of principle, we selected 20 of 29 patients in the study and isolated EVs from the selected patients' plasma samples at diagnosis and during different treatment blocks (Fig. 3). When comparing the particle numbers before and after treatment (all patients combined at each therapy block), lower particle numbers (although non-significant) were observed after treatment than before treatment ["p" = 0.084] (Fig. 3a). The number of leukemic blast cell burden was also declined during therapy; however, there was no correlation between the decrease of the amount of EVs and blasts (Table 1) (Supplementary Figure 4, and Supplementary Table S2). Analysis of EV numbers in individual patients before treatment and during different treatment blocks revealed a fluctuation of EV numbers in the plasma of patients, with a trend of an initial sharp decline and gradual increase during the time course of treatment (Fig. 3b–e).

EV samples from pediatric AML patients contain dsDNA reflecting AML-specific mutations

To establish the diagnostic relevance of EV-dsDNA isolated from pediatric AML patients, we isolated total DNA from EV fractions of our AML patients and, for comparison, from the unfractionated and fractionated plasma. Prior digestion of the DNA bound to the outside of EVs was not a pre-requirement in this study, as removing this DNA would have no diagnostic advantage for detecting mutations in EV-associated dsDNA. The DNA concentration was measured using Qubit (Fig. 4a) to quantify the amount for downstream NGS analysis. Additionally, dsDNA Quantifluor (Fig. 4b) was used to establish the double-stranded nature of EV-DNA, as demonstrated by Thakur et al. 2014 [13]. After isolation of EVs, we observed a lower concentration of dsDNA in EV-depleted (fractionated) plasma in comparison to unfractionated and EV fractions in the majority of patients in this study (Fig. 4a, b), validating the recent paper suggesting that majority of previously considered human blood plasma cell-free DNA is localized in EVs [24]. Additionally, in around 50% of the samples, the EV fractions contained more dsDNA than the initial unfractionated plasma (Fig. 4a, b). This could reflect that the DNA inside of the EVs is more protected than the cell-free DNA, which is vulnerable to degradation by nucleases in the plasma. In the next step, the dsDNA extracted from the initial samples, unfractionated plasma and EVs from patients 1, 2, 3, and 4, was sequenced using Illumina MiSeqDx (Supplementary Table S3). In three patients, identical mutations were detected in the EV-dsDNA and in the genomic DNA from the primary sample (gDNA) (Table 3). In one patient, mutations were only detectable

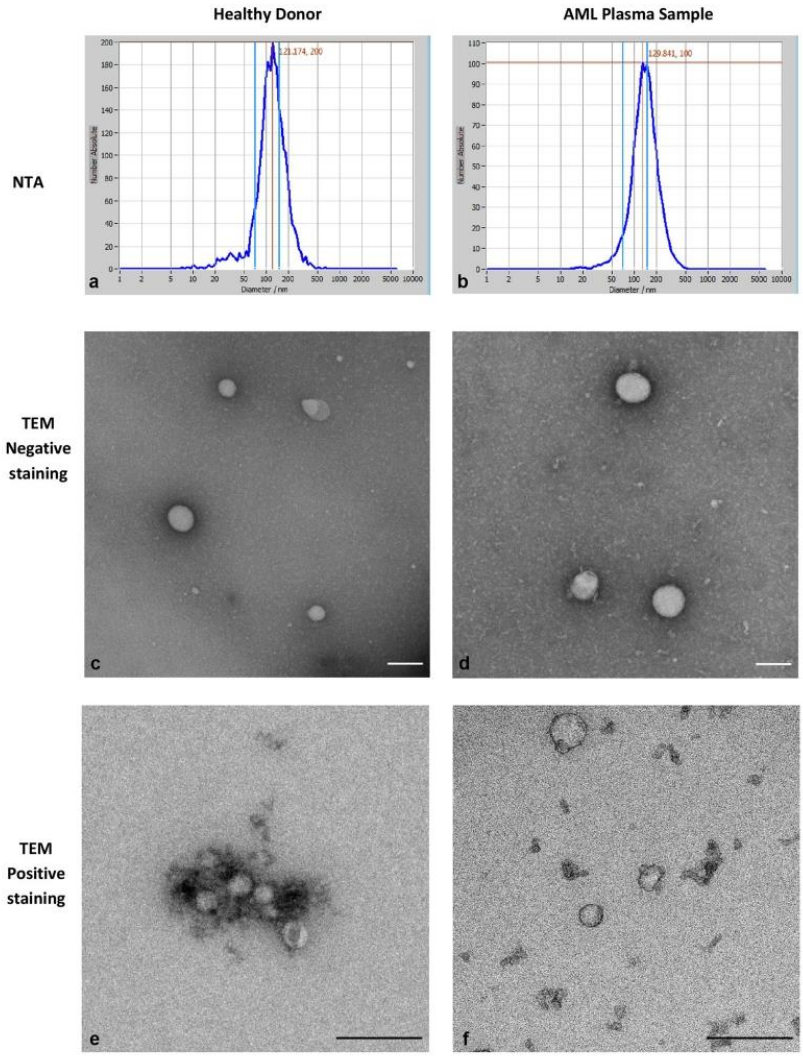


Fig. 1 Detection and characterization of EVs in healthy donor samples and patient samples. **a**, **b**) Exemplary data of Diameter size and number of particles per mL of plasma from healthy donor (HD) and AML patient samples (P), as measured by NTA. The size distribution is in the range of extracellular vesicles (30–150 nm). **c**, **d**) Exemplary data of TEM of negatively stained EVs from plasma of healthy donor samples and AML patient samples. Scale bar 200 nm. **e**, **f**) Exemplary data of TEM of positively stained EVs from plasma of healthy donor samples and AML patient samples. Scale bar 200 nm

in the gDNA and not in unfractionated plasma or EV-dsDNA (Table 3).

EV-derived dsDNA reflects therapy status based on mutational signature in pediatric AML

To gain insight into whether EV-dsDNA can be used to monitor the treatment response in pediatric AML patients, EV-dsDNA was isolated from patients before and after treatment. We observed that almost all AML patients in our study had higher dsDNA levels in EVs before therapy, compared with the EVs harvested from the same patients after therapy (Fig. 4c, d). The mutational background of the patients appeared to play a role in the EV-dsDNA concentration, as the samples from patients carrying only the FLT3-ITD mutation revealed a higher amount of dsDNA after treatment, while samples from patients that had an NPM1 mutation only or combined FLT3-ITD and NPM1 mutations showed decreased dsDNA concentration after treatment (Fig. 4e). The same trend was also observed with the EV number and RNA concentration [23]. In addition, to further characterize the EV-DNA, the DNA before and after treatment was analyzed using a bioanalyzer. This analysis revealed that in AML patients, four distinct DNA fragment sizes could be detected in EVs before treatment (Fig. 4f), but not after treatment

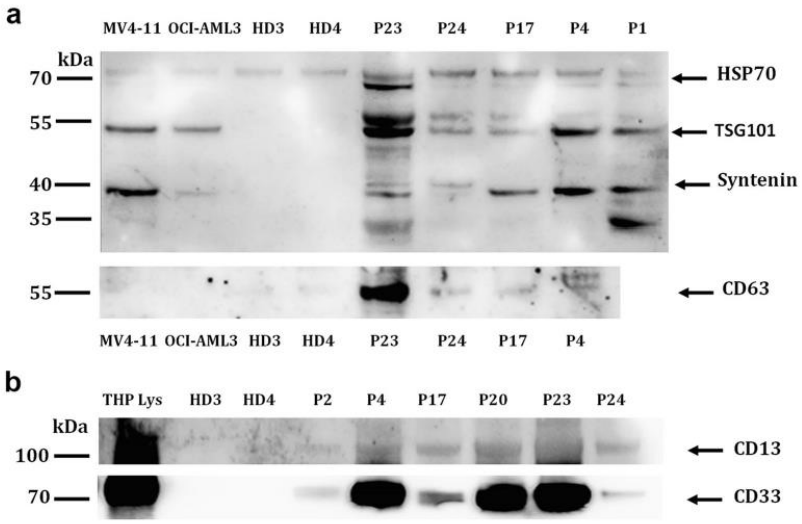


Fig. 2 Western blot analysis of EV-specific markers. **a** Western blot analysis of leukemia cell lines (MV4-11 and OCI-AML3 as positive control), healthy donors and AML patient samples for EV-specific antibodies. **b** Western blot analysis of THP1 lysate (positive control), healthy donors, and AML patient samples for myeloid-specific antibodies

(Fig. 4g). Healthy donor EV-DNA was also analyzed for comparison and it was found that healthy donor DNA reflected that of the after treatment samples (Fig. 4h). This result could reflect that the DNA fragment sizes are in some way affected by the AML disease state, or that the amount of DNA in healthy donor EVs or after treatment EVs is too low to detect these distinct groups. Furthermore, in the initial four patient samples where mutational status was analyzed using NGS analysis, the initially discovered AML-specific mutations were no longer detectable in the EV-dsDNA in two out of four patients after treatment (P1 and P4), suggesting that the decline in EV number after therapy is associated with a reduction in the number of cancer cells that contain AML-specific mutations (Table 1). Subsequently, sixteen more patient samples (P14-P29) were analyzed for specific AML mutations using GeneScan-based fragment-length analysis. These results supported the previous results obtained from NGS. AML-specific mutations were detectable in EV-DNA before treatment (except patient 15) but not after treatment, except in four patient samples (P17, P20, P21, and P29) where the mutations were still detectable in one after treatment sample (Table 1). In the majority of samples, the EV-dsDNA findings were corresponding to the gDNA mutational status provided by the AML diagnostic laboratory (Table 1). Additionally, in two patients (P2 and P15) no AML-specific mutations were detectable before treatment; therefore, it was obvious that after treatment, the mutations were still undetectable. Patient sample P3 had only single-nucleotide polymorphisms (SNPs) and no AML-specific mutations, logically serving as a positive control in our study. As expected, we observed that the SNPs were detectable in EV-dsDNA before and after treatment (Table 1), again suggesting that therapy in patients leads to a reduction of EVs released by leukemia cells without compromising the EVs released by healthy cells in blood circulation.

Discussion

In this study, we aimed to evaluate leukemia-derived EVs for their diagnostic importance, based on the fact that EVs derived from tumor cells or leukemia blasts illustrate specific protein, lipid, and nucleic acid signatures that represent the pathological state of the respective parental cells [25].

Firstly, we provided evidence that EVs were indeed present in our samples and some had a myeloid origin. Then, we showed that the number of EVs could perhaps be a useful diagnostic indicator, as EV numbers declined accumulatively after treatment. Secondly, after demonstrating that EVs in tumor patients contain dsDNA, we revealed a potential correlation between the mutational background and the dsDNA concentration before and after treatment. After testing our samples for their DNA quality, we wondered whether EV-dsDNA had the diagnostic potential to detect AML. In proof of principle experiments, we demonstrated that discovered somatic mutations were detectable in the EV-dsDNA of seventeen AML patients before treatment but not in EV-dsDNA after treatment, with an exception of three cases (P2, P3, and P15).

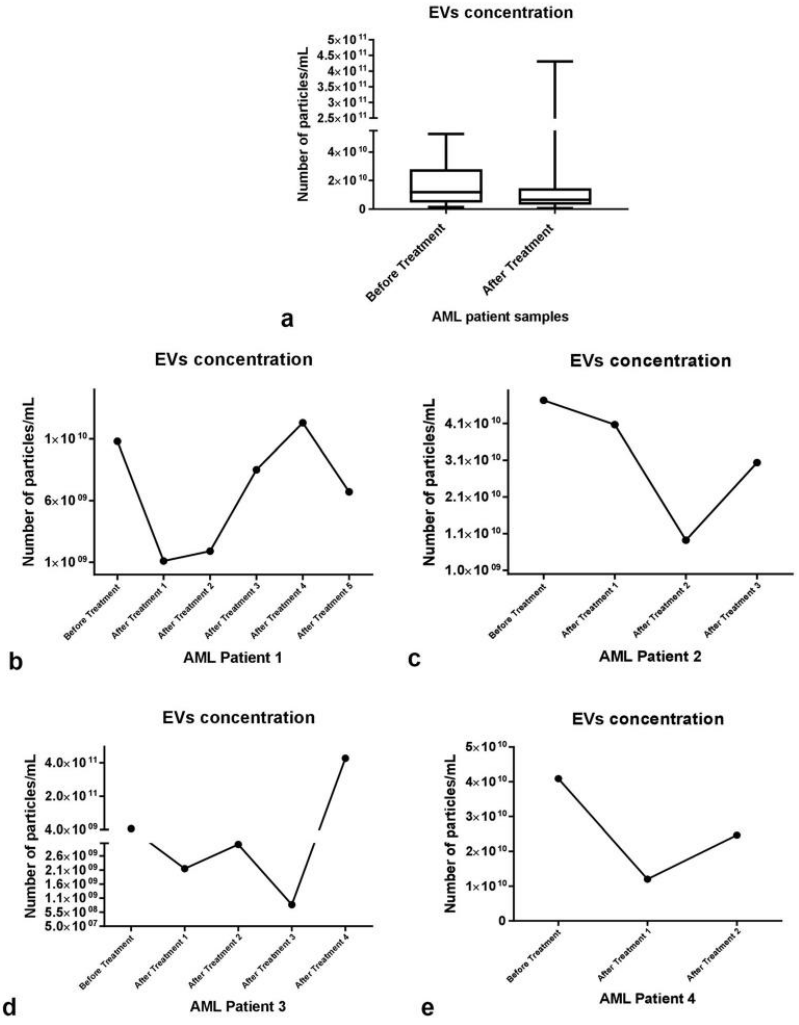


Fig. 3 Comparison of EVs before and after treatment. **a** Comparison of EV concentration in before and after treatment samples from 20 AML patients by NTA analysis. Higher concentration of EVs in before treatment samples was observed. **b**–**e** Individual monitoring of EV number using NTA. EVs isolated from the plasma of AML patients P1–P4 from before and after treatment

When considering the average EV concentration of all patients together, the observed overall reduction in EV concentration after treatment was expected, as the treatment should eliminate leukemia cells and, therefore, the number of leukemia-derived EVs in circulation. When considering the EV concentration results for each individual patient, fluctuation in the number of EVs after each treatment time point was observed which was not correlated to any other blood value such as the number of blasts. Consequently, the reason for these individual fluctuations are unclear; however, it could be related to normal myelopoiesis which may be a rich source of myeloid-derived EVs depending on regenerative activity and/or phases of cell destruction. Additionally, the result could be related to each person's individual response to the treatment, physical conditions, genetic background, etc. In future, to validate these results, it will be necessary to repeat this study using a large cohort of patients with well-chosen, age appropriate control subjects. In addition, the lifestyle factors which could influence the EV status of the participants in the study, e.g., circadian rhythm, exercise, nutrition, and stress, should be taken in to consideration at the time of the blood draw. Previously, it has been published that EVs released by cells of highly metastatic, malignant tumor cells contain high amounts of dsDNA [26]. Consequently, in this study, DNA isolated from EV fractions was detectable, and sometimes at an even higher concentration than the DNA of unfractionated

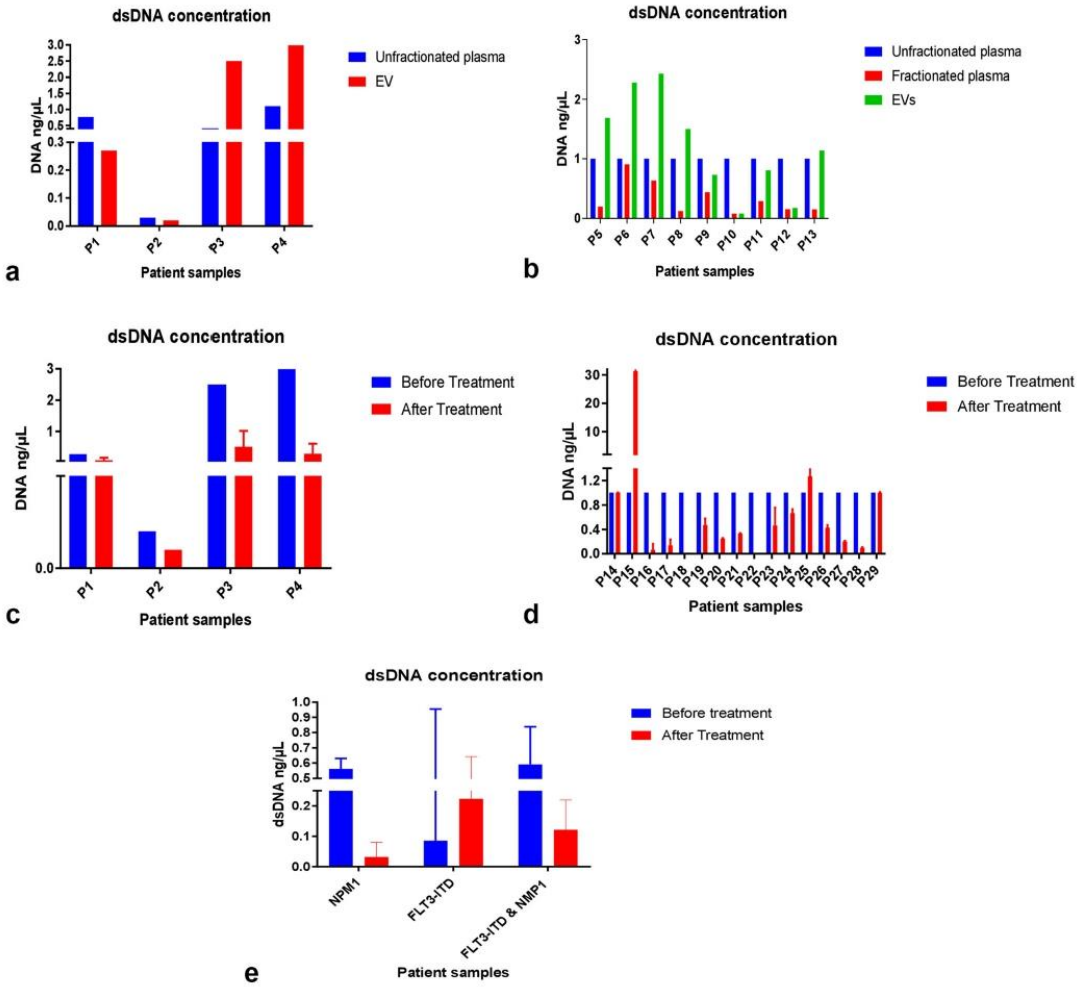
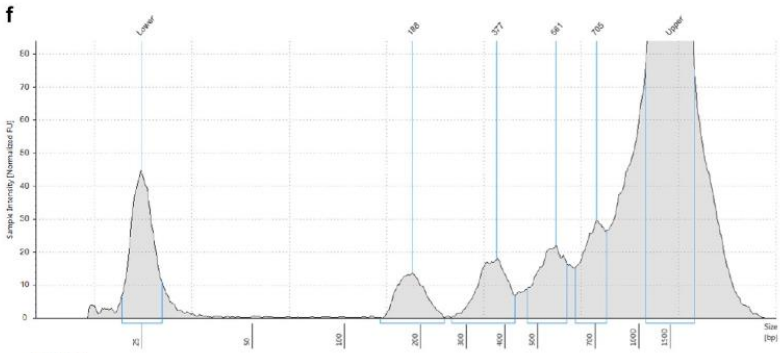


Fig. 4 Comparison of dsDNA from different plasma fractions and EV fraction in patient samples. **a** Comparison of dsDNA concentration that was extracted from unfractionated plasma and EV fractions of AML patients P1–P4. Qubit was used for the quantification of the samples. No specific trend was observed. **b** Comparison of dsDNA concentration that was extracted from unfractionated plasma, fractionated plasma, and EV fractions of AML patients P5-P13. dsDNA quantifluor was used for quantification and, again, no specific trend was observed. **c** Comparison of dsDNA concentration that was extracted from EV fractions of AML patients P1–P4 from before and after treatment. **d** Comparison of dsDNA concentration that was extracted from EV fractions of AML patients P14–P29 from before and after treatment. **e** Comparison of dsDNA concentration that was extracted from EV fractions of AML patients P14–P29 from before and after treatment, according to their mutational background. **f** Qualitative analysis of dsDNA of a patient sample before treatment. **g** Qualitative analysis of dsDNA of the same patient after treatment. **h** Qualitative analysis of dsDNA of a healthy donor sample

samples. EV-DNA and unfractionated DNA were sequenced to compare the mutational status with that of the corresponding patient gDNA that was recorded at primary diagnosis. As expected, the sequencing data revealed that the unfractionated plasma DNA and EV-DNA from the same individual patients had the exact same mutations, showing that, diagnostically speaking, the unfractionated DNA and the EV-DNA could complement each other. Routinely, after treatment, follow-up sequencing is performed using patient gDNA to analyze the mutational status of the patient. Therefore, in some cases, we were able to compare the mutational status between the gDNA and EV-DNA of patients after treatment. This analysis revealed that the EV-DNA and gDNA after treatment both showed the same absence of AML-specific mutations or the presence of SNPs in the majority of the samples, with mutational absence being presumably due to

f



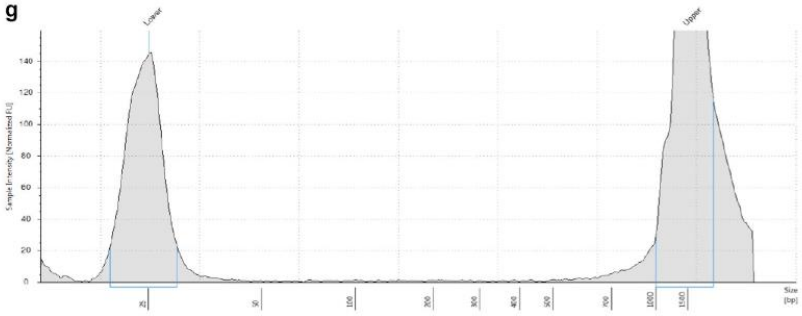
Peak Table

Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	Peak Molarity [pmol/l]	%Integrated Area	Peak Comment	Observations
25	22.4	-	1380	-		Lower Marker
188	8.72	-	71.2	17.58		
377	12.0	-	49.1	24.25		
561	13.4	-	36.9	27.08		
705	15.4	-	33.7	31.10		
1500	250	250	256	-		Upper Marker

Region Table

From [bp]	To [bp]	Average Size [bp]	Conc. [pg/µl]	Region Molarity [pmol/l]	%of Total	Region Comment	Color
50	1350	660	87.1	345	72.38	whole	

g



Peak Table

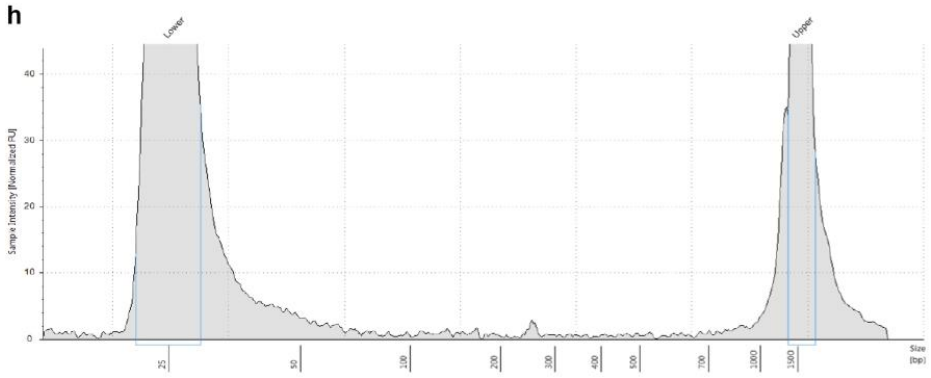
Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	Peak Molarity [pmol/l]	%Integrated Area	Peak Comment	Observations
25	105	-	6450	-		Lower Marker
1500	250	250	256	-		Upper Marker

Region Table

From [bp]	To [bp]	Average Size [bp]	Conc. [pg/µl]	Region Molarity [pmol/l]	%of Total	Region Comment	Color
50	1350	688	12.3	125	13.08	whole	

Fig. 4 continued.

the number of leukemic cells/EVs being reduced to such a low level that they no longer approached the detection threshold of the assay. Given that this method was mostly successful, in future, the plan would be to perform this assay with more sensitive sequencing methods, in order to further optimize the diagnostic potential of EV-DNA in AML. Due to the complexity of the disease, we are convinced that an elaborated understanding of clonal evolution in AML and an earlier detection of evolving AML sub-clones will help to improve diagnostic and therapeutic strategies. Accordingly, using the results from this current project and described future studies, the ultimate future aim would be to develop and establish a novel, highly sensitive EV-dsDNA-based analysis platform to serve as an additional approach to current leukemia diagnostic platforms, for obtaining additional/complementary information on leukemia cell populations. We believe that a diagnostic approach that combines multi-compartment blood



Peak Table

Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	Peak Molarity [pmol/l]	% Integrated Area	Peak Comment	Observations
25	342	-	21000	-		Lower Marker
1500	250	250	256	-		Upper Marker

Region Table

From [bp]	To [bp]	Average Size [bp]	Conc. [pg/µl]	Region Molarity [pmol/l]	% of Total	Region Comment	Color
50	1350	752	30.8	248	30.78	whole	

Fig. 4 continued.

profiling in pediatric AML will prove to be superior to the current individual molecular diagnostic approaches that mainly focus on one class of diagnostic factors derived from leukemia blasts.

In conclusion, in this study, we demonstrated that AML-derived EVs contain DNA that reflects the mutational status of the cells of origin. To the best of our knowledge, this is the first study demonstrating the mutational analysis of EV-dsDNA in primary AML pediatric patient samples. Although these are very preliminary results, with further optimisation and additional large cohort studies this method could potentially enhance or

Table 3 Next generation sequencing analysis of four patient samples

Patient	Cancer type	Mutation in gDNA of AML cells	Primary leukemia cells	Unfractionated Plasma	EV-DNA
P1	AML-M1	NPM1 FLT3/TKD WT1 GATA2 ETV6 (SNP) ZRSR2(SNP)	NPM1 FLT3/TKD WT1 GATA2 ETV6 (SNP) ZRSR2(SNP)	NPM1 FLT3/TKD WT1 GATA2 ETV6 (SNP) ZRSR2(SNP)	NPM1 FLT3/TKD WT1 GATA2 ETV6 (SNP) ZRSR2(SNP)
P2	AML-M2	FLT3/ITD RAD21 KIT EZH2	FLT3/ITD RAD21 KIT EZH2	n.d n.d n.d n.d	n.d n.d n.d n.d
P3	AML-M3	GATA2 (SNP) NOTCH1 (SNP)	GATA2 (SNP) NOTCH1 (SNP)	GATA2 (SNP) NOTCH1 (SNP)	GATA2 (SNP) NOTCH1 (SNP)
P4	AML-M4	NRAS KIT (SNP) PHF6 (SNP)	NRAS KIT (SNP) PHF6 (SNP)	NRAS KIT (SNP) PHF6 (SNP)	NRAS KIT (SNP) PHF6 (SNP)

Next-generation sequencing analysis of known mutations was performed to investigate the diagnostic potential of DNA that was extracted from primary leukemia cells, unfractionated plasma, and EVs. The results were compared with the actual mutational status of the patients that was obtained from the already existing primary leukemia database in the AML-BFM lab. Four initial patient samples (P1–P4) before therapy were used for this analysis. For the sequencing, 50 ng or a maximum volume of 15 µL was used from each sample depending on their concentration. AML-specific mutations were detectable in all fractions of patient samples, except in patient 2. SNPs were present in all analyzed samples. Any "positive" or "negative" entries are mentioned by the name of the mutations or a note that the mutations were not detectable (n.d)

support classic AML detection methods that are currently used today, and will permit the development of new improved strategies for better diagnosis, prognosis, and therapy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Informed consent Informed consent was obtained from all individual participants (or their parents) included in the study. Each patient was consented following institutional review board approval AML-BFM 2004 (3VCreutzig1).

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Statement of welfare of animals This article does not contain any studies with animals performed by any of the authors.

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3. DISCUSSION

AML represents about 20% of all pediatric leukemias, showing relapse rates of more than 30% in all patients. Its biological heterogeneity at the level of gDNA is one of the challenges for clinically targeting AML and accomplishing a disease-free state in patients (Aziz et al. 2017; Grove and Vassiliou 2014). In spite of the ongoing advances in the treatment and diagnosis of AML, a significant portion of patients relapse, which remains the greatest reason for death in AML (Hornick et al. 2015; Paietta 2012). These features highlight the ongoing requirement for the development of a delicate methodology with the capacity to recognize and screen the disease all the more adequately. All the while, this methodology ought to include the benefit of reducing pain and inconvenience for the patients brought about by the present standard bone marrow puncture-based strategies. In the present project, we endeavored to make the primary strides towards the improvement of a tool that would satisfy these criteria by assessing the diagnostic capability of both plasma-derived EV-RNA and EV-dsDNA in pediatric AML, as they reflect the diseased condition of the cells of origin by depicting explicit nucleic acid, protein, and lipid signatures (Whiteside 2016).

3.1 Cell lines- derived EVs and their RNA information reflection

Two of the most common mutations in pediatric AML are NPM1 (Braoudaki et al. 2010), and FLT3-ITD (Sexauer and Tasian 2017), which are also observed in the majority of our patient samples. Consequently, in order to firstly establish the methodology, cell lines carrying these mutations were selected prior the use of our important patient samples. Mutational analysis revealed that both mutations were detectable in RNA extracted from both cells and EVs, showing a higher expression in EVs with FLT3-ITD mutations in comparison to the RNA of the cells of origin. These data revealed a possible relation between the RNA levels in EVs and their mutational background. Cells with FLT3-ITD mutation could release EVs with higher amounts of RNA, possibly due to higher gene expression, which could show a related mechanism between the mutation and the EV-RNA. Unfortunately, there is no other similar study with findings that could be comparable. In addition, there is an expanding assemblage of proof supporting that fragments of non-coding RNA (ncRNA) are implicated in gene regulation, as well as many other biological processes (De Lay and Garsin 2016; Anderson and Ivanov 2014; Chen et al. 2016; Sharma et al. 2016).

It has been proven that numerous fragments obtained from mRNAs and ncRNAs revealed a noticeable amelioration in EVs in comparison to their cells of origin, confirming the relation between the EVs and several of the particular fragments (van Balkom et al. 2015; Vojtech et al. 2014; Nolte-'t Hoen et al. 2012; Tosar et al. 2015; Lunavat et al. 2015). This means that a FLT3-ITD mutation could be related to higher levels of these RNA fragments in the EV preparations. Hence, as there is no published evidence connecting the mutational background with the amount of the EV-RNA, additional experiments in a larger study cohort of patients carrying the current mutation, showing any possible mechanistic relationship between the mutational background and the EV-RNA, is necessary. This could provide us with useful pathophysiological and mechanistic information, which could help us better understand how the mutation can affect the RNA levels, potentially leading to an increase in the efficiency of treatment methods for patients carrying this mutation. However, mutational detection was performed successfully in all cases in spite of their background. These positive outcomes reflected that our proposed techniques were substantially important and useful at the cell line level, and warranted further investigation with clinical samples.

3.2 EVs in pediatric AML patient samples

As it has been previously published, EVs are shed constantly from both healthy and diseased cells. They have been proven to regulate many biological processes such as intercellular signal transmission, immune responses, infections, antigen presentation, and cancer progression (Li et al. 2006; S et al. 2013; Yanez-Mo et al. 2015; Park et al. 2013; Lee et al. 2011). Apart from direct intercellular interaction between healthy and/or cancerous cells, they also mediate the communication between tumor and distant cells, metastasis and immune responses (Zhang and Grizzle 2014). Depending on the cell of origin, EVs appear to have different molecular signatures by carrying many important biomolecules such as nucleic acids, proteins and lipids into the extracellular space (Ratajczak et al. 2006; Deregibus et al. 2007; Valadi et al. 2007). Therefore, EVs are involved in both beneficial and pathological functions. They can be used as a useful tool for the evaluation of health status as well as cancer diagnosis and prognosis, as they can be analyzed and examined regarding their composition from body fluids such as breast milk, blood and more (Borges, Reis, and Schor 2013; Kim et al. 2015). These publications confirmed our successful validation of EV presence in our patient samples, which revealed a significantly lower

amount of EVs in the control group in comparison to the AML group. Such a finding is supported by previous studies showing that in the vast majority of malignancies, a higher amount of EVs is released when compared to healthy cells (Lazaro-Ibanez et al. 2014; Went et al. 2004; Galindo-Hernandez et al. 2013). This also explains why markers of myeloid origin were undetectable in the EVs originating from healthy cells, as this could be related to the decreased amount of EVs that healthy cells release. A general reduction in the EV concentration after therapy takes place as the exploitation in the secretion of EVs succeeding the short cytotoxic cell exposure after a therapy is transient due to the loss of many tumor cells (Ab Razak et al. 2019). After therapy many cancer cells have been rapidly killed by undergoing apoptosis, and, consequently, the number of tumor-derived EVs has been reduced. These results are in agreement with our findings, which in both studies showed that the quantity of particles was consistently lower in the after-treatment EV samples than in the before-treatment samples. This outcome suggests that a decrease in the number of leukemia cells after-therapy corresponds to the decrease in the total number of EVs.

However, when it comes to each individual patient, it is obvious that there is a fluctuation in the EV amount that is not related to any other blood value. Subsequently, the explanation behind these individual fluctuations are unclear; in any case, it could be identified with typical myelopoiesis, which might be a rich wellspring of myeloid-derived EVs relying upon regenerative action and additional cell destruction periods. Moreover, the outcome could be identified with every individual reaction to the treatment, physical conditions, diet, body mass index and so forth (Robbins 2017; Danielson et al. 2016). These factors could affect the release rate or even the quality of the EVs leading to different results each time. Consequently, in future, in order to approve these outcomes, it will be important to rehash this examination utilizing an increased number of patients with well-chosen factors among groups. Furthermore, the way-of-life factors that could impact the EV status of the members in the study, e.g., circadian rhythm, exercise, nutrition, and stress, ought to be considered at the time point of the blood draw. As we found that there was a higher EV number in the after-treatment samples carrying FLT3-ITD mutation in comparison with the samples with NPM1 or both NPM1 and FLT3-ITD, it is clear that the number of EVs is not only affected by the treatment and the number of leukemia cells, but also by the mutational background of the cells, a status that is statistical significant revealing a relationship between the mutational group and the number of released EVs. However, there is no published study with relative outcome to compare with ours. Therefore, further investigations are needed in

order to check whether there is a correlation between the mutations and the released EV amount, which could help us to understand better how the mutational status can affect the progress of the disease with regards to EV level.

3.3 EV-RNA and EV-dsDNA findings

Despite a better perception regarding the EV heterogeneity and the non-EV RNA-carrying structure contaminants, whether RNA is embraced by all EV types as well as how the various EV subpopulations can affect and differentiate the RNA content are still underexplored. Several investigations have demonstrated that RNA is highly heterogeneous and differs among the various subpopulations along with the cell type of cell of origin. This high heterogeneity is also affected by other facts. For instance, it has been shown that miRNA in EVs is dependent on the immune cell type (Mittelbrunn et al. 2011). Furthermore, an interesting study supported RNA heterogeneity by revealing that sex affects the miRNA content in EVs isolated from urine (Ben-Dov et al. 2016). Another important factor that has been proven to affect the RNA content is the separation of EVs in several fractions by a variety of g-forces (Crescitelli et al. 2013). Moreover, an important RNA analysis complication in EVs is the contamination of non-EVs extracellular RNA structures such as large proteins or lipoprotein complexes (LDL and HDL which carry miRNA) (Vickers et al. 2011), viral particles, and ribonucleoprotein complexes (RNPs), that can be co-isolated when performing ultracentrifugation (Arroyo et al. 2011; Shelke et al. 2014; Wei et al. 2016). Hence, it is clear that not all the RNA forms are present in every EV population, and it could be possible that in some cases non-EVs RNA can be co-isolated leading to a completely different EV-RNA outcome. On top of that, there is no confirmation showing how the EV-RNA pieces are constructed, what the formation process is and how to ensure that they are not co-isolated contaminants which could tamper with the outcome. There are two scenarios regarding the cleavage of EV-RNA, either the process takes place in the cytoplasm of the cells of origin and follows the compound of the formed fragments into EVs or instead the generation of the fragments is completed inside the EVs during a maturation process (Mateescu et al. 2017). However, to address this issue, many *in vitro* experiments of EV-RNA transfer have been designed, with a remaining challenge to find out how EVs and EV-RNA enter the target cells.

As the main purpose of studying EV-RNA is its diagnostic potential in the concept of liquid biopsy, blood despite its complexity, is the most generally utilized biological fluid for EV research and

biomarker discovery. However, the process of EV isolation remains challenging and it seems to be of high importance. In plasma, EVs can be used as a tool for studying (patho)physiological processes, while in serum most of the EVs have been shed by platelets, and could have a biomarker potential for many pathological actions (Antwi-Baffour et al. 2015). Regardless of its wide research use, there is constrained information on the differences in RNA level between plasma and serum samples coming from the same source (Cheng et al. 2014).

Our plasma analysis of 16 AML patients (73 samples) reflected again a relation between the amount of EV-RNA and the mutational background by presenting higher concentration of EV-RNA in the before-treatment samples than in the after-treatment samples in AML patients with the NPM1 mutation only or with a combined mutation of NPM1 and FLT3-ITD. In contrast, the group carrying FLT3-ITD mutation, showed higher concentrations in the after-treatment in comparison with the before-treatment samples. Conclusively, the higher EV-RNA concentration in the samples of patients with FLT3-ITD mutation could be affected by a higher amount of RNA contaminants or could be related to a different way of RNA endorsement in the EVs. Both possibilities seem to be connected to the mutational background, although there is no published evidence to evaluate this. Nevertheless, the field continues to encounter numerous technical challenges: the large variety in EV isolation procedures, the small size of EVs, the minute amounts of RNA that are recovered from these vesicles, and biases in the analysis of these trace amounts of RNA. Consequently, it is considered necessary and important to improve the currently used EV isolation methods as well as the quantification techniques of EV-RNA, which will foster progress towards a new era of EV analysis by providing more reliable results and identification of explicit biomarkers.

Besides RNA, EVs contain also DNA, which can be present as single-stranded DNA (ss-DNA), mitochondrial DNA (mtDNA), or dsDNA (Thakur et al. 2014; Guescini et al. 2010; Wang et al. 1987) and whose size varies from 100 bp to fragments of up to 2 million bp (Thakur et al. 2014; Vagner et al. 2018). It has been demonstrated that EV-DNA is detected either on the EV surface (Fischer et al. 2016; Nemeth et al. 2017; Grigor'eva et al. 2016), or inside the EVs, where it is well protected from degradation due to the lipid membrane (Thakur et al. 2014; Vagner et al. 2018). Interestingly, there are studies supporting that EVs that are shed from tumor cells contain gDNA fragments, which represent the entire genome with a spread over all chromosomes. They are resistant to enzymes e.g. DNase and they mirror the mutational background of the corresponding

tumor cells (Kahlert et al. 2014; Lee et al. 2014; Thakur et al. 2014; Lazaro-Ibanez et al. 2014). However, extracellular DNA, that is present in many biological fluids such as plasma (Kondratova et al. 2005), shows high sensitivity to enzymatic activities like digestion. It is connected to the EV surface and plays an important role in EV zeta potentials, as well as the EV internalization and aggregation (Fischer et al. 2016; Nemeth et al. 2017).

Similarly to the EV-RNA, EV-DNA shows high heterogeneity. *Lázaro-Ibáñez E et al*, proved that prostate cancer EV-DNA illustrates mutations that are identical to the cell of origin mutations. However, they showed that their DNA cargos were varied in distinct populations and were connected to the parental cells as well as the EV types (Lazaro-Ibanez et al. 2014).

A variety of studies have brought to light many important information in the field of EV-DNA. EV-DNA has been proven to extend across all chromosomes of gDNA (Thakur et al. 2014; Vagner et al. 2018; Kahlert et al. 2014). Interestingly, mtDNA is not present in all EVs, revealing a connection between this type of DNA and the cells of their origin (Thakur et al. 2014). Surprisingly, EV-DNA originating from bacteria as well as a non-consistent illustration of the human genome was shown in EVs of healthy donors (Grigor'eva et al. 2016).

Factors contributing to the limitation of EV-DNA analysis are related to the highly heterogeneous EV populations, the presence of circulating cell-free DNA (cf-DNA) or other contaminant structures which can be co-isolated with the EVs, and the DNA packaging or selective DNA sorting mechanisms inside EVs, which are lacking principal information and evidence (Lazaro-Ibanez et al. 2019).

In the current situation and study, removing the DNA that is bound to the outside of EVs was not a prerequisite, as removing this DNA would not have any diagnostic advantage for detecting mutations in EV-associated dsDNA. However, a comparison of the dsDNA concentration in different collected fractions in the AML patients revealed a decreased concentration of dsDNA in EV-depleted (fractionated) plasma in comparison to unfractionated. Besides, EV fractions was observed in the majority of patients of this study, confirming the recent finding showing that the majority of previously considered human blood plasma cf-DNA is localized in EVs (Fernando et al. 2017). In EV fractions, higher amounts of dsDNA were detected than in the initial unfractionated plasma, which reflects a higher protection of the DNA inside the EVs than the vulnerable cf-DNA degraded by the plasma nucleases (Cai et al. 2013; Kahlert et al. 2014; Lee et al. 2014; Thakur et al. 2014; Lazaro-Ibanez et al. 2014).

In almost all AML patients in our study, dsDNAs were found at higher levels in EVs before-therapy, in comparison to the ones after-therapy, which could be explained by the fact that in before-treatment samples the number of EVs were higher than in after-treatment samples, showing a relation between the stage of disease, the number of EVs and the amount of the dsDNA. Similar to our RNA findings, patients carrying only the FLT3-ITD mutation revealed a higher amount of dsDNA after-treatment, while samples from patients that had an NPM1 mutation only or combined FLT3-ITD and NPM1 mutations showed decreased dsDNA concentrations after-treatment. The higher EV-dsDNA concentration obtained by the FLT3-ITD patient samples could be affected by a higher amount of DNA contaminants or could be related to a different way of DNA sorting or packaging inside the EVs. However, both reasons are related with the mutational background, which cannot be confirmed due to the lack of available studies. Consequently, it is considered of great importance to improve not only the current EV isolation techniques, but the purification and the quantification methods of EV-DNA as well, which will lead to a more reliable way of obtaining prominent scientific results needed to validate the biomarker potential of EV-DNA.

Except the quantity of EV-DNA, its quality also remains a crucial challenge in the field providing main information regarding its origin and role. It is important to obtain pure EV-DNA and not fragments of DNA from apoptotic bodies or other contaminants in order to use it as a diagnostic or even a treatment tool. Our samples revealed a different pattern in before-treatment, in comparison to the healthy donor EV-DNA as well as of the after-treatment sample. This could be explained as a relation between the DNA fragment sizes and the AML disease state, or as a consequence of the lower amount of DNA in healthy donor and after-treatment EVs which could make it difficult to detect these distinct groups. Nevertheless, due to the lack of equivalent studies, further investigations are needed for ensuring the quality of EV-DNA obtained from different sources, which could help us to understand how the cells of origin can be related to the size pattern of EV-DNA, which would help in establishing advanced techniques in the medical field.

3.4 EV-RNA and EV-dsDNA mutational analysis

A quantitative (Vagner et al. 2018; Balaj et al. 2011) and qualitative (Thakur et al. 2014; Kahlert et al. 2014; Yang et al. 2017; San Lucas et al. 2016; Allenson et al. 2017; Castellanos-Rizaldos et al. 2018; Garcia-Romero et al. 2017; Mohrmann et al. 2018) representation of gDNA of the cells of origin has be proven to be mirrored in the EV-DNA, whose mutational analysis, due to the

higher stability in comparison to the EV-RNA, is of greater diagnostic potential and can lead to improved results.

NPM1 and FLT3-ITD mutations, identical to the ones of primary leukemia blasts, were present and detectable in the vast majority of our AML patients' before-therapy samples. Any AML-specific mutation was no longer detectable in all patient samples after-therapy. The difficulty in detecting any AML-specific mutation in the after-treatment samples can be related to the lower amount of EVs which were present in the after-treatment samples and which, in turn, affected the levels of the EV-RNA, as has been previously discussed. In addition to the lower amount of EV-RNA in the after-treatment samples, the highly sensitive nature of RNA, in terms of stability, could play an important role in the inadequacy of mutational detection.

It is reported that cell-released EVs contain dsDNA that covers the entire genome and, in the context of cancer, also allows detection of the oncogenic mutations known to occur in the parental cancer cells (Garcia-Romero et al. 2017; Thakur et al. 2014; Kahlert et al. 2014). In comparison to the cf-DNA, that has been described as a novel marker in MRD prognosis (Mussolin et al. 2013), EV-dsDNA is likely to be more stable due to its protection from DNases in the serum by the outer EV membrane (Jin et al. 2016). The combination of all the previous mentioned properties of tumor-derived EVs and EV-DNA make them important candidates as advanced tools in personalized medicine on top of their potential as cancer biomarkers (Thakur et al. 2014; Li et al. 2017; Balaj et al. 2011).

The analysis of the mutational status of EV-dsDNA revealed similar results to those of EV-RNA. The initially discovered AML-specific mutations in before-treatment samples were no longer detectable in the after-treatment EV-dsDNA with the exception of a few samples. The EV-dsDNA findings echo gDNA mutational status provided by the AML diagnostic laboratory, in the majority of the cases. This outcome suggests once more that the reduction of the cancer cells' number after-therapy leads to a decline in the EV number that contain AML-specific mutations. Furthermore, the heterogeneous background of EVs, due to the fact that are shed by both healthy and diseased cells into the blood circulation, could be partly responsible for the inadequacy of the mutational detection in patient samples (Lazaro-Ibanez et al. 2014). Alternatively, it could also be that these findings are reflecting the real lack of those mutations in this after-treatment patient cohort (Lazaro-Ibanez et al. 2014). As it is of great importance, further investigation is needed in order to establish new methods to successfully distinguish and isolate the desired EV population in high

purity from biological fluids such as blood. Finally, as anticipated, SNPs were detectable in EV-dsDNA in all before- and after-treatment samples, further supporting that therapy in patients leads to a reduction of EVs released by leukemia cells probably without compromising the EVs released by healthy cells in to the blood circulation.

3.5 Diagnostic potentials of EV-RNA and EV-dsDNA

Due to the complexity of the AML disease, we believe that an elaborated understanding of clonal evolution in AML and an earlier detection of evolving AML sub-clones will help to improve diagnostic and therapeutic strategies.

Many unsolved issues and remaining technical challenges such as the EV isolation methods, technical concerns, sample handling and reproducibility of EV-based assays have to be improved. However, forthcoming utility and incorporation of all 'omics-based' technologies and biological systems are discussed to provide significant comprehension, which will introduce EVs into the clinical scene and personalized medicine.

A promising idea comes from the field of biomarkers, in which EVs originating from tumors, these small particles could play a key role in the monitoring of cancer at every stage of the disease as well as in future therapy advancements against cancer by serving as novel targets (Becker et al. 2016). This function of EVs is based on their release and circulation into the blood stream. In this respect, they represent a valid and stable source of a disease's important genetic information at the level of prognosis, diagnosis and of course treatment (Lazaro-Ibanez et al. 2014). Moreover, their molecular cargo and constitution have shown distinctive characteristics that could result in the advancement and development of biomarker tools with prognostic, diagnostic, and predictive potentials (Bracht et al. 2018; Konig et al. 2017; Overbye et al. 2015; Tovar-Camargo, Toden, and Goel 2016). However, before this final step of employing EVs in cancer diagnostics, further investigation is needed in order to obtain and characterize more potential disease specific EV biomarker characteristics, in addition to their association with any clinical parameter (Lazaro-Ibanez et al. 2014).

Currently, there are several studies supporting the role of EV genetic material as a potential diagnostic tool for cancer and metastasis. It has been proven that tumor-related EV-mRNA originating from glioblastoma patients mirrors the mutational background of EGFRvIII (Pelloski et al. 2007; Skog et al. 2008), while tumor-specific EV-dsDNA reflects the genetic information of

cells of origin (Thakur et al. 2014; Kahlert et al. 2014; Melo et al. 2015). In addition, Balaj *et al*., 2011 showed that ssDNA can be also carried by EVs, summing up genomic defects in the primary tumor (Balaj et al. 2011). The cancer type in melanoma is related to metastasis and, consequently, to the amount of dsDNA, revealing that the more aggressive the cancer is the higher the EV-dsDNA level is (Thakur et al. 2014). These findings highlight the biomarker potential of the EV genetic cargo as a beneficial clinical diagnostic tool in mutational analysis.

However, the present proposed approach, with the current sensitivity, does indeed have its limitations. However, to our knowledge, the current project is the first study demonstrating the mutational analysis of EV-RNA and EV-dsDNA in primary AML pediatric patient samples. Despite the successful mutational detection of the method in almost all patients at the stage of diagnosis, it was not possible to detect the AML-specific mutations in the majority of the after-therapy samples. These sensitivity issues must be addressed by the advancement of improved EV-isolation and enrichment methods, which will help us to isolated more and highly pure EV populations of our interest, as it remains of utmost importance when designing diagnostic detection methods. The current results' restriction could be attributed to the lower amount of RNA/DNA that was obtained from these samples; possibly due to a reduction in the EV production by mutation-containing cells or a reduction of the cells themselves. Otherwise, they could also be related to the EV heterogeneity in the blood circulation (Lazaro-Ibanez et al. 2014).

Despite the already mentioned limitations, the main advantage of this method over the current diagnostic methods is obvious, in terms of patient welfare. A liquid biopsy approach could indeed be a valuable diagnostic tool, offering a fast, pain-free, and hassle-free addition to the present painful bone marrow biopsies (Raimondi et al. 2017). With regards to pediatric patients, being able to diagnose and monitor AML simply by drawing blood and isolating EVs instead of having to undergo bone marrow puncture would be much more convenient and less stressful.

Although the current results are preliminary, the findings could provide supplementary biochemical basis to monitor and predict treatment responses, after a further sensitivity optimization and additional recapitulation in larger study cohorts. In addition, our study could potentially provide an adjunctive overview of the status of MRD while enhancing or supporting classic AML detection methods that are currently used today, leading to the development of new improved strategies for better diagnosis, prognosis, and therapy.

3.6 Future Aims

Considering the complex nature of AML, and our current findings as well as the abovementioned future studies, the ultimate aim would be to develop and establish a novel, a highly sensitive EV-dsDNA and/or EV-RNA-based analysis platform to serve as a complementary approach to the present leukemia diagnostic platforms. This would contribute to the collection of supplementary information on leukemia cell populations. A diagnostic method that combines multi-compartment blood profiling in pediatric AML would prove to be more useful in comparison to the available molecular diagnostic approaches that mainly focus on one class of diagnostic factors derived from leukemia blasts.

As EVs are populations of high heterogeneity, many questions remain unresolved. For a potential implementation of EVs in the clinical field, further studies need to be performed to investigate the following: what is the involvement of each EV population in cancer progression, what is the tumor cell of origin such as tumor stem cells and the targets of these nanoparticles, and what is the role of EVs in the formation of the premetastatic niche as well as the metastatic microenvironment.

Therefore, the suggested method requires a period of optimization before any implementation as a supplementary, clinically routine method. In the future, this study should be reproduced using a larger cohort of patients, in combination with an enhanced EV isolation method that is capable of specifically sorting for AML-derived EVs. In addition, it is of great importance to perform the assay with more sensitive sequencing methods, in order to further optimize the diagnostic potential of EV-RNA and EV-dsDNA in AML. Moreover, whether and how the EVs influence the healthy recipient cells through their content coming from tumor cells will help us to understand the mechanism of promoting the premetastatic niche, and how invasion and metastasis occur. Furthermore, the mechanistic analysis of tumor-derived EVs will lead to the control of those EVs, which will improve their diagnostic and therapeutic potential and will, consequently, contribute to a better standard of care for cancer patients.

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5. LIST OF ABBREVIATIONS

ABL2	Abelson-related gene
ADxE	Cytarabine, liposomal daunorubicin and etoposide
AI	Cytarabine and idarubicin
AIE	Cytarabinr, idarubicin and etoposide
Alpha-MEM	Alpha Modified Eagle's Medium
AML	Acute Myeloid Leukemia
ATRO	Arsenic trioxide
ATRA	All-Trans-Retinoic Acid
BCA	Bicinchoninic acid
BFM	Berlin-Frankfurt-Münster
BM	Bone Marrow
bp	Base pairs
CBF	Core Binding Factor
CdxA	Clofarabine (40mg/m^2)
CEBPA	CCAAT Enhancer Binding Protein Alpha
cf-DNA	Circulating cell-free DNA
CML	Chronic Myelogenous Leukemia
Dag-21	Diacyl Glycerol
DNase	Deoxyribonuclease
dsDNA	Double Stranded-DNA
DPBS	Dulbecco's Phosphate-Buffered Saline
DT	Diphtheria Toxin
DXN-FLA	Doxorubicin /Fludarabine and high-dose cytarabine and G-CSF
EDTA	Ethylene diamine tetra-acetic acid
EMU	Electron Microscopy Unit
ETV6	ETS Variant Transcription Factor 6
EVs	Extracellular Vesicles
EZH2	Enhancer of Zeste Homolog 2
FBS	Fetal bovine serum
FLT3	fms related tyrosine kinase 3
FLT3-ITD	fms related tyrosine kinase 3-internal tandem deletion
FLT3-TKD	fms related tyrosine kinase 3- tyrosine kinase domain
GATA2	GATA Binding Protein 2
gDNA	Genomic DNA
HAE	HD-cytarabine and etoposide
haM	HD-cytarabine (1g/m^2) and mitoxantrone
HAM	HD-cytarabine (3g/m^2) and mitoxantrone
HDL	High-density Lipoprotein
HSCs	Hematopoietic Stem Cells
IMCES	Imaging Center Essen
ISEV	International Society of Extracellular Vesicles
JMML	Juvenille Myelomonocytic Leukaemia
KIT	KIT Proto-Oncogene Receptor Tyrosine Kinase

K-RAS	KRAS Proto-Oncogene
LDL	Low-density Lipoprotein
LSCs	Leukemic Stem Cells
MDS	Myelodysplastic Disorders
MDB	Membrane Desalting Buffer
MRD	Minimal Residual Disease
mRNA	Messenger-RNA
miRNA	Micro-RNA
MSCs	Mesencymal Stem Cell
mtDNA	Mitochondrial-DNA
MVB	Multi Vesicular bodies
MVs	Multicellular Vesicles
MV4-11	Cell line of biphenotypic B myelomonocytic leukemia
M1	Acute myeloblastic leukemia with minimal maturation
M2	Acute myeloblastic leukemia with maturation
M3	Acute promyelocytic leukemia
M4	Acute myelomonocytic leukemia
M5	Acute monocytic leukemia
M6	Acute erythroid leukemia
ncRNA	Non-coding RNA
NGS	Next Generation Sequencing
NOTCH1	Notch homolog-1, translocation-associated
NPM1	Nucleophosmin-1
NTA	Nano Particle Tracking Analysis
OCI-AML3	Cell line of acute myeloid leukemia
PB	Peripheral Blood
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
Pen/Strep	Penicillin/streptomycin
PHF6	plant homeodomain finger protein-6
PTA	Phosphotungstic Acid
RAD21	Double-strand-break repair protein rad-21 homolog
RNA	Ribonucleic Acid
Rnase	Ribonuclease
RNPs	RiboNucleoProtein complexes
RPMI 1640	Roswell Park Memorial Institute 1640
RT	Room Temperature
RT-PCR	Real Time PCR
SNPs	Single Nucleotide Polymorphism
ss-DNA	Single-stranded DNA
TCEP	tris(2-carboxyethyl)phosphine
TEM	Transmission Electron Microscopy
TGF-β1	Transforming growth factor beta-1
THP1-lysate	Human Acute Monocytic Leukemia cells
TSM	TruSight Myeloid

WT	Wild Type
WT1	Wilms-Tumor-1 gene
ZRSR2	U2 small nuclear ribonucleoprotein auxiliary factor 35 kDa subunit-related protein-2

6. LIST OF FIGURES

8. EIDESSTATTLICHE ERKLÄRUNGEN

Erklärung:
Hiermit erkläre ich, gem. § 7Abs. (2) d) + f)der Promotionsordnung der Fakultät für Biologiezur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient,bei der Abfassung der Dissertation nur die angegeben Hilfsmittel benutzt und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

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Erklärung:

Hiermit erkläre ich, gem. § 7Abs. (2) e) + g)der Promotionsordnung der Fakultät für Biologiezur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner anderen Fakultät/Fachbereichabgelehnt worden ist.

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Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. (2) g) der Promotionsordnung der Fakultät für Biologie zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „The diagnostic potentials of extracellular vesicles in pediatric acute myeloid leukemia“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Evangelia Kontopoulou befürworte und die Betreuung auch im Falle eines Weggangs, wenn nicht wichtige Gründe dem entgegenstehen, weiterführen werde.

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