BASIC SCIENCE ARTICLE

Longitudinal assessment of peripheral blood *BRAFV600E* levels in patients with Langerhans cell histiocytosis

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BACKGROUND: Langerhans cell histiocytosis (LCH) is a histiocytic disorder driven by a constitutive activation of the MAPK signaling pathway in myeloid cells. In 50–60% of cases, it is caused by the *BRAFV600E* mutation. There is evidence that levels of *BRAFV600E* in the peripheral blood of patients with LCH correlate with disease burden and could be used as marker for disease extent and response to therapy. However, there is currently no consensus on how testing for minimal disseminated disease should be performed.

METHODS: Different approaches to determine the mutation load in patients with LCH were assessed and longitudinal evaluation of patient DNA during treatment with chemotherapy and/or the RAF inhibitor vemurafenib was performed. DNA was isolated from whole blood, different leukocyte subsets, and circulating cell-free DNA (ccf-DNA).

RESULTS: We show that determining BRAF levels from whole blood is superior to using ccfDNA. Furthermore, it is important to identify the clinically relevant BRAF-mutated cellular subpopulations such as CD14⁺ monocytes or CD1c⁺ DCs, since other blood cells can also harbor the mutation and therefore confound whole blood or ccfDNA measurements.

CONCLUSION: Our data support the view that single-agent treatment with an RAF inhibitor reduces disease activity but does not cure LCH.

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INTRODUCTION

Langerhans cell histiocytosis (LCH) is a myeloid neoplasm characterized by the accumulation of CD1a⁺CD207⁺ (langerin⁺) positive cells resembling histiocytes and a prominent inflammatory infiltrate.^{1,2} Any age group may be affected, but its most severe clinical course predominantly affects young children. The clinical course of LCH presents in a wide range, including both spontaneous regression of single lesions as well as rapid dissemination and life-threatening multisystem disease that needs to be treated with systemic chemotherapy and in some cases hematopoietic stem cell transplantation. Targeted therapies with RAF or MEK inhibitors are emerging as important part of the treatment, although it is still unclear how they should be incorporated in current treatment regimens. One major problem in the management of LCH is the very high number of patients who are refractory to initial treatment or reactivate within a few years.³ The current international treatment protocol for LCH in children ("LCH-IV", EudraCT Nr.: 2011-001699-20) comprises empiric multimodal chemotherapy with treatment decisions that solely rely on clinical presentation. Biological markers that could assess disease burden and measure treatment response would significantly improve decision-making and treatment of these patients by enabling more personalized treatment of children with LCH.

Genomic analyses revealed constitutive activation of MAPK signaling in a significant number of LCH patients.^{4,5} In 50–60% of cases, the MAPK activation is caused by the *BRAFV600E* mutation. The *BRAFV600E* mutation has been described in several cancer types, predominantly in melanoma, papillary thyroid tumors, and colorectal cancer. During the last years, the detection of mutated *BRAF* was introduced as a marker for prognosis and treatment outcome, therefore several detection methods were established.^{6–10} Recent studies have presented several approaches using *BRAFV600E* levels in children with LCH to monitor disease burden and to predict treatment outcome.^{6,11–14} In addition, an association of *BRAFV600E* status with a more severe clinical outcome and increased resistance to first-line chemotherapy was presented by Héritier et al.¹¹ The same group evaluated cell-free *BRAFV600E* as a biomarker for monitoring the response to therapy and obtained promising results.¹² In our previous reports we used a method called Intplex, a refined allele-specific quantitative PCR using SYBRgreen based on the protocol published by the group of Thierry.^{6,7}

There is no consensus on how BRAF should be determined in the peripheral blood of patients with LCH: it is unclear whether ccf DNA, whole-blood DNA, or cellular DNA should be determined. Furthermore, there are different methods to determine BRAF in the peripheral blood, among them were allele-specific SYBR-Green PCR, Taqman Mutation Detection Assay and digital droplet

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PCR (ddPCR). In view of the large impact BRAF measurements could have on future treatment protocols for children with LCH and the necessity to align protocols between different laboratories, we have assessed different methods and approaches and propose an approach that could be used by different laboratories to assess BRAF in patients with histiocytoses.

MATERIALS AND METHODS

Patients

This study was conducted in accordance with the Declaration of Helsinki after approval by the local IRB, and written and informed consent of the parents.

Molecular analysis

Peripheral blood mononuclear cells (PBMCs) from patients were isolated by density-gradient centrifugation using Ficoll-Paque (GE Healthcare, UK). DNA was isolated either from whole blood or flow cytometric sorted cell subsets using the QIAamp DNA blood mini kit (Qiagen, Germany). ccfDNA was isolated using the ChargeSwitch gDNA 1 ml Serum Kit (CS11040, ThermoFisher Scientific, MA).

Detection of *BRAF* V600E

*BRAF*V600E allele-specific PCR using SYBRgreen was performed as described previously.⁶ Briefly, qRT-PCR was performed using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific). Assays were performed in triplicate using primers specific for *BRAF*wt and *BRAF*V600E. ABI 7500 Fast Detection System (Applied Biosystems, Foster City, CA) was used. Percentage of mutated allele was calculated as $m\% = (\text{Conc MUT} / \text{Conc REF})$. For the Taqman® mutation detection assays: Mutation Allele Assay: *BRAF*_476_mu -4465804 Hs00000111_mu and wild-type allele reference Assay - *BRAF*_476_wt -4465804 Hs00000110_wt (ThermoFisher Scientific) were used according to the manufacturer's description. Assays were performed in triplicate using the Applied Biosystems 7500 Fast Detection System (Applied Biosystems, Foster City, CA). ddPCR was performed using the Mutation Assay *BRAF* p.V600E c.1799T-A, Human (dHsaMDV2010027 Biorad, Germany) and the QX200 Droplet Digital PCR System (Biorad). For samples containing 1% or less *BRAF*V600E-mutated cells, three wells were pooled, for amounts above 1% single wells were analyzed according to the manufacturer's description.

Cell sorting, flow cytometric techniques and chimerism analysis
All chimerism analyses were performed on flow-sorted CD45⁺ leukocyte subtypes defined by their unique antigen co-expression. Myeloid cells were CD14⁺ monocytes and HLA-DR⁺CD1c⁺CD11c⁺ DCs; T-cells were defined by their positivity for CD3⁺CD4⁺ and CD3⁺CD8⁺. Cell sorting was performed on a FACSria instrument (BD Biosciences, San Jose, CA). The FACSDiVa software (BD Biosciences) was used for data evaluation. The number of cells isolated for subsequent FISH analysis ranged from 1000 to 10,000. Chimerism analyses from gender-mismatched donor were analyzed using Dual-color FISH performed according to the standard procedures¹⁵ with commercially available probes specific for the centromeric and heterochromatic regions of the X and Y chromosomes, respectively. Depending on the number of cells available, up to 500 cells were analyzed within each sorted cell fraction.

RESULTS

In a first set of experiments, we compared three widely used methods to determine levels of mutated BRAF: an allele-specific PCR method,⁷ a commercially available Taqman Mutation Detection Assay which was already used in several studies,^{10,16} and a commercially available digital droplet PCR Kit.¹⁷ To evaluate the

Fig. 1 Comparison of three allele-specific PCR methods. The percentage of measured *BRAF*V600E mutated cells (Y-axis) is depicted versus the percentage of *BRAF*V600E mutated cells used in the PCR reactions (X-axis) for **a** 100 to 0.01%, **b** 2 to 0.01% and **c** 0.1 to 0.01% *BRAF*V600E-positive cells used. Black: calculated amount of cells used; Green: PCR reaction using SYBRGreen; Purple: PCR reaction using Taqman Mutation Detection Assay; Red: ddPCR reaction

three methods, we diluted genomic DNA from the A673 cell line, which contains a heterozygous *BRAF*V600E mutation with wild-type DNA extracted from blood of healthy donors ranging from 100% mutated cells to 0.01% mutated cells and performed the PCR reactions in parallel. As depicted in Fig. 1a, the SYBRGreen-based allele-specific PCR and ddPCR correlate well to the cell

Fig. 2 Longitudinal assessment of *BRAF*V600E-positive cells in patients with LCH. Patients **a** 134 **b** 107 and **c** 73 with MS-LCH. Patients **d** 202 and **e** 216 were diagnosed with multifocal bone lesions. All patients were *BRAF*V600E positive in their biopsy samples. Y-axis represents the percentage of circulating *BRAF*V600E mutated cells during therapy. X-axis represents days after therapy start. SCT Stem cell transplantation, IC1 Initial Course 1, IC2 Initial Course 2, CR Clinical Remission, R Relapse

Table 1. Patient information summary

Patient ID	SS/MS	RO	Age at diagnosis (d)	Gender	BRAF inhibitor
73	MS	—	115	m	—
107	MS	+	762	m	—
134	MS	+	292	m	vemurafenib
202	SS, multifocal bone	—	1089	m	—
216	SS, multifocal bone	—	160	m	—
262	MS	+	866	f	vemurafenib
289	MS	+	250	f	vemurafenib

SS single system, MS multisystem, RO risk organ involvement, +risk organs involved, — no risk organs involved, m male, f female

amounts used in the reaction. In contrast, an overestimation of the mutational load was observed with the Taqman Mutation Detection Assay, especially in the lower range (Fig. 1b). Lower levels of the mutated allele ($\leq 0.05\%$ of cells) are below the detection limit of the Taqman method (Fig. 1b, c). In contrast, ddPCR and the allele-specific SYBRGreen PCR precisely reflect the amount of cells used down to a level of around 0.01% mutated *BRAF* allele. Therefore, both these methods are equally suited to determine *BRAF*V600E in the peripheral blood.

We then performed sequential sample analysis during therapy and follow-up of five *BRAF*V600E biopsy-positive patients using the allele-specific PCR (Fig. 2). Clinical characteristics of the studied patients are summarized in Table 1.

Patient 134 was diagnosed with multisystem LCH (MS-LCH) with involvement of skin and liver and low levels of mutated *BRAF* in his PB (0.19%) were detected (Fig. 2a). Chemotherapy with vinblastine and prednisolone for 12 weeks (IC 1 and 2) resulted in improvement of skin and remission of liver disease. After additional 24 weeks of treatment with vincristine, prednisolone, and cytarabine (Stratum II), his skin improved even further. When continuation therapy was started, the patient was clinically in remission but his *BRAF*V600E levels had increased to 3.5%. About 40 days later, the patient relapsed and deteriorated clinically with involvement of skin, liver, spleen, and the hematopoietic system. At the molecular level, *BRAF*V600E peaked at 15.1% (Fig. 2a). Because of his critical condition, treatment with vemurafenib was started, which led to a rapid clinical response and a moderate decrease of *BRAF*V600E levels. With vemurafenib as single-agent, stable very good partial remission of skin, liver, spleen, and the hematopoietic system over a period of 20 months is observed, but *BRAF*V600E is still detectable (7.2%). Up to now, the patient is still treated with the inhibitor.

Patient 107 was reported previously until the timepoint he received a hematopoietic stem cell transplantation (HSCT).⁶ This patient had MS-LCH, which was refractory to chemotherapy, subsequently received vemurafenib, and was then transplanted from a matched sibling donor using a reduced intensity-conditioning (RIC) regime. One month after HSCT, the patient was clinically well, showed no signs of disease and the amount of *BRAF*V600E-positive cells was low (between 0.01 and 0.7%), but still detectable. Vemurafenib was discontinued 1 month after transplantation. While the patient stayed clinically stable for 9 months, *BRAF*V600E levels started to increase steadily 1 month after cessation of treatment with vemurafenib. Nine months after vemurafenib discontinuation (day 755 after initial start with vemurafenib) the patient relapsed, presenting osteolytic lesions in the skull. Therapy with vinblastine/prednisolone according to

the LCH-IV protocol was re-administered, but the clinical condition worsened involving febrile neutropenia, clostridia enteritis, EBV reactivation and the mutational load increased up to 10%. At that time (day 822 after initial vemurafenib start) vemurafenib was prescribed again and the patient stabilized soon accompanied by a drop in his *BRAF*V600E levels. He is now clinically well and is treated with vemurafenib every other day (Fig. 2b). The patient still has measurable levels of *BRAF*V600E in the peripheral blood.

Patient 73 was diagnosed with MS-LCH with involvement of lungs, skin, bone, and gingiva. Before treatment, 0.3% of his peripheral blood cells were *BRAF*V600E positive. During IC1 and 2, bone involvement was stable, but the skin disease got worse. According to the LCH-IV protocol, the patient was therefore switched to Second-line Initial Therapy (Stratum II in LCH-IV). With this therapy his skin and bone LCH improved, accompanied by a drop of *BRAF*V600E detectable in his PB (0.02%). At the end of therapy he was in clinical remission and *BRAF*V600E levels in the peripheral blood had decreased below the detection limit (0.01%) (Fig. 2c).

Patient 202 presented with multifocal bone lesions and 0.034% *BRAF*V600E-positive cells. After IC 1 and 2 the patient clinically improved and accordingly continuation therapy was initiated. He was in clinical remission after completion of the therapy. At the same time *BRAF*V600E was below the detection limit (0.009%) (Fig. 2d).

Patient 216 was diagnosed with LCH with multifocal bone lesions. Administration of IC1 and 2 resulted in an improved clinical condition. After continuation treatment the patient was disease free. Although the *BRAF*V600E mutation was identified in the biopsy, the mutation was never detected in the patients peripheral blood (Fig. 2e).

Taken together, patients 73, 202, and 216, who all were *BRAF*V600E negative at the end of their therapy, have not relapsed until now (9 months, 10 months and 4 months after the end of therapy). In contrast, patients relapsed if *BRAF*V600E was still detectable at the end of therapy, even though *BRAF*V600E levels dropped drastically and no clinically apparent signs of disease were observed. These observations suggest that *BRAF*V600E mutation load could serve as a predictive marker of LCH relapse and should therefore be carefully monitored. Furthermore, vemurafenib reduced *BRAF*V600E levels, but did not eliminate *BRAF*V600E in two patients even after long-term therapy.

Next, we longitudinally assessed *BRAF*V600E levels in circulating cell-free DNA of three patients with *BRAF*V600E-positive MS-LCH (Fig. 3). Overall, the pattern of cf *BRAF*V600E levels reflects the pattern observed with DNA isolated from whole peripheral blood. In patient 134, while ccf mutational load was as low as in PB at time of diagnosis, *BRAF*V600E levels increased up to 31% after administration of vemurafenib. While ccf*BRAF*V600E varied between 4 and 31%, *BRAF*V600E remained at comparable values in DNA isolated from whole blood (between 6 and 10%) during treatment, reflecting the stable clinical presentation of this patient (Fig. 3a). For patients 107 (Fig. 3b) and 262¹⁸ (Fig. 3c), amounts of ccf*BRAF*V600E again varied to a much higher degree compared to PB levels. The most prominent variation occurred around day 100 after the start of vemurafenib treatment of patient 262. In contrast to the whole blood *BRAF*V600E level, which was 5.4%, ccf mutation load was 31.2%. Blood sampling was performed using standard EDTA tubes and not cell-free DNA collection tubes, which preserve ccfDNA and inhibit the lysis of nucleated cells, which would contaminate ccfDNA with cellular DNA.¹⁹ Therefore these data have to be interpreted with caution and underline the necessity of a strictly regulated protocol, preferably using ccf-DNA collection tubes and short and temperature-stable transportation.

In patients with LCH, mutated *BRAF* can be detected in different cell types such as monocytes, dendritic cells, or lymphocytes.^{10,20} It is unclear, which and if one of these populations contribute to LCH-pathogenesis and might contain precursors of the lesional

Fig. 3 Comparison of *BRAF*V600E levels in whole blood and plasma. DNA was isolated either from whole blood, or circulating cell-free DNA from plasma of three patients **a** 134, **b** 107, and **c** 262 with MS-LCH. Y-axis represents the percentage of circulating *BRAF*V600E-mutated cells during therapy. X-axis represents days after therapy start. SCT Stem cell transplantation, IC1 Initial Course 1, IC2 Initial Course 2, whole blood in black, plasma in red, CR Clinical Remission, R Relapse

LCH cells. We therefore tested whether specific cell populations reflected the clinical situation of a given patient better than others. To this end, we sorted defined cell populations using flow cytometry and analyzed the mutational load in these populations in three patients whenever possible. Limitations in this case were either low volume of blood drawn, coagulated blood, or cytopenia at time of blood sampling. As described earlier, the amount of mutated *BRAF*V600E in the peripheral blood of patient 134 was initially very low (0.19%). However, when we analyzed CD14⁺ monocytes and CD1c⁺ DCs—two cell populations that might contain the progenitors of the CD1a⁺CD207⁺ LCH cells^{10,21,22}—3.9% of CD14⁺ monocytes and 1.3% of CD1c⁺ DCs (mDCs) were *BRAF*V600E positive. The highest observed level of *BRAF*V600E in these populations was detected at about 40 days on vemurafenib (CD14⁺ 26.2%, CD1c⁺ DC 12.0%). A drop of mutated *BRAF* levels in whole PB was accompanied by a small reduction of *BRAF* in monocytes and CD1c⁺ DC at day 130 after start with vemurafenib,

but mutated *BRAF* nevertheless stayed relatively high (day 356 CD14⁺ 17.3%, CD1c⁺ 14.1%) (Fig. 4a).

However, the possibly most interesting results were obtained when analyzing different peripheral blood cell populations of patient 107. In contrast to patient 134, the mutational load in monocytes and CD1c⁺ DC was lower compared to whole blood after initial treatment with vemurafenib (Fig. 5a). If CD14⁺ monocytes and CD1c⁺ DC account only for a minor proportion of *BRAF*V600E-positive cells, which other cell populations might be mutated? To address this question, we analyzed CD16⁺ monocytes, granulocytes, and T-cell subpopulations in this patient. While CD16⁺ monocytes and granulocytes showed only minimal positivity (data not shown), a high percentage of T-cells carried the *BRAF* mutation. After the first cessation of vemurafenib when total *BRAF* in whole blood was rising to around 10%, 12.4% of CD8⁺ and 46.8% of CD4⁺ T-cells were *BRAF*V600E positive (Fig. 5b). These levels increased further and reached their peak at the time

Fig. 4 Determination of *BRAFV600E* levels in $CD14^{+}$ monocytes and $CD1c^{+}$ DCs. *BRAFV600E* mutational load for patient 134 was determined from whole blood and flow cytometry sorted $CD14^{+}$ monocytes (CD14) and $CD1c^{+}$ DCs (mDC). Y-axis represents the percentage of circulating *BRAFV600E*-mutated cells during therapy. X-axis represents days after therapy start. IC1 Initial Course 1, IC2 Initial Course 2, $CD14^{+}$ monocytes in green, $CD1c^{+}$ DC in red, CR Clinical Remission, R Relapse

when the percentage of total *BRAFV600E*-mutated cells was at a maximum, about 850 days after initial start of vemurafenib (13.2% in PB, 79.2% in $CD4^{+}$, 23.0% in $CD8^{+}$). When the mutational load in the peripheral blood decreased after re-administration of vemurafenib to 3.3%, also the amount of *BRAFV600E* in $CD4^{+}$ (28.6%) and $CD8^{+}$ (5.8%) declined. Therefore, the levels of *BRAFV600E* in the peripheral blood of this patient are caused primarily by *BRAFV600E*-positive T-cells. This finding is important when considering that LCH is currently categorized as myeloid neoplasm that might originate from $CD14^{+}$ monocytes or $CD1c^{+}$ DCs.^{10,20,22,23}

We therefore took a closer look at the $CD14^{+}$ monocytes in this patient. Low levels of *BRAFV600E* were detected in monocytes after transplantation during the time the patient was in a clinical stable condition, but were elevated (0.9%) at time of relapse. In contrast, during an increase of *BRAFV600E* in T-cells (and consequently also in whole blood) around day 822, the *BRAFV600E* levels were low in $CD14^{+}$ monocytes, perhaps reflecting the clinical remission of the patient (Fig. 5a). Taken together, these data show that while the dynamics of total *BRAFV600E* and its levels in the T-cell subpopulations are similar, they do not necessarily correlate with the clinical picture, which might be reflected more accurately by the *BRAFV600E* content in myeloid cells. Chimerism in peripheral blood populations showed that $CD14^{+}$ monocytes and $CD1c^{+}$ DCs were mainly from the donor, whereas, T-cells were predominantly from the recipient (Fig. 5c). Furthermore, 3.4% of $CD34^{+}$ stem cells were from the recipient, indicating that a pool of potentially *BRAFV600E*-positive cells remained after transplant, which could potentially induce an LCH relapse.

To assess whether *BRAFV600E* can also be detected in other patients, we analyzed $CD4^{+}$ and $CD8^{+}$ T-cells, $CD14^{+}$ monocytes, and $CD1c^{+}$ DCs in two children with MS-LCH at timepoints when they were clinically stable but had high levels of *BRAFV600E* in whole blood analysis. (134: 8.2% ~1 year after clinical remission, 289: 12.5% about 180 days after clinical remission). Patient 289, presented with MS-LCH with involvement of skin, liver, spleen, bone marrow and lymph nodes and initial *BRAFV600E* levels of 30% in BM. Two weeks after start with vemurafenib, the patient went into clinical remission except for hepatosplenomegaly, which resolved after two more weeks of treatment. The amount of total *BRAFV600E* levels in the bone marrow decreased to 14% after two weeks, and varied between 8 and 12% during the next 178 days. However, no signs of disease were apparent during this time and the patient is still clinically well.

When we analyzed *BRAFV600E* levels in those two patients, we also observed high levels in $CD4^{+}$ and $CD8^{+}$ T-cells (134: 10.9% in $CD4^{+}$ and 6.2% in $CD8^{+}$ cells; 289: 34.5% in $CD4^{+}$ and 39.0% in $CD8^{+}$ cells Fig. 5d). Together, these data show that non-myeloid cells that carry the mutation can contribute to high levels of *BRAFV600E* in the peripheral blood.

DISCUSSION

LCH is a highly heterogeneous disease with a broad range of clinical manifestations unified by the accumulation of $CD1a^{+}CD207^{+}$ histiocytes and constitutive activation of the MAPK signaling pathway.²³ In 50–60% of cases, the MAPK activation is caused by the *BRAFV600E* mutation.⁴ Promising studies demonstrate the use of *BRAFV600E* measurements in patients to assess therapy response and predict treatment outcome.¹² Therefore, a prospective international trial that incorporates the analysis of *BRAFV600E* in the peripheral blood of LCH patients during therapy is urgently needed. In order to do this, the question which detection method should be used for the assessment of circulating *BRAFV600E* needs to be solved. Here we tested three different qPCR-based methods. While the commercially available Taqman Mutation Allele Assay leads to an up to 7-fold overestimation of the mutational load at percentages above 1%, *BRAFV600E* mutations are not detected at all below 0.05%, which is in line with the detection limit of 0.1%, stated by the company. The method based on the previously published method Intplex⁷ using a phosphate-modified blocking oligonucleotide targeting the wild-type allele and ddPCR both demonstrated high accuracy also at low levels of mutated allele.

In addition to whole blood and cellular DNA, we have also evaluated the use of ccfdNA, an established biomarker in a wide range of different solid tumors.^{9,24} Although the overall pattern of *BRAFV600E* levels correlated with the pattern determined using whole-blood DNA, we observed several outliers showing elevated *BRAFV600E* amounts at times of clinical stable conditions. These discrepancies might be explained by pre-analytical inaccuracies such as longer time spans between drawing of the blood and isolation of ccfdNA and or changes in temperature during transportation to the laboratory. The influence of time on DNA concentration was previously reported with almost a 3-fold increase of ccfdNA amount 24 h post blood drawing and an increase to over 60-fold by 96 h in one study.²⁵ For the present study, blood sampling was performed using standard EDTA tubes (similarly to the study of Heritier¹²) and not ccfdNA collection tubes. The use of these collection tubes would be highly

Fig. 5 Determination of *BRAFV600E* levels in cell subpopulations and chimerism analysis after SCT. **a** *BRAFV600E* mutational load for patient 107 was determined from whole blood and flow cytometry sorted $CD14^{+}$ monocytes (CD14), $CD1c^{+}$ DCs (mDC) and together with **b** $CD3^{+}$, $CD4^{+}$, and $CD8^{+}$ T-cells. Y-axis represents the percentage of circulating *BRAFV600E*-mutated cells during therapy. X-axis represents days after therapy start. **c** Chimerism analysis for flow cytometry sorted $CD14^{+}$ monocytes (CD14), $CD1c^{+}$ DCs (mDC), $CD3^{+}$, $CD4^{+}$ and $CD8^{+}$ T-cells. Y-axis represents the percentage of % cells from the recipient. X-axis represents days after therapy start. **d** *BRAFV600E* mutational load for patients 107 (black), 134 (gray) and 289 (white) were determined from whole blood (107, 134) and bone marrow aspirate (289) and flow cytometry sorted $CD14^{+}$ monocytes (CD14), $CD1c^{+}$ DCs (mDC), $CD4^{+}$, and $CD8^{+}$ T-cells. SCT stem cell transplantation, IC1 initial course 1, CR clinical remission, R relapse

recommended for further studies that aim to include patients from a large number of treatment centers. Alternatively, EDTA tubes could be used if blood samples are immediately processed and frozen plasma (-80°C) is shipped to the analyzing institution. This requires a standardized centrifugation protocol, which has to be implemented at each hospital sending blood or plasma for analysis. Children in this study were on average 592 days old (ranging from 115 days to 1089 days) and the amount of blood received was on average 2 ml. Swinkels and colleagues reported that in addition to pre-analytical factors including centrifugal force and pipetting efficacy, also the amount of blood collected influences efficiency of removal of contaminating cellular DNA from plasma.²⁶ One problem is the low concentration of ccfDNA isolated from plasma. Although the plasma of cancer patients in general contains higher amounts of ccfDNA in comparison to healthy controls, the amount varies considerably depending on the type and stage of cancer. For instance, in adults with LCH a mean quantity of 316.5 pg/ml (median, 290.4 pg/ml) was detected.¹⁴ In our opinion however, the major disadvantage of *BRAF*V600E analysis from both plasma and whole blood is the loss of the information which of the cell populations contain mutated *BRAF* in a given patient. *BRAF*V600E can be detected in several cell subsets, and the implication of the presence of a mutated allele for LCH pathogenesis in the specific cell subsets is not known yet. For example, in patient 107 the highest *BRAF* levels were detected in T-cells (up to 70% of CD4^{+} T-cells), which also seemed to be the largest contributing cell subset to the amount of mutated *BRAF* measured in peripheral blood and plasma. Therefore this patient had high levels of mutated *BRAF* when just analyzing total DNA or ccf-DNA from the PB, even though he was clinically stable. Since the relevance of the mutation in T-cells in LCH is unclear (it is unlikely that *BRAF* mutated T cells contribute to the development of $\text{CD1a}^{+}\text{CD207}^{+}$ LCH cells), it is likely that measurement of ccf-DNA or whole blood is inaccurate in some patients to measure disease activity. Therefore, precise monitoring of LCH-relevant populations such as CD14^{+} monocytes or CD1c^{+} DCs might be necessary in some patients to obtain accurate results. We propose that in future collaborative trials that study *BRAF* levels in the peripheral blood of patients with LCH should take this into account and investigate subset-specific levels in addition to bulk *BRAF*.

Nevertheless, there is a correlation of *BRAF*V600E levels and clinical outcome and response. In accordance with the results of Héritier et al.¹¹ and our own recent study,⁶ we observed that some patients who were treated by conventional chemotherapy or *BRAF* inhibitors and showed clinical remission accompanied by a decrease of *BRAF*V600E but still remained positive for *BRAF*V600E, relapsed after the cessation of their therapy. This suggests that treatment reduces disease activity but does not lead to an eradication of the disease at the molecular level, which could lead to a relapse. In line, patients with no detectable *BRAF*V600E in the peripheral blood at the end of therapy (below 0.01%) remained in remission so far (maximum time of observation 10 months).

In our patient who was transplanted, 3.4% of CD34^{+} stem cells were from the recipient, which might be the cause of the relapse after SCT. We therefore question whether reduced intensity conditioning is the best treatment approach for patients with high-risk LCH that receive allogeneic HSCT. The view of LCH as hematologic malignancy by many²⁷ (but not all²⁸), could support this view. Perhaps some patients with high-risk LCH should be treated with myeloablative conditioning.

Taken together, we suggest EDTA blood should be collected from patients with LCH. This enables both determining mutational content from whole blood and from different, sorted cell subsets. In our hands there is no difference in the sensitivity between ddPCR and the Intplex PCR, therefore both methods can be used

to trace minute amounts of mutated *BRAF* in the peripheral blood. As next step, protocols need to be aligned between different laboratories and a collaborative study that tests whether *BRAF* can be used as marker for minimal disseminated disease in patients with LCH has to be initiated. Such efforts will ultimately improve clinical decision-making and help patients to receive the appropriate therapy.

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ADDITIONAL INFORMATION

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