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Targeting SAMHD1 with hydroxyurea in first-line cytarabine-based therapy of newly diagnosed acute myeloid leukaemia: Results from the HEAT-AML trial

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Abstract. Jädersten M, Lilienthal I, Tsesmetzis N, Lourda M, Bengtzén S, Bohlin A, et al. Targeting SAMHD1 with hydroxyurea in first-line cytarabine-based therapy of newly diagnosed acute myeloid leukaemia: Results from the HEAT-AML trial. *J Intern Med.* 2022;**292**:925–940.

Background. Treatment of newly diagnosed acute myeloid leukaemia (AML) is based on combination chemotherapy with cytarabine (ara-C) and anthracyclines. Five-year overall survival is below 30%, which has partly been attributed to cytarabine resistance. Preclinical data suggest that the addition of hydroxyurea potentiates cytarabine efficacy by increasing ara-C triphosphate (ara-CTP) levels through targeted inhibition of SAMHD1.

Objectives. In this phase 1 trial, we evaluated the feasibility, safety and efficacy of the addition of hydroxyurea to standard chemotherapy with cytarabine/daunorubicin in newly diagnosed AML patients.

Methods. Nine patients were enrolled and received at least two courses of ara-C (1 g/m^2/2 h b.i.d. d1-5, i.e., a total of 10 g/m^2 per course), hydroxyurea (1–2 g d1-5) and daunorubicin (60 mg/m^2 d1-3). The primary endpoint was safety; secondary endpoints were complete remission rate and measurable residual disease (MRD). Additionally, pharmacokinetic studies of ara-CTP and *ex vivo* drug sensitivity assays were performed.

Results. The most common grade 3-4 toxicity was febrile neutropenia (100%). No unexpected toxicities were observed. Pharmacokinetic analyses showed a significant increase in median ara-CTP

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levels (1.5-fold; $p = 0.04$) in patients receiving doses of 1 g hydroxyurea. *Ex vivo*, diagnostic leukaemic bone marrow blasts from study patients were significantly sensitised to ara-C by a median factor of 2.1 ($p = 0.0047$). All nine patients (100%) achieved complete remission, and all eight (100%) with validated MRD measurements (flow cytometry or real-time quantitative polymerase chain reaction [RT-qPCR]) had an MRD level <0.1% after two cycles of chemotherapy. Treatment was well-tolerated, and median time to neutrophil recovery $>1.0 \times 10^9$/L and to platelet recovery $>50 \times 10^9$/L after the start of cycle 1 was 19 days and 22 days, respectively. Six of nine patients underwent allogeneic haematopoietic stem-cell transplantation (allo-HSCT). With a median follow-up of 18.0 (range 14.9–20.5) months, one patient with adverse risk not fit for HSCT experienced a relapse after 11.9 months but is now in second complete remission.

Conclusion. Targeted inhibition of SAMHD1 by the addition of hydroxyurea to conventional AML therapy is safe and appears efficacious within the limitations of the small phase 1 patient cohort. These results need to be corroborated in a larger study.

Keywords: acute myeloid leukaemia, cytarabine, hydroxyurea, precision medicine, SAMHD1, targeted therapy

Introduction

Acute myeloid leukaemia (AML) has a yearly incidence of 3–5 per 100,000 and occurs at a median age of ~70 years [1, 2]. The overall prognosis is poor but varies with risk group and age. In patients fit for intensive chemotherapy, the 2-year overall survival (OS) is ~50% in patients <65 years but only 25% in those ≥65 years [1, 2]. This stems both from challenges of administering intensive chemotherapy in frail patients and from differences in disease biology, with more myelodysplasia-related features in the elderly [3]. The standard-of-care for non-promyelocytic AML consists of intensive induction and consolidation chemotherapy with cytarabine (ara-C) and an anthracycline, frequently daunorubicin [4, 5]. Multiple attempts have been made to improve the outcome by adding novel components to the daunorubicin + ara-C backbone, but with little success. Important exceptions are the FLT3 inhibitor midostaurin and gemtuzumab ozogamicin in patients with somatic mutations in *FLT3* and core-binding factor AML (CBF-AML), respectively [4, 6–8]. Risk-adapted allogeneic haematopoietic stem-cell transplantation (allo-HSCT) should be considered after achieving complete remission [4, 6, 9].

High-dose ara-C is a critical part of AML therapy, particularly during post-remission consolidation [4, 6, 10, 11]. Ara-C is a deoxycytidine analogue that is transported into leukemic cells by the equilibrative nucleoside transporter 2 (hENT2), and is subsequently mono-, di- and triphosphorylated by deoxycytidine kinase (dCK), UMP/CMP kinase and nucleotide diphosphate kinases 1 and 2 (NDPK1/2), respectively. Ara-C and ara-C monophosphate (ara-CMP) can be inactivated through deamination by cytidine deaminase (CDA) and dCMP deaminase, and ara-CMP can be dephosphorylated by cytosolic 5′-nucleotidases. Ara-C triphosphate (ara-CTP) is a substrate for DNA polymerases and can be incorporated into DNA during replication, perturbing further DNA elongation and triggering DNA damage responses [12]. Failure to accumulate the active metabolite ara-CTP in leukemic blasts is correlated to inferior clinical response [13–18]. We and others have recently shown that the deoxynucleoside triphosphate triphosphohydrolase SAMHD1 can hydrolyse ara-CTP, and expression levels of SAMHD1 in leukaemic blasts were shown to negatively correlate with event-free and overall survival following high-dose ara-C-containing regimens [19–23]. Drug screening to alleviate SAMHD1-mediated resistance to ara-C resulted in the identification of the non-competitive ribonucleotide reductase inhibitor (RNRi) hydroxyurea as a potent synergistic drug with ara-C with the ability to increase intracellular ara-CTP accumulation [24, 25]. Mechanistically, we have shown that hydroxyurea causes dNTP imbalances by reducing dATP and dGTP levels without affecting dCTP. In parallel, dCK is activated [24]. Hence, neither allosteric inhibition of dCK nor substrate competition at DNA polymerase by dCTP are expected effects of hydroxyurea treatment. Hydroxyurea has pleiotropic effects [26], with potent SAMHD1 inhibition already at concentrations of 20 μM

[14], while reversible S-phase cell-cycle arrest is increasingly seen at concentrations in the millimolar range [27]. The plasma levels reached in adults dosed with 1 g hydroxyurea is expected to fully inhibit SAMHD1 while only having a limited effect on cell cycling [28].

In support of the addition of RNRi to ara-C, the competitive RNRi fludarabine had been reported to clinically increase ara-CTP accumulation [29], which led to the establishment of AML treatment strategies combining fludarabine and ara-C [30]. However, more recent data suggest that fludarabine, in contrast to hydroxyurea, does not increase the ara-C sensitivity or the ara-CTP levels in cellular models of AML [24]. Another previously employed strategy to increase leukaemic ara-CTP concentrations was inhibition of the ara-C deaminating enzyme CDA [31]. However, a clinical trial in relapsed AML patients incorporating a CDA inhibitor did not achieve significant inhibition of ara-C deamination [32].

Therefore, in this phase 1 trial, we evaluated the feasibility, safety and efficacy of hydroxyurea to enhance ara-C-based AML-directed conventional therapy in newly diagnosed AML patients by the addition of hydroxyurea. Analysis of the phase 1 run-in part reported here was preplanned and specified in the protocol (see Methods and Supplementary Materials). Whereas hydroxyurea monotherapy has a long-standing role in cytoreductive and palliative treatment [33], toxicity in combination with intensive chemotherapy including high-dose ara-C and daunorubicin in treatment-naïve patients has not been studied. Rather than a dose escalation to a maximum tolerated dose, this phase 1 trial was designed as a run-in for a subsequent phase 2 trial evaluating the tolerability of hydroxyurea in the context of highly intensive chemotherapy at doses expected to yield SAMHD1 inhibition based on preclinical data [24] and known pharmacokinetics of hydroxyurea [28].

While definite conclusions regarding enhanced efficacy of hydroxyurea-augmented ara-C therapies will require larger patient cohorts, this phase 1 trial incorporated translational endpoints of *in vivo* ara-CTP measurements and *ex vivo* drug treatment to provide a proof of principle alongside the reported clinical outcomes (HEAT-AML trial; EudraCT-number: 2018-004050-16).

Methods

Clinical study design and eligibility

This national open-label, phase 1 trial is part of a phase 1/2 trial and was run at two sites (Karolinska University Hospital and Uppsala University Hospital, Sweden). The aim was to evaluate the feasibility, safety, and efficacy of adding hydroxyurea to standard AML-directed therapy according to national guidelines. Eligibility criteria included age >18 years, newly diagnosed non-promyelocytic AML and fitness for intensive chemotherapy. Patients with CBF-AML eligible for treatment with gemtuzumab ozogamicin were excluded. Treatment comprised 2 to 4 cycles of ara-C 1 g/m^2/2 h i.v. b.i.d. on days 1–5 during all four cycles and daunorubicin 60 mg/m^2/8 h i.v. q.d. on days 1–3 during cycles 1 and 2, and on days 1–2 during cycle 3. Patients with *FLT3*-mutated AML received midostaurin 50 mg b.i.d. on days 8–21 of each cycle, but no maintenance was given after completed consolidation, in line with national guidelines. Risk-adapted allo-HSCT was performed at the discretion of the treating haematologist. Hydroxyurea was given 1 h prior to the start of the ara-C infusion b.i.d. on days 1–5, and the dose was escalated in a 3 + 3 design: 500 mg b.i.d (level 1), 1000 mg q.d. + 500 mg q.d (level 2) and 1000 mg b.i.d. (level 3). Full inclusion/exclusion criteria and details regarding cycle intervals/dose reductions are provided in the protocol in the Supplementary Material.

Clinical study oversight

The clinical study protocol was designed by the study investigators. The sponsor of the clinical study was Karolinska University Hospital. The study was monitored by the Center for Clinical Cancer Studies, Karolinska University Hospital. Study oversight was performed by an independent data and safety monitoring committee. Data were collected and analysed by the study investigators. The protocol was reviewed by national and institutional ethics and regulatory bodies (Swedish Ethical Review Authority Dnr 5.1-2019-4650, Swedish Medical Products Agency, Research Council at Karolinska University Hospital). The study has been conducted in accordance with the Declaration of Helsinki and International Council for Harmonisation on Good Clinical Practice guidelines, and all patients provided written informed consent.

Endpoints

Primary endpoints were safety and tolerability (frequency and severity of toxicities according to CTCAE v5), including time to haematopoietic recovery (absolute neutrophil count [ANC] 0.5 and 1.0 $\times$ 10^9/L; platelets 50 $\times$ 10^9/L) after each chemotherapy cycle, defined as the time from the start of the cycle until recovery.

Secondary endpoints were response according to European LeukemiaNet (ELN) criteria [4] and measurable residual disease (MRD) <0.1% following treatment cycle 2, and ara-CTP accumulation in peripheral mononuclear cells during the first chemotherapy cycle with or without hydroxyurea.

An additional explorative endpoint not predefined in the protocol was *ex vivo* drug screening of patient AML cells.

Comparison of SAMHD1 gene expression in healthy bone marrow and AML blasts

SAMHD1 gene expression and annotations in healthy bone marrow cells and AML samples were accessed using the publicly available BloodSpot portal [34]. The following datasets were retrieved: Human Normal Hematopoiesis—GSE42519 and Human AML cells—GSE13159, GSE15434, GSE61804, GSE14468, The Cancer Genome Atlas. Human AML cells were grouped into cytogenetic risk groups according to ELN criteria [4]. Data were visualised using Qlucore Omics Explorer version 3.8(1).

SAMHD1 protein expression

Expression of SAMHD1 was assessed using a double immunostaining method for SAMHD1/CD68 at diagnosis or SAMHD1/CD34 at remission, an autostainer system (BenchMark Ultra, Ventana, Rotkreuz, Switzerland) and previously validated protocols [21]. $CD68^+$/$SAMHD1^+$ histiocytes (macrophages) served as internal controls in the diagnostic bone marrow biopsies assessed. CD34 costaining at remission allowed assessment of SAMHD1 expression in haematopoietic stem and progenitor cells (HSPCs). The percentage of SAMHD1-positive blasts was calculated by counting at least 500 blasts in each case, assessed by two haematopathologists independently.

MRD measurement

MRD was measured by flow cytometry after cycle 2 (according to the EuroFlow and NOPHO-AML protocols, Table S1) or with real-time quantitative polymerase chain reaction (RT-qPCR) if validated genetic markers were available (such as mutated *NPM1* or *DEK*::*NUP214* fusion transcript, Table S3) [35–37]. To ensure additional information on mutational clearance, patients without a standard validated genetic marker for MRD were analysed by sensitive deep sequencing after cycle 2, as previously described [36].

Pharmacokinetic study

On day 1 of treatment cycle 1, patients received hydroxyurea 1 h prior to one of two ara-C infusions. Patients 1–3 received 500 mg hydroxyurea prior to the first, patients 4–6 received 1000 mg hydroxyurea prior to the first and patients 7–9 received 1000 mg hydroxyurea prior to the second ara-C infusion. The reason to allocate hydroxyurea either prior to the first or second administration of ara-C was to control for possible accumulation of ara-CTP after two doses of ara-C, which might introduce a bias towards higher ara-CTP levels following the second ara-C infusion. In that case, the efficacy of hydroxyurea to increase ara-CTP might be overestimated. Eighteen millilitres of peripheral blood was drawn directly following the first and second infusion of ara-C, and mononuclear cells (MNCs) were isolated using Lymphoprep (STEMCELL Technologies, Cambridge, UK). Following methanol extraction, ara-CTP was quantified using chromatography tandem mass spectrometry [24] with a modified liquid chromatography (LC) method (column: Hypercarb [100 $\times$ 2.1 mm, 5 μm, Thermo Scientific]; mobile phase A: 5 mM hexylamine and 0.4% dimethylhexylamine [v/v], pH 10; mobile phase B: 50 % acetonitrile; LC gradient: mobile phase B increased from 5% to 50% in 13 min, 50% to 80% in 1 min and then returned to the initial condition in 1 min; flow rate 0.4 ml/min).

Ex vivo *drug sensitivity analysis*

AML MNCs, healthy CD34+ haematopoietic stem and progenitor cell (HSPC) donor cells and THP-1 SAMHD1-wt or THP-1 SAMHD1-knockout cells [19] were dispensed in 384-well tissue culture plates (Corning) at a concentration of 2.5 $\times$ 10^5 cells per millilitre reconstituted in RPMI 1640 media (ThermoFisher) supplemented with penicillin, streptomycin, 10% foetal calf serum

and 12.5% conditioned cell culture supernatant (obtained from confluent HS-5 cells [CRL-11882, ATCC]) [38]. Cells were incubated for 72 h with the drugs hydroxyurea (10–100,000 nM) or gemcitabine (0.1–1000 nM) together with ara-C (1–10,000 nM) in five combinations. For analysis of drug combinations, all concentrations of hydroxyurea or gemcitabine were tested against all concentrations of ara-C in duplicates. The drugs were preplated using acoustic dispensing (Echo 550, Labcyte). Following incubation for 72 h, cell viability was measured using CellTiter-Glo (Promega). Data were collected on an EnSight (PerkinElmer) system. Data on each plate were normalised to a plate-specific negative control (vehicle) and a positive control (100 μM benzethonium chloride). Quality control and calculation of half-maximal inhibitory concentrations (IC_{50}) and zero interaction potency (ZIP) [39] and data analysis was performed using custom scripts in R and Breeze pipeline (breeze.fimm.fi) [40].

Ex vivo *ara-CTP measurements*

AML MNCs were cultured in Iscove Modified Dulbecco Medium (IMDM) supplemented with penicillin, streptomycin, 10% foetal calf serum and 20 ng/ml interleukin 3, 20 ng/ml interleukin 6 (both R&D Systems), 20 ng/ml human recombinant Granulocyte-monocyte colony-stimulating factor and 100 μg/ml human recombinant thrombopoietin (both Stem Cell Technologies) and treated with 500 nM ara-C with or without 60 μM hydroxyurea for 24 h. Samples were further processed and analysed as described above (Pharmacokinetic Study).

Statistical analysis

For ara-CTP measurements from MNCs and ara-C IC_{50} values in diagnostic bone marrow mononuclear cells with or without prior hydroxyurea, paired two-tailed Student's *t*-tests were performed prior to normalization. For comparison of ara-CTP levels or ara-C IC_{50} values with respect to SAMHD1 expression levels, Kruskal–Wallis tests were performed (Prism 9.2.0, GraphPad Software, San Diego, CA, USA).

Role of the funding sources

The funding sources had no role in the study design; in the collection, analysis and interpretation of data; in the writing of the report and in the decision to submit the paper for publication.

Results

Patients

In total, nine patients were enrolled between October 2020 and March 2021 (Table 1 and Fig. 1). During the recruitment period, a total of 31 patients with newly diagnosed AML received intensive chemotherapy at our institution. Of the 22 patients who were not screened for inclusion, the main reasons were frailty (such as age >75 years with comorbidities) that precluded full-dose induction and hyperleukocytosis requiring pretreatment with hydroxyurea, which was not allowed in the phase 1 part of the study. In addition, two patients were ineligible due to having CBF-AML.

The patients were of all ELN risk categories: favourable (n = 3), intermediate (n = 1) and high (n = 5). *FLT3* mutation was seen in five patients (two ITD, three TKD) and they all received midostaurin 50 mg b.i.d. days 8–21 after each cycle of chemotherapy. All patients received at least two cycles of therapy according to protocol. For patient 1106, the dose of ara-C was reduced to 80% during cycle 2 due to high age (76 years) and severe sepsis after cycle 1.

Safety and tolerability

There were no non-haematological grade 4 toxicities. The most common grade 3 adverse event was febrile neutropenia, occurring in all patients. Other grade 3 infections were sepsis (n = 2), catheter-related infection (n = 3) and wound infection (n = 1). Gastrointestinal grade 3 events included colitis (n = 1), rectal pain (n = 1) and bleeding (n = 1). The remaining adverse events of grade 3 were back pain (n = 1), epistaxis (n = 1), bronchial obstruction (n = 1) and urinary tract obstruction (n = 1). Grade 1 and 2 events were as expected for patients undergoing intensive chemotherapy.

Specific adverse events known to be related to ara-C were monitored in detail. No cerebellar or other central nervous adverse events occurred. Two patients, both concomitantly treated with midostaurin according to national guidelines, had grade 1 (patient 1109) or 2 (patient 1105) palmar–plantar erythrodysesthaesia. Both patients continued treatment according to protocol, and the skin lesions were successfully treated with topical steroids. Conjunctivitis was not reported above grade 1.

Table 1. *Patient characteristics and outcome*

Patient	Karyotype	Mutations, fusions	ELN category	SAMHD1 IHC	CR cycle 1/2	MRD after cycle 2, flow cytometry/RT-qPCR/deep sequencing	Allo-HSCT	Time to relapse (months)	Overall survival (months)
1101 Female 26 years	47,XX,t(8;19)(p21;p10),+der(8)t(8;19)[14] /46,idem,-7 [3] /48,idem,+21 [2] /46,XX [1]	*RUNX1*	Adverse	25%–75%	Yes/Yes	0.02%/NA/*RUNX1* 8.9%	Yes	NA	20.5+
1102 Female 27 years	46,XX [20]	*NRAS, U2AF1*	Intermediate	<25%	Yes/Yes	0.07%/NA/*U2AF1* 0.4%, *NRAS* <0.02%	Yes	NA	20.1+
1103 Male 26 years	46,XY [20]	*FLT3*-TKD, *RUNX1, IKZF1*	Adverse	25%–75%	Yes/Yes	0.09%/NA/*RUNX1* 0.74%, *FLT3*-TKD 0.049%	Yes	NA	19.2+
1104 Female 26 years	46,XX,t(6;9)(p22;q34) [17] /46,XX [3]	*DEK*::*NUP214*	Adverse	<25%	Yes/Yes	NA/<0.01%/NA	Yes	NA	18.6+
1105 Female 48 years	46,XX [20]	*NPM1, FLT3*-TKD, *IDH2, DNMT3A*	Favourable	>75%	Yes/Yes	NA/<0.001%/NA	No	NA	18.0+

(*Continued*)

Table 1. *(Continued)*

Patient	Karyotype	Mutations, fusions	ELN category	SAMHD1 IHC	CR cycle 1/2	MRD after cycle 2, flow cytometry/RT-qPCR/deep sequencing	Allo-HSCT	Time to relapse (months)	Overall survival (months)
1106 Male 76 years	46,XY [20]	*RUNX1*, *BCORL1*, *BCOR*, *FLT3*-TKD	Adverse	25%–75%	No[a]/Yes	0.004%/NA/ *RUNX1* 10.5%, *FLT3*-TKD 0.004%	No	11.9	16.8+
1107 Male 52 years	46,XY [20]	*NPM1*, *FLT3*-ITD low ratio, *IDH2*	Favourable	<25%	Yes/Yes	NA/0.0017/ NA	No	NA[b]	16.7+
1108 Female 61 years	45,X,-X [10]/46,XX [10]	*RUNX1*, *SF3B1*, *CBL*	Adverse	<25%	Yes/Yes	NA[c]/NA/ *RUNX1* 0.79%, *CBL* 0.77%	Yes	NA	16.0+
1109 Female 61 years	46,XX t(5;6)(q31;q25) [6] /46,XX [16]	*NPM1*, *FLT3*-ITD low ratio, *TET2*	Favourable[d]	<25%	Yes/Yes	NA/0.017%/ NA	Yes	NA	14.9+

Abbreviations: Allo-HSCT, allogeneic haematopoietic stem cell transplantation; *BCOR*, BCL6 Corepressor; *BCORL1*, BCL6 Corepressor Like 1; *CBL*, Cbl Proto-Oncogene; CR, complete remission; *DEK::NUP214*, fusion of DEK Proto-Oncogene and Nucleoporin 214; *DNMT3A*, DNA Methyltransferase 3α; ELN, European LeukemiaNet; *FLT3*-ITD, Fms Related Receptor Tyrosine Kinase 3 internal tandem duplication; *FLT3*-TKD, Fms Related Receptor Tyrosine Kinase 3 tyrosine kinase domain; *IDH2*, Isocitrate Dehydrogenase 2; IHC, immunohistochemistry; *IKZF1*, IKAROS Family Zinc Finger 1; MRD, measurable residual disease; NA, not available; *NPM1*, Nucleophosmin 1; *NRAS*, Neuroblastoma RAS viral oncogene homolog; *RUNX1*, Runt-related transcription factor 1; *SF3B1*, Splicing Factor 3b Subunit 1; *TET2*, Tet Methylcytosine Dioxygenase 2; *U2AF1*, U2 Small Nuclear RNA Auxiliary Factor 1.

[a]Blasts 5.5%; Granulocyte colony-stimulating factor use prior to sampling may have increased the morphologic blast count.

[b]At 9 months, MRD *NPM1* switched from negative to positive at 0.00024%, when double-checked then negative. Prior to receiving the confirmatory MRD result, the patient was put on AZA-VEN and received three cycles.

[c]Patient 1108 lacked a leukaemia-specific phenotype for flow cytometry.

[d]This patient had a prior history of myeloproliferative neoplasia with osteosclerosis and extramedullary haematopoiesis, and therefore better resembled a higher-risk secondary AML.

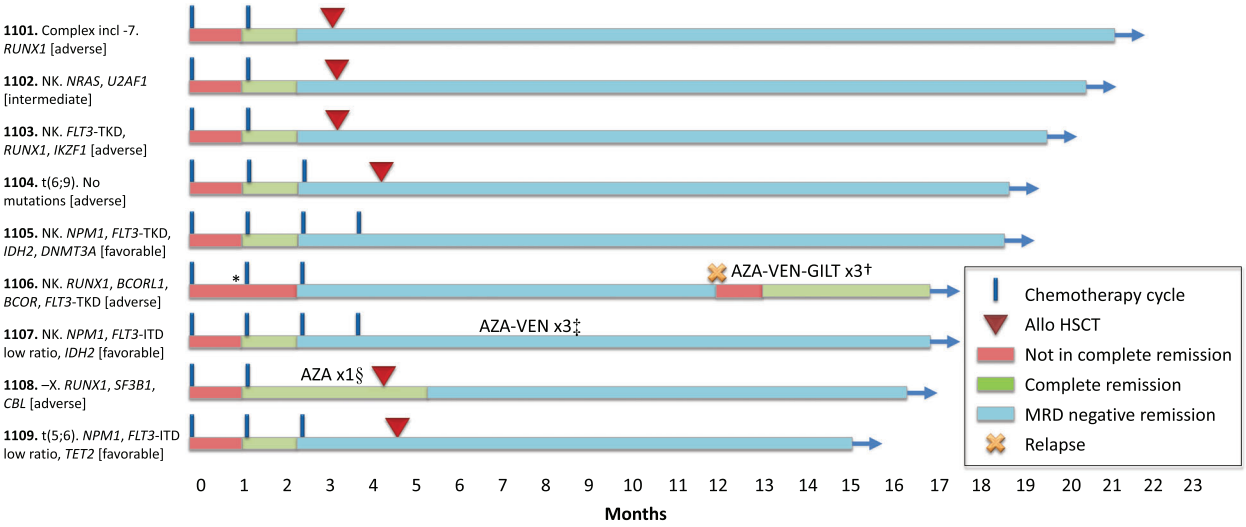

Fig. 1 *Treatment outcome. The nine patients have been followed for 14.9–20.5 months. Six patients have undergone allogeneic haematopoietic stem cell transplantation (allo-HSCT). Measurable residual disease (MRD) was performed in accordance with national guidelines after cycle 2 and cycle 4 or prior to allo-HSCT. In non-transplanted patients with NPM1, MRD by real-time quantitative polymerase chain reaction (RT-qPCR) was performed every 3 months. After allo-HSCT, patients were routinely monitored every 3 months either by RT-qPCR (NPM1) or flow cytometry (all others). Eight of nine patients remain in MRD negative remission (as defined by MRD <0.1% by flow cytometry or RT-qPCR). *, Patient 1106 had bone marrow blasts 5.5% after cycle 1 and did not fulfil complete remission (CR) criteria until after cycle 2. However, blasts may have been elevated by granulocyte colony-stimulating factor (G-CSF) usage prior to sampling; †, patient 1106 relapsed after 11.9 months and was treated with a combination of azacitidine–venetoclax–gilteritinib (AZA-VEN-GILT) and is now in a second CR; ‡, in patient 1107, NPM1 RT-qPCR MRD increased from negative to 0.00024%, was rechecked and was then negative again. Before receiving the result of the confirmatory MRD, the patient was put on azacitidine–venetoclax (AZA-VEN), and three cycles were given as an additional consolidation due to the inconsistent results; §, patient 1108 received one cycle of AZA as bridging while waiting for allo-HSCT.*

To estimate the likelihood of a negative impact of hydroxyurea-mediated SAMHD1 inhibition on haematological recovery, we analysed SAMHD1 expression in a large set of publicly available AMLs and healthy bone-marrow cells. While SAMHD1 expression is similar in different cytogenetic risk groups of AML, the HSC and other bone marrow progenitor cells with the exception of the granulocyte–monocyte progenitor expressed much lower levels of SAMHD1 (Fig. S1). This was also reflected by the general negativity for SAMHD1 in CD34+ HSCPs in the immunohistochemical evaluation of the patients' bone marrow during remission (see below). The time-to-neutrophil recovery with ANC $\geq$0.5 and $\geq$1.0 $\times$ 10^9/L was similar, in median 19 (range 16–23) days after cycle 1. The rapid increase in ANC was likely attributed to the fact that all nine patients received G-CSF support during the neutropenic phase, in accordance with local routines during the COVID-19 pandemic. Time to platelet recovery $\geq$50 $\times$ 10^9/L was in median 22 (17–25) days after the first cycle. Similar haematological recovery was seen during the subsequent cycles (Table S2).

No dose-limiting toxicities were found for the evaluated hydroxyurea doses, and the recommended phase 2 dose (RP2D) for the subsequent phase 2 part of the study was therefore set to 1000 mg b.i.d.

Response and leukaemic clearance

All nine patients achieved complete remission (CR) after cycle 2 (eight of nine already after cycle 1). One patient who received G-CSF had 5.5% blasts after cycle 1 and hence was in partial remission per definition, despite full peripheral regeneration. Validated MRD measurements after cycle 2 were negative (<0.1%) in eight of eight evaluable patients (four of four by flow cytometry, four of four by RT-qPCR). Patient 1108 lacked a specific leukaemia-associated immunophenotype (LAIP) and MRD could therefore not be robustly determined with routine methods.

Targeted deep sequencing was used to further evaluate the mutational clearance after cycle 2 in the four patients for whom MRD was determined by flow cytometry. This demonstrated a reduction in variant allele frequency (VAF) to below 0.02 and up to 10.5% depending on the mutation analysed. In patient 1108 who lacked an LAIP, the VAF for *RUNX1* was reduced to 0.79% after cycle 1. *FLT3*-TKD was observed in two of four patients with mutated *RUNX1*, and the *FLT3*-mutated clone was cleared <0.05% (corresponding to an MRD <0.1%) in both patients after cycle 2 (Fig. 2).

Patient 1107 showed a low-level MRD positivity for *NPM1* at month nine. However, resampling could not confirm detectable MRD. While the confirmatory results were pending, the patient received azacitidine and venetoclax, and received in total three cycles as additional consolidation.

Allo-HSCT was performed in six of nine patients, after cycle 2 ($n = 2$), cycle 3 ($n = 2$) or after cycle 2 with one cycle of azacitidine as bridging ($n = 1$; patient 1108).

The median follow-up was 18.0 (range 14.9–20.5) months. One elderly patient with adverse risk and not fit for HSCT relapsed after 11.9 months. The patient initially only received three cycles of chemotherapy, with cycle 3 being dose-reduced. At the time of relapse, the patient received azacitidine–venetoclax–gilteritinib and is now in second complete remission (Fig. 1).

Enhanced ara-CTP levels in leukaemic blasts in vivo and increased sensitivity to ara-C ex vivo

Protein expression levels of SAMHD1 in diagnostic bone marrow biopsies varied with low (<25%), intermediate (25-75%) and high expression (>75%) in five, three and one patient, respectively (Table 1 and Fig. 3a). At remission, SAMHD1 was largely absent particularly in CD34+ HSPCs (with the exception of histiocytes and reactive lymphocytes) (Fig. 3b). In the absence of hydroxyurea, there was no significant association of SAMHD1 expression levels with ara-CTP levels in vivo ($n = 9$, $p = 0.83$) or ara-C IC_{50} values ex vivo ($n = 7$; $p = 0.13$) (Fig. S2).

Paired comparisons of ara-CTP levels in peripheral blood mononuclear cells (PBMCs) [41–45] treated with or without hydroxyurea showed no significant differences at a dose of 500 mg ($n = 3$; $p = 0.45$, Fig. S3). The median percentage of blasts in the PBMC fraction was 40% (range 0%–92% [Table S4]). At a dose of 1 g hydroxyurea (given either prior to the first or the second ara-C infusion to control for increases in ara-CTP that might stem from accumulation rather than effects of hydroxyurea), median ara-CTP levels increased to 150% ($n = 6$; $p = 0.04$, range 100%–311%; Fig. 4). In addition, we measured ara-CTP levels in bone marrow-derived blasts *ex vivo* treated with ara-C plus/minus hydroxyurea. A modest but significant increase of ara-CTP could be detected ($n = 8$; $p = 0.02$, range 99%–139%; Fig. S4). Bone marrow–derived MNCs from seven patients could be subjected to *ex vivo* drug testing. The addition of hydroxyurea decreased the IC_{50} values of ara-C by a median factor of 2.1 ($p = 0.0047$, range 1.6–8.3; Fig. 4). Similar results were obtained for gemcitabine, another non-competitive inhibitor of RNR, with a factor of 1.6 ($p = 0.01$, range 1.3–5.4, Fig. S5). As a comparison, hydroxyurea decreased IC_{50} values of ara-C by a factor of 8.9 in the SAMHD1-positive THP-1 cell line but had no effect in a SAMHD1-knockout THP-1-derivative or in healthy CD34+ bone marrow cells (Fig. S6). The median ZIP in patient cells as another measure of ara-C synergy with hydroxyurea and gemcitabine was 2.6 and 3.6, respectively, in line with the ZIP of 3.3 and 4.7 in the AML cell line THP-1 (Fig. S7).

Discussion

Based on preclinical data suggesting that the addition of hydroxyurea potentiates cytarabine efficacy by increasing ara-CTP levels through inhibition of SAMHD1, this trial was designed to assess the feasibility from a toxicity perspective and to investigate the efficacy of such addition of hydroxyurea to conventional daunorubicin/ara-C (DA)-based chemotherapy. In parallel, the effects of hydroxyurea on ara-CTP pharmacokinetics and *ex vivo* ara-C sensitivity were assessed to evaluate the study rationale translationally [24, 27, 28]. The nine patients were representative of the general AML population eligible for intensive chemotherapy, with a median age of 48 years (range 26–76), albeit with a skewing towards adverse risk. Partially, this skewing can be explained by exclusion of CBF-AML. Adverse genetics according to ELN were seen in five of nine (56%) patients, and *FLT3*-mutation was present in five of nine (56%). The corresponding figures according to the Swedish population-based AML registry were 30%–40% and 25%–30%, respectively [46].

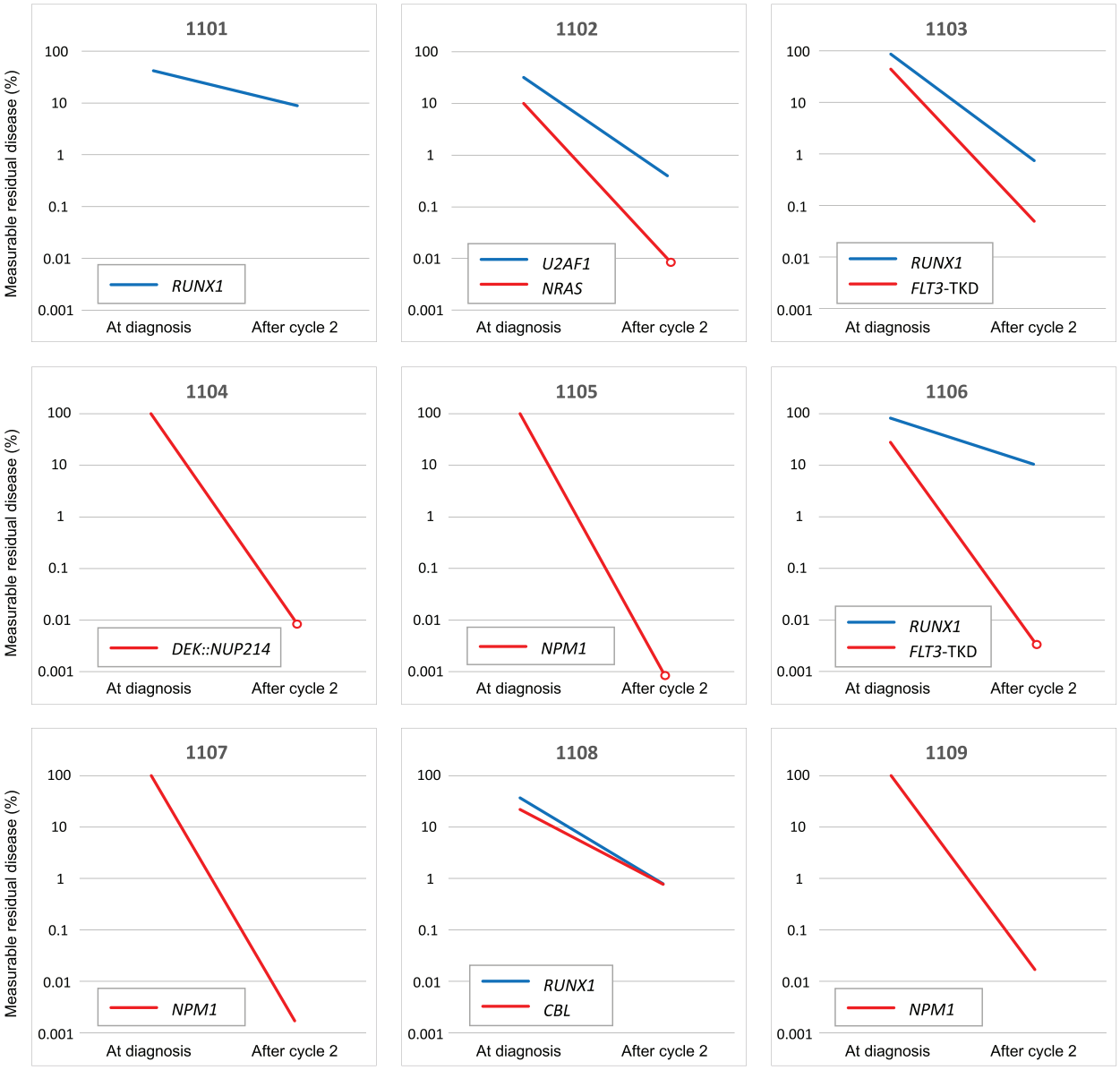

Fig. 2 *Leukemic clearance after cycle 2. Validated real-time quantitative polymerase chain reaction (RT-qPCR) markers for NPM1 and DEK::NUP214, respectively, were analysed in four patients, and all showed mutational clearance <0.1% (relative to the diagnostic level) after cycle 2. In the remaining five patients, one or two mutations were monitored for measurable residual disease (MRD) using deep sequencing (reporting the variant allele frequency). Mutation in RUNX1 was seen in four patients and was reduced to 0.74%–10.5% after cycle 2. In two of four patients with RUNX1, a FLT3-TKD was also present, but was reduced below 0.05% after two cycles of treatment. After cycle 2, MRD levels below the detection limit for the method are indicated with open circles.*

It is conceivable that the addition of hydroxyurea potentiates the risk of ara-C-related adverse events. However, the observed toxicities and time to haematopoietic recovery were in line with expected outcomes for patients undergoing intensive chemotherapy for AML [47] and did not delay subsequent cycles of chemotherapy or time to allo-HSCT. This might at least partially be explained by the low expression levels of SAMHD1 in healthy bone marrow progenitors including HSPC. The lack

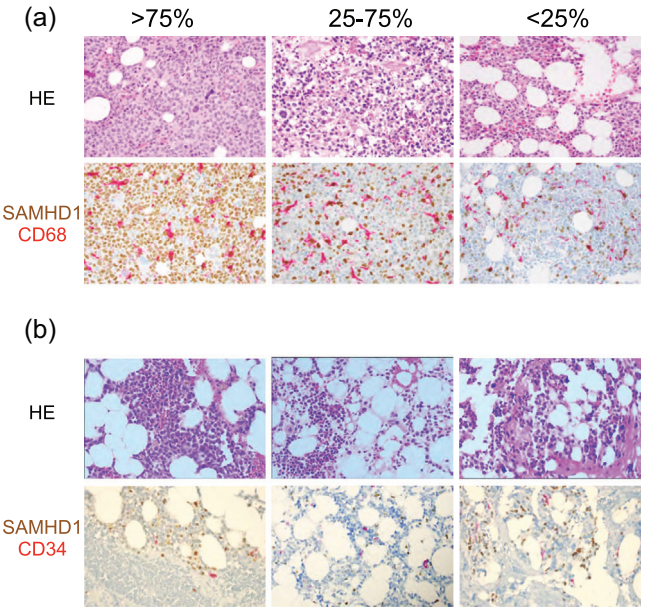

Fig. 3 *SAMHD1 protein expression in acute myeloid leukaemia (AML) blasts and in remission bone marrow. (a) Upper images show haematoxylin and eosin routine staining in diagnostic bone marrow biopsies. The lower images show the corresponding SAMHD1-CD68 double immunohistochemical staining. SAMHD1 (in brown) is mainly expressed in the nucleus of the AML blasts, while CD68^{+} macrophages (magenta) are strongly positive for SAMHD1 and serve as an internal positive control. Low, intermediate and high expression levels were defined as <25%, 25%–75% and >75% positive cells, respectively, as previously described [21]. (b) Upper images show haematoxylin–eosin routine staining in remission bone marrow biopsies from patients 1105 (left), 1101 (middle) and 1104 (right), respectively, with cellularity within normal range and morphology suggestive of reactive bone marrow. Lower images show SAMHD1/CD34 double immunostaining without double-positive blasts. Occasionally, SAMHD1+/CD34− normal blasts are present. The SAMHD1+/CD34− cells are morphologically mostly histiocytes and reactive lymphocytes. All images are shown at 400× magnification.*

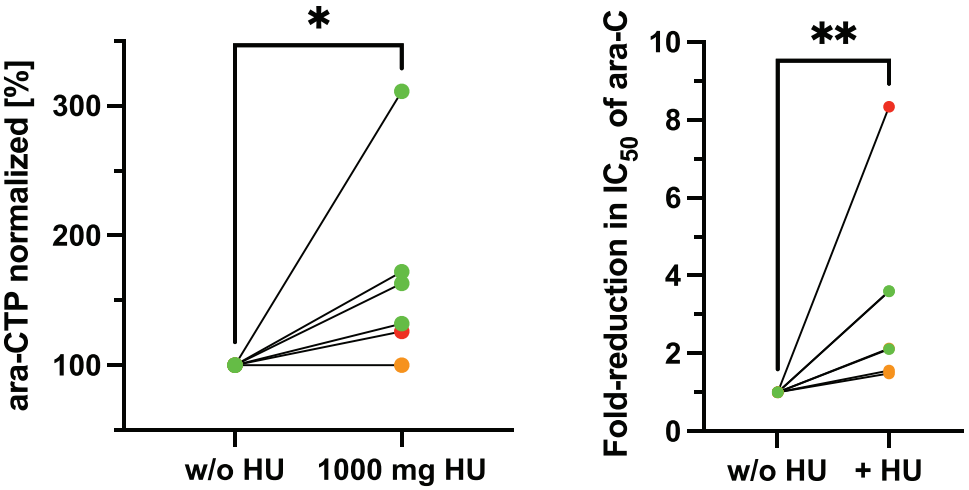

Fig. 4 *Effect of hydroxyurea on peak ara-C triphosphate (ara-CTP) levels in circulating mononuclear cells and ex vivo sensitivity to ara-C. The left panel shows ara-CTP levels measured in circulating mononuclear cells without hydroxyurea (w/o HU) as compared to ara-CTP with hydroxyurea 1000 mg taken p.o. 1 h prior to start of ara-C infusion, normalised to without hydroxyurea (n = 6). The right panel shows the fold reduction in IC_{50} values of ara-C in diagnostic bone marrow mononuclear cells treated ex vivo in the presence or absence of 100 μM hydroxyurea, normalised to the absence of hydroxyurea (n = 7). Individual dots correspond to individual patients; colours represent levels of SAMHD1 expression at diagnosis (green, <25%, orange 25%–75% and red >75%).*

of excess myelosuppression together with our previous reports on the negative role of SAMHD1 during ara-C-based consolidation courses [19, 21] hence justifies the addition of hydroxyurea even in post-remission treatments. Two patients experienced palmar–plantar erythrodysesthaesia, a well-known side effect of ara-C. Both patients were on concomitant midostaurin, and both were able to continue treatment according to protocol (four cycles and three cycles + allo-HSCT, respectively). Toxicities were consistent with previous studies in which both hydroxyurea and ara-C were

administered at varying schedules and dosing to a heterogenous population of relapsed or refractory patients (summarised in [24]). Time to recovery of neutrophils and platelets was on par with what is expected after intensive chemotherapy according to the Swedish AML Registry.

Albeit the limitations of the small phase 1 cohort, the efficacy appeared highly promising, with all nine patients achieving CR, including five of five with high-risk genetics. All eight patients evaluable for MRD assessment with flow cytometry or RT-qPCR reached negativity (defined as <0.1%) after cycle 2. According to data from the Swedish AML Registry, the expected CR/MRD-negativity rates with an identical chemotherapy regimen but without hydroxyurea is 92%/80% in favourable-risk, 84%/64% in intermediate-risk and 71%/60% in adverse-risk patients [48]. The treatment efficacy will be further evaluated in the phase 2 extension part of the study, where an additional 60 patients with AML will be included. Addition of midostaurin for *FLT3*-mutated AML patients contributes to therapeutic efficacy [7] and consequently is recommended by national guidelines for patients fit for intensive chemotherapy. While this might confound interpretation of the effects of added hydroxyurea in our study, real-world data in *FLT3*-mutated patients treated with or without the addition of midostaurin did not show any difference of complete remission rates, consistent with the seminal trial [7, 49]. This argues against the notion that midostaurin would confound the early surrogate endpoints of complete remission and MRD negativity.

To gain a deeper understanding of the degree of molecular clearance after cycle 2 in the five patients lacking validated genetic MRD markers, targeted deep sequencing was performed on selected driver mutations. VAF reduction varied from five-fold to 8000-fold with remaining VAFs from <0.02% to 10.5%. However, selecting relevant genetic markers for MRD in AML can be challenging as they may occur in premalignant clones. The two patients with detectable driver mutations above 1% after cycle 2 (patients 1101 and 1106) had flow cytometry based MRD <0.1%. Importantly, both patients had genetic alterations characteristic for MDS-AML [50]—*RUNX1*, in combination with complex karyotype including monosomy 7 and multiple mutations including *RUNX1* and *BCOR*, respectively, where incomplete mutational clearance is often observed [51]. In both patients with *RUNX1*-mutation and concomitant *FLT3*-TKD (1103 and 1106), the *FLT3*-mutated clone was cleared below 0.1%. Taken together, the data indicate that the leukaemic clones may be more potently eradicated than premalignant clones.

Neither absolute peak intracellular ara-CTP levels nor ara-C IC_{50} values differed significantly with respect to SAMHD1 expression, which is consistent with a previous report in primary AML cells [19]. It is a limitation of the study that no sequential ara-CTP measurements were performed, as this would have allowed calculation of half-life and area under the curve, both of which correlate with clinical responses whereas peak ara-CTP levels do not [17]. However, administering 1000 mg of hydroxyurea 1 h prior to ara-C infusion significantly increased relative peak ara-CTP levels in circulating patient blasts by a factor of 1.5, and those results could be confirmed by treating diagnostic patient blasts with ara-C and hydroxyurea ex vivo. As hydroxyurea was either given before the first or the second administration of ara-C (12 h apart), we controlled for ara-CTP accumulation caused by accumulation due to repetitive dosing of ara-C. Furthermore, *ex vivo* supplementation of hydroxyurea reduced IC_{50} values for ara-C by a factor of 2.1. While more studies are needed, we suggest using *ex vivo* drug testing rather than ara-CTP measurements as a putative predictor for treatment benefit of ara-C/hydroxyurea combinations. This strategy is faster, less expensive and requires at least 100-fold fewer cells per tested condition. The latter allows testing of a wide range of concentrations, which is necessary to correctly predict synergy of ara-C and hydroxyurea [24].

A recent publication suggests that treatment of AML with nucleoside analogues might induce differentiation through hyperactivation of ribonucleotide reductase, leading to a dNTP pool imbalance that could be further aggravated by depletion of SAMHD1 [52]. Hence, ara-C-mediated differentiation that is increased by SAMHD1 inhibition might be an alternative explanation for the observed efficacy to achieve MRD negativity in our trial. However, in our previous studies, we neither observed effects of SAMHD1 depletion on protein levels of RNR subunits nor on dNTP pool balances [19, 24].

In conclusion, the high rate of complete remission and MRD negativity together with the pharmacokinetic and *ex vivo* evidence suggest that

the efficacy of cytarabine-based AML treatment can be enhanced by the addition of hydroxyurea as a targeted inhibitor of SAMHD1. Importantly, orally administered hydroxyurea may provide a safe, inexpensive and broadly accessible strategy to improve outcome in AML. These results need to be validated in a larger patient cohort.

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Conflicts of interest

T. S. is employed by Heidelberg ImmunoTherapeutics, not relevant to this work. J. I. H. is a consultant for SOBI, not relevant to this work. The other authors declare no conflicts of interest.

Author contributions

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Data availability statement

The clinical trial protocol is included in the Supplementary information. Individual participant data that underlie the results reported in this article, after de-identification (text, tables, figures and appendices), will be made available upon request.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. *In silico* analysis of *SAMHD1* mRNA expression in healthy bone marrow progenitors and AML blasts
Figure S2. Ara-CTP levels in circulating blasts and *ex vivo* sensitivity to ara-C with respect to SAMHD1 expression

Figure S3. Effect of 500 mg hydroxyurea on peak ara-CTP levels in circulating mononuclear cells
Figure S4. Effect of 60 μM hydroxyurea on ara-CTP levels in diagnostic bone-marrow mononuclear cells *ex vivo*
Figure S5. Effect of gemcitabine on *ex vivo* sensitivity to ara-C
Figure S6. Effect of hydroxyurea on *ex vivo* sensitivity to ara-C in THP-1 cells and healthy CD34+ HPSCs
Figure S7. Zero Interaction Potency of ara-C and hydroxyurea in diagnostic bone-marrow mononuclear cells *ex vivo* and THP-1 cells

Table S1. Antibody panels for flow cytometry
Table S2. Haematologic recovery
Table S3. Mutations assessed with deep sequencing
Table S4. White blood cell composition at time of ara-CTP measurements

Supplementary References ■

