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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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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/joim.13553.
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ABSTRACT

Background

Treatment of newly diagnosed acute myeloid leukaemia (AML) is based on combination chemotherapy with cytarabine 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-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.

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Methods

Nine patients were enrolled and received at least two courses of ara-C (1g/m^2/2 h b.i.d. d1-5, i.e. a total of 10/m^2 g 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 levels (1.5-fold; *P*=0.04) in patients receiving doses of 1 g hydroxyurea. *Ex vivo*, diagnostic leukemic 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 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 x 10^9/L and to platelet recovery >50 x 10^9/L after start of cycle one was 19 days and 22 days, respectively. Six of nine patients underwent hematopoietic stem-cell transplantation. 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 CR2.

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.

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Keywords: Acute myeloid leukaemia; SAMHD1; hydroxyurea; cytarabine; targeted therapy

INTRODUCTION

AML has a yearly incidence of three-five 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 two-year overall survival (OS) is ~50% in patients <65 years but only 25% ≥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]. 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 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 hematopoietic 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 deoxytidine kinase (dCK), UMP/CMP kinase, and Nucleotide diphosphate kinases 1 and 2 (NDPK1/2), respectively. Ara-C and ara-CMP can be inactivated through deamination by cytidine deaminase (CDA) and dCMP deaminase (DCTD), and ara-CMP can be dephosphorylated by cytosolic 5′-nucleotidases. 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

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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 leukemic blasts were shown to negatively correlate with event-free survival (EFS) and OS 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-allosteric 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, deoxycytidine kinase (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 higher concentrations, reported 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 allosteric 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 leukemic ara-CTP concentrations was inhibition of the ara-C deaminating enzyme cytidine deaminase (CDA)[31]. However, a clinical trial in relapsed AML patients incorporating a CDA inhibitor did not achieve significant inhibition of ara-C deamination[32].

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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 pre-planned 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 (MTD), 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 pre-clinical 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

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comprised 2 to 4 cycles of ara-C 1 g/m^2/2 h i.v. b.i.d. on day 1-5 during all 4 cycles and daunorubicin 60 mg/m^2/8 h i.v. q.d. on day 1-3 during cycles 1 and 2, and on day 1-2 during cycle 3. Patients with *FLT3*-mutated AML received midostaurin 50 mg b.i.d. on day 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 hour prior to the start of the ara-C infusion b.i.d. on day 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 hematopoietic recovery (ANC 0.5 and 1.0 x 10^9/L; platelets

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50 x 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 Leukemia Net (ELN) criteria[4] and measurable residual disease (MRD) <0.1% following treatment cycle 2, and ara-CTP accumulation in peripheral blasts during the first chemotherapy cycle with or without hydroxyurea.

An additional explorative endpoint not pre-defined in the protocol was ex-vivo drug screening of patient AML cells.

Comparison of SAMHD1 gene expression in healthy bonemarrow 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 (TCGA). Human AML cells were grouped into cytogenetic risk groups according to ELN criteria[4]. Data was 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 diagnostic bone-marrow biopsies assessed. CD34 co-staining at remission allowed assessment of SAMHD1 expression in haematopoietic stem cells. The percentage of SAMHD1-positive blasts was calculated by counting at least 500 blasts in each case, assessed by two haematopathologists independently.

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MRD measurement

MRD was measured by flow cytometry after cycle 2 (according to the EuroFlow and NOPHO-AML protocols, Supplementary Table S1) or with RT-qPCR if validated genetic markers were available (such as mutated *NPM1* or *DEK*::*NUP214* fusion transcript, Supplementary 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 hour 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, respectively. 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. 18 mL of peripheral blood was drawn directly following the first and second infusion of ara-C, and mononuclear cells were isolated using Lymphoprep (STEMCELL Technologies, Cambridge, UK). Following methanol extraction, ara-CTP was quantified using chromatography–tandem mass spectrometry[24] with a modified LC method (Column: Hypercarb [100 x 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 initial condition in 1 min. Flow rate 0.4 mL/min.).

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Ex-vivo drug sensitivity analysis

AML mononuclear cells (MNCs), healthy CD34+ HPSC 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 x 10^5 cells per mL reconstituted in RPMI 1640 media (ThermoFisher) supplemented with penicillin, streptomycin, 10% foetal calf serum, and 12.5% CM culture supernatant (obtained from confluent HS-5 cells [CRL-11882, ATCC]) [38]. Cells were incubated for 72 hours with the drugs hydroxyurea (10-100 000 nM) or gemcitabine (0.1-1000 nM) together with ara-C (1-10 000 nM) in 5 points. For analysis of drug combinations, all concentrations of hydroxyurea or gemcitabine were tested against all concentrations of ara-C in duplicates. The drugs were pre-plated using acoustic dispensing (Echo 550, Labcyte). Following incubation for 72 hours, cell viability was measured using CellTiter-Glo (Promega). Data was collected on an EnSight (PerkinElmer) system. Data on each plate were normalized to a plate-specific negative control (vehicle) and a positive control (100 µmol/L benzethonium chloride). Quality control and calculation of half-maximal inhibitory concentrations (IC_{50}) and Zero Interaction Potency[39] and data analysis was performed using custom scripts in R and Breeze pipeline (breeze.fimm.fi)[40].

Ex-vivo ara-CTP measurements

AML mononuclear cells (MNCs) were cultured in IMDM supplemented with penicillin, streptomycin, 10% foetal calf serum, and 20 ng/mL IL-3, 20 ng/mL IL-6 (both R&D Systems), 20 ng/mL GM-CSF), 100 µg/mL TPO (both Stem Cell Technologies) and treated with 500 nM ara-C with or without 60 µM hydroxyurea for 24 hours. Samples were further processed and analysed as described above (Pharmacokinetic Study).

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Statistical analysis

For ara-CTP measurements from MNCs and ara-C IC_{50} values in diagnostic bone-marrow blasts 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 (2 ITD, 3 TKD), and they all received midostaurin 50 mg b.i.d. day 8-21 after each cycle of chemotherapy. All patients received at

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least two cycles of therapy according to protocol. For patient 1106, the dose of ara-C was reduced to 80% during cycle two due to high age (76 years) and severe sepsis after cycle one.

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 GI bleeding (n=1). 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.

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 haematopoietic stem cell (HSC) and other bone-marrow progenitor cells with the exception of the Granulocyte-Monocyte Progenitor expressed much lower levels of SAMHD1 (Supplementary Fig. S1). This was also reflected by the general negativity for SAMHD1 in CD34+ HSCs in the immunohistochemical evaluation of the patients' bone marrow during remission (see below).

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The time-to-neutrophil recovery with ANC ≥0.5 and ≥1.0 x 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 ≥50 x 10^9/L was in median 22 (17-25) days after the first cycle. Similar haematological recovery was seen during the subsequent cycles (Supplementary 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 LEUKEMIC CLEARANCE

All nine patients achieved complete remission (CR) after cycle two (eight of nine already after cycle one. One patient who received G-CSF had 5.5% blasts after cycle one and hence was in partial remission per definition, despite full peripheral regeneration. Validated MRD measurements after cycle two 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 two in the four patients for which 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 a LAIP, the VAF for RUNX1 was reduced to 0.79% after cycle one. 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 two (Fig. 2).

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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 two (n=2), cycle three (n=2), or after cycle two 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 three being dose-reduced. At the time of relapse, the patient received azacitidine-venetoclax-gilteritinib and is now in CR2 (Fig. 1).

ENHANCED ARA-CTP LEVELS IN LEUKEMIC 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+ HSCs (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) (Supplementary 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, Supplementary Fig. S3). The median percentage of blasts in the PBMC fraction was 40% (range 0 to 92% (Supplementary Table S4). At a dose of 1 g hydroxyurea

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(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%; Supplementary Fig. S4). Bone marrow-derived blasts 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-allosteric inhibitor of RNR, with a factor of 1.6 (*P*=0.01, range 1.3-5.4, Supplementary 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 (Supplementary Fig. S6). The median zero interaction potency (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 (Supplementary 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 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

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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].

It is conceivable that the addition of hydroxyurea potentiates the risk of ara-C-related adverse events. However, the observed toxicities and time to hematopoietic 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-HSCT. This might at least partially be explained by the low expression levels of SAMHD1 in healthy bone-marrow progenitors including HSC. The lack 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 (summarized 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 two. According to data from the Swedish AML Registry, the expected CR/MRD-negativity rates with an identical chemotherapy regimen but without

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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 two 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 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 two 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 VAF frequencies from <0.02% to 10.5%. However, selecting relevant genetic markers for MRD in AML can be challenging as they may occur in pre-malignant clones. The two patients with detectable driver mutations above 1% after cycle two (patient 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, with known incomplete clearance[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 leukemic clones may be more potently eradicated than pre-malignant 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

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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 (AUC), both of which correlate with clinical responses whereas peak ara-CTP levels do not[17]. However, administering 1000 mg of hydroxyurea one hour 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 hours 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 >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 which 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

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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.

Data sharing

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.

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.

Acknowledgement

The authors would like to express their gratitude to Henrik Hasle, Björn Wahlin and Per-Ola Andersson for their critical data and safety assessment. The authors want to thank Katja Wiklund and Yvonne Larsén for monitoring the study. The authors are grateful to Janelle Cederlund for support with trial patient management and Indranil Sinha for assistance with visualization of gene expression data. This work would not have been possible without the support of the following sources: CIMED 2019 (to M.J.), Barncancerfonden (TJ2018-0128, PR2019-0100, TJ2021-0080, OA2011-0001, KP2018-0005, PR2018-0016, PR2020-0077, TJ2019-0072 to M.L., C.A., B.S.L., T.E., P.Ö., J.I.H., and N.H.), Knut and Alice Wallenberg Stiftelse (KAW 2015.0291 to C.A., B.S.L., T.E., and P.Ö.), Stiftelsen för Strategisk Forskning (SSF SB16-0058 to C.A., B.S.L., T.E., and P.Ö.), Swedish Research Council (2017-06095, 2020-01184 to C.A., B.S.L., T.E., P.Ö., and N.H.), NIH (AI162633, AI136581,

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MH116695 to B.K. and R.F.S.), Cancerfonden (21 1494 Pj, CAN 2017/517 to N.H.), Jeanssons Stiftelser (to N.H.), Märta and Gunnar V Philipson Foundation (to N.H.), Region Stockholm (20200246, K2892-2016 to N.H.), Sjöbergstiftelsen (2020-008 to N.H.), Radiumhemmets Forskningsfonder (191112, 211143 to N.H.) and Svenska Läkaresällskapet (SLS-875361, SLS-961737 to N.H.).

Author contributions

M.J., T.S., S.D., M.H., J.I.H. and N.H. designed the clinical trial. I.L., N.T., M.L., S.B., A.B., C.A., T.E., B.S.L., G.G., S.T., P.Ö., R.F.S., B.K., and S.L. collected and processed clinical samples, and performed experiments and data analysis for ara-CTP measurements and *ex vivo* drug testing. S.B., A.B., G.S.B., S.L. and L.F. collected and processed clinical samples, designed and performed patient-based NGS experiments. L.S. and G.R.Z. performed analysis of SAMHD1 immunohistochemistry. G.J. contributed epidemiological analyses. M.J. and N.H. wrote the initial manuscript. All authors provided feedback on the initial manuscript.

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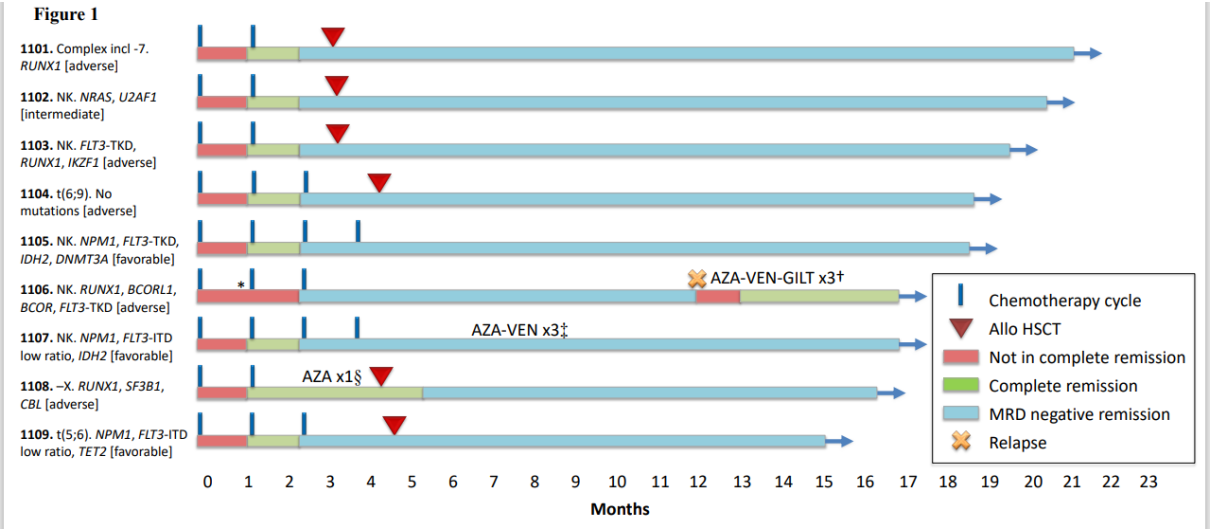

Figure 1: Treatment outcome.

The nine patients have been followed for 14.9-20.5 months. Six patients have undergone allogeneic stem cell transplantation. MRD was performed in accordance with national guidelines after cycle two and cycle four or prior to allo-HSCT. In non-transplanted patients with *NPM1*, MRD by RT-qPCR was performed every three months. After allo-HSCT, patients were routinely monitored every three 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 one and did not fulfil CR criteria until after cycle two. However, blasts may have been elevated by 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,

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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.

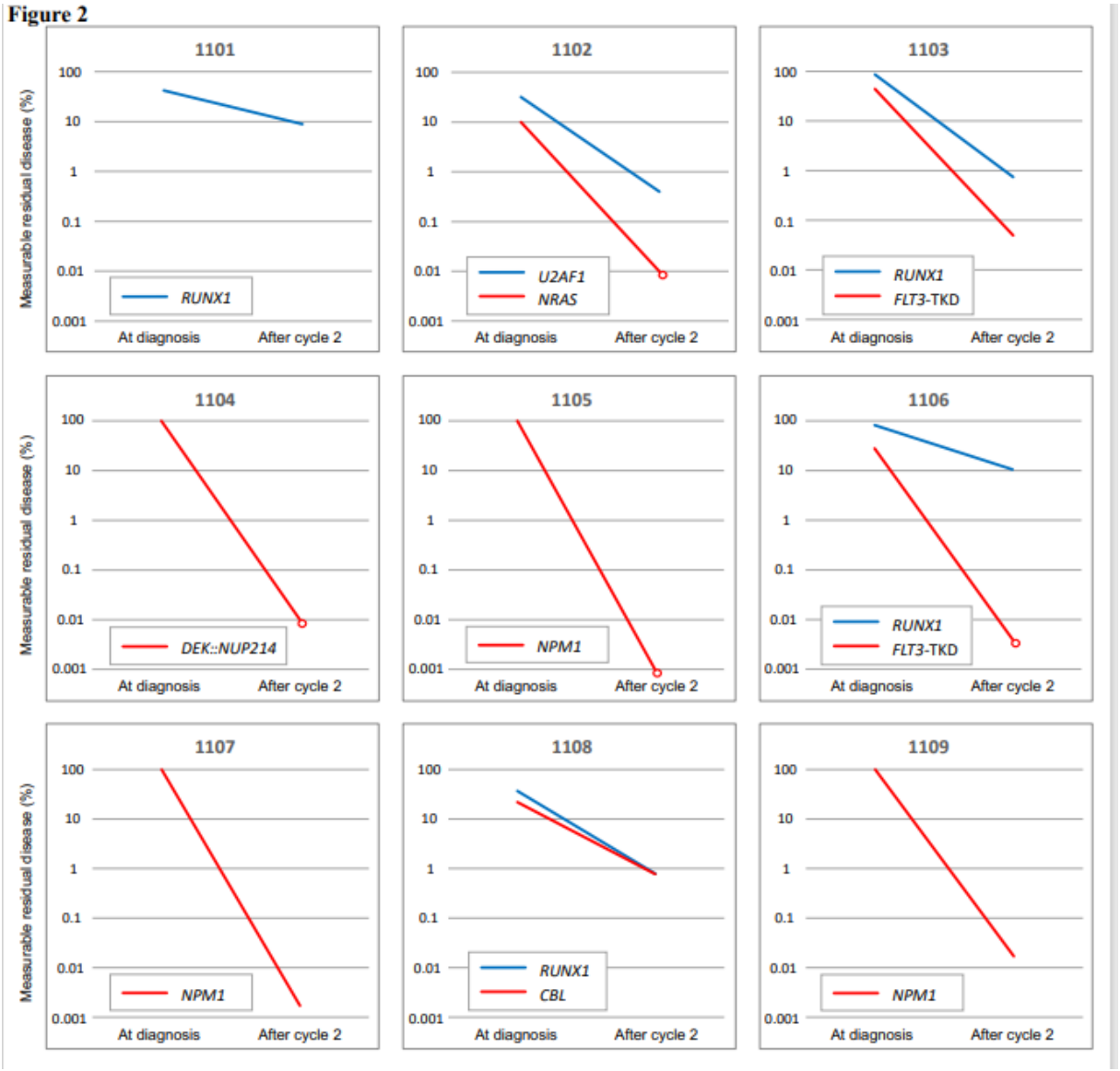

Figure 2: Leukemic clearance after cycle two

Validated 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 two. In the remaining five patients, one or two mutations were monitored for MRD using deep sequencing (reporting the variant allele frequency). Mutation in *RUNX1*

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was seen in four patients and was reduced to 0.74 – 10.5% after cycle two. 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 two, MRD levels below the detection limit for the method are indicated with open circles.

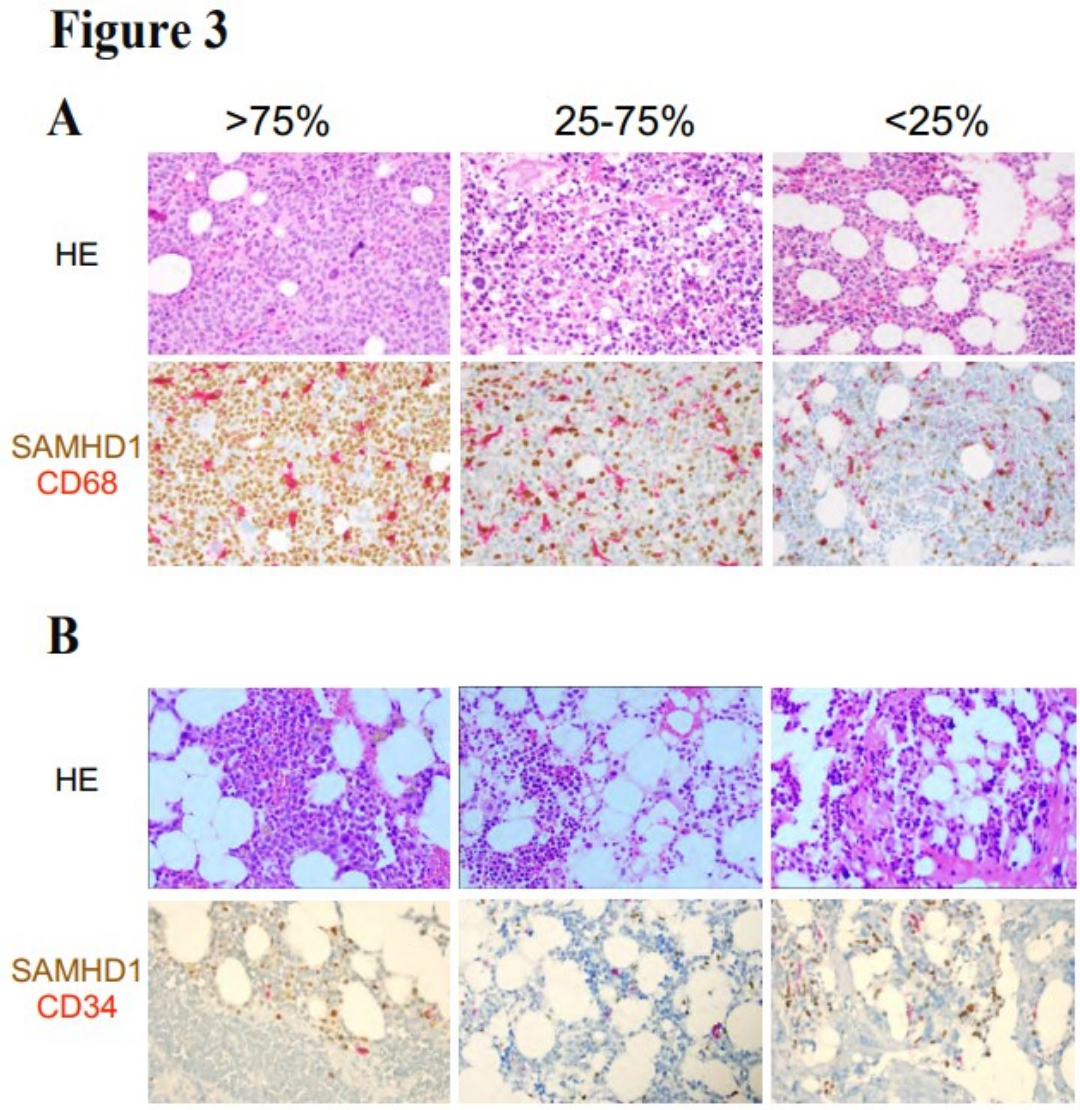

Figure 3: SAMHD1 protein expression in AML blasts and in remission bone marrow

(A) Upper images show haematoxylin and eosin routine staining in diagnostic bone-marrow biopsies. The lower images show the corresponding SAMHD1-CD68 double

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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 400x magnification.

Figure 4

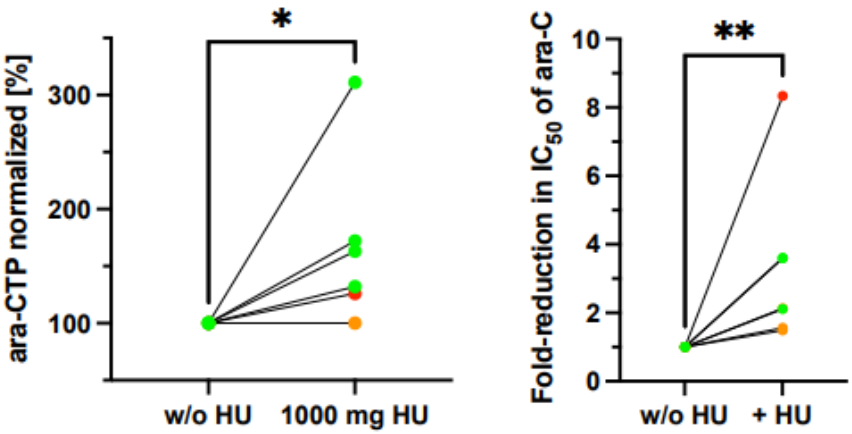

Figure 4: Effect of hydroxyurea on peak ara-CTP levels in circulating mononuclear cells and *ex vivo* sensitivity to ara-C

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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. one hour prior to start of ara-C infusion, normalized to without hydroxyurea (n=6).

The right panel shows the fold-reduction in IC_{50} values of ara-C in diagnostic bone-marrow blasts treated *ex vivo* in the presence or absence of 100 µM hydroxyurea, normalized to absence of hydroxyurea (n=7).

Individual dots correspond to individual patients; colours represent levels of SAMHD1 expression at diagnosis (green, <25%, orange 25-75%, red >75%).

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+
1106 Male 76 years	46,XY[20]	*RUNX1, BCORL1, BCOR, FLT3*-TKD	Adverse	25-75%	No* / 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†	16.7+
1108 Female 61 years	45,X,-X[10]/46,XX[10]	*RUNX1, SF3B1, CBL*	Adverse	<25%	Yes / Yes	NA‡ / 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§	<25%	Yes / Yes	NA / 0.017% / NA	Yes	NA	14.9+

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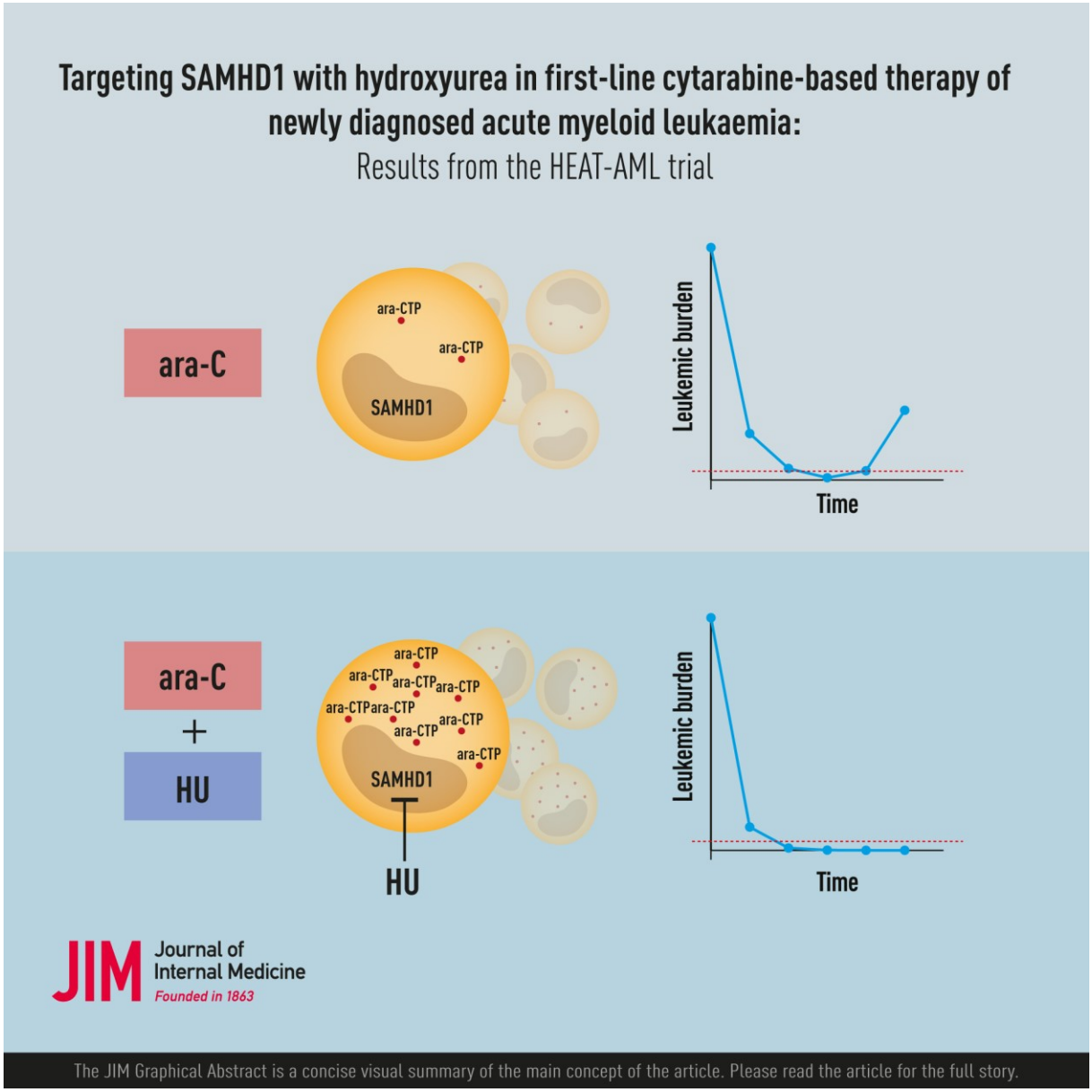
* Blasts 5.5%; G-CSF use prior to sampling may have increased the morphologic blast count
† At 9 months MRD *NPM1* switched from negative to positive at 0.00024%, when double checked then negative. Prior to receiving the confirmatory MRD result, the patient was put on AZA-VEN and received 3 cycles.
‡ Patient 1108 lacked a leukaemia-specific phenotype for flow cytometry
§ This patient had a prior history of myeloproliferative neoplasia with osteosclerosis and extramedullary hematopoiesis, and therefore better resembled a higher risk secondary AML



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