Acute myeloid leukaemia niche regulates response to L-asparaginase

Ilaria M. Michelozzi[1], Valentina Granata[1], Giada De Ponti[1], Gaia Alberti[1], Chiara Tomasoni[1], Laura Antolini[2], Carlo Gambacorti-Passerini[3], Bernhard Gentner[4], Francesco Dazzi[5], Andrea Biondi[1,6], Tiziana Coliva[6], Carmelo Rizzari[6], Alice Pievani[1*], Marta Serafini[1*].

[1]M. Tettamanti Research Center, Department of Paediatrics, University of Milano-Bicocca, Monza, Italy

[2]Centro di Biostatistica per l'epidemiologia clinica, Department of Health Sciences, University of Milano-Bicocca, Monza, Italy

[3]Department of Haematology, San Gerardo Hospital, University of Milano-Bicocca, Monza, Italy

[4]San Raffaele Telethon Institute for Gene Therapy (SR-TIGET), IRCCS San Raffaele Scientific Institute, Milan, Italy

[5]Department of Haemato-Oncology, Rayne Institute, King's College London, London, UK

[6]Department of Paediatrics, Pediatric Hematology-Oncology Unit, Fondazione MBBM/San Gerardo Hospital, Monza, Italy

[*]These authors contributed equally to this work

Corresponding author

Marta Serafini,
M. Tettamanti Research Center,
Department of Paediatrics,
University of Milano-Bicocca,
San Gerardo Hospital/Fondazione MBBM,
Via Pergolesi, 33
Monza, 20900,
Italy
Tel: +39-039-2333661
Fax: +39-039-2332167
Email: serafinim72@gmail.com

Conflict of interest

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Summary

Eradicating the malignant stem cell is the ultimate challenge in the treatment of leukaemia. Leukaemic stem cells (LSC) hijack the normal haemopoietic niche in which they are largely protected from cytotoxic drugs. The anti-leukaemic effect of L-asparaginase (ASNase) has been extensively investigated in acute lymphoblastic leukaemia, but only partially in acute myeloid leukaemia (AML). We explored the susceptibility of AML-LSC to ASNase as well as the role of the two major cell types that constitute the bone marrow (BM) microenvironment, i.e., mesenchymal stromal cells (MSC) and monocytes/macrophages. Whilst ASNase was effective on both $CD34^{+}CD38^{+}$ and $CD34^{+}CD38^{-}$ LSC fractions, MSC and monocytes/macrophages partially counteracted the effect of the drug. Indeed, the production of cathepsin B, a lysosomal cysteine protease, by BM monocytic cells and by AML cells of the FAB M5 subtype is related to the inactivation of ASNase. Our work demonstrates that, while MSC and monocytes/macrophages may provide a protective niche for AML cells, ASNase has a cytotoxic effect on AML blasts and, importantly, LSC subpopulations. Thus, these features should be considered in the design of future clinical studies aimed at testing ASNase efficacy in AML patients.

Keywords: Acute Myeloid Leukaemia, Asparaginase, Leukaemic Stem Cells, Bone Marrow Microenvironment, Cathepsin B

Running title: L-asparaginase effect within AML niche

Introduction

Acute myeloid leukaemia (AML), a heterogeneous blood cancer, represents the most frequently diagnosed leukaemia in adults (25%) and it accounts for 15-20% cases in children.(Siveen et al., 2017) Despite continuous progress in the comprehension of AML pathogenesis and in AML diagnosis and stratification, patients are still subject to a high rate of relapse and to a poor overall survival.(Siveen et al., 2017)

Biologically, AML cells could be represented as a hierarchy at the top of which there are leukaemic stem cells (LSC).(Bonnet and Dick, 1997) LSC are a heterogeneous group of cells, with stemness properties, which are responsible for initiating and maintaining the disease giving rise to more differentiated blasts.(Bonnet and Dick, 1997; Eppert et al., 2011; Hope et al., 2004) Moreover, LSC refractoriness to conventional chemotherapies determines AML relapse.(Ishikawa et al., 2007; Siveen et al., 2017; Pollyea and Jordan, 2017) This is due to their peculiar characteristics (e.g., quiescence and expression of efflux pumps)(Siveen et al., 2017) and to the protection provided by the bone marrow (BM) microenvironment.(Korn and Mendez-Ferrer, 2017; Ninomiya et al., 2007)

Undeniably, stromal cells in the BM niche contribute to establish a sanctuary in which LSC can acquire a drug-resistant phenotype and thereby evade chemotherapy-induced death. Particularly, mesenchymal stromal cells (MSC) can favour AML blast and LSC survival upon chemotherapy through several mechanisms, including release of factors (e.g., CXCL12/CXCR4 and VCAM-1/VLA-4 axis), modification of leukaemic metabolism, and enhancement of the expression of c-myc.(Korn and Mendez-Ferrer, 2017) In addition, BM contains various mature immune cell types, such as T and B cells, dendritic cells and macrophages that participate in protective environment for leukaemic cells. (Isidori et al., 2014; Riether et al., 2015)

According to the importance of LSC in AML pathogenesis, therapeutic approaches aiming at targeting LSC are necessary to eradicate these cells, thus preventing their further evolution and consequent AML relapse.(Pollyea and Jordan, 2017)

L-Asparaginase (ASNase) is a deamidating enzyme that catalyses the hydrolysis of L-asparagine and L-glutamine causing L-asparagine depletion in blood and in BM(Steiner et al., 2012; Tong et al., 2013), L-glutamine reduction(Steiner et al., 2012) and leukaemic cell death under condition of these amino acids deprivation.(Asselin et al., 1989; Willems et al., 2013)

Although ASNase has been widely exploited in the treatment of acute lymphoblastic leukaemia (ALL) since 1960s,(Egler et al., 2016) it has been partially investigated in the context of AML both *in vitro* and in clinical trials.(Emadi et al., 2014) Despite the evidence of a higher efficacy of ASNase on ALL than AML blasts,(Okada et al., 2003; Zwaan et al., 2000) some specific subtypes and a subgroup of AML were reported to be more susceptible to ASNase as compared to others.(Bertuccio et al., 2017; Okada et al., 2003; Zwaan et al., 2000) It has been recently demonstrated that AML cells are addicted in particular to glutamine for their energetic and biosynthetic metabolism.(Jacque et al., 2015; Matre et al., 2016; Willems et al., 2013) Consequently, *Erwinia* ASNase, with 10-fold higher glutaminase activity as compared to *E. coli* ASNase(Avramis, 2012), understandingly exhibits greater cytotoxicity on AML cells.(Willems et al., 2013)

Resistance to ASNase has been suggested to occur in ALL due to the L-asparagine and L-glutamine secreted by MSC and adipocytes surrounding blasts in BM.(Ehsanipour et al., 2013; Iwamoto et al., 2007) A further mechanism proposed as cause of therapy failure is the inactivation of ASNase mediated by cellular lysosomal cysteine proteases.(Patel et al., 2009) Microenvironment cells such as macrophages can produce cathepsin B (CTSB) and contribute to ASNase turnover *in vivo* in mice.(van der Meer et al., 2017)

In this study, we aimed at investigating the effects of ASNase on AML blasts, focusing on the role of different players of the leukaemic microenvironment, e.g., LSC, MSC and monocytes/macrophages, in susceptibility to ASNase. Herein, we demonstrated that, while MSC and monocytes/macrophages contribute to provide a protective microenvironment to AML cells, ASNase exerts an effect on LSC subpopulations, as well as AML leukaemic blasts.

Materials and Methods

Cell lines, patients and healthy donor samples

Peripheral blood or BM samples of 37 AML patients at diagnosis were collected after having obtained an informed consent. Mononuclear cells were isolated using a Ficoll-Paque^{TM} Plus (GE Healthcare, Little Chalfont, Buckinghamshire, UK) density gradient separation and used either fresh or after cryopreservation for experiments. The study was approved by the Ethics Committee of San Gerardo Hospital-Monza (LMA ASNASE 2900). Clinical and biological patients' features are reported in Table SI. Details of cell lines and healthy donor samples are described in Supplementary Methods.

Reagents and compounds

We tested two formulations of L-asparaginase: *E. coli* ASNase (Kidrolase®) and *E. chrysanthemi* ASNase (Erwinase®) (Jazz Pharmaceuticals, Dublin, Ireland).

StemRegenin1 (SR1) and UM729 (StemCell^{TM} Technologies, Vancouver, BC, CA) were used at a final concentration of 250 nM and 1 μM, respectively.

ASNase cytotoxicity

To determine the half maximal inhibitory concentration (IC50) of each ASNase formulation on AML cell lines, $4x10^4$ cells/well were seeded in 96-well plates in complete culture medium with different concentrations of *E. coli* (0.1-300 iu/ml) and *Erwinia* (0.0001-100 iu/ml) ASNase. After 48 hours of treatment, live cells were counted by flow cytometry. IC50 was calculated using CompuSyn Software (www.combosyn.com).

For primary AML samples, $2x10^5$ cells/well were plated in 96-well plates in complete Advanced RPMI 1640 medium with or without 1 iu/ml of *Erwinia* ASNase and cell viability was evaluated by flow cytometry after 48 hours (Supplementary Methods). These experiments were also performed in LSC supportive culture conditions using complete medium supplemented with SR1 and UM729.

RQ-PCR

RQ-PCR assays were used to determine asparagine synthetase (*ASNS)* expression in healthy donor (HD)- and AML-MSC, and cathepsin B (*CTSB*) expression in full healthy BM and AML BM (samples with >70% blast cell content), and in $CD14^+$ and respective $CD14^-$ BM fractions purified from healthy donors using MIDIMACS immunoaffinity columns (Miltenyi Biotec, Bergisch Gladbach, Germany). Full details are provided in Supplementary Methods.

Degradation of ASNase

Washed cell pellets from primary healthy or AML BM samples were lysed by freeze-thawing in digestion buffer (50 mM trisodium citrate buffer, pH 4.5, 5 mM Dithiotreitol), clarified by centrifugation and stored at -80°C. 7.5 iu/ml ASNase was incubated overnight at 37°C with 20 µg of whole-cell lysate. For inhibition of ASNase cleavage, lysates were incubated with protease inhibitor cocktail P8340 (PIC; Sigma-Aldrich, St. Louis, Missouri, USA) or CTSB-specific inhibitor Ca-074 (10 µM; Sigma-Aldrich) before addition of ASNase.

After incubation, the residual ASNase activity was measured by spectrophotometric determination of the released ammonia after reaction with Nessler's reagent (Supplementary Methods).

Statistical analyses

Data were analysed using GraphPad Prism 7 (GraphPad Software, LA Jolla, CA, USA). Differences between groups were compared with the Mann-Whitney test or Wilcoxon matched-pairs signed rank test in the case of matched values. All tests were two-sided with a 5% significance level.

Results

Patient-derived AML cells are susceptible to ASNase

We tested the inhibitory effect on cell proliferation of two different formulations of ASNase (*E. coli* and *Erwinia* ASNase) on different AML cell lines (THP-1, KG-1 and HL-60) and on the 697 ALL cell line, used as a control. As shown in Fig 1A, the half maximal inhibitory concentration (IC50) of *Erwinia* ASNase was lower as compared to *E. coli* ASNase for each cell line tested, particularly for AML lines (*Erwinia vs E. coli* ASNase IC50 values: 697, 0.12 *vs* 0.26 iu/ml; THP-1, 2.89 *vs* 12.75 iu/ml; KG-1, 0.13 *vs* 0.65 iu/ml; HL-60, 0.11 *vs* 0.91 iu/ml).

The superior efficacy of *Erwinia* ASNase was observed in all AML cell lines tested also in terms of induction of apoptosis. Indeed, *Erwinia* ASNase was able to induce apoptosis in THP-1, KG-1 and HL-60 at comparable levels to that of *E. coli* ASNase, used at doses respectively 4-, 5- and 8-fold lower than the latter (data not shown).

Then, the effects of *Erwinia* ASNase on primary AML samples representative of various leukaemia subtypes according to French-American-British (FAB) classification (Table SI) were evaluated. Within specimens, we distinguished between blast and non-blast populations according to side scatter profile combined with CD45 intensity. Interestingly, treatment with 1 iu/ml ASNase for 48 h

caused within AML blast population a significant decrease in the number of live cells (median reduction treated *vs* untreated: 47.35%, P<0.0001) along with an increase in the percentage of apoptotic cells (median apoptosis treated *vs* untreated: 47.53% *vs* 22.33%, P<0.0001) (Fig 1B). Instead, the number of non-blast cells was only minimally reduced after exposure to ASNase (median reduction treated *vs* untreated: 5.62%, P=0.0286) and the percentage of apoptosis after treatment was almost unaffected (Fig 1B).

Comparing the 3 AML FAB-type subgroups including more than 3 samples (FAB M1/M2, FAB M4, and FAB M5), there were no statistically significant differences with respect to the median reduction of live cell number and the median percentage of apoptosis of blasts after treatment (Fig. S1).

ASNase affects leukaemic precursors within patient-derived AML cells

Next, with the aim of understanding the ability of ASNase to target the AML tumour-initiating cells, the cytotoxic effect of ASNase on leukaemic clonogenic cells within primary AML specimens was evaluated. Colony growth was determined by colony forming unit (CFU) assay in the presence or in the absence of ASNase. The exposure of the cells to low dose of ASNase (0.01 iu/ml) was able to significantly reduce the clonogenic potential of AML cells as compared to untreated controls (median colony number treated *vs* untreated: 2.75 *vs* 28.75, P=0.0001) (Fig 2A). Notably, colony formation was completely blocked by higher drug concentrations (0.1-1 iu/ml) (data not shown).

Moreover, the effects of the drug on LSC subpopulations within AML samples, identified according to the expression of CD34 and CD38 markers,(Eppert et al., 2011) were investigated.

Notably, the $CD34^+$ population was significantly susceptible to 48h treatment with 1 iu/ml ASNase (P<0.0001 *vs*. untreated control). Particularly, a significant decrease in the number of live cells after treatment was observed on both $CD34^+CD38^+$ (median reduction treated *vs* untreated: 57.97%, P<0.0001) and $CD34^+CD38^-$ subpopulation (median reduction treated *vs* untreated: 53.13%,

P<0.0001) (Fig 2B, top). Percentages of live cells after treatment in these subpopulations were comparable to those obtained on the bulk population of the same sample (P=0.4282 calculated by Friedman test, Fig 2B, bottom).

To deeply investigate the specific effect of ASNase on LSC, the culture conditions of primary AML specimens were modified by adding the SR1 and UM729 small molecules, which have been previously described for their capability to better maintain the survival and stemness of AML-LSC *in vitro*.(Pabst et al., 2014) As shown in Figure S2, the two compounds acted on the $CD34^+CD38^-$ fraction significantly enhancing their viability as compared to a control population incubated without small molecules (median fold change of $CD34^+CD38^-$ in LSC supporting culture conditions *vs* control: 1.38, P=0.0244).

Of note, also in these LSC supportive culture conditions, $CD34^+CD38^+$ and $CD34^+CD38^-$ subpopulations displayed high sensitivity to ASNase (median $CD34^+CD38^+$ reduction treated *vs* untreated: 41.45%, P=0.0015; median $CD34^+CD38^-$ reduction treated *vs* untreated: 46.71%, P=0.0005). Similarly, the drug effect was maintained also on the bulk population (P=0.0005 *vs* untreated) (Fig 2C).

Furthermore, we observed a concomitant reduction of mIR-126, a regulator involved in governing LSC self-renewal and quiescence,(Lechman et al., 2016) for four out of five AML samples treated with ASNase in LSC supporting culture conditions (Fig 2D).

MSC show a protective role on AML cells against ASNase cytotoxicity by asparagine synthetase expression

In order to elucidate whether the BM microenvironment could exert an effect against the action of ASNase on AML blasts, primary AML samples were maintained in culture in the presence either of a normal or of a patient-derived MSC layer and treated with ASNase. The effect due to the coculture with MSC has been determined comparing both the number of live cells and the

proportion of apoptotic cells in AML blast cultures treated with the drug in the presence or not of MSC and normalised to the respective untreated control.

MSC derived from both healthy and AML BM were poorly sensitive to ASNase. Indeed, neither cell growth nor apoptosis were affected after exposure for 48 h at the higher ASNase concentration tested (3 iu/ml) (Figure S3).

We showed that MSC derived from healthy donors (HD-MSC) were able to counteract ASNase cytotoxicity on AML blasts, significantly increasing the number of live cells (median increase of live cells number w HD-MSC *vs* w/o HD-MSC: 35.43%, P=0.0010) and decreasing the percentage of apoptotic cells (median decrease of apoptosis w HD-MSC *vs* w/o HD-MSC: 33.73%, P=0.0005) induced by treatment (Fig 3A).

Similarly, the presence of HD-MSC significantly enhanced the viability of both $CD34^+CD38^+$ (median percentage of remaining live cells w HD-MSC *vs* w/o HD-MSC: 62.67% *vs* 37.3%, P=0.0078) and $CD34^+CD38^-$ fractions (median percentage of remaining live cells w HD-MSC *vs* w/o HD-MSC: 72.29% *vs* 23.68%, P=0.0039) upon ASNase treatment (Fig 3B), demonstrating that MSC protect also these primitive populations from the drug cytotoxicity.

Considering that several microenvironment features could be modified by the disease, additional experiments were performed using cocultures of primary AML samples and AML-MSC derived from the same patient. Also in this autologous setting, AML-MSC significantly enhanced the number of leukaemic live cells (median increase of live cells number w AML-MSC *vs* w/o AML-MSC: 47.37%, P=0.0078) and reduced the percentage of leukaemic apoptotic cells (median decrease of apoptosis w AML-MSC *vs* w/o AML-MSC: 39.23%, P=0.0391) in treated AML samples (Fig 3C).

In a similar fashion, the presence of AML-MSC significantly decreased ASNase cytotoxicity against the $CD34^+CD38^+$ subpopulation (median percentage of remaining live cells w HD-MSC *vs* w/o HD-MSC: 59.96% *vs* 38.02%, P=0.0078) and showed an effect on the $CD34^+CD38^-$ cells

where a positive trend was found in the majority of the performed experiments, almost approaching significance (P=0.0547, Fig 3D). Furthermore, considering that the protective capacity of MSC may be dependent on the release of asparagine within the microenvironment, we evaluated the expression of *ASNS* in patient-derived MSC. The mean $2^{-\Delta\Delta Ct}$ value of *ASNS* mRNA in AML-MSC was comparable to HD-MSC (respectively: 4.86 *vs* 3, P=0.0952) (Fig 3E).

CTSB over-expressed in BM monocytes/macrophages and in FAB M5 subtype of AML blasts degrades ASNase

Another mechanism of ASNase resistance could be the drug clearance mediated by lysosomal cysteine proteases such as CTSB expressed by cells of the BM microenvironment or by blasts themselves.

In this respect, we found that in normal BM, *CTSB* was expressed primarily by $CD14^+$ monocytes/macrophages. Indeed, the median $2^{-\Delta\Delta Ct}$ value of *CTSB* mRNA of $CD14^+$ samples (26.08) was observed to be higher as compared to the median $2^{-\Delta\Delta Ct}$ value of *CTSB* mRNA in $CD14^-$ samples (5.96; P=0.0005; Fig 4A). To determine whether proteases present in BM $CD14^+$ monocytes/macrophages, in particular cathepsin B, degrade ASNase, cell lysates were incubated with the drug. The mean residual ASNase activity after incubation with $CD14^+$ samples was reduced to 43% and was only slightly affected (83% of activity) after incubation with CD14-depleted BM cell lysates (P=0.03; Fig 3B). Moreover, the degradation mediated by $CD14^+$ samples was prevented by addition of a protease inhibitor cocktail (residual ASNase activity: 81%, P=0.03 *vs.* control) and also by the CTSB-specific inhibitor Ca-074 (residual ASNase activity: 73%, P=0.03 *vs.* control), confirming the potential role exerted on ASNase cleavage by CTSB present in $CD14^+$ BM monocytes/macrophages (Fig 4B).

AML BM exhibited an average of 3.4-fold higher *CTSB* mRNA as compared to normal BM. When examining relative expression levels among the 28 AML patients, *CTSB* was upregulated by 4 to12-

fold in 10 out of 28 (35.7%) patients in comparison to controls. Among these *CTSB* overexpressing patients, 9 (90%) belong to the FAB M5 subtype.

The relative expression of *CTSB* mRNA was elevated in 9 out of 12 (75%) FAB M5 AML samples, with a median $2^{-\Delta\Delta Ct}$ value of 30.55. Instead, *CTSB* was constitutively expressed at lower levels by FAB M0/M1 (median $2^{-\Delta\Delta Ct}$ value of 4.82; P *vs* AML-M5=0.0037), FAB M2 (median $2^{-\Delta\Delta Ct}$ value of 4.86; P *vs* AML-M5=0.0044) and FAB M4 (median $2^{-\Delta\Delta Ct}$ value of 5.8; P *vs* AML-M5=0.0039) AML samples (Fig 4C).

As for healthy BM monocytic cells, also in the case of leukaemic blast cells we found that cell lysates degraded ASNase through proteases such as CTSB. As shown in Fig 3D, ASNase activity was reduced when incubated with whole cell lysates from blasts characterised by high *CTSB* expression but was only slightly decreased in the case of low *CTSB* expressing blasts. Also in this case, PIC and especially the CTSB-specific inhibitor reduced degradation (Fig 4D).

Discussion

In the last few years it has been clarified that, in order for any AML therapy to be curative, it needs to be effective against the cells that propagate and sustain the disease, the so called LSC which reside in BM microenvironment.

As a consequence, to assess the potential of new compounds, it is pivotal to investigate their toxicity on leukaemia and progenitor cell populations in relation with other cell types contained within the leukaemic niche.

Here, we report the susceptibility of AML-LSC and progenitors to ASNase as well as the role of two cell types that constitute the BM microenvironment, e.g., MSC and monocyte/macrophages in counteracting the effect of the drug.

Even though the anti-leukaemic effect of ASNase has been extensively demonstrated in ALL therapy (Faderl et al., 2011; Pession et al., 2005), it has been used only occasionally, with different modalities and with alternate effects, to treat other haematological malignancies like AML (Emadi et al., 2014; Ahmed et al., 2015; Buaboonnam et al., 2013; Emadi et al., 2018; Gibson et al, 2011) and solid tumours, these latter with scarce results.(Taylor et al., 2001)

ASNase acts firstly by inhibiting the proliferation of leukaemic cells and, subsequently, by inducing their apoptosis.(Ueno et al., 1997) For this reason, we analysed the drug effect in terms of both absolute number of live cells and percentage of apoptotic cells.

ASNase, used at clinically attainable dose, was able to reduce the number and to induce apoptosis of AML blasts, while minimally affecting non-blast cells. The susceptibility of FAB M5 subgroup to ASNase appeared to be higher compared to other FAB subtypes but this was not statistically significant. A further analysis on a larger number of samples is warranted to address the question of selectivity amongst different FAB/molecular subtypes. Effects of ASNase on AML primitive cell fractions have not been examined so far. In this work, we analysed the effect of the drug on clonogenic capacity, as an *in vitro* measure of the self-renewal in AML cells. Treatment with ASNase reduced the clonogenicity of primary AML specimens.

Next, we investigated the susceptibility of AML-LSC to ASNase. We considered the $CD34^+CD38^-$ (LSC-enriched) and $CD34^+CD38^+$ fractions since LSC are not characterised by an unique phenotype and both compartments were reported to contain most of the LSC.(Eppert et al., 2011) Notably, we showed that ASNase could reduce the number of live cells within $CD34^+CD38^+$ and $CD34^+CD38^-$ fractions in a proportion similar to the bulk blast population.

Recently, two small molecules, StemRegenin1 (SR1) and UM729 that support human LSC activity *ex vivo* have been identified. Their use in culture systems preserves AML-LSC by inhibiting their spontaneous differentiation *in vitro* and retaining their engraftment capacity.(Pabst et al., 2014) Also in these culture conditions favourable for LSC maintenance, we were able to further support

329 the cytotoxic effect of ASNase on AML compartments containing LSC and progenitor cells. In
330 addition, the concomitant reduction of mIR-126, a regulator implicated in governing the stemness
331 state of human LSC,(Lechman et al., 2016) offered an additional evidence of the effect of ASNase
332 on LSC frequency.
333 The capability of ASNase to act on cancer stem cells and to reduce their clonogenic potential has
334 been observed in solid tumours and was related to its glutaminase activity.(Liao et al., 2017)
335 Indeed, in the absence of glutamine, the levels of reactive oxygen species augmented through
336 attenuation of glutathione synthesis, leading to the downregulation of the β-catenin pathway and,
337 subsequently, to the reduction of cancer stem cells.(Liao et al., 2017) As LSC show susceptibility to
338 oxidative stress and to alterations in the β-catenin pathway,(Wang et al., 2010) we can speculate
339 that the cytotoxic effect of ASNase on $CD34^{+}CD38^{+}$ and $CD34^{+}CD38^{-}$ compartments observed in
340 our work could be linked to the glutaminase activity of the drug.
341 Concerning ASNase activity within microenvironment, Iwamoto et al.,(Iwamoto et al., 2007)
342 proposed that MSC might support ALL blasts during ASNase treatment through local amino acid
343 secretion. They demonstrated that coculture with MSC protected ALL cells from the cytotoxicity
344 caused by ASNase, and this protective effect correlated with *ASNS* levels. Therefore, *ASNS*
345 silencing decreases the protection, whereas enforced expression gives enhanced
346 protection.(Iwamoto et al., 2007) Laranjeira et al. showed that insulin-like growth factor-binding
347 protein 7 (IGFBP7) released by leukaemic cells boosts asparagine synthesis by stromal
348 cells.(Laranjeira et al., 2012)
349 In accordance with these works, we found that MSC exert a protective role also in AML blasts
350 against the cytotoxic effects of ASNase. Primary AML cells varied in their susceptibility to the
351 protective effects of MSC, probably because of differences in the capacity of leukaemic cells to
352 interact with the microenvironment. Not only bulk AML cells, but especially the $CD34^{+}CD38^{+}$ and
353 $CD34^{+}CD38^{-}$ fractions, containing *bona fide* LSC, showed an increased viability upon ASNase

354 treatment in the presence of MSC. This suggests that protective signals within the stromal
355 microenvironment could maintain residual leukaemic cells, in particular LSC, relatively insensitive
356 to ASNase therapy, potentially responsible for the recurrence of the disease.
357 We tested the protective activity of MSC using different BM specimens derived both from healthy
358 donors and from AML patients. This approach eliminated the potential heterogeneity inherent in
359 allogeneic human MSC. Furthermore, it is known that AML-MSC present in their transcriptome
360 alterations supporting leukaemogenesis and chemoresistance, due to the leukaemia-induced
361 remodelling of the BM microenvironment.(Kim et al., 2015) Similarly to HD-MSC, also AML-
362 MSC significantly increased the resistance to ASNase of $CD34^{+}CD38^{+}$ cells, whereas in the
363 $CD34^{+}CD38^{-}$ compartment a similar trend was found in the majority of experiments performed
364 (7/9), approaching, but not reaching, statistical significance. This result could be explained by the
365 limited number of samples analysed rather than by defects in blasts supportive capabilities of AML-
366 MSC.
367 The role of the microenvironment in the regulation of the response to chemotherapy in AML is
368 already known. Indeed, Matsunaga et al. found that the interaction between VLA-4 on AML cells
369 and fibronectin on MSC was essential for the persistence of cytarabine-resistant disease and that the
370 VLA-4 expression is an adverse prognostic factor in patients with AML.(Matsunaga et al., 2003)
371 Moreover, Konopleva et al., observed that MSC increased the expression of anti-apoptotic proteins
372 and augmented the resistance to cytarabine in AML cells.(Konopleva et al., 2002) In the case of
373 ASNase, protection seems to be attributable to asparagine released by MSC in the
374 microenvironment. We observed that *ASNS* gene expression levels in MSC were variable but
375 similar between HD- and AML-derived cells. The development of appropriate techniques to reduce
376 the expression of *ASNS* by the AML-MSC could then improve the effect of ASNase therapy.
377 Cytarabine, a first-line AML chemotherapeutic, has been reported to induce downregulation of
378 *ASNS* transcription.(Takagaki et al., 2003) Thus, the combination of ASNase with conventional

chemotherapy may provide a potentially synergistic effect. Even if the MRC AML12 trial did not show any outcome benefit in patients randomized to receive an additional single consolidation course including high-dose cytarabine and a very limited amount of ASNase,(Gibson et al, 2011) a more appropriate use of ASNase (in terms of dosing and timing) could potentially provide a clinical benefit. Indeed, in ALL a more intensive and prolonged exposition to ASNase has been very often associated with a better survival, being the extended asparagine depletion the mainstay of its therapeutic efficacy.(Silverman LB et al, 2001)

Furthermore, proteolytic inactivation of ASNase could have a potential role in the modulation of its effect within the malignant niche. Indeed, it has been previously reported that lysosomal CTSB and asparaginyl endopeptidase (AEP) hydrolyse ASNase, resulting in inactivation and exposure of immune epitopes.(Patel et al., 2009) AEP and CTSB are expressed by lymphoblasts, in particular by Philadelphia positive (Ph+) and iAMP21 leukaemia cells, two high-risk cytogenetic subtypes.(Patel et al., 2009; Strefford et al., 2006) Moreover, a germ line mutation in the gene encoding CTSB has been linked with a strongly prolonged ASNase turnover in a patient.(van der Meer et al., 2014)

Increased CTSB activity has been described in solid tumours, and it derives not only from the tumour mass but also from the cells surrounding the tumour, with a role in cancer progression and metastasis.(Rakashanda et al., 2012) In particular, tumour-associated macrophages have been identified as the primary source of high levels of cathepsin activity in pancreatic islet cancers, mammary tumours, and lung metastases.(Gocheva et al., 2010) Phase I and phase I-II clinical trials using ASNase were conducted in patients with solid tumours showing that a large proportion of patients was not responsive to the treatment, mainly because the active dose of the drug quickly decreased after administrations, probably due to the proteolytic inactivation.(Taylor et al., 2001)

In healthy human BM samples, we found that the expression of CTSB is attributable to monocytic $CD14^+$ cells. Moreover, lysates from BM $CD14^+$ specifically degraded ASNase. This is consistent with findings of *in vivo* ASNase distribution showing that the drug is rapidly cleared from the serum by murine BM-resident phagocytic cells.(van der Meer et al., 2017) Therefore, BM-resident monocytes/macrophages may collaborate on the establishment of a protective niche for leukaemic cells by effectively removing ASNase from the BM through the release of CTSB. It is known that macrophages in tumour microenvironment can protect tumour cells from cell death induced by a range of additional chemotherapeutic drugs (e.g. taxol, etoposide and doxorubicin), via a cathepsin-dependent mechanism.(Shree et al., 2011)

Moreover, myeloid blasts themselves can produce CTSB. Increased expression of CTSL and CTSB in AML patients seems to be associated with reduced overall survival.(Jain et al., 2010) Notably, we found that in primary AML, the majority of FAB M5 samples specifically overexpressed *CTSB*, showing a 5.1-fold increase in mRNA levels compared to the other subtypes. Although the above mentioned data had been assayed in unsorted AML BM samples, we included in the analysis only specimens with >70% of blast cell content, assuming that the results will remain roughly the same even in purified blast cells.

We showed that lysates from overexpressing CTSB FAB M5 blasts specifically degrade ASNase. Given these data, some previous results need to be reconsidered. Indeed, there is a general agreement that FAB M5 blast cells are responsive to ASNase *in vitro*.(Okada et al., 2003) Zwaan et al., reported that FAB M5 is equally sensitive *in vitro* to ASNase as ALL and this can be explained by the low level of *ASNS* in FAB M5.(Dubbers et al., 2000; Zwaan et al., 2000) Nevertheless, it should be considered that the *in vitro* response to ASNase could not reliably match with the *in vivo* clinical response because other factors, such as the levels of expression of proteases, e.g., CTSB, are likely to significantly modulate the therapeutic response to ASNase. Thus, the elimination of

ASNase from the systemic circulation can be subject to the presence of enzymes produced by leukaemic cells. This suggests the possibility that patients with a high leukaemic burden, who positively respond to anti-leukaemic drugs before ASNase administration, may release more of these proteases in serum, which would then reduce the stability of ASNase.

To improve the outcome and decrease morbidity in patients undergoing chemotherapy an option would be to use specific protease inhibitors in association with ASNase therapy.(Olson and Joyce, 2015) Another option can be the generation of novel modified versions of ASNase. Indeed, several studies have shown that the structure of ASNase permits the introduction of modifications to resist proteolytic cleavage without impairment of enzymatic function.(Maggi et al., 2017; Offman et al., 2011)

In conclusion, we demonstrated that, whilst ASNase was effective on AML bulk blasts and LSC fractions, MSC have a protective effect, nurturing blast survival. Moreover, monocytes/macrophages and FAB M5 blasts themselves can partially counteract the effect of the drug via CTSB-dependent mechanism.

Thus, our work highlights crucial aspects, which should be considered in the design of future clinical studies aimed at testing ASNase efficacy in AML patients. Based on the well known ASNase mechanism of action, pharmacokinetic and pharmacodynamic characteristics, clinical advantages routinely reported in front-line and relapsed ALL and sparsely in relapsed AML, and the new insights of its mechanism of action in BM niche here described, it is possible that a more extensive use of ASNase might be beneficial in the treatment of patients with AML when combined with conventional AML-directed chemotherapy and could hopefully ensure the desired favourable effects without increasing haematological toxicity.

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Author contributions

I.M.M. and V.G. performed research and analysed the data; G.D., G.A., and C.T. performed research; L.A. supervised the statistical analyses; C.G.P. provided patient samples and edited the manuscript; T.C. and C.R. designed research, provided patient samples and contributed to the writing of the paper; B.G., F.D., and A.B. interpreted the data and edited the manuscript; A.P. and M.S. designed research, interpreted the data, and wrote the manuscript.

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Figures

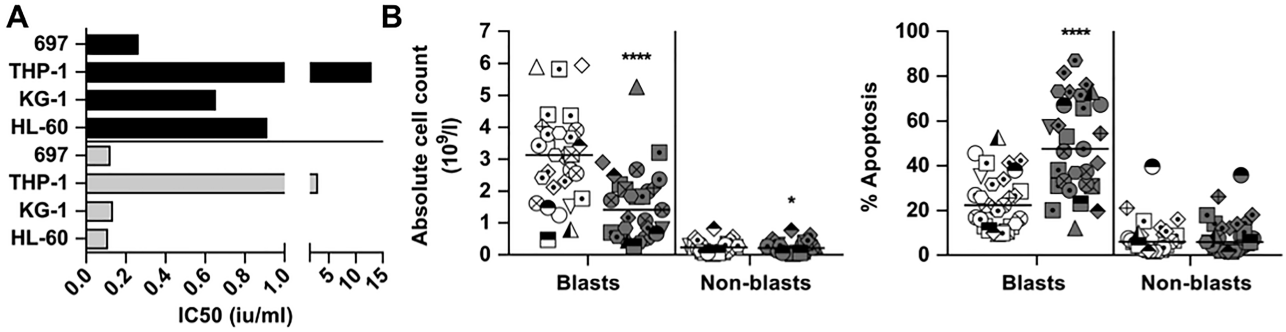


Fig 1. ASNase cytotoxicity on AML cell lines and on primary AML patient-derived cells

(A) IC50 values of *E. coli* (black) and *Erwinia* (grey) ASNase obtained for 697, THP-1, KG-1 and HL-60 cell lines, evaluating the reduction of cell number after 48 h of treatment.

(B) Cell viability of primary AML patient-derived cells after incubation for 48h without (white) or with ASNase (1 iu/ml) (grey). We evaluated within blast and non-blast populations the absolute live cell count (10^9/l) (on the left) and the percentage of apoptotic cells (on the right). Each symbol represents an individual AML patient (mean of technical triplicates). Bar indicates the median for each group. 29 independent experiments performed on 17 different patients are shown. *P<0.05; ****P<0.0001: Wilcoxon matched-pairs signed rank test.

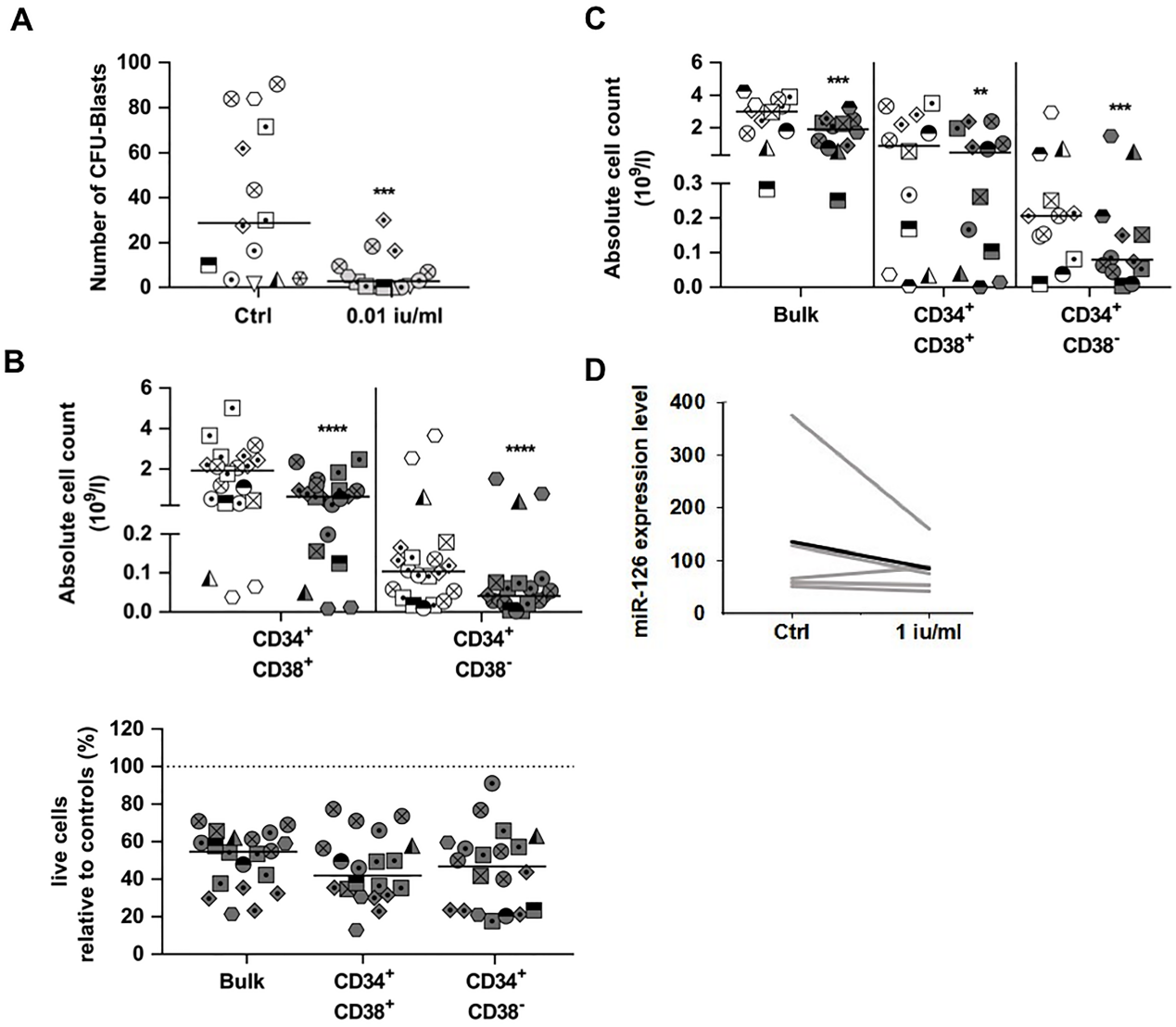


Fig 2. ASNase treatment affects primitive populations within primary AML patient-derived samples

(A) The effect of the drug on the clonogenic potential of 9 primary AML patient-derived samples was assayed after 14 days of culture on methylcellulose without (white) or with 0.01 iu/ml of ASNase (grey). Numbers of CFU-Blasts (mean of technical duplicates) counted in 14 independent experiments are shown.

(B) The effect of the drug on cell viability was analysed also in primitive $CD34^+CD38^+$ and $CD34^+CD38^-$ fractions of 9 primary AML patient-derived samples. We evaluated the absolute live

cell count (10^9/l) (on the top) and the proportion of live cells in treated samples relative to the vehicle control (on the bottom). 20 independent experiments are shown. The dotted horizontal line represents the control. P=0.4282: Friedman test.

(C) Primary AML patient-derived cells were incubated without (white) or with (1 iu/ml) ASNase (grey) in the presence of SR1 and UM729. The absolute live cell count (10^9/l) was evaluated in the bulk population, and in the $CD34^+CD38^+$ and $CD34^+CD38^-$ fractions. 12 independent experiments performed on 10 different patients are shown.

(D) mIR-126 expression levels evaluated by quantitative ddPCR in primary AML patient-derived cells after being incubated for 48 h without (Ctrl) or with (1 iu/ml) ASNase in the presence of SR1 and UM729. 5 independent experiments performed on 5 different patients (grey lines) and the mean (black line) are shown.

**P<0.01; ***P<0.001; ****P<0.0001: Wilcoxon matched-pairs signed rank test (Panels A, B top, and C). Each symbol represents an individual AML patient.

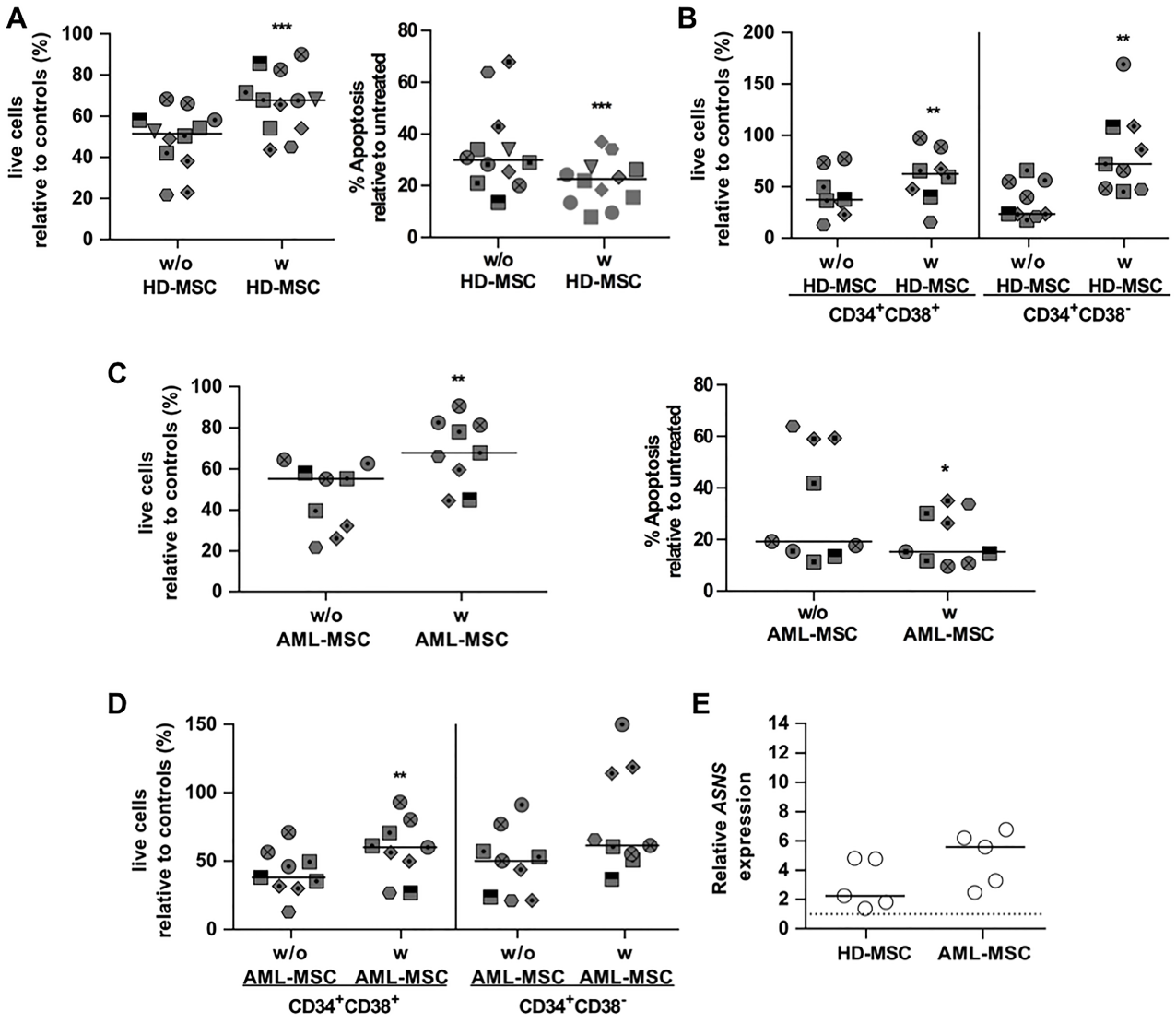
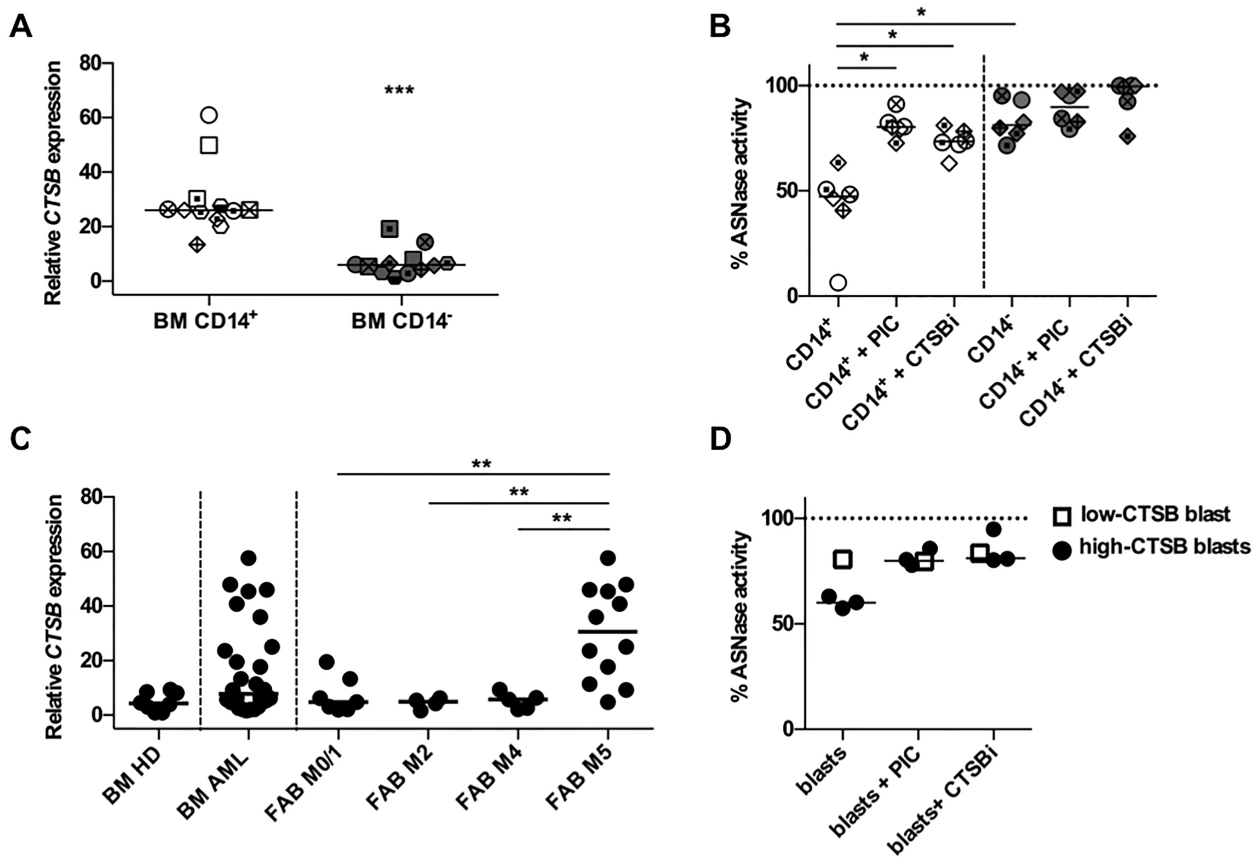


Fig 3. Protective role of MSC against ASNase cytotoxicity

(A) Primary AML patient-derived cells were cultured without or with HD-MSC in the presence of ASNase (1 iu/ml) for 48 h. The number of live cells (left) and the percentage of apoptosis (right) normalised to untreated control in the presence or in the absence of HD-MSC are represented. Each symbol represents an individual AML patient (mean of technical triplicates). Bar indicates the median for each group. 12 independent experiments performed on 9 different AML patient-derived cells and 2 HD-MSC lines are shown. ***P<0.001: Wilcoxon matched-pairs signed rank test.

(B) Analysis of ASNase effect in the presence or in the absence of HD-MSC on number of live cells (relative to controls) in primitive $CD34^+CD38^+$ and $CD34^+CD38^-$ AML fractions. 9 independent

699 experiments performed on samples derived from 6 different AML patients are shown. **P<0.01:
700 Wilcoxon matched-pairs signed rank test.
701 (C) Primary AML patient-derived cells were cultured without or with AML-MSC in the presence of
702 ASNase (1 iu/ml) for 48 h. The number of live cells (left) and the percentage of apoptosis (right)
703 normalised to untreated control in the presence or in the absence of AML-MSC are represented. 9
704 independent experiments performed on autologous cocultures of blasts and MSC, both derived from
705 6 different AML patients are shown. *P<0.05; **P<0.01: Wilcoxon matched-pairs signed rank test.
706 (D) Analysis of ASNase effect in the presence or in the absence of AML-MSC on number of live
707 cells (relative to controls) in primitive $CD34^{+}CD38^{+}$ and $CD34^{+}CD38^{-}$ AML fractions. 9
708 independent experiments performed on samples derived from 6 different AML patients are shown.
709 **P<0.01: Wilcoxon matched-pairs signed rank test.
710 Each symbol represents an individual AML patient.
711 (E) Expression of *ASNS* in HD- *vs* AML-MSC. The expression is showed as fold change, calculated
712 as $2^{-\Delta\Delta Ct}$ using the 697 cell line as the reference ($2^{-\Delta\Delta Ct}$ =1, dotted horizontal line). 10 different MSC
713 donors (5 HD-MSC and 5 AML-MSC) were analysed. P=0.0952: Mann Whitney test.



714

715 **Fig 4. CTSB expressed by BM CD14$^+$ monocytes/macrophages and by FAB M5 subset of**
716 **AML degrades ASNase**

717 (A) *CTSB* expression by CD14$^+$ and CD14$^-$ cells isolated from healthy donor BM. The expression
718 was analysed by RQ-PCR and is showed as fold change, calculated as $2^{-\Delta\Delta Ct}$ using the REH cell line
719 as the reference ($2^{-\Delta\Delta Ct}$=1). CD14$^+$ samples (average purity 86%) and CD14$^-$ samples (average
720 purity 85%) from 12 different donors were analysed. ***P<0.001: Wilcoxon matched-pairs signed
721 rank test.

722 (B) Residual ASNase activity following incubation of the drug with whole-cell lysates from BM
723 CD14$^+$ and CD14$^-$ cells was quantified by Nessler assay. To prevent ASNase cleavage, lysates were
724 incubated with protease inhibitor cocktail (PIC) or CTSB-specific inhibitor Ca-074 prior to
725 incubation with the drug. CD14$^+$ and CD14$^-$ samples from 6 different BM donors were analysed.
726 *P<0.05: Wilcoxon matched-pairs signed rank test.

(C) *CTSB* expression by primary AML cells. AML (n=28, with >70% of blast cell content) and HD BM samples (n=8) were analysed by RQ-PCR. Within AML patients, 7 belonged to FAB M0/M1, 4 belonged to FAB M2, 5 belonged to FAB M4, and 12 belonged to FAB M5. **P<0.01: Mann-Whitney test.

(D) Residual ASNase activity following incubation with whole-cell lysates obtained from 3 different primary AML blasts with high *CTSB* expression (black circle) and from one AML sample with low *CTSB* expression (white square) in the presence of PIC and Ca-074.

Supplementary Methods

Cells

The human AML cell lines KG-1, THP-1 and HL-60 were obtained from ATCC and the human ALL cell line 697 was purchased from DSMZ. Cells were cultured according to manufacturer's recommendations in complete RPMI 1640 medium (EuroClone, Milan, Italy) or complete Advanced RPMI 1640 medium (Gibco™, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10-20% of heat-inactivated foetal bovine serum (FBS) (Biosera, Ringmer, UK), 2 mM L-glutamine, 50 iu/ml penicillin and 50 μg/ml streptomycin (EuroClone).

MSC were isolated from BM aspirates of AML patients at diagnosis (AML-MSC) and of healthy donors (HD-MSC), as previously described.[1] Cells were grown in DMEM-low glucose (1 g/l; Gibco™, Thermo Fisher Scientific), supplemented with 10% FBS, 2 mM L-glutamine and antibiotics (50 iu/ml penicillin and 50 μg/ml streptomycin). MSC were not used for more than 7 passages.

Analysis of cell viability

For analysis of apoptosis, cells were stained with AnnexinV/7-AAD (Apoptosis/Necrosis Detection Kit, Enzo Life Sciences, Farmingdale, NY, USA). The percentage of $AnnexinV^{+}/7\text{-}AAD^{-/+}$ early and late apoptotic cells was evaluated by FACS analysis. Apoptosis relative to untreated control has been calculated as reported.[2]

To evaluate the number of viable cells, counting beads (CountBright™ absolute counting beads, Invitrogen™, Thermo Fisher Scientific) were added to samples before the acquisition and the absolute cell count (10^9/l) was calculated following manufacturer's protocol.

Primary patient-derived AML cells were labelled with pacific orange-anti CD45 (clone HI30; Invitrogen, Thermo Fisher Scientific), phycoerythrin-cyanine™ 7-anti CD34 (clone 8G12; BD Biosciences, Franklin

Lakes, NJ, USA) and allophycocyanin-Alexa Fluor® 750-anti CD38 (clone LS198-4-3; Beckman Coulter Inc., Brea, CA, USA) to perform the analysis gating on the non-blast cells within the sample, the bulk blast population and the leukaemic $CD34^{+}CD38^{-}$ and $CD34^{+}CD38^{+}$ subpopulations.[3]

Experiments were performed on a FACSCanto™ II (BD Biosciences) and analysed with FACSDiva™ software v.6.1.3 (BD Biosciences).

Coculture experiments

The susceptibility of MSC to ASNase was previously determined seeding HD- and AML-MSC (passage 5 to 6) at 1.7-2x10^4 cells/well in 96-well plates in complete RPMI 1640 medium and, when confluent (typically in 1-2 days), treating with *Erwinia* ASNase at different concentrations (0.1-1-3 iu/ml). After 48 hours, cells were trypsinised and their viability was evaluated by flow cytometry. All experiments were performed in triplicate.

Coculture experiments were performed seeding HD-MSC (passage 4 to 7) at 1.7-2x10^4 cells/well in 96-well plates. When confluent, primary AML patient-derived cells (2x10^5 cells/well) were added to the culture in complete Advanced RPMI 1640 medium with or without 1 iu/ml of *Erwinia* ASNase. After 48 hours of treatment, the bottom of the wells was scraped and the harvested cells were passed through a 18-gauge needle, to eliminate MSC aggregates. Then, cell suspensions were analysed for viability by flow cytometry.

We executed the same experiment coculturing primary AML cells with the autologous AML-MSC (passage 3 to 6). All experiments were performed in triplicate.

Clonogenic assay

1x10^4 of primary AML cells were resuspended with 1 mL of MethoCult™ H4434 classic (StemCell™ Technologies) in the presence or in the absence of 0.01 iu/ml of ASNase. The mixture was plated in 35 mm

low-adherence plastic dishes (Thermo Scientific™ Nunc™, Thermo Fisher Scientific) and maintained at 37 °C and 5% CO_2. After 14 days, colonies were counted on an inverted microscope. Experiments were performed in duplicate.

mIR-126 expression

Primary AML cells after 48 hours of incubation in the presence of SR1 and UM729 with or without ASNase, as previously described, were resuspended in TRIzol™ reagent (Invitrogen™, Thermo Fisher Scientific) and frozen. Total RNA, including miRNA, was extracted from samples using miRNeasy Micro Kit (Qiagen, Hilden, Germany), following manufacturer's instructions. RNA concentration was measured using Quantus™ Fluorometer. cDNA was synthetised using Universal cDNA synthesis kit II (Exiqon, Copenhagen, Denmark) following the company's guidelines for miRNA profiling. UniSP6 spike-in was included in each reaction as a retrotranscription and PCR plate-loading control. Digital droplet PCR (ddPCR) was performed using EvaGreen supermix (Bio-Rad, Hercules, CA, USA) and one of the following miRCURY LNA PCR primer sets (Exiqon): hsa-miR-126-3p (ID 204227), hsa-let-7a-5p (ID 205727), hsa-miR-16-5p (ID 205702), SNORD24 (ID 206999), SNORD48 (ID 203903), UniSP6 (ID 203956). Droplets were generated using Automated Droplet Generator (Bio-Rad). Recommended thermal cycling conditions for EvaGreen assays were used, except for annealing step optimisation. Droplets were analysed using QX200 Droplet Reader (Bio-Rad) and QuantaSoft™. miR-126-3p levels were normalised by the geometric mean of let-7a-5p, miR-16-5p, SNORD24 and SNORD48.

RQ-PCR

Total cellular RNA was isolated using TRIzol™ reagent according to manufacturer's protocol. One μg of total RNA was reversely transcribed using the SuperScript II Reverse Trascriptase (Invitrogen™, Thermo Fisher Scientific).

824 Quantitative RT-PCR experiments were performed using Light Cycler 480II with Universal Probe Master
825 system (Roche Diagnostics, Rotkreuz, Switzerland).

826 *ASNS* and *CTSB* primers were designed through the Software Probe Finder (Roche Diagnostics) and are the
827 following: h*ASNS*upl-left 5'-GATGAACTTACGCAGGGTTACA-3'and h*ASNS*upl-right 5'-
828 CACTCTCCTCCTCGGCTTT-3'; h*CTSB*upl-left 5'-CAGCCACCCAGATGTAAGC-3' and h*CTSB*upl-
829 right 5'-GCCGGATCCTAGATCCACTA-3'. As reference, housekeeping gene *ABL1* was used (h*ABL1*upl-
830 left: 5'-AGGAATCCAGTATCTCAGACGAA-3' and h*ABL1*upl-right: 5'-GGAGGTCCTCGTCTTGGTG-
831 3'). UPL probe number 2 or 30 and 57 were used in combination to detect *ASNS* and *CTSB* expression.

832 Three independent replicates were performed. RQ-PCR data were calculated with the ΔΔCt method using as
833 a reference the 697 and REH cell lines.

834

835 **Nessler assay**

836 The colorimetric Nessler method consists of the reaction between Nessler's reagent (potassium
837 tetraiodomercurate(II), Sigma-Aldrich) and the ammonia released during the conversion of L-Asn into L-
838 Asp, providing a characteristic yellow reaction mixture that can be quantified by spectrophotometry. Briefly,
839 60 µl of 44 mM L-asparagine (Sigma-Aldrich) dissolved in 15 mM Tris-HCl buffer, pH 7.3, supplemented
840 with 0.015% w/v BSA was added to 15 µl of sample and incubated at 37°C for 30 minutes. The reaction was
841 stopped by the addition of 50 µl trichloroacetic acid (24.5% w/v, Sigma-Aldrich). After centrifugation, 15 µl
842 of the supernatant was plated and 120 µl Nessler's solution diluted with ddH_2O (1:8) was added. The
843 adsorbance of the reaction product was read at 450 nm using the Tecan GENios microplate reader
844 fluorometer (Tecan, Mannedorf, Switzerland).

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847

Supplementary References

1. Michelozzi, I. M. *et al.* Human aplastic anaemia-derived mesenchymal stromal cells form functional haematopoietic stem cell niche in vivo. *Br. J. Haematol.* **179,** 669–73 (2017).

2. Podhorecka, M. *et al.* Resveratrol increases rate of apoptosis caused by purine analogues in malignant lymphocytes of chronic lymphocytic leukemia. *Ann. Hematol.* **90,** 173–83 (2011).

3. Lara-Castillo, M.C. *et al*. Repositioning of bromocriptine for treatmernt of acute myeloid leukemia. *J. Trans. Med.* **14**, 261 (2016).

Supplementary Figures

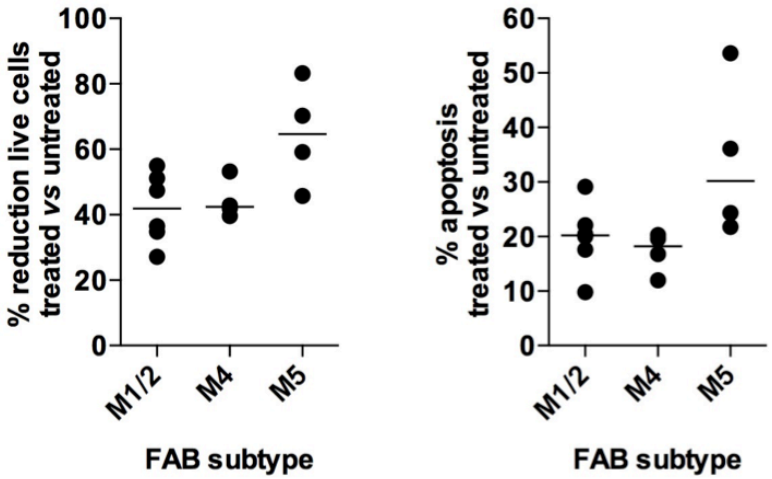

Figure S1. Comparison of ASNase susceptibility between samples from different FAB subtypes.

Distribution and median values of reductions of live cell number (on the left) and percentage of apoptosis (on the right) of blasts after treatment in 3 AML FAB subtypes (FAB M1/M2, FAB M4, and FAB M5) were shown. Each dot represents an individual AML patient. Horizontal bar in the graph indicates the median for each subtype. Mann-Whitney test was used for comparison between pairs of groups.

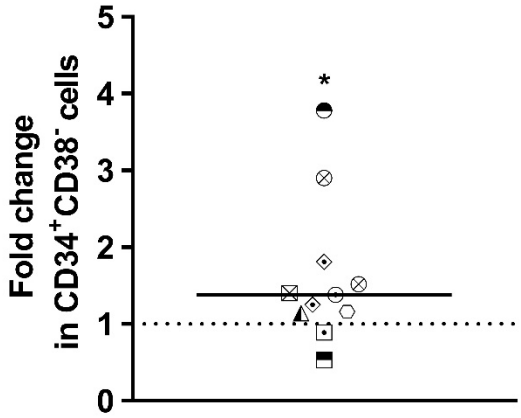

Figure S2. Effect of SR1 and UM729 on maintenance of primary $CD34^{+}CD38^{-}$ AML cells in culture

Primary AML patient-derived cells were cultured with or without SR1 and UM729 for 48 hours. The effect of these two reagents on $CD34^{+}CD38^{-}$ subpopulation was expressed as fold change relative to the control cultured for 48 hours without the two compounds. Each symbol represents an individual AML patient (mean of technical triplicates). 11 independent experiments performed on 9 different patients are shown. Bar indicates the median and the dotted horizontal line represents the control. *P<0.05: Wilcoxon signed rank test.

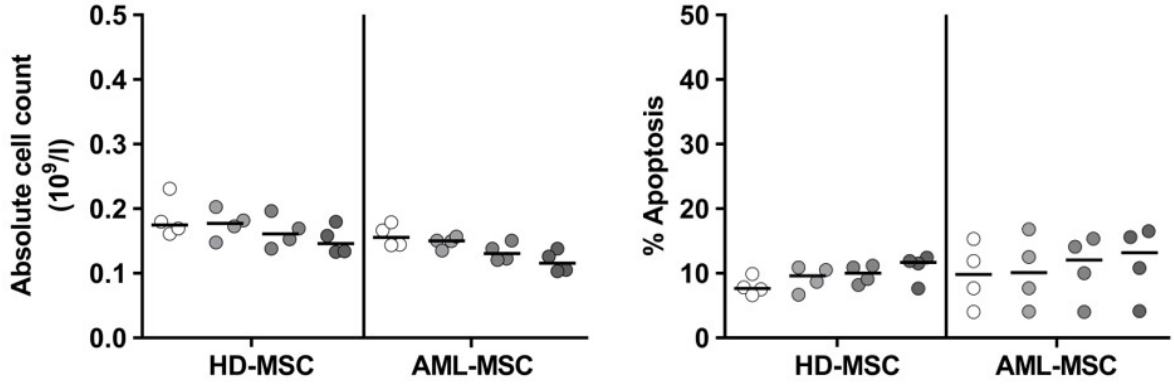

Figure S3. ASNase cytotoxicity on HD- and AML-MSC

HD- and AML-MSC were incubated for 48 hours in the absence (white) or in the presence of 0.1-1-3 iu/ml of ASNase (represented by darker shades of grey as the dose increase). The absolute cell count (10^9/l) (on the left) and the percentage of apoptotic cells (on the right) were evaluated by flow cytometry. 4 independent experiments were performed on 8 different MSC donors (4 HD and 4 AML). Each dot represents the mean of technical triplicates and bar indicates the median for each group.

Supplementary Table

Table SI. Clinical and biological patients' details.

Patient code	Age (y)	Sex	AML type	WBC 103 /uL	% Blasts in BM	% Blasts in PB	Cellular source	Molecular status	Karyotype
AML1	51	F	M5a, de novo	64	90	70	BM (fresh)/ (thawed)	*FLT3-ITD*, *NPM1* mut	46,XX[25]
AML2	64	M	M0, de novo	33.03	90	90	BM (fresh)/ (thawed)	none	48,XY,+8,+13[10]/46,XY [2]
AML3	30	M	M5a, de novo	72.61	90	94	BM (fresh)/ (thawed)	none	46,XY,del(11)(q23)[20]
AML4	17	M	M1, de novo	12.58	60	70	BM (thawed)	none	46,XY[15]
AML5	13	M	M2, de novo	28.35	40	58	BM (thawed)	*FLT3-ITD*	46,XY[20]
AML6	8	F	M4, de novo	22.42	85	64	BM (thawed)	*FLT3-ITD*, *DEK-CAN* – t(6;9)	47,XX,+8[18]/47,idem,iso (13)(q11)[2]
AML7	9	F	M4, de novo	4.57	90	40	BM (thawed)	*MLL-ELL*	46,XX,t(11;19)(q23;p13)[18]/46,XX[2]
AML8	1	M	M5a, de novo	39.42	80	51	BM (thawed)	*MLL-AF10*	46,XY,t(10;11)(p12;q23), der(14)t(1;14)(q?21;q11)[20]
AML9	3	M	M2, de novo	14.28	80	42	BM (thawed)	*NUP98-NDS1* - t(5;11)	46,XY[20]
AML10	71	F	M1/M 2, de novo	N.A.	50	N.A.	PB (fresh)	none	N.A.

AML11	48	F	M0, de novo	31.46	>70	N.A.	BM (thawed)	*FLT3-ITD*, *NPM1* mut	46,XX[20]
AML12	3	M	M5a, de novo	378.92	N.A.	92	PB (fresh)	none	47,XY,+9,t(11;17)(q23;q12 or q21)[20]
AML13	65	M	M0, de novo	49.86	90	N.A.	BM (fresh)/ (thawed)	none	46,XY,del(5)(q13q31)[20]
AML14	48	M	M4, de novo	104.43	80	N.A.	BM (thawed)	*FLT3 D835*, *CBFB-MYH11*	46,XY,inv(16)(p13q22)[20]
AML15	70	F	M4, secondary	6.45	50	30	BM (thawed)	none	44~45,XX,?+X,t(1;22)(q12;q11),+1,del(5)(q13q34),?inv (7)(q14q22),tas(8;15)(q24;p13),-17,-21,del(22)(q11)der(22)t(1;22)(q12;q11),+mar[cp18]/45,XX,t(1;22)(q12;q11),+1,del(5)(q13q34),-7,der(17)t(7;17)(q11;q25),-21,del(22)(q11)der(22)t(1;22)(q12;q11)[7]
AML16	62	F	M1, de novo	1.91	70	5	BM (fresh)	*NPM1* mut	46,XX,del(13)(q14q22)[3]/46,XX[17]
AML17	30	F	M1/M2, de novo	23	94	90	BM (fresh)/ (thawed)	*FLT3 D835*, *CEBPA* mut	46,XX[20]
AML18	58	M	M1/M2, de novo	130	95	88	BM (fresh)	*FLT3-ITD*, *NPM1* mut	46,XY,i(7)(q10)[9]/46,XY[12]
AML19	85	F	M4eo, secondary	42.90	80	65	BM (thawed)	N.A.	47,XX,del(7)(q22),+?22[20]
AML20	56	F	M4, de novo	33.81	80	N.A.	BM (thawed)	*FLT3-ITD*, *NPM1* mut	46,XX,del(9)(q21)[20]
AML21	14	M	M1, de novo	9.73	95	80	BM (thawed)	none	47,XY,+?13[11]/46,XY[9]
AML22	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
AML23	12	M	M1, de novo	56.24	95	77	PB (thawed)	*FLT3 D835*, *NPM1* mut	46,XY[25]

AML24	13	F	M2, de novo	42.40	80	71	BM (thawed)	*AML1-ETO*, *cKIT* mut	46,XX,t(3;7)(?q25;?q22),t(8;21)(q22;q22)[2]/46,idem, del(9)(q12q22)[18]
AML25	12	F	M2, de novo	13.70	90	N.A.	BM (thawed)	*AML1-ETO*	46,XX,t(8;21)(q22;q22)[20]
AML26	39	M	M1, de novo	6.29	50	43	BM (thawed)	*NPM1* mut	46,XY[20]
AML27	13	M	M1, de novo	8.83	85	75	BM (thawed)	none	46,XY[19]
AML28	15	M	M5a, de novo	97.32	N.A.	93	PB (thawed)	none	46,XY,del(17)(p11.2)[3]/46,XY,del(9)(p21), del(17)(p11.2)[16]
AML29	9 months	F	M5b, de novo	353.69	N.A.	90	PB (thawed)	*MLL-AF6*	51,XX,+3,+6,t(6;11)(q27;q23),+7,+8,+12[20]
AML30	16	M	M5a, de novo	73.85	N.A.	89	PB (thawed)	*NPM1* mut	46,XY[20]
AML31	77	M	M5b, de novo	24.80	95	67	BM (thawed)	N.A.	47,XY,+8[20]
AML32	4	F	M5a, de novo	235.32	95	N.A.	BM (thawed)	N.A.	47-48,XX,del(2)(p12),del(5)(p12),?t(6;7)(q21;q32),t(9;?) (q34;?),-11,del(12)(p11),+19,+4markers[cp9]/46,XX[3]
AML33	1	F	M5a, de novo	372.79	N.A.	85	PB (thawed)	*MLL-AF10* – t(10;11)	46,XX[20]
AML34	12	M	M5a, de novo	1.73	90	25	BM (fresh)	*MLL-AF9* – t(9;11)	48,XXY,+21c[22]
AML35	41	F	M5b, de novo	85.17	90	N.A.	BM (fresh)	*FLT3-ITD*, *NPM1* mut	47,XX,+8[20]
AML36	66	M	M5b, de novo	83.96	90	N.A.	BM (fresh)	*FLT3-ITD*, *NPM1* mut	46,XY[25]
AML37	3	M	M4, de novo	22.34	80	38	BM (fresh)	*CBFB-MYH11*, *cKIT* mut	46,XY,inv(16)(p13q22)[20]

Abbreviations: WBC, white blood cells; BM, bone marrow; PB, peripheral blood; none, negative for mutations and translocations analysed; N.A., not analysed.