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$MgSO_4$ anticoagulant prevents pseudothrombocytopenia by preserving the integrity of the platelet GPIIb-IIIa complex

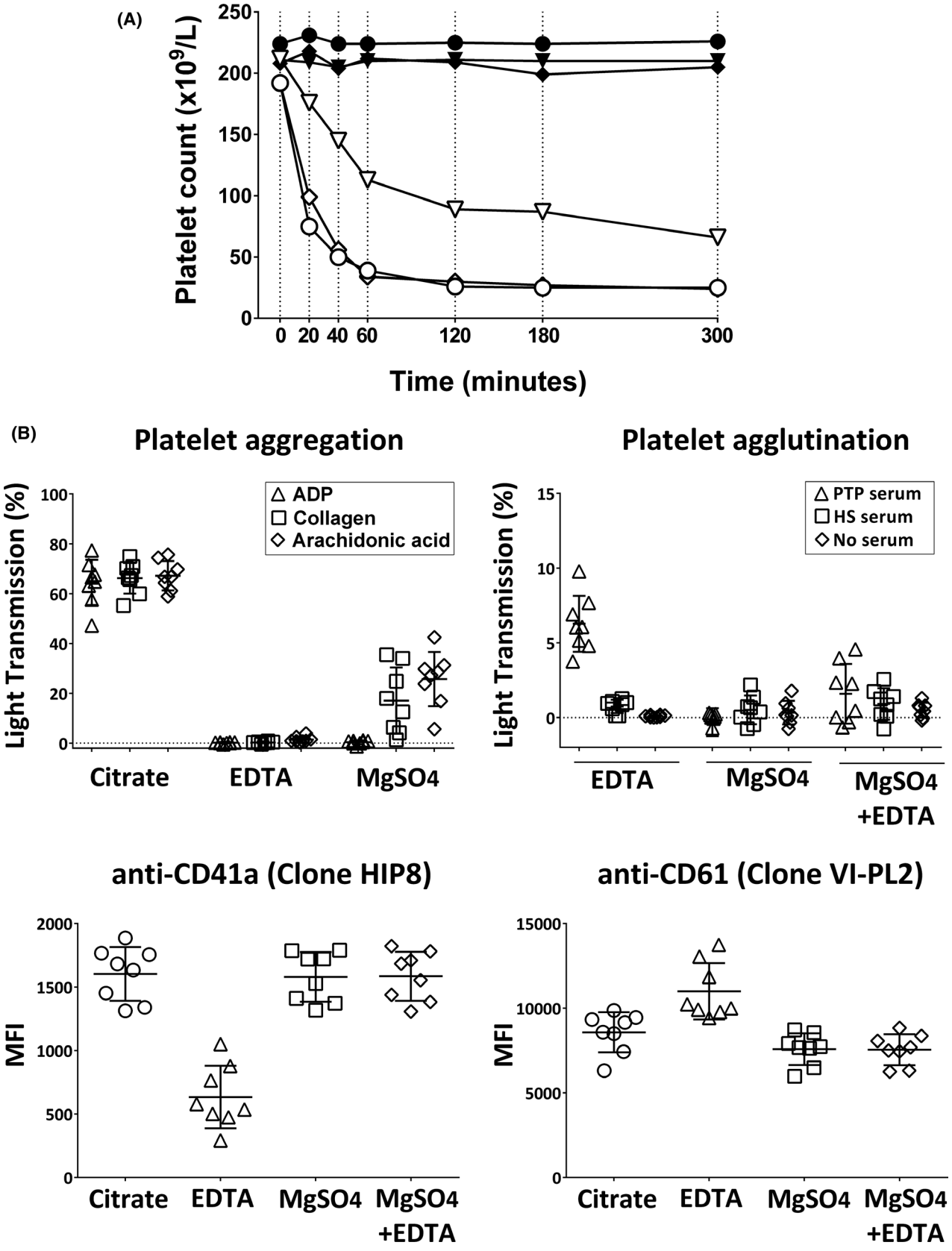
Ethylenediaminetetra-acetic acid (EDTA) is the *in vitro* anticoagulant commonly used to collect blood samples for cell counts.[1] One drawback is the development of pseudothrombocytopenia (PTP), a spuriously low platelet count caused by platelet clumping *in vitro* in 0·07–0·27% of EDTA samples during storage at room temperature.[2] PTP is caused by the binding of (auto)antibodies to cryptic epitope(s) in the platelet glycoprotein (GP)IIb/IIIa complex that become(s) accessible in the presence of EDTA.[3,4] Although PTP is an *in vitro* artefact without clinical relevance, failure to diagnose it may trigger unnecessary and expensive diagnostic investigations and potentially dangerous treatments.[2]

It has been proposed to substitute EDTA with magnesium sulfate ($MgSO_4$) which prevents the development of PTP.[5–7] Excess Mg^{2+} inhibits platelet aggregation,[8,9] likely interfering with the essential role of Ca^{2+},[10,11] and this property has been advocated as the mechanism by which it prevents PTP.[5,12] However, this suggestion appears incongruous, because EDTA is a very potent inhibitor of platelet aggregation, as it is a strong Ca^{2+} chelator:[11] if PTP is prevented by inhibitors of platelet aggregation, then EDTA would prevent, rather than cause it.

We compared $MgSO_4$ and EDTA under different experimental conditions, to characterize better the mechanism of PTP prevention by $MgSO_4$. A detailed description of the methods is published in the online supplement.

First of all, we confirmed that $MgSO_4$, when used instead of EDTA, effectively prevents the development of PTP:[5,12] The platelet counts of three PTP patients decreased with time in EDTA blood samples kept at room temperature, while they remained stable in $MgSO_4$ samples (Fig 1A). However, the results of additional experiments were not compatible with the suggestion that the effect of $MgSO_4$ is mediated by inhibition of platelet aggregation.[5,12] Indeed, we showed that platelet aggregation, compared to that observed in citrate platelet-rich plasma (PRP), was abolished in EDTA-PRP with any tested agonist [adenosine diphosphate (ADP), collagen and arachidonic acid], while it was abolished with ADP and only partially inhibited with collagen and arachidonic acid in $MgSO_4$-PRP

Fig 1. (A) Effects of the *in vitro* anticoagulants ethylenediaminetetra acetic acid (EDTA) and $MgSO_4$ on the stability of platelet counts in samples from three pseudothrombocytopenia (PTP) subjects. Cell counts were performed immediately after sampling (time 0) and after 20, 40, 60, 120, 180 and 300 min storage at room temperature in EDTA or $MgSO_4$ anticoagulant. Statistical analysis: EDTA (T0) *versus* $MgSO_4$ (T0) – *t* test, $P = 0{\cdot}2418$; EDTA (T0) *versus* EDTA (T300) – *t* test, $P = 0{\cdot}002$; $MgSO_4$ (T0) *versus* $MgSO_4$ (T300) – *t* test, $P = 0{\cdot}6914$; Open symbols: samples in EDTA anticoagulant; closed symbols: samples in $MgSO_4$ anticoagulant. (B) Effects of different *in vitro* anticoagulants on platelet aggregation, agglutination and binding of monoclonal antibodies. Upper left panel: platelet aggregation induced by ADP (4 μmol/l), collagen (2 μg/ml) or arachidonic acid (1 mmol/l) in citrate-, EDTA- and $MgSO_4$-platelet-rich plasma (PRP). Statistical analysis: ADP – ANOVA, $P < 0{\cdot}0001$; internal contrasts: citrate *versus* EDTA or $MgSO_4$, $P < 0{\cdot}0001$; EDTA *versus* $MgSO_4$, $P = 0{\cdot}7111$. Collagen – ANOVA, $P < 0{\cdot}0001$; internal contrasts: citrate *versus* EDTA, $P < 0{\cdot}0001$; citrate *versus* MgSO4, $P = 0{\cdot}0003$; EDTA *versus* MgSO4, $P = 0{\cdot}0239$. Arachidonic acid – ANOVA, $P < 0{\cdot}0001$; internal contrast: citrate *versus* EDTA, $P < 0{\cdot}0001$; citrate *versus* $MgSO_4$,$P = 0{\cdot}0002$; EDTA *versus* $MgSO_4$, $P = 0{\cdot}001$. Upper right panel: platelet agglutination measured in EDTA-, $MgSO_4$- and $MgSO_4$ plus EDTA-anticoagulated PRP in the presence of serum from healthy subjects (HS), from a subject with PTP or without added serum. Statistical analysis: PTP serum – ANOVA, $P < 0{\cdot}0001$; internal contrasts: EDTA *versus* $MgSO_4$, $P < 0{\cdot}0001$; EDTA *versus* $MgSO_4$ + EDTA, $P = 0{\cdot}003$; $MgSO_4$*versus* $MgSO_4$ + EDTA, $P = 0{\cdot}0612$. HS serum – ANOVA, $P = 0{\cdot}3965$; no serum – ANOVA, $P = 0{\cdot}3258$. Lower left panel: flow cytometry analysis of platelet-binding of monoclonal antibody anti-CD41a (clone HIP8), which binds to glycoprotein (GP)IIb when it is coupled to GPIIIa to form the GPIIb/IIIa complex. Statistical analysis: ANOVA, $P < 0{\cdot}0001$; internal contrasts: EDTA *versus* citrate or $MgSO_4$ or $MgSO_4$ + EDTA, $P = 0{\cdot}0003$; citrate *versus* $MgSO_4$ or $MgSO_4$ + EDTA, $P = 0{\cdot}8325$; $MgSO_4$*versus* $MgSO_4$ + EDTA: $P = 0{\cdot}9519$. Lower right panel: flow cytometry analysis of platelet binding of monoclonal antibody anti-CD61 (clone VI-PL2), which binds GPIIIa. Statistical analysis: ANOVA, $P < 0{\cdot}0001$; internal contrasts: EDTA *versus* citrate or $MgSO_4$ or $MgSO_4$ + EDTA, $P = 0{\cdot}0075$; citrate *versus* $MgSO_4$ or $MgSO_4$ + EDTA, $P = 0{\cdot}002$; $MgSO_4$*versus* $MgSO_4$ + EDTA, $P = 0{\cdot}9879$. All data are represented as means ± SD of eight experiments.



(Fig 1B). Were PTP caused by platelet aggregation, then EDTA would be more effective than $MgSO_4$-PRP in preventing it. Failure of $MgSO_4$ to inhibit platelet aggregation induced by collagen and arachidonic acid completely could be attributed to the fact that, at variance with ADP, which induces platelet aggregation without causing platelet secretion directly,[13] they induce platelet secretion directly, thus providing Ca^{2+} from dense granules at the platelet plasma membrane level at sufficiently high concentrations to partially overcome the inhibitory effect of Mg^{2+}. In additional experiments, we showed that serum from a subject with PTP, but not serum from a healthy subject, induced an increase in light transmission through EDTA-PRP, which was not prevented by 1 μmol/l prostaglandin-E1 (not shown), indicating that it was not caused by platelet aggregation, but likely by platelet agglutination (Fig 1B). In contrast, PTP serum or healthy serum did not induce appreciable platelet agglutination in $MgSO_4$-PRP (Fig 1B). Therefore, when platelets were exposed to an antibody causing PTP, the opposite picture of that observed with platelet agonists was evident: EDTA allowed platelet clumping to occur, while $MgSO_4$ completely prevented it. The addition of EDTA to $MgSO_4$-PRP before the challenge with sera only slightly increased the extent of platelet agglutination induced by PTP serum although the difference with the effects of healthy serum was not statistically significant (Fig 1B). The failure of added EDTA to restore platelet agglutination is likely the result of it being unable to chelate Ca^{2+} completely in $MgSO_4$ samples, due to the presence of high concentrations of competing Mg^{2+}, which is also chelated by EDTA. Finally, we showed that the binding of the monoclonal antibody (MoAb) anti-CD41a (clone HIP8) (Fig 1B) or anti-CD41 (clone P2) (not shown), which bind to GPIIb when it is coupled to GPIIIa to form the GPIIb/IIIa complex, was significantly lower in EDTA samples than in citrate, $MgSO_4$ or $MgSO_4$ + EDTA. In contrast, the

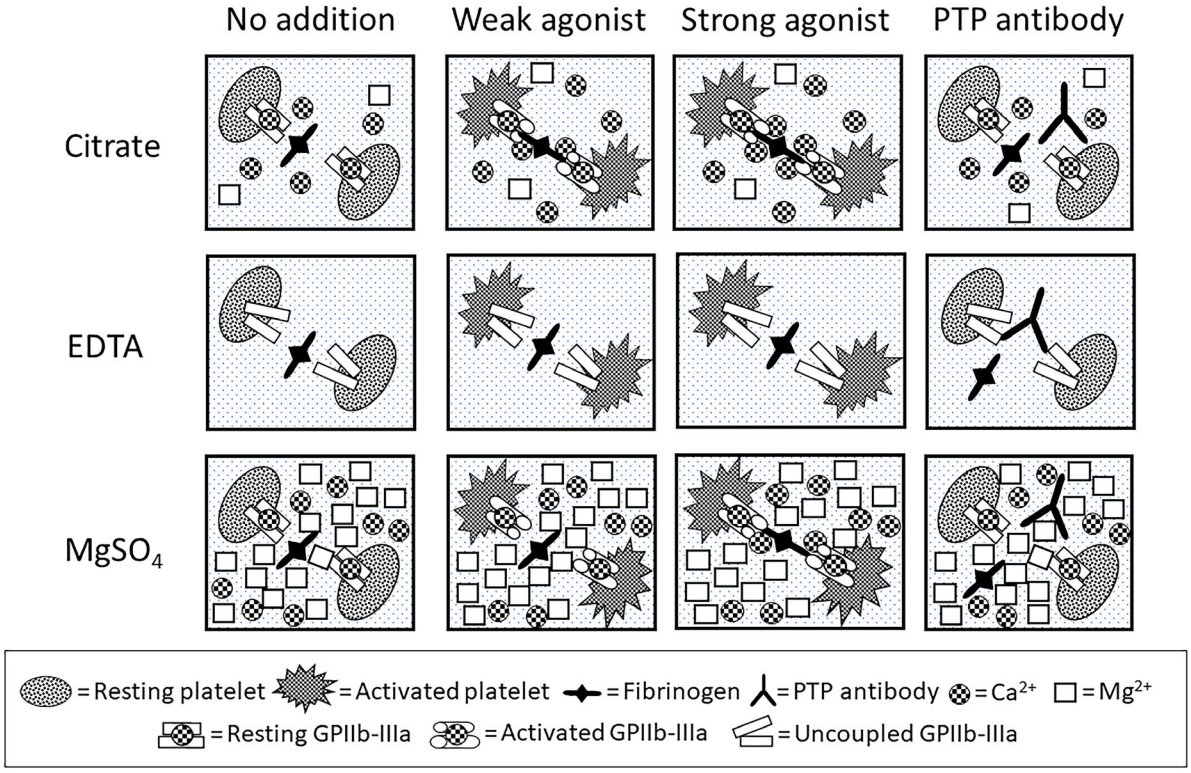


Fig 2. Schematic representations of the effects of different *in vitro* anticoagulants on agonist-induced platelet aggregation and PTP serum-induced platelet agglutination in human platelet-rich plasma (PRP). (1) Citrate partially chelates divalent cations, such as Ca^{2+} and Mg^{2+}. In citrate-anticoagulated blood, the Ca^{2+} concentration is high enough to prevent the dissociation of the GPIIb/IIIa complex and to support fibrinogen binding to activated glycoprotein (GP)IIb/IIIa; therefore, in citrate-PRP platelet aggregation occurs (induced by both weak and strong agonists) and PTP, due to the binding of PTP antibody to dissociated GPIIb/IIIa, does not occur. Similar results would be obtained with anticoagulants, such as hirudin, that do not chelate divalent cations. (2) Ethylenediaminetetra-acetic acid (EDTA) is a very strong chelator of divalent cations. In its presence, the concentration of Ca^{2+} is extremely low and GPIIb dissociates from GPIIIa. As a consequence, platelet aggregation does not occur, independently of the type of platelet agonist (weak *versus* strong agonist), and the PTP antibody, when present, can bind to GPIIb, thus bridging platelets to form platelet agglutinates. (3) In the presence of excess Mg^{2+}, such as when high concentrations of $MgSO_4$ are used to anticoagulate blood samples, fibrinogen does not bind to weak agonist-stimulated platelet GPIIb/IIIa and platelet aggregation does not occur, because Mg^{2+} negatively interferes with Ca^{2+}, which is necessary for the process. When platelets are stimulated by strong agonists, the high concentration of Ca^{2+} that is released from platelet delta granules can compete locally with Mg^{2+} in supporting fibrinogen binding to GPIIb/IIIa, thus allowing some platelet aggregation to occur. Mg^{2+}, even at very high concentrations, does not dissociate the Ca^{2+}-dependent GPIIb/IIIa complex, thus preventing platelet agglutination that is dependent on the binding of PTP antibody to GPIIb.

binding of anti-CD61 (clone VI-PL2) (Fig 1B), which binds to GPIIIa independently of the integrity of the GPIIb/IIIa complex, was higher in EDTA-anticoagulated samples compared to other samples. These data are compatible with the uncoupling of the GPIIb/IIIa complex by EDTA, which chelates also the Ca^{2+} that holds the two glycoproteins in the complex, thus disrupting it.[14] This would lead to the unmasking of the epitopes that are recognized by PTP antibodies,[4] while allowing an easier accessibility of the MoAb against GPIIIa. The fact that, in contrast to EDTA, $MgSO_4$ did not affect the binding of any tested antibody indicates that it does not uncouple the glycoprotein complex, thus preventing the binding of the PTP antibody. This finding also contributes to accounting for the lesser inhibitory effect of $MgSO_4$ on platelet aggregation, compared to EDTA, which depends on the integrity of the GPIIb/IIIa complex.

Figure 2 summarizes the effects of the tested anticoagulants on divalent cations and the platelet GPIIb/IIIa complex, with the aim of illustrating the consequent effects on platelet aggregation induced by agonists and on platelet agglutination induced by PTP antibodies.

In light of the evidence that $MgSO_4$ does prevent PTP, should we continue using EDTA or switch to $MgSO_4$ to collect blood samples for cell counts, as has been suggested?[5,12] We wonder whether the routine use of $MgSO_4$ instead of EDTA would imply relevant and expensive organizational consequences. In addition, it must be emphasized that EDTA is used also to prevent platelet aggregation stimulated by released or synthesized agonists during cumbersome procedures of blood sampling. $MgSO_4$ would likely be less effective in this respect, as it is a weaker inhibitor of platelet aggregation than EDTA. We believe that the most convenient way to prevent the misdiagnosis of PTP would be to continue using EDTA, being well aware of the possibility of encountering spuriously low platelet counts indicative of PTP. In suspected cases of PTP, we suggest that the blood cell count should be repeated in $MgSO_4$-anticoagulated samples, because other alternative anticoagulants do not prevent PTP to occur in all cases,[2,15] or again in EDTA-anticoagulated samples, but immediately after blood sampling, considering that PTP develops time-dependently.[15]

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Author contributions

MS contributed to the design of the study, performed laboratory analyses, analysed the data, contributed to writing the manuscript and critically reviewed it; EB contributed to the design of the study and performed laboratory analyses; GMP contributed to the design of the study, enrolled the subjects, analysed the data and critically reviewed the manuscript; MC designed the study, coordinated the group, contributed to data analysis and interpretation and wrote the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors report no conflicts of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. Supplementary data.

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Phase I study of F16IL2 antibody–cytokine fusion with very low-dose araC in acute myeloid leukaemia relapse after allogeneic stem cell transplantation

Besides multiple functions in cellular immunity, interleukin 2 (IL2) causes tumour regression.[1-5] Proleukine® [aldesleukin, human recombinant IL2 (rhIL2)] was approved for renal cancer and melanoma. Response rates were low and clinical application was limited by rapid clearance and serious dose-limiting toxicities (DLT), e.g. vascular leakage.[6] Thus, reducing systemic toxicity by targeting IL2 to tumour-associated structures and thereby guiding cellular immune responses to the tumour seems promising.

F16IL2, a recombinant fusion protein of a human monoclonal antibody fragment F16 and rhIL2, binds the A1 domain of tenascin C (TnC), spliced into TnC during angiogenesis and tissue remodeling, which is expressed in the vasculature of solid and haematological tumour types, but virtually absent in normal tissues.[7] F16 and the murine homolog F8 can localize therapeutic payloads to tumours and leukaemias.[7-9] Targeting, biodistribution of F8 in mouse leukaemia models, and therapeutic activity of F8IL2 against syngeneic murine acute myeloid leukaemia (AML) models is promising.[9] In combination with low-dose cytarabine, F8IL2 eradicates AML, an effect mediated by natural killer (NK) cells and $CD8^+$ T cells.[9] A patient with myeloid sarcoma relapse upon multiple therapies including two allogeneic haematopoietic stem cell transplantations (alloHSCT) achieved a near complete response for several months with a combination of F16IL2 and very low-dose cytarabine (VLDAC).[9] Further remissions occurred in cases of medullary AML relapse after alloHSCT.[10]

We report a phase I study (see Supporting Information) combining F16IL2 (Figure S1) with VLDAC in patients with AML relapse after alloHSCT. Eight patients (characteristics in Table SI) received at least one dose and were evaluable for safety. Two received only one dose of F16IL2 due to rapidly progressing AML, were therefore replaced and not evaluable for DLT. Risk categorization and response criteria were according to European LeukemiaNet (ELN).[11] Patients had multiple prior lines of therapy (Tables SII and SIII) and AML relapse after alloHSCT, three patients after two and one after three alloHSCTs. One patient had additional donor lymphocyte infusions (DLI). Cohort 1 received F16IL2 at 30 MioIU IL2 equivalents on days 1, 8, 15 and 22 and VLDAC (5 mg twice daily, first 10 days of the cycle). In cohort 2, the F16IL2 dose on days 8, 15 and 22 was 50 MioIU with VLDAC. The most frequent treatment-related adverse events (AE) of grades 1 and 2 (CTCAE) were chills (seven patients), pyrexia (seven patients), nausea (five patients), vomiting (four patients), flare-ups of skin GVHD (three patients), headache (three patients), fatigue (two patients), and abdominal pain upper (two patients). All AEs were completely reversible. Cytokine release syndrome (CRS; three patients, grades 2 and 3) and acute graft-*versus*-host-disease (GVHD) in liver (two patients, grades 2 and 3) were observed; two of those were grade 3 serious adverse events (SAE; one GVHD, one CRS; Table SIII). Both occurred at 30 MioIU, were treatment-related, and both patients had received haploidentical alloHSCT before. Since GVHD was promptly and fully reversible and the second patient had to be replaced before occurrence of the CRS, both SAEs did not meet DLT criteria. Thus, we proceeded with the 50 MioIU level. All grade 4 AEs observed were interpreted as being related to cytarabine, although myelosuppression was reported for IL2 and combined effects cannot be excluded (Table SIII). There was no death on trial. We did not further increase the dose