Enhanced labile plasma iron in hematopoietic stem cell transplanted patients promotes *Aspergillus* outgrowth

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**Key Points**
- Serum-enhanced labile plasma iron in patients undergoing allogeneic HSCT is critical for *Aspergillus fumigatus* growth in vitro.
- Transferrin iron in serum is inaccessible for *A fumigatus*, and uptake of iron in the form of eLPI involves fungal siderophores.

**Introduction**

Apart from relapse and graft-versus-host disease, prevention of life-threatening infections remains the most important challenge in clinical care of patients undergoing hematopoietic stem cell transplantation (HSCT). Despite improved antifungal prophylaxis regimens, invasive aspergillosis (IA) still poses a major threat, with mortality rates up to 80% among infected HSCT patients.1 Thus, novel biomarkers to identify patients at risk and a better understanding of underlying pathomechanisms are needed.

In healthy individuals, plasma iron accessible for cells is bound to transferrin (Tf). However, under pathologic conditions, once Tf’s binding capacity is exceeded, enhanced labile plasma iron (eLPI) develops.2 Indeed, iron-overloaded patients with myelodysplastic syndrome or acute myeloid leukemia undergoing HSCT are at risk of developing eLPI as a result of chemotherapy-induced shut-down of erythropoiesis and tissue damage causing iron release from apoptotic cells, as well as frequent red blood cell transfusion therapy for anemia.3-6 Recently, the Allogeneic Iron Investigators (ALLIVE) trial demonstrated a causative link between the occurrence of eLPI and an increased incidence of infection-associated nonrelapse mortality in patients undergoing allogeneic HSCT.3 In particular, eLPI’s promoting role for the dissemination of selected bacteria has been determined.7,8 Referring to IA, studies have proposed iron overload as a risk factor for the occurrence of IA after transplantation.9,10 Furthermore, iron is known to be essential for *Aspergillus fumigatus (AFU)* growth and virulence.11 However, the relevance of eLPI for *AFU* as an iron source is unknown. Here we specifically investigated the role of eLPI for *AFU* outgrowth, using serum samples of patients with acute myeloid leukemia and myelodysplastic syndrome who underwent HSCT within the ALLIVE trial.

**Methods**

**Study design**

Serum samples of a selected cohort of patients who participated in the ALLIVE trial have been analyzed. All patients gave written informed consent to participate in the trial, which was approved by the ethics committee of the Technical University Dresden (registration number EK338102012). This trial was registered at www.clinicaltrials.gov as #NCT01746147.

**Fungal in vitro assay and procedures**

The scheme of fungal growth assay is shown in supplemental Figure 1. All further details are given in the supplemental Data.
Statistics
Statistical analysis was performed using R. Data were analyzed using generalized linear models with a logit link function. P < .05 is considered significant. All further details are given in the supplemental Data.

Results and discussion
To study the relationship between AFU infection and iron parameters in HSCT patients, we developed an in vitro assay to investigate the development of fungal outgrowth in sera after inoculation with AFU (supplemental Figure 1A-B). Longitudinally collected serum samples during the consecutive phases of HSCT of 29 representative ALLIVE participants were screened (details given in supplemental Table 2). Of importance, eLPI development of our cohort resembled the one seen in the whole ALLIVE cohort: Mean eLPI concentration and percentage of patients showing measurable eLPI levels increased during conditioning, being highest on day 7 (C7; 1.1 ± 0.7; 100%), at transplantation (0; 0.9 ± 0.8; 75.9%), and 7 days after transplantation (7; 0.8 ± 0.6; 79.3%); supplemental Figure 2A-B).

Strikingly, we found that AFU outgrowth was almost exclusively observed when eLPI was present (Figures 1A-B). Therefore, the probability of fungal outgrowth was dependent on the presence of eLPI per se (defined as eLPI ≥ 0.2 arbitrary units; odds ratio [OR], 2235; 95% confidence interval [CI], [337-28 638]; P = 4 × 10^{-12}), rather than on the absolute eLPI amount (OR, 285; 95% CI, 66-1707; P = 7 × 10^{-12}; Figure 1C). Importantly, when considering other clinical confounders including sex, disease type, liver iron content, and transfusion burden, eLPI positivity remained a highly significant predictor for fungal outgrowth (OR, 778; 95% CI, 197-4676; P = 6.2 × 10^{-17}; supplemental Figure 2C).

As mentioned earlier, Tf-saturation (Tf-Sat) and eLPI are mutually interconnected parameters. In our cohort, eLPI and fungal outgrowth were clearly associated with Tf-Sat exceeding 75% (supplemental Figure 2D). Comparing receiver-operator curves, outgrowth were clearly associated with Tf-Sat exceeding 75% interconnected parameters. In our cohort, eLPI and fungal As mentioned earlier, Tf-saturation (Tf-Sat) and eLPI are mutually interconnected parameters. In our cohort, eLPI and fungal outgrowth were clearly associated with Tf-Sat exceeding 75% (supplemental Figure 2D). Comparing receiver-operator curves, outgrowth were clearly associated with Tf-Sat exceeding 75% interconnected parameters. In our cohort, eLPI and fungal As mentioned earlier, Tf-saturation (Tf-Sat) and eLPI are mutually interconnected parameters. In our cohort, eLPI and fungal outgrowth were clearly associated with Tf-Sat exceeding 75% (supplemental Figure 2D). Comparing receiver-operator curves, outgrowth were clearly associated with Tf-Sat exceeding 75% interconnected parameters. In our cohort, eLPI and fungal As mentioned earlier, Tf-saturation (Tf-Sat) and eLPI are mutually interconnected parameters. In our cohort, eLPI and fungal outgrowth were clearly associated with Tf-Sat exceeding 75% (supplemental Figure 2D). Comparing receiver-operator curves, outgrowth were clearly associated with Tf-Sat exceeding 75% interconnected parameters. In our cohort, eLPI and fungal As mentioned earlier, Tf-saturation (Tf-Sat) and eLPI are mutually interconnected parameters. In our cohort, eLPI and fungal outgrowth were clearly associated with Tf-Sat exceeding 75% (supplemental Figure 2D). Comparing receiver-operator curves, outgrowth were clearly associated with Tf-Sat exceeding 75%

Next, we tried to elucidate the mechanism by which AFU accesses eLPI. Notably, deficiency in extracellular siderophores (ΔsidF mutant) led to an impaired outgrowth in the presence of eLPI, whereas lack of extra- and intracellular siderophores (ΔsidA mutant) blocked even spore germination, independent of the presence of eLPI (Figure 2D). Together, these results indicate that iron in the form of eLPI can promote AFU outgrowth without extracellular siderophores, albeit at a considerably slower rate, unless the intracellular iron stores (represented by intracellular siderophores) are depleted. These results are in accordance with ΔsidF displaying attenuated virulence and ΔsidA being avirulent in murine aspergillosis models. Considering that IA still represents a major hindrance to a favorable outcome in patients undergoing HSCT, and wide use of antifungal regimes elicit resistant strains, new therapeutic targets are warranted. Although apo-Tf has already been effectively tested in conditions with eLPI presence, its medical use is limited. Moreover, eLPI can be targeted by iron chelators. Yet, application of these drugs in the setting of HSCT is still controversial.

In agreement with previous reports, deferoxamine served as xenosiderophore for AFU, thus promoting fungal outgrowth even in samples, which were negative for eLPI. In contrast, outgrowth was absent in cultures containing deferasirox, proposing that iron chelated by deferasirox does not serve as a xenosiderophore, at least for AFU (Figure 2E). Deferasirox application has previously been shown to have an activity against AFU in vitro and in vivo models. Notably, application of deferasirox for mucormycosis in mice and humans yielded inconsistent outcomes, so that further work up is warranted and data must be interpreted with caution. Moreover, pharmacologic increase of hepcidin levels, being the master regulator of systemic iron homeostasis, may also turn out to be a possible treatment option for the prevention of eLPI. Preclinical studies have shown the potential of minihepcidin therapy in reversing bacterial infections.

In summary, our study provides evidence on the putative role of eLPI for promoting IA, being the most common invasive fungal infection in patients undergoing allogeneic HSCT. To further validate the value of eLPI as a biomarker for IA, eLPI measurements in patients undergoing HSCT suffering from IA vs noninfected patients are warranted, maybe including quantitative measurements instead of semiquantitative, as used in this study and in the ALLIVE trial. Our studies recommend eLPI measurement as a predictor for the risk for IA and will potentially pave the way for therapeutic interventional trials aiming at scavenging eLPI, and thereby improving the outcome of HSCT patients at risk.
**Figure 1.**

A. Images showing the eLPI levels (0 eLPI: 1.1, 7 eLPI: 2.1, 14 eLPI: 0.0, 21 eLPI: 0.0, 100 eLPI: 0.0).

B. Diagrams illustrating fungal outgrowth and eLPI presence.

C. Graphs depicting the OR and CI values for eLPI presence, eLPI concentration, and Tf-Sat >75%.
Figure 1. Presence of enhanced labile plasma iron in sera of patients undergoing allogeneic hematopoietic stem cell transplantation determines AFU outgrowth. Human serum samples of 29 representative ALLIVE participants, which were collected during the consecutive phases of allogeneic hematopoietic stem cell transplantation (HSCT) were screened for AFU outgrowth. AFU spores (5 × 10⁶ spores/mL) were seeded in RPMI containing 10% ferric iron-spiked human plasma (0-50 μM Fe³⁺) supplemented with 25 mg/mL apo-Tf or holo-Tf, respectively (original magnification x20; bars represent 30 μm). (B) Removal of iron from holo-Tf by AFU was studied. AFU spores (5 × 10⁶/mL) were inoculated into RPMI containing 5 μM Fe³⁺ and 2 mg/mL apo- or holo-Tf in 6-well plates. Holo-Tf:apo-Tf conversion in culture supernatant was monitored by absorbance measurements at 280 nm (A₂₈₀corr) and 460 nm (A₄₆₀corr) (B) and urea-polyacrylamide gel electrophoresis (C) at indicated times. Uninoculated RPMI media with apo- and holo-Tf were used as controls in both assays. (D) eLPI use by AFU mutant strains with defects in iron acquisition systems. Representative microscopy images showing the growth pattern of wild-type (Wt), ΔsidF, and ΔsidA conidia in RPMI containing 10% ferric iron-spiked human plasma after 48-hour culture (original magnification x20; bars represent 30 μm). (E) Influence of clinically applicable iron chelators on AFU outgrowth in eLPI-deficient and eLPI-positive serum cultures. AFU spores (5 × 10⁶/mL) were cultured in RPMI containing 10% ferric iron-spiked human plasma supplemented with 100 μM deferoxamine (DFO) or 200 μM deferasirox (DFX). Control cultures were DMSO treated. Photographs were taken 48 hours after inoculation. Representative images of fungal cultures are shown (original magnification x20; bars represent 30 μm). Experiments shown in panels A, D, and E were performed at least in 6 replicates, all showing consistent results.

Figure 2. AFU cannot retrieve iron from holo-transferrin, and enhanced labile plasma iron uptake depends on fungal siderophores. (A) Role of holo-Tf as an iron source for AFU growth. Representative microscope images of AFU cultures taken 48 hours after inoculation (5 × 10⁴ spores/mL) of RPMI containing 10% ferric iron-spiked human plasma (0-50 μM Fe³⁺) supplemented with 25 mg/mL apo-Tf or holo-Tf, respectively (original magnification x20; bars represent 30 μm). (B) eLPI use by AFU mutant strains with defects in iron acquisition systems. Representative images of fungal cultures are shown (original magnification x20; bars represent 30 μm).
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Authorship
Contribution: V.P., M.W., P.T., H.H., U.P., and I.T. conceived the project, designed experiments, and analyzed and interpreted data; V.P., P.T., H.H., and I.T. wrote the manuscript; V.P., P.T., M.S., R.O., S.B., and L.L. performed experiments; G.W. provided intellectual input and edited the manuscript; D.O.-H. provided A fumigatus wild-type isolates and intellectual input; M.W., D.W., and U.P., designed and supervised the ALLIVE trial, provided patients’ material and details, and provided intellectual input; and all authors read and corrected the manuscript.

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