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Human plasma protein levels alter the in vitro antifungal activity of caspofungin: An explanation to the effect in critically ill?

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Abstract

Background: Recent studies have shown low caspofungin concentrations in critically ill patients. In some patients, the therapeutic target, area under the total plasma concentration curve in relation to the minimal inhibition concentration (AUC_{tot}/MIC), seems not to be achieved and therapeutic drug monitoring (TDM) has been proposed. Caspofungin is highly protein-bound and the effect of reduced plasma protein levels on pharmacodynamics has not been investigated.

Objectives: Fungal killing activity of caspofungin in vitro was investigated under varying levels of human plasma protein.

Methods: Time-kill studies were performed with clinically relevant caspofungin concentrations of 1-9 mg/L on four blood isolates of *C. glabrata*, three susceptible and one strain with reduced susceptibility, in human plasma and plasma diluted to 50% and 25% using Ringer's acetate.

Results: Enhanced fungal killing of the three susceptible strains was observed in plasma with lower protein content ($p < .001$). AUC_{tot}/MIC required for a 1 $\log_{10}$ CFU/ml kill at 24 h in 50% and 25% plasma was reduced with 36 + 12 and 80 + 9%, respectively. The maximum effect was seen at total caspofungin concentrations of 4–9 × MIC. For the strain with reduced susceptibility, growth was significantly decreased at lower protein levels.

Conclusions: Reduced human plasma protein levels increase the antifungal activity of caspofungin in vitro, most likely by increasing the free concentration. Low plasma protein levels in critically ill patients with candidemia might explain a better response to caspofungin than expected from generally accepted target attainment and should be taken into consideration when assessing TDM based on total plasma concentrations.

KEYWORDS
antifungal activity, caspofungin, free concentration, plasma protein, protein-binding

1 | INTRODUCTION

Candidemia, the most common form of invasive candidiasis, has a major impact on morbidity and mortality in intensive care unit (ICU) patients.[1] Caspofungin, an antifungal agent with a high protein binding of approximately 97%,[2-4] is currently together with other echinocandins recommended as first-line therapy in invasive candidiasis.[5,6] However, the critically ill have altered pathophysiological processes that may influence antifungal pharmacokinetics (PK), including changes in drug-protein binding. Albumin is the major drug-binding protein, and hypoalbuminemia is common in this patient group due to protein losses via capillary leakage, decreased production and dilution by fluid resuscitation.[7-11]

Recent pharmacokinetic/pharmacodynamic (PK/PD) analyses have indicated that caspofungin has a relatively high degree of target attainment expressed as area under the free concentration curve divided by the MIC (AUC_{free}/MIC) for a net stasis effect.[12] However, clinical studies on intensive care patients at considerable risk for candidemia indicate lower AUCs calculated from total plasma concentrations (AUC_{tot})[8-10] than those employed in the PK/PD analysis above.[12] Furthermore, these studies found that concentrations necessary to reach a killing effect were not always achieved.[9,10]

Total concentration is the sum of free and protein-bound concentrations by which only the unbound free fraction of the drug is generally considered pharmacologically active.[4,13,14] Therefore, one explanation to the favourable outcome in the clinical studies[15-17] could be a higher free drug concentration than deduced from the total concentration due to a reduction in the number of binding sites in hypoalbuminaemia. The importance of free concentration for the antimicrobial effect has been well demonstrated for antibiotics,[13] whereas studies with antifungals are limited. There are in fact data questioning the importance of free antifungal drug concentrations. An analysis of pooled data from six prospective clinical studies found echinocandin treatment effective in candidemia caused by *Candida parapsilosis* despite MIC values well above calculated free drug concentrations.[18,19] Similarly, it has been experimentally shown that the highly protein-bound antifungal posaconazole exerts its effect on different *Candida* spp. at calculated free concentrations lower than the MIC values, suggesting a rapid and direct flux of the drug from plasma proteins to the fungal target.[20]

For these reasons, analysis of the free concentration of caspofungin at different levels of albumin and other plasma proteins is of interest. At present, such an analysis is hampered by technical difficulties and commercial methods are not available. The present study aimed to investigate the influence of plasma protein levels on the PD activity of caspofungin in human plasma to explore the impact of free antifungal concentration. Time-kill experiments were performed using four clinical blood isolates of *C. glabrata* and a range of clinically relevant caspofungin concentrations. In addition, experiments were performed to assess the potential impact of pH on caspofungin PD, as hydrogen ion concentration may affect protein binding in vitro as well as clinically in critically ill patients.

2 | MATERIAL AND METHODS

2.1 | Strains and antifungal susceptibility testing

Candida glabrata strains were used as representatives for the *Candida* spp. due to their inability to develop pseudohyphae, thereby increasing the precision of the viable count. Four clinical blood isolates, three susceptible strains and one with reduced susceptibility, were obtained from the Department of Clinical Microbiology, Karolinska University Hospital, Stockholm, Sweden. Antifungal susceptibility testing was performed using Sensititre Yeast One® (Thermo Fisher Diagnostics AB). MIC values for the isolates were 0.06 (ARU459, ARU455), 0.12 (ARU767) and 1 mg/L (ARU792).

2.2 | Antifungal agent

Stock solutions of caspofungin (Sigma-Aldrich AB) were prepared in sterile water according to the manufacturer's instructions and diluted to the desired concentration in phosphate-buffered saline (PBS) immediately before use.

2.3 | Plasma

Plasma from three blood donors was frozen without any previous treatment and used for all the time-kill experiment. Prior to the start of each experiment, plasma was thawed and carefully warmed to $36 \pm 1°C$.

2.4 | Time-kill experiments

2.4.1 | Effects of plasma protein levels

Initially, the isolates were cultured on Sabouraud dextrose agar (SDA) plates in $36 \pm 1°C$ for 48 h. Three colonies were transferred to 7.5 ml yeast extract peptone dextrose (YPD) medium and incubated with agitation overnight at $36 \pm 1°C$. An aliquot of 200 μl from the YPD-containing tube was serially diluted in PBS and transferred to the plasma-containing time-kill tubes resulting in a starting inoculum of $2–7 \times 10^4$ CFU/ml. Caspofungin was added to clinically relevant total concentrations of 1.0, 2.0, 5.0 and 9.0 mg/L resulting in a final volume of 2 ml. Identical set-ups were performed in plasma diluted using a Ringer's Acetate solution (Ri-Ac) to 50% and 25%. The time-kill tubes were placed on a shaker and incubated at $36 \pm 1°C$. Aliquots of 250 μl were sampled at 0, 2, 6 and 24 h, serially diluted in PBS and 100 μl inoculated onto SDA plates for viable counts. The plates were incubated at $36 \pm 1°C$ for 24 h. The lower limit of detection was 10 CFU/ml. All experiments were performed in triplicate and on separate days. Antifungal carryover effects were assessed by placing the aliquots as a drop on the SDA plates, allowed to soak into the agar, after which the sample was spread. If colony count was reduced adjacent

to where the aliquot was placed, the plate was divided into sections and the section with reduced colony count was excluded.

In separate experiments with identical set-ups as above, caspofungin concentrations of 0.06, 0.12, 0.25 and 0.50 mg/L were used to define the concentration resulting in a net static effect, that is neither killing nor growth had occurred at 24 h in relation to the starting inoculum. These concentrations are referred to as subclinical concentrations.

2.4.2 | Effect of changes in pH

The effect of pH on PD was explored to detect potential effects caused by Ri-Ac and carbon dioxide release from the plasma in vitro, as well as possible pH changes in critically ill patients. In screening experiments, the pH range was established by measurements in each medium planned for the time-kill experiments, that is 100%, 50% and 25% plasma, with and without caspofungin. To assess the impact of pH on caspofungin PD, time-kill experiments with caspofungin were performed, as described above, with concentrations of 0.5 and 1.0 mg/L expected to give free concentrations close to the MICs of the susceptible strains. The initial pH was set to 6.80, 7.15, 7.40 and 7.70, with experiments executed in 100% and 25% plasma. Hydrochloric acid or sodium bicarbonate was added as needed to achieve the desired pH.

2.5 | Statistics and calculations

Colony counts displayed in text and figures are expressed as mean $\log_{10}$ CFU/ml ±standard deviation (±SD) unless otherwise indicated. $\log_{10}$ values of samples with a viable count <10 CFU/ml were set to 0.5. The 24-h AUC based on total concentrations required for 1 $\log_{10}$ CFU/ml kill at 24-h (AUC_{tot}) for the susceptible strains was calculated by identifying two caspofungin concentrations: the highest concentration that did not attain 1 $\log_{10}$ CFU/ml kill compared to the starting inoculum and the lowest caspofungin concentration that resulted in more than 1 $\log_{10}$ CFU/ml kill. Utilising the results from these two concentrations, the caspofungin concentration resulting in a 1 $\log_{10}$ CFU/ml kill was derived by linear extrapolation. From this concentration, the 24-h AUC then was computed by multiplying the concentration by 24.

Mixed models with the unstructured covariance structure for the repeated effect were used to statistically analyse differences in growth. Mixed models were preferred to repeated measure ANOVA since Mauchly's test of sphericity demonstrated that at least one of the assumptions for the compound symmetry covariance structure was violated. Differences in viable count at the end of the experiment at 24 h for the various caspofungin concentrations were additionally analysed using the independent t test, two-tailed. Statistical significance was defined as a p-value of <.05. SPSS software (version 24; IBM®Armonk) was employed to analyse the data.
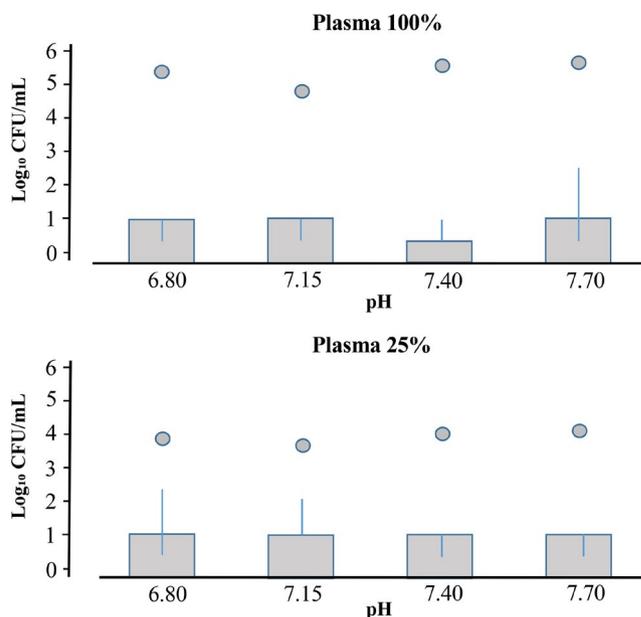

FIGURE 1 Fungal growth after 24 h exposure to caspofungin 1 mg/L in 100% and 25% plasma at different pH at 0 h using a starting inoculum of $\log_{10}$ 4.3 ± 0.2 CFU/ml. Because values are close to the detection limit and based on only one experiment per strain, results are presented as median (bars) and range (whiskers) in the three strains with MICs of 0.06-0.12 mg/L (ARU455, ARU459 and ARU767). Individual values are presented for the strain with MIC of 1 mg/L (ARU792) (circles)

2.6 | Ethics statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as the research in this article related to microorganisms.

3 | RESULTS

3.1 | Time-kill experiments

3.1.1 | The effect of pH

The screening experiments showed a difference in pH at 0 h with values of 7.60 ± 0.01, 7.54 ± 0.03 and 7.09 ± 0.09 in 100%, 50% and 25% plasma, respectively. Addition of varying amounts of caspofungin did not affect the pH. Fungal growth of the four strains was not affected by manipulating the pH at 0 h. Growth after 24 h exposure to caspofungin at the lowest clinical concentration of 1 mg/L in 100% and 25% plasma showed no significant differences in viable count (Figure 1). Similar results were observed at 2 and 6 h and at all time points with caspofungin in the subclinical concentration of 0.5 mg/L. Detailed information on the effect of pH is presented in the Supplementary data (Supplementary information and Figures

S1A–C). The effect of pH was minimal; therefore, it was left unadjusted in the pivotal time-kill experiments.

3.1.2 | The effect of plasma protein levels

The results of lowering the plasma fraction from 100% to 50% and 25% in time-kill experiments with ARU455, 459 and 767 are displayed in Figure 2. The growth of the three strains in caspofungin-free controls did not differ based on the plasma protein content. The enhanced fungal killing in media with lower plasma fractions increased over the 24-h period of the experiments and differences in killing were highly significant when the three strains were analysed together ($p < .001$). When analysed separately, the effect was also significant ($p < .05$) for the strains ARU455 and ARU767. ARU459 exhibited a similar trend, albeit non-significant. The effect of decreasing the plasma protein content was more evident at the lower clinical caspofungin concentrations of 1 and 2 mg/L than at higher concentrations. To reduce

inter-strain variation, the results for ARU792 with reduced susceptibility are shown separately in Figure 3. There was no fungal killing but, similarly to ARU455 and ARU767, growth was significantly reduced at the lower protein levels ($p < .05$).

Time-kill curves for ARU455, 459 and 767 after exposure to subclinical caspofungin concentrations are depicted in Figure 4. There was an increased killing in all strains when plasma was diluted also at these concentrations. At concentrations, at which a net static effect occurred, inhibition of growth was similarly affected (Table 1).

Total caspofungin concentration achieving a 1 $\log_{10}$ CFU/ml reduction from the starting inoculum at 24 h in the different protein-containing media and the effect on AUC_{tot}/MIC in the fully susceptible strains are listed in Table 2. The AUC_{tot}/MIC in 50% was reduced with 36 ± 12 and in 25% plasma with 80 ± 9%. For the strain with reduced susceptibility, ARU792, 1 $\log_{10}$ CFU/ml kill at 24 h was not achieved even at the highest concentration of 9 mg/L. Although >1 $\log_{10}$ CFU/ml lower than the control, there was an increase of 0.2 $\log_{10}$ CFU/ml at this concentration in 100% plasma (Figure 3). This
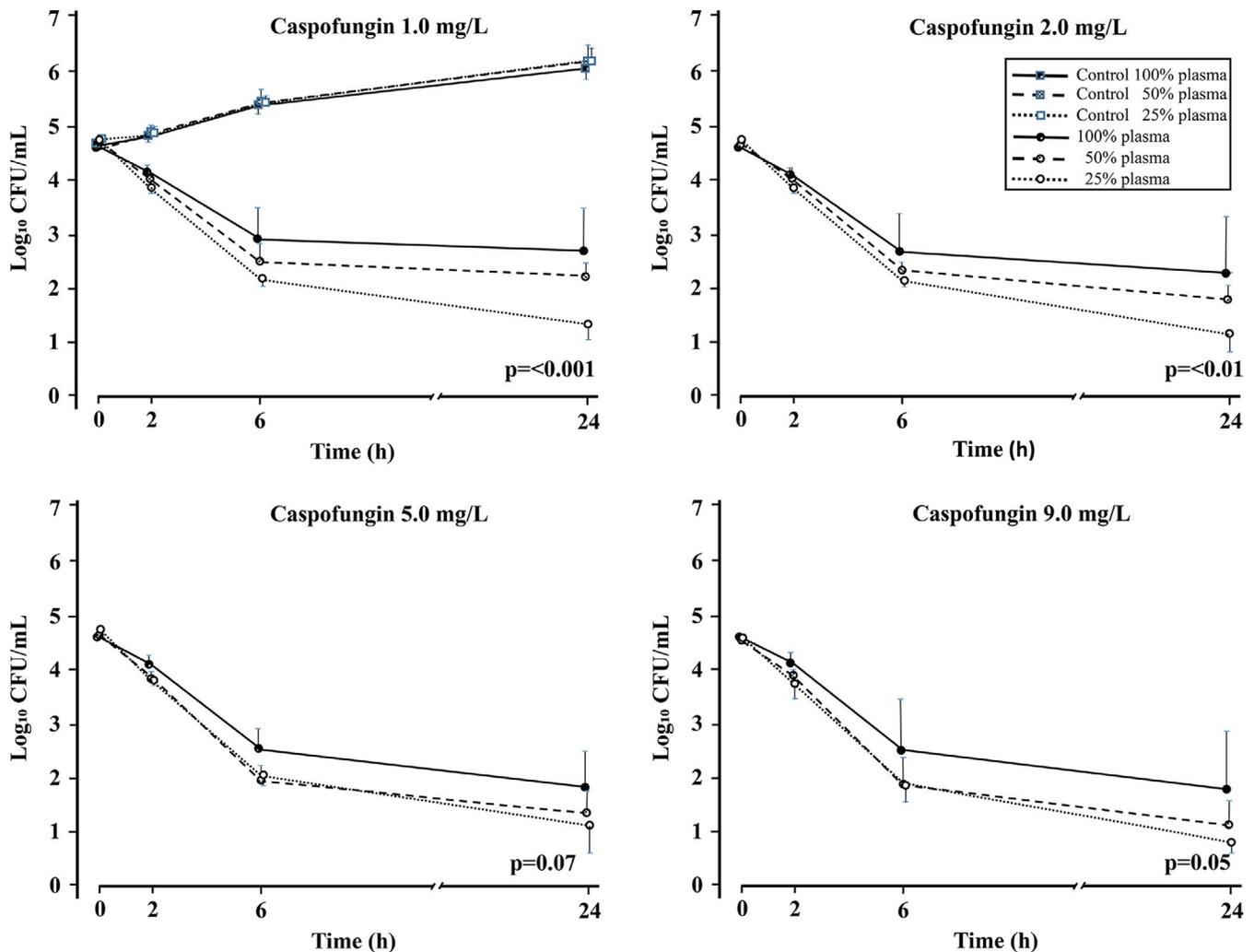

FIGURE 2 Twenty-four-hour time-kill curves for ARU455, ARU459 and ARU767 in medium containing plasma 100%, 50% and 25% and caspofungin at clinically relevant concentrations of 1.0, 2.0, 5.0 and 9.0 mg/L. The drug-free controls are depicted in the upper left section of the figure. For each strain, the experiments in different media were performed in triplicate. Data are mean ± SD for the three strains. The *p*-value represents differences in growth in the varying media at 24 h (unpaired *t* test)
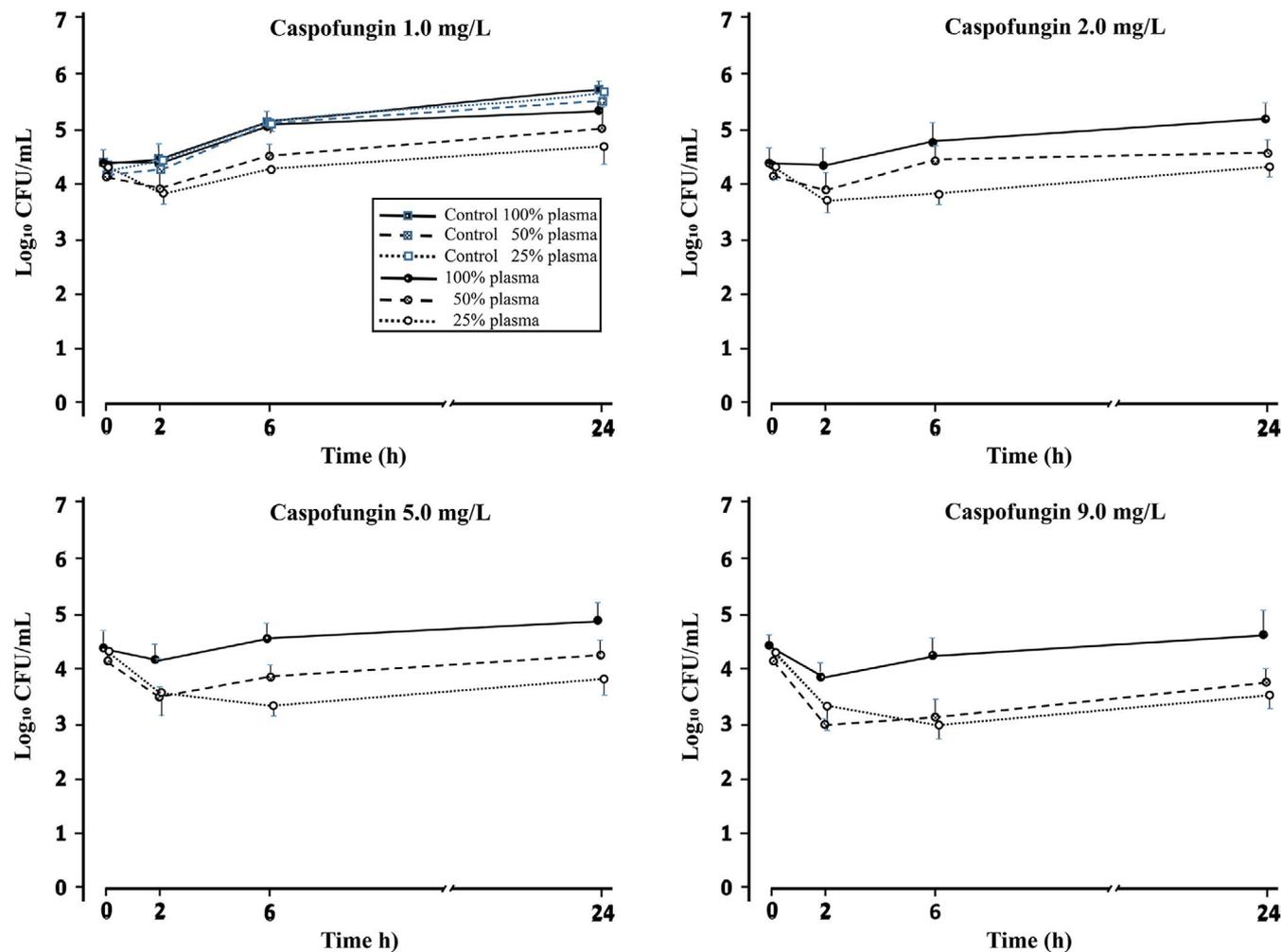
FIGURE 3 Twenty-four-hour time-kill curves for ARU792 with MIC 1 mg/L in medium containing plasma 100%, 50% and 25% and caspofungin at clinically relevant concentrations of 1, 2, 5 and 9 mg/L. The drug-free controls are depicted in the upper left section of the figure. The experiments in different media were performed in triplicate. Data are mean ± SD for the three experiments with this strain demonstrating the interexperimental variation in these experiments

magnitude of response was seen at a concentration of 5 mg/L in 50% plasma and of 1–2 mg/L in 25% plasma, suggesting a similar plasma protein-dependent effect also in this strain.

4 | DISCUSSION

Our results indicate that caspofungin has an increased fungal killing activity in terms of a lower AUC_{tot}/MIC to attain a 1 $\log_{10}$ CFU/ml kill at 24 h in the presence of lower plasma protein concentrations. This effect is most likely caused by a higher fraction of free unbound caspofungin as the total concentration of caspofungin is the same. In vivo unbound caspofungin is more available for elimination, probably explaining the association with low AUCs and low plasma albumin levels seen in previous studies.[10,21] However, our data indicate that fungal killing is increased in relation to what can be expected from the low AUC_{tot} at low plasma protein levels.

Critically ill patients treated with caspofungin have maximal total plasma concentrations (C_{max}) in the range of 1–9 mg/L and trough

concentrations in the range of 0.5–2.5 mg/L.[10] The effect of reducing protein levels is most pronounced at low caspofungin concentrations, (Figures 2 and 4). If the differences in growth between 100% and 25% plasma at different caspofungin concentrations for the four strains are depicted, a gradual relationship emerges (Figure 5). The enhanced activity for the susceptible strains is limited at the lowest and the highest concentrations, 0.06 and 9 mg/L, respectively. This is likely due to limited or no antifungal activity at the lowest concentration and a near-maximal activity already achieved in undiluted plasma at the highest concentration. There is an increase in antifungal enhancement in the 25% plasma up to a maximal effect at concentrations of 4–9 × MIC after which there is a gradual fall. There is a similar MIC-dependent manifestation in the strain with reduced susceptibility, but concentrations above 10 × MIC for this particular strain are not clinically relevant and therefore not tested. Our results support the discussion by Zeitlinger et al.[13] that the most pronounced PD effect of protein binding occurs at concentrations relatively close to the MIC.

Plasma protein levels are mirrored by the albumin concentration, the major drug transporter with concentrations in healthy individuals
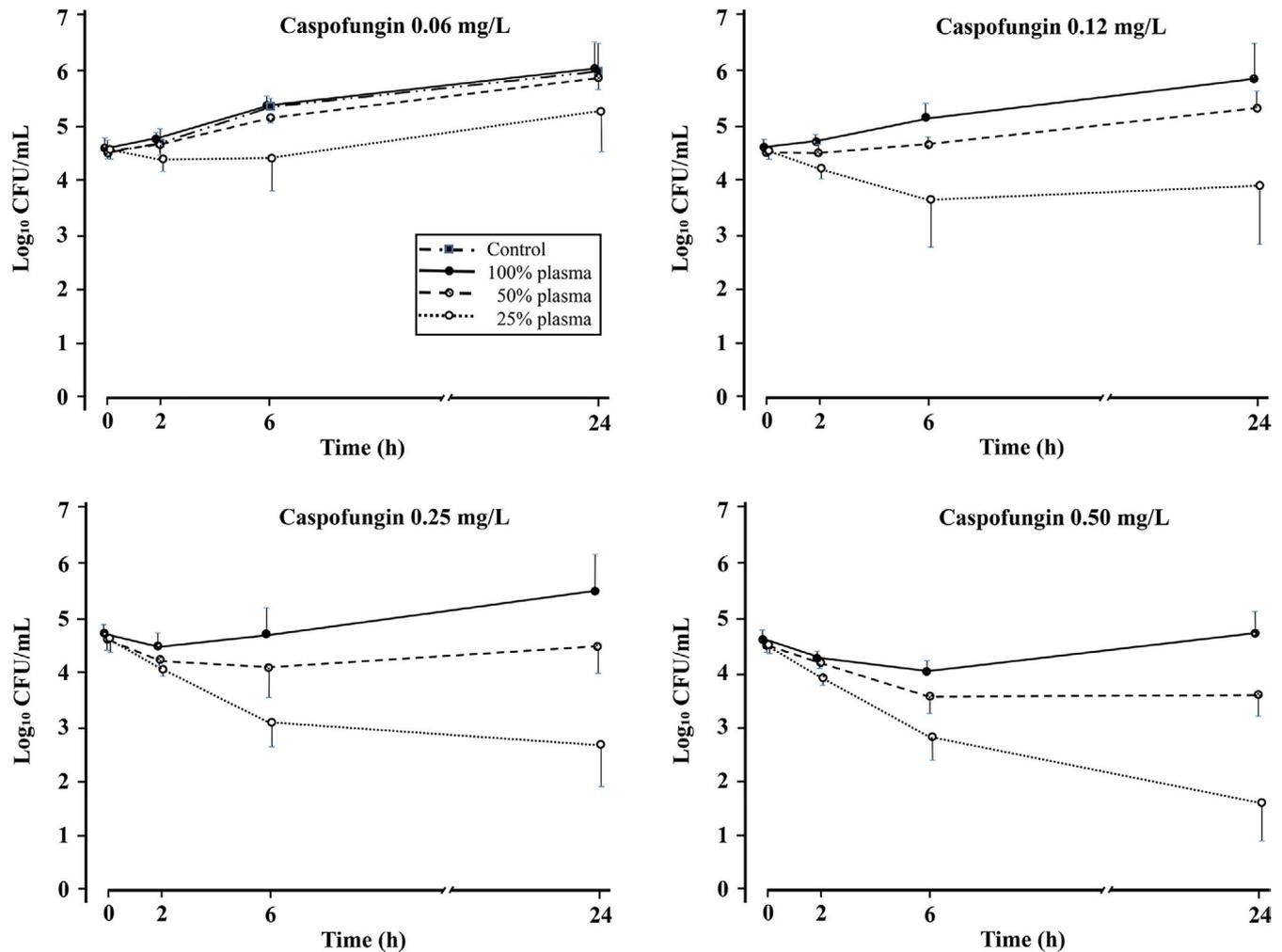
FIGURE 4 Twenty-four-hour time-kill curves for ARU455, ARU459 and ARU767 in medium containing plasma 100%, 50% and 25% and caspofungin at subclinical concentrations of 0.06, 0.12, 0.25 and 0.5 mg/L. Data are mean ± SD for the three strains. Because there were no differences in growth in the drug-free controls in the respective medium, only one mean of the three controls is depicted in the upper left section of the figure. No formal statistics were applied since this was a secondary analysis and only one experiment was performed for each strain in the respective medium

TABLE 1 Caspofungin concentration interval resulting in net static effect at 24 h for the strains ARU455, 459 and 767

	ARU455 MIC 0.06 mg/L	ARU459 MIC 0.06 mg/L	ARU767 MIC 0.12 mg/L
Plasma 100%, mg/L	0.25–0.5[a]	0.25–0.5	0.5–1
Plasma 50%, mg/L	0.12–0.25	0.12–0.25	0.25–0.5
Plasma 25%, mg/L	0.06–0.12	0.06–0.12	0.06–0.12

[a]The intervals cover the caspofungin concentrations that resulted in a net static effect, that is neither killing nor growth had occurred at 24 h in relation to the starting inoculum.

of 35–50 g/L.[11] In the general ICU population with suspected severe sepsis, albumin levels are decreased to values often lower than 30 g/L and even more so in patients with suspected fungal infection.[7] Muilwijk et al.[8] found that more than 80% had albumin values of <24 g/L. In a similar study by our group most patients had values below 20 g/L, some close to 10 g/L,[10] that is albumin levels corresponding to a plasma protein reduction of approximately 75%, the magnitude of the lowest plasma protein content used in the current study.

The PD 24-h target can be expressed as either a net stasis or a 1 $\log_{10}$ CFU/ml kill effect.[4] In the present study, a 1 $\log_{10}$ CFU/ml kill was chosen based on the assumption that the superior effect of echinocandins is due to their fungicidal capacity as opposed to fluconazole's fungistatic effect.[17] The AUC_{tot}/MIC to attain a 1 $\log_{10}$ CFU/ml kill at 24 h in 100% plasma in our study is of similar magnitude as previously shown by Andes et al in a neutropenic mouse model, in which it was calculated from the free unbound concentration and the AUC_{free}/MIC.[4] In the clinical study previously published by our

TABLE 2 Change in pharmacodynamics as represented by 1 $\log_{10}$ CFU/ml kill 24 h at different plasma protein levels in the three caspofungin susceptible *Candida glabrata* strains: ARU455, ARU459 and ARU767

Plasma protein levels	ARU455 MIC 0.06 mg/L			ARU459 MIC 0.06 mg/L			ARU767 MIC 0.12 mg/L			All three strains mean ± SD		
	100%	50%	25%	100%	50%	25%	100%	50%	25%	100%	50%	25%
Total caspofungin concentration achieving 1 $\log_{10}$ CFU/ml reduction[a] at 24 h, mg/L	0.67	0.54	0.21	0.75	0.44	0.09	1.25	0.66	0.20	0.89 ± 0.31	0.55 ± 0.11	0.17 ± 0.07
1 $\log_{10}$ CFU/ml kill 24-h AUC$_{tot}$, mg × h/L	16.2	13.0	5.1	18.0	10.5	2.1	29.9	15.9	4.8	21.4 ± 7.4	13.1 ± 2.7	4.0 ± 1.7
1 $\log_{10}$ CFU/ml kill 24-h AUC$_{tot}$/MIC	270	217	85	300	175	35	273	175	40	273 ± 25	175 ± 42	54 ± 27
Reduction of AUC$_{tot}$ from 100% plasma	–	20%	68%	–	42%	88%	–	47%	84%	–	36 ± 12%	80 ± 9%
Increase in fungal killing	–	24%	216%	–	71%	746%	–	88%	521%	–	61 ± 27%	494 ± 217%

[a]From the starting inoculum.

group, all patients reached this target if the MIC of the causative *C. glabrata* strain was ≤0.06 mg/L. However, this covered only 80% of the *C. glabrata* strains.[10] Because there was a significant correlation between AUC$_{tot}$ and albumin values in that study, most of the patients with low AUCs had albumin levels of <20 g/L. The results from our time-kill experiments show an increase in killing of approximately 60% at a plasma protein reduction of 50% and an almost fivefold increase at a reduction of 75% (Table 2). On the other hand, caution is recommended in quantitative comparisons between static in vitro conditions, devoid of elimination processes and the more complex in vivo situation, where metabolic clearance of the unbound drug determines equilibrium concentrations. Hence, if we assume that the metabolic clearance of unbound caspofungin during the aforementioned disease conditions remains the same, a limited change of free concentration at steady state would be expected, whereas the total concentration, as monitored by therapeutic drug monitoring (TDM), would decrease appreciably with lowering of binding protein. In line with this, our data indicate that a higher antifungal activity than can be expected from AUC$_{tot}$/MIC might occur in patients with low albumin values. These data may explain that a proportion of patients not reaching the AUC$_{tot}$/MIC target have sufficient free concentrations and AUC$_{free}$/MIC to eliminate their infection. Presumably, this will mainly be of importance for the effect on *C. glabrata* strains with MICs within the higher range of the MIC distribution.

C. glabrata strains were used in the current study because of their lack of pseudohyphae formation resulting in higher precision of the viable count. However, it seems plausible that similar effects in the PD activity will be achieved also in other *Candida* species. The results further indicate that protein levels affect the activity on strains with reduced susceptibility.

The possibility of poor target attainment at low concentrations, especially in infections with *Candida* spp. with reduced susceptibility and the risk of a paradoxical effect at high concentrations has led to TDM recommendations of caspofungin and other echinocandins.[9,10,22] Our findings suggest that plasma protein levels should also be considered in the clinical assessment of total plasma caspofungin concentrations when performing TDM. Based on the discussion above, dose adjustment might not be needed despite an observed decrease in the total concentration.

If the current results are extrapolated to *C. parapsilosis*, the increase in killing caused by less protein binding is insufficient to explain Kontoyiannis et al.'s[18] positive results. Only 50% of the *C. parapsilosis* strains have MICs of ≤0.25 mg/L and the AUC/MIC for *C. parapsilosis* has been shown to be twice as high as that for *C. glabrata*, yet, with large variations among the strains.[4,19] The large deviation in target concentrations may explain the positive effect in some *C. parapsilosis* strains. However, low virulence and catheter removal are probably other factors for favourable outcome despite poor target attainment.[23,24] Another possibility might be a flux from the protein-bound fraction to the fungal target enzyme, as hypothesised for posaconazole.[20]

The abundance of charged amino acid residues on the albumin molecule makes it an effective plasma buffer and the presence or
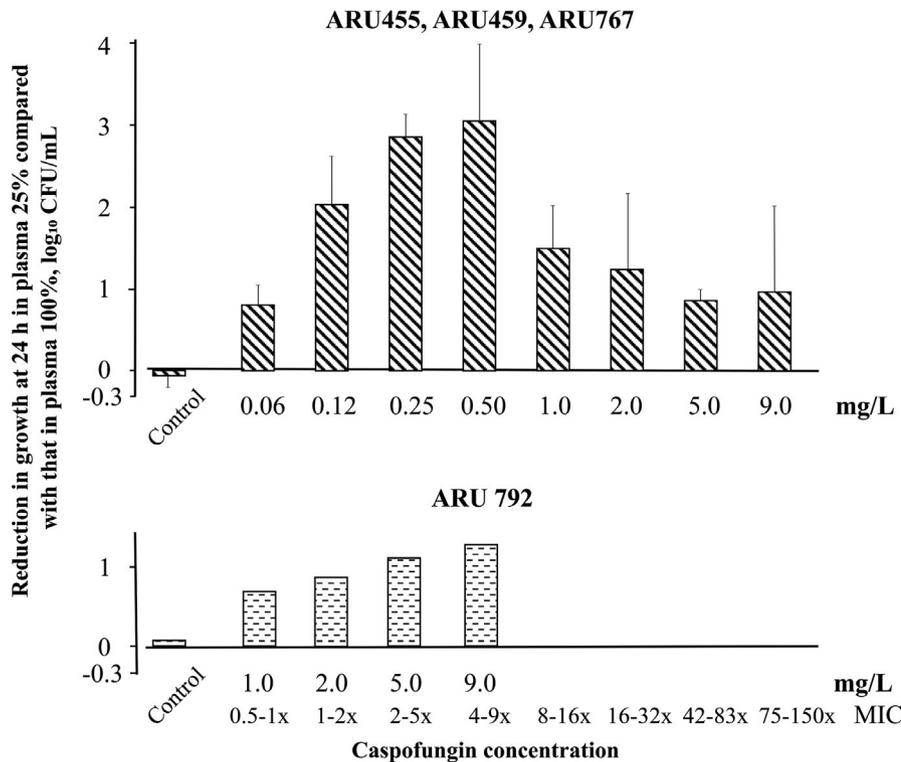
FIGURE 5 Increased killing expressed as reduction in growth at 24 h in plasma 25% compared with that in plasma 100% for the four *C. glabrata* strains at different caspofungin concentrations, expressed in both mg/L and in reference to the MICs of the strains to which the x-axes have been adjusted. The MICs for the fully susceptible strains ARU455, ARU459 and ARU467 are 0.06, 0.06 and 0.12 mg/L, respectively, and for the ARU792 strain with reduced susceptibility 1 mg/L

absence of hydrogen ions could potentially affect the protein binding of caspofungin.[11] However, despite using near-net static caspofungin concentrations of 0.5 and 1 mg/L, the effects of which are vulnerable to protein binding changes,[13] differences in pH did not result in any substantial differences in fungal killing in any of the strains. Our results imply that acid-base disturbances, often identified in critically ill patients, will not significantly affect PD and the relationship between free and unbound caspofungin.

Our study has some limitations that may have influenced the results. One limitation is that free and unbound caspofungin was not analysed but the assumption that the free fraction is increased when plasma proteins levels are reduced seems reasonable and clinically, the effect on fungal growth is a parameter of substantial interest. In this in vitro model, the reduction of plasma proteins is achieved by dilution. A limitation when extrapolating our results to the in vivo situation is that in the critically ill, the magnitude of reduction of the different plasma proteins is not the same and albumin may be more reduced than other plasma proteins. Conversely, balanced solutions, such as Ri-Ac used in the present study, are often applied for fluid resuscitation in septic patients and often in large volumes.[25] Moreover, it is not in detail known to which proteins caspofungin binds. On this point, a strength with our model is that all plasma proteins are reduced to a similar degree by the dilution. Finally, our findings offer one possible explanation to an effect in these medically complex patients not reaching target concentrations. Other theoretically potential explanations with some in vitro evidence might be interactions with the host defence, rapid flux from plasma proteins to the fungal enzyme, or in the case of hyphae-producing species, albumin-induced delivery to germinating hyphae.[20,26-28] Furthermore, a human target has not been established but is derived

from studies in mice in which the deviation was substantial making possible lower target than previously stated.[4]

In conclusion, reduced plasma protein levels in vitro increase the antifungal activity of caspofungin and lower the pharmacodynamic target AUC_{tot}/MIC, most likely by increasing the free concentration. Thus, low plasma protein levels may constitute one explanation to the successful response in clinical trials on patients with candidemia that exceeds what can be expected from generally accepted target attainment. If TDM is performed, the present results suggest that plasma protein levels should be considered when assessing the expected PD activity based on the total caspofungin concentration. Ultimately, free drug concentrations should be measured when possible.

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CONFLICT OF INTEREST
JS has received lecture fees from Gilead and Unimedic Pharma and reimbursement for expert testimonies from Unimedic Pharma. MF has received lecture fees from Pfizer, Gilead and Unimedic Pharma. All other authors: none to declare.

AUTHOR CONTRIBUTIONS
Siri Kurland: Conceptualization (equal); Data curation (equal); Formal analysis (lead); Investigation (equal); Methodology (lead); Project administration (lead); Writing-original draft (equal); Writing-review & editing (equal). **Elisabeth Löwdin:** Conceptualization (equal); Methodology (equal); Writing-review & editing (equal). **Mia Furebring:** Conceptualization (equal);

Methodology (equal); Project administration (equal); Writing-review & editing (equal). **Ayda Shams:** Data curation (equal); Investigation (equal); Methodology (lead); Writing-review & editing (equal). **Erja Chryssanthou:** Formal analysis (equal); Methodology (equal); Writing-review & editing (equal). **Pernilla Lagerbäck:** Project administration (equal); Resources (equal); Writing-review & editing (equal). **Thomas Tängdén:** Funding acquisition (supporting); Resources (equal); Writing-review & editing (equal). **Olof Breuer:** Formal analysis (equal); Writing-review & editing (equal). **Jan Sjölin:** Conceptualization (equal); Formal analysis (lead); Funding acquisition (lead); Supervision (lead); Writing-original draft (equal); Writing-review & editing (equal).

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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