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Using (1,3)-β-D-glucan concentrations in serum to monitor the response of azole therapy in patients with eumycetoma caused by *Madurella mycetomatis*

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Abstract

Introduction: (1,3)-β-D-glucan is a panfungal biomarker secreted by many fungi, including *Madurella mycetomatis*, the main causative agent of eumycetoma. Previously we demonstrated that (1,3)-β-D-glucan was present in serum of patients with eumycetoma. However, the use of (1,3)-β-D-glucan to monitor treatment responses in patients with eumycetoma has not been evaluated.

Materials and Methods: In this study, we measured (1,3)-β-D-glucan concentrations in serum with the WAKO (1,3)-β-D-glucan assay in 104 patients with eumycetoma treated with either 400 mg itraconazole daily, or 200 mg or 300 mg fosravuconazole weekly. Serial serum (1,3)-β-D-glucan concentrations were measured at seven different timepoints. Any correlation between initial and final (1,3)-β-D-glucan concentrations and clinical outcome was evaluated.

Results: The concentration of (1,3)-β-D-glucan was obtained in a total of 654 serum samples. Before treatment, the average (1,3)-β-D-glucan concentration was 22.86 pg/mL. During the first 6 months of treatment, this concentration remained stable. (1,3)-β-D-glucan concentrations significantly dropped after surgery to 8.56 pg/mL. After treatment was stopped, there was clinical evidence of recurrence in 18 patients. Seven of these 18 patients had a (1,3)-β-D-glucan concentration above the 5.5 pg/mL cut-off value for positivity, while in the remaining 11 patients, (1,3)-β-D-glucan concentrations were below the cut-off value. This resulted in a sensitivity of 38.9% and specificity of 75.0%. A correlation between lesion size and (1,3)-β-D-glucan concentration was noted.

Conclusion: Although in general (1,3)-β-D-glucan concentrations can be measured in the serum of patients with eumycetoma during treatment, a sharp decrease in β-glucan concentration was only noted after surgery and not during or after antimicrobial treatment. (1,3)-β-D-glucan concentrations were not predictive for recurrence

and seem to have no value in determining treatment response to azoles in patients with eumycetoma.

KEYWORDS
azole therapy, biomarkers, eumycetoma, glucans, *Madurella mycetomatis*, prognostic

1 | INTRODUCTION

Eumycetoma is a neglected tropical disease of the subcutaneous tissue that is endemic in tropical and subtropical regions.[1] It is most commonly caused by the fungus *Madurella mycetomatis*.[2,3] The disease is characterized by the presence of painless tumorous lesions which can discharge grains.[4] Eumycetoma is treated by a combination of antifungal therapy and surgery.[5,6] Typically, once a diagnosis has been made, treatment is initiated with an azole for a period of 6 months. After this, the lesion is surgically removed and then another 6 months of azole treatment is given to prevent recurrent infection.[6] The recommended azole is itraconazole, which is given in a daily dose of 400 mg.[6] Fosravuconazole was under clinical investigation in a DND*i*-sponsored phase II clinical trial (NCT03086226)[7] where it was being given in weekly doses of 200 mg or 300 mg for a duration of 12 months.

Currently, therapeutic response is monitored clinically by ultrasound and evaluation of lesion closure. However, it is difficult to assess whether grains are still present, and an invasive biopsy would be needed to obtain the grains in order to culture them to determine their viability.

(1–3)-β-D-glucan is a fungal cell wall component that is used as a broad-based marker for invasive fungal infections.[8,9] Fungal (1,3)-β-D-glucan has been detected in the serum of patients with eumycetoma[10,11] and a correlation has been observed between higher (1,3)-β-D-glucan concentrations and larger lesions. Furthermore, in other fungal infections, (1,3)-β-D-glucan concentrations have been shown to decline during therapy.[12,13] The use of (1,3)-β-D-glucan for therapeutic monitoring had not been evaluated in patients with eumycetoma. In this study, we evaluated the use of the serum (1,3)-β-D-glucan as a prognostic marker for therapeutic response in patients with eumycetoma and treated with either 400 mg itraconazole daily or 200 mg or 300 mg fosravuconazole weekly.

2 | MATERIALS AND METHODS

2.1 | Patients

Patients with PCR-confirmed eumycetoma caused by *M. mycetomatis* were enrolled in a DNDi-sponsored, randomized, double-blinded, phase II proof-of-concept trial comparing fosravuconazole therapy with itraconazole for the treatment of eumycetoma. Written informed consent was obtained from participants prior to enrolment, according to guidelines from the ethical committee at the Soba University Hospital, Khartoum, Sudan. All patients were ≥ 15 years old and had a single eumycetoma lesion in one anatomical area, or multiple lesions in one anatomical area ranging between 2.67 cm^2 and 89.32 cm^2, caused by *M. mycetomatis*. The size of the lesions was calculated using the following formula: S = π*0.25*length*width, where S is size and results are expressed in cm^2. Patients were treated for 12 months with either 400 mg itraconazole daily or 200 mg or 300 mg fosravuconazole weekly. In the 6th month of treatment, patients underwent surgical removal of the encapsulated lesion. The primary endpoint was complete cure at the end of treatment, defined as the absence of mycetoma mass, a normal lesion site or only fibrosis as observed by ultrasound, and a negative culture from a surgical biopsy.

2.2 | Collection and storage of serum samples

Blood samples were taken at seven different timepoints at 2-month intervals: at visit 0 (day 0, timepoint 1), before receiving treatment; visits 4 and 6 (timepoints 2 and 3), after receiving treatment for 22 (±3) and 85 (±7) days, respectively; visit 7, after receiving treatment for 176 (±7) days but before the lesion was surgically removed; visits 8 and 9 after receiving treatment for 267 (±14) and 358 (±7) days, respectively; and visit 10, which was a follow-up visit 90 (±7) days after end of treatment (Figure 1).

To obtain serum, blood was allowed to clot for 20–30 min and centrifuged for 5 mins at 3000 RPM (EBA 20 Hettich centrifuge). 1 mL of the serum was aliquoted into a 2 mL cryovial within 2 h of collection. The serum was frozen at −80°C and transferred to the Erasmus MC University Medical Center, Rotterdam for (1,3)-β-D-glucan analysis.

2.3 | (1,3)-β-D-glucan detection

Serum (1,3)-β-D-glucan concentrations were measured using the WAKO (1,3)-β-D-glucan assay as previously described.[10] Briefly, following thawing and mixing, 100 µL of serum was added to 900 µL of pretreatment solution and incubated for 10 min at 70°C in a Thermostation TS-70/16 (WAKO). After incubation, the sample was transferred to an ice box for 3 min. 200 µL of the pretreated sample were then transferred to the LAL reagent and mixed by vortexing

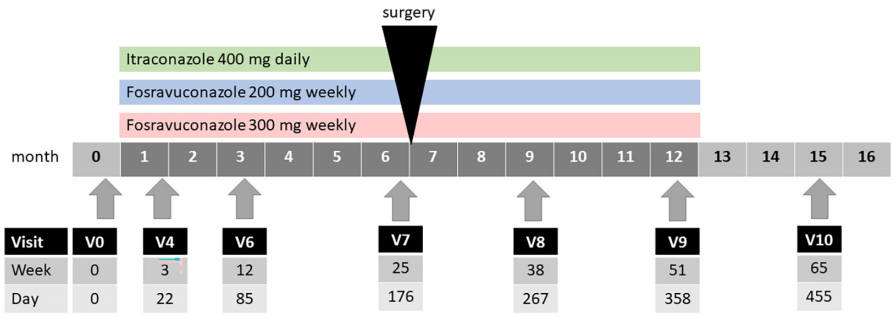

FIGURE 1 Serum collection during the azole treatment. Patients were treated for 12 months with either 400 mg/day itraconazole, 200 mg/week fosravuconazole or 300 mg/week fosravuconazole. At visit 0 (V0), before start of treatment the first serum sample was taken for (1,3)-β-D-glucan measurement. Subsequent serum samples for (1,3)-β-D-glucan measurement were taken at visit 4 (V4: 22 days after start treatment), visit 6 (V6: 85 days after start treatment), visit 7 (V7: 176 days after start treatment), visit 8 (V8: 267 days after start treatment), visit 9 (V9: 358 days after start treatment) and visit 10 (V10: 455 days after start treatment). Surgical removal of the lesion was performed just after V7 at 6 months after start treatment.

until the LAL reagent was completely dissolved. The tube was transferred to a MT-6500 toxinometer for turbidimetric measurement for 90 min at 37°C. The (1,3)-β-D-glucan concentration in picograms per millilitre (pg/mL) was obtained by comparing the gelation time to that of an internal calibration curve provided by the manufacturer. Two different cut-off values for positivity were used. The first cut-off value was 7 pg/mL, as recommended by the manufacturer, the second cut-off value was 5.5 pg/mL, as previously determined in a cohort of mycetoma patients; in the final analysis we used 5.5 pg/mL as the cut-off value.[10]

2.4 | Statistical analysis

Serial (1,3)-β-D-glucan levels were plotted over time, and Graphpad was used to calculate the best linear fit. In addition, the means (± standard error of the mean (SEM)) of the (1,3)-β-D-glucan concentration and lesion size were compared using the Kruskal–Wallis test and results were considered statistically significant if $p < .05$. The correlation between (1,3)-β-D-glucan concentration and lesion size was determined using Pearson's correlation.

3 | RESULTS

3.1 | Study population

In total, 104 patients were included in the study. The patients were on average 28 years (ranging from 15 to 77 years) old and the male-to-female ratio was 4.8:1. During the course of the study, five patients were lost to follow-up, two patients withdrew after the start of the study and one died. All patients received azole therapy, 36 patients received 400 mg itraconazole daily, 34 patients received 200 mg fosravuconazole weekly and 34 patients received 300 mg fosravuconazole weekly. Overall, 654 of the 728 samples expected from the seven visits were obtained and analyzed (Table 1).

TABLE 1 Demographic characteristics of the 104 patients with eumycetoma caused by *M. mycetomatis* included in the trial.

Characteristic	Patients with eumycetoma
Mean age (years) (range)	28 (15–77)
Median (years)	25
Gender	
Male	86
Female	18
Treatment group	
Healthy controls	28
Itraconazole (400 mg daily)	36
Fosravuconazole (200 mg weekly)	34
Fosravuconazole (300 mg weekly)	34

3.2 | Reduction in (1,3)-β-D-glucan concentration over time

At the start of treatment, one serum sample was available for all 104 patients. Of those 104 patients, 76 (73.1%) had a (1,3)-β-D-glucan concentration >5.5 pg/mL (Figure 2A). The remaining 28 (26.9%) patients had no detectable (1,3)-β-D-glucan in serum. Of these 28 patients, four patients remained negative throughout the treatment. The remaining 24 patients became positive at visit 4 ($n = 6$), visit 6 ($n = 3$), visit 7 ($n = 5$), visit 8 ($n = 4$), visit 9 ($n = 5$) or visit 10 ($n = 1$); 14 of these 24 patients were positive only at a single timepoint.

The mean baseline (1,3)-β-D-glucan concentration was 22.83 (±51.52) pg/mL. At visits 4 and 6, nonsignificant decrease of 28.5% of the initial (1,3)-β-D-glucan concentration was noted, with (1,3)-β-D-glucan concentrations of 14.94 (±19.52) pg/mL and 13.54 (20.33) pg/mL, respectively (Table 2). At the time of surgery (visit 7), the average (1,3)-β-D-glucan concentration was 18.99 (±29.46) pg/mL (Table 2, Figure 2B), again not significantly different from the concentration observed at baseline. At the first visit after surgery (visit 8), a sharp and significant decrease in (1,3)-β-D-glucan concentration was noted. At visit 8 the average

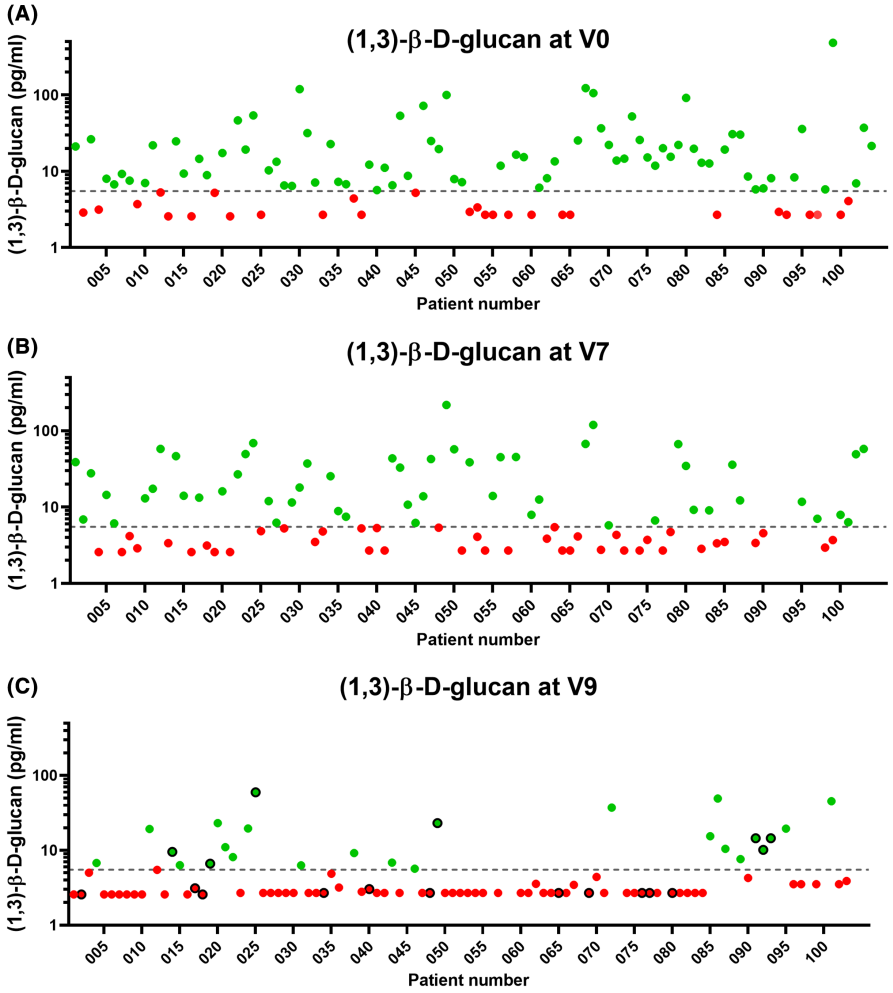

FIGURE 2 Assessing (1,3)-β-D-glucan levels in serum of 104 patients with eumycetoma. (A) At baseline at visit 0 (V0), 76/104 patients had concentration above cut-off. (B) 6 months after beginning of treatment and at the time of the surgery, 53/95 patients had concentration above cut-off. (C) At visit 9, at the end of treatment 25/91 patients had concentration above cut-off. The green circles represent samples with concentrations above the cut-off value of 5.5 pg/mL (the dashline). The red samples present samples with (1,3)-β-D-glucan concentrations below the cut-off. The 18 circles with black rims represent patients with recurrence, for which seven had positive (1,3)-β-D-glucan concentrations.

(1,3)-β-D-glucan concentration was 8.56 (±14.13) pg/mL, which further lowered to 7.03 (±10.16) pg/mL at visit 9 and 4.87 (±4.23) pg/mL at visit 10 (Table 2), which was statistically significant when compared to visit 0 (Mann–Whitney, $p < .0001$ for visit 8; $p = .004$ for visit 9 and $p = .003$ for visit 10). The pattern of (1,3)-β-D-glucan concentrations was similar in patients treated with 400 mg/day itraconazole or 200 mg/week or 300 mg/week fosravuconazole (Figure 3, Table 3).

3.3 | (1,3)-β-D-glucan concentration and recurrence

Despite the significant lowering of the average (1,3)-β-D-glucan concentration after surgery, 30/85 (35.3%) patients at visit 8, 25/91 (27.5%) at visit 9 and 14/76 (18.4%) at visit 10 still had a (1,3)-β-D-glucan concentration above 5.5 pg/mL, suggesting a remaining or recurrent mycetoma infection. Seven of the 25 (28.0%) patients with a (1,3)-β-D-glucan concentration above 5.5 pg/mL at the end of treatment (visit 9) had clinical evidence of recurrence. Four of the 14 (28.6%) patients who had a (1,3)-β-D-glucan concentration above 5.5 pg/mL at the final visit 10 (3 months after final treatment) had clinical evidence of recurrence. On the contrary, of the 18 patients who did have clinical evidence of recurrence, only seven had a (1,3)-β-D-glucan above 5.5 pg/mL at visit 9 and four at visit 10 (Table 2 and Figure 2C). Three of the patients with a recurrence and a (1,3)-β-D-glucan below the cut-off, where already negative at baseline. The (1,3)-β-D-glucan assay therefore resulted in a sensitivity of 38.9%, specificity of 75.3%, positive predictive value of 28.0% and a negative predictive value of 83.3% for visit 9, and a sensitivity of 22.2%, specificity of 82.8%, positive predictive value of 28.6% and a negative predictive value of 77.4% for visit 10 for diagnosing recurrence.

3.4 | Correlation between (1,3)-β-D-glucan concentrations and lesion size

Next, we assessed whether there was an association between lesion size and serum (1,3)-β-D-glucan concentrations. When all 654 serum (1,3)-β-D-glucan concentrations were plotted against their corresponding lesion sizes, a significant positive correlation was noted (Pearson's correlation, $p < .0001$, $r = 0.377$), as shown in Figure 4A. The overall lesion size was 13.47 cm^2. At visit 0, a significant positive correlation was found (Pearson's correlation, $p = .027$, $r = 0.217$) (Figure 4B) with an average lesion size of 22.18 cm^2. Furthermore,

TABLE 2 Overall serial concentrations of β-glucan across different timepoints.

Variable	V0	V4	V6	V7	V8	V9	V10
Overall patients ($n=104$)	104	103	101	94	85	91	76
Median (range) (1,3)-β-D-glucan (pg/mL)	9.27 (<2.57–482.2)	7.01 (<2.54–91.80)	5.21 (<2.57–112.50)	6.79 (<2.57–217.90)	3.29 (<2.45–87.01)	2.69 (<2.57–59.81)	2.69 (<2.57–23.84)
Mean ± (SD) β-glucan (pg/mL)	22.86 (51.52)	14.94 (19.52)	13.54 (20.33)	18.99 (29.46)	8.56 (14.13)	7.03 (10.16)	4.87 (4.23)
No of positives (5.5 pg/mL) (%)	76 (73.1)	62 (60.2)	45 (44.6)	52 (55.3)	30 (35.3)	25 (28.6)	14 (18.4)
Positive predictive value (%)						28	28.6
Negative predictive value (%)						83.3	77.4

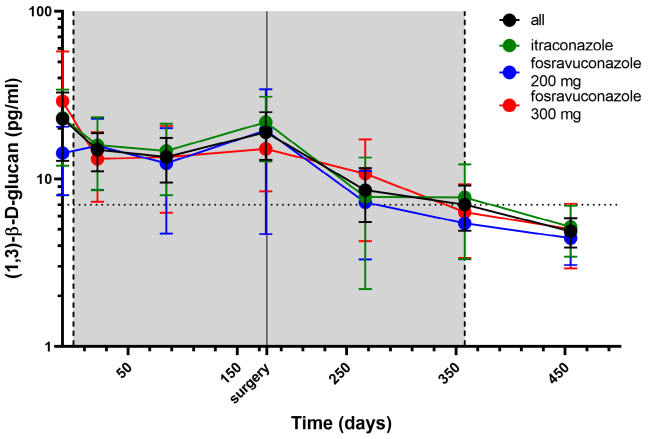

FIGURE 3 (1,3)-β-D-glucan concentration response over time associated with mycetoma treatment. The highest average (1,3)-β-D-glucan concentration is at V0, after which there is a decline, especially following surgical removal. At the final visit (Visit 10) the average (1,3)-β-D-glucan concentration was below the cut-off value. Each point on the figure represents a sampling time, and the bars represent standard error of the mean.

after 6 months of treatment just before surgery at visit 7, a strong significant (Pearson's correlation, $p=.004$) positive correlation ($r=0.36$) was found (average lesion size of 22.22cm^2). After surgery, at Visit 10, most patients had no measurable lesion. Of the three patients with a remaining small lesion, there was no correlation between serum (1,3)-β-D-glucan concentration and lesion size ($p=.443$, $r=-0.09$), with an average lesion size of 0.09cm^2.

4 | DISCUSSION

In this study, we evaluated whether serum (1,3)-β-D-glucan concentrations could be used to monitor treatment response in patients with eumycetoma. Before the start of therapy, a (1,3)-β-D-glucan concentration above the WAKO recommended cut-off value of 7 pg/mL was noted in 62% of the patients, and in 73% of the patients, the (1,3)-β-D-glucan concentration was above the 5.5 pg/mL cut-off value we previously proposed for mycetoma patients.[10] In 28 patients, no detectable (1,3)-β-D-glucan levels were found at visit 0. Although disappointing, these findings are in line with previous studies performed in other fungal infections.[8,13–15] For candidemia, aspergillosis and rare fungal diseases (including mycetoma), (1,3)-β-D-glucan concentrations were found to be above the cut-off value for positivity in only 64%, 52% and 61% of patients, respectively.[15] In that study, only five patients with eumycetoma were tested and all were positive for serum (1,3)-β-D-glucan.[15] The reason for the negative (1,3)-β-D-glucan results in a subset of patients in our study is not clear, but individual differences in (1,3)-β-D-glucan release from lesions into the bloodstream or clearance from the bloodstream could have played a role, as suggested by Damiani and colleagues.[16] (1,3)-β-D-glucan is cleared from the bloodstream via immune cells, such as neutrophils, natural killer cells, dendritic cells

TABLE 3 Mean (± SE) (1,3)-β-D-glucan concentration over time.

	Mean (± SE) (1,3)-β-D-glucan concentration					
Variable	Itraconazole 400 mg (*N* = 36)	*N*	Fosravuconazole 200 mg/week (*N* = 34)	*N*	Fosravuconazole 300 mg/week (*N* = 34)	*N*
V0	24.99 (5.63)	36	14.25 (3.07)	34	29.12 (13.97)	34
V4	15.92 (3.63)	35	15.73 (3.51)	34	13.15 (2.86)	34
V6	14.71 (3.29)	34	12.39 (3.76)	33	13.50 (3.55)	34
V7	21.82 (4.47)	34	19.50 (7.25)	31	15.13 (3.27)	29
V8	7.81 (2.75)	31	7.25 (1.92)	27	10.74 (3.15)	27
V9	7.76 (2.18)	33	5.43 (1.03)	30	7.87 (2.07)	28
V10	5.18 (0.84)	25	4.43 (0.66)	26	5.02 (1.02)	25

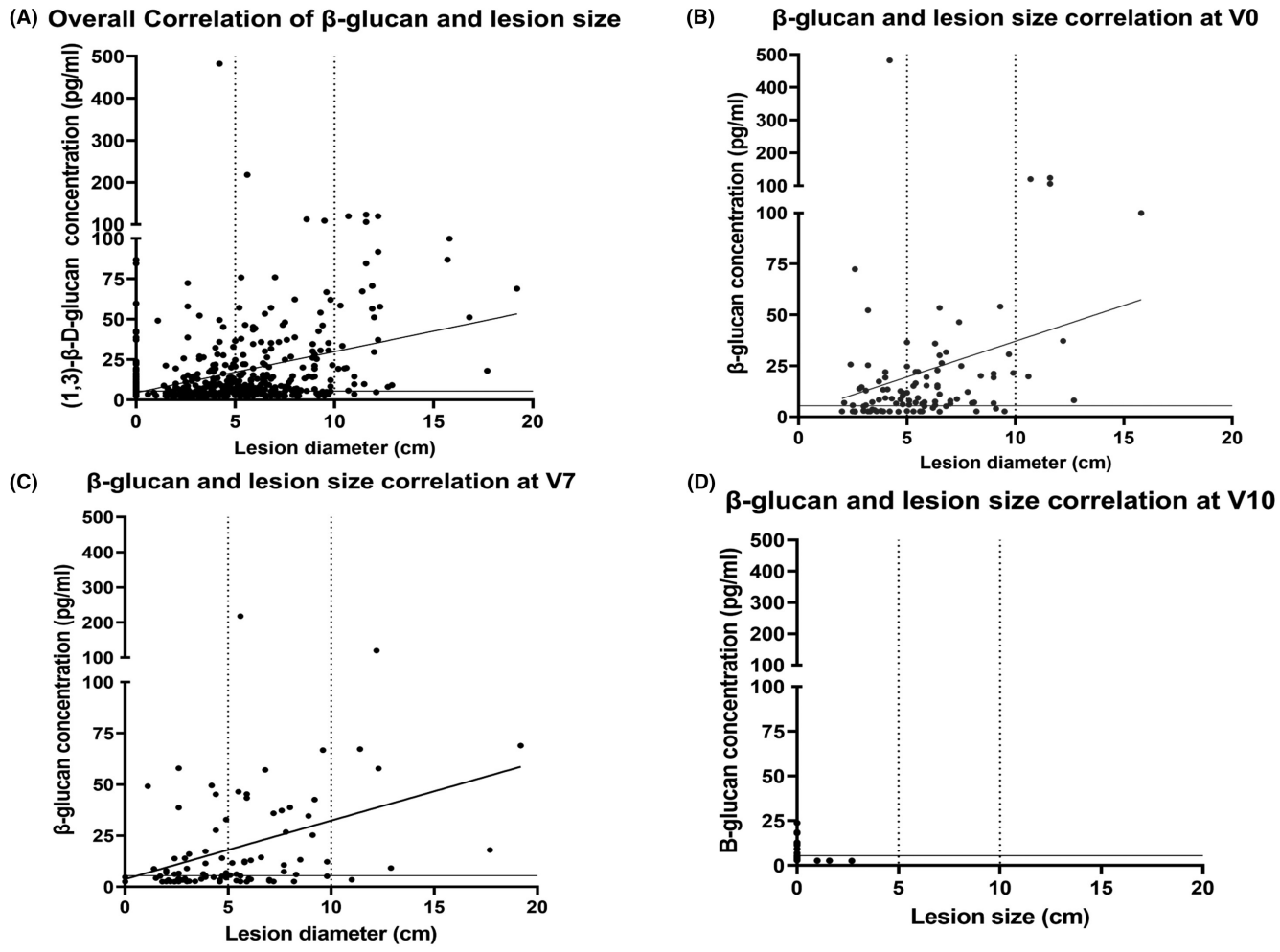

FIGURE 4 Correlation between (1,3)-β-D-glucan concentration and lesion size. (A) For the 654 serum samples, there was a significant positive correlation between (1,3)-β-D-glucan concentrations and lesion sizes ($p < .0001$, $r = 0.3772$). The average lesion size was 13.47cm^2 (B) For the 103 patients at the beginning of the trial, there was a significant positive correlation between (1,3)-β-D-glucan concentrations and lesion size ($p < .027$, $r = 0.2174$). The average lesion size was 22.18 cm^2 (C) For the 92 patients at visit 7 prior to surgical removal, there was a significant positive correlation between (1,3)-β-D-glucan concentrations and lesion size ($p = .004$, $r = 0.360$). The average lesion size was 22.22cm^2. (D) At the end of the clinical trial at visit 10, almost all patients had closed lesions, and there was a slightly negative correlation between (1,3)-β-D-glucan concentrations and lesion size ($p = .443$, $r = -0.09$). The average lesion size was 0.09 cm^2.

and macrophages,[17] and clearance seems not to be uniform among patients, resulting in differences in the (1,3)-β-D-glucan concentrations measured.[16]

Of the patients who did have a measurable (1,3)-β-D-glucan concentration in serum at the start of treatment, the concentration tended to decrease at 22 (±3) and 85 (±7) days after start treatment. It has been reported that treatment with azoles, particularly itraconazole, enhances encapsulation of the lesion resulting in a thicker capsule, which makes surgical removal of the lesion easier.[5,18] It could be hypothesized that this encapsulation and thickening of the capsule could be responsible for the decrease in (1,3)-β-D-glucan concentrations noted 22 (±3) and 85 (±7) days after start treatment, by limiting the release of (1,3)-β-D-glucan into the bloodstream. Subsequently, a nonsignificant increase in (1,3)-β-D-glucan concentrations was noted between 85 (±7) and 176 (±7) days, the timepoint at which the lesion was surgically removed. This increase was patient-dependent and not due to the surgery, as surgery was performed after obtaining the serum sample. However, the increase was mainly noted in patients in which an increase in lesion size was noted, indicating that the individual increase in (1,3)-β-D-glucan concentration was most likely due to the increase in lesion size.

The rapid reduction in (1,3)-β-D-glucan concentration after surgery may be associated with a sharp decline in *M. mycetomatis* fungal load. The decline in (1,3)-β-D-glucan concentrations varied per patient and 55 of 85 (64.7%) patients had (1,3)-β-D-glucan below the cut-off at 91 days after surgery. Furthermore, for other invasive fungal diseases (eumycetoma, deep dermatophytosis and phaeohyphomycosis) it has been demonstrated that positive levels of (1,3)-β-D-glucan can be observed for more than 25 weeks after treatment.[16] Indeed, as more time passed after surgery, a larger number of patients became negative for (1,3)-β-D-glucan. For those patients which constantly remained positive for (1,3)-β-D-glucan but which had no other signs of recurrence it remains difficult to say, if a subclinical mycetoma recurrence is present. In these cases, one might need to monitor the patients for a longer time to monitor any signs of recurrence on a later timepoint.

Detection of (1,3)-β-D-glucan at visit 10, 90 days after the end of treatment, did not predict recurrence. The lack of detectable (1,3)-β-D-glucan concentrations in true recurrent infections can be attributed to several factors. First, we demonstrated in earlier studies that smaller lesions have undetectable (1,3)-β-D-glucan levels more often than the larger lesions. Since the recurrences in this study were noted while they were still very small, their size could have prevented the rise of detectable (1,3)-β-D-glucan levels in serum. Second, all patients underwent surgery and the resulting scar formation surrounding the original lesion might have prevented the release of (1,3)-β-D-glucan into the bloodstream.

For patients with eumycetoma, serial (1,3)-β-D-glucan measurement was not a good marker for treatment response in this study, and other studies have also had varying results. For instance, in a study focusing on candidiasis, a negative slope in (1,3)-β-D-glucan levels correlated with a positive clinical outcome following antifungal therapy.[13] In patients with invasive aspergillosis, high levels of (1,3)-β-D-glucan decreased with successful response to treatment and (1,3)-β-D-glucan concentrations were negative at the end of successful treatment.[19] However, in another study, 7 (17%) of 42 people living with HIV with *Pneumocystis jirovecii* pneumonia had normal (1,3)-β-D-glucan concentrations following treatment.[20] In a similar study with 17 people living with HIV with *Pneumocystis jirovecii*, (1,3)-β-D-glucan levels dropped below the cut-off only in three patients following treatment.[21] Since we did observe a steep decline after removing the mycetoma lesion surgically, serial measurement of (1,3)-β-D-glucan in serum might become more predictive when more active fungicidal drugs are developed for the treatment of mycetoma.

In conclusion, during the first 6 months of azole treatment, (1,3)-β-D-glucan concentrations were generally stable when either itraconazole or ravuconazole are used. After surgical removal of lesions, (1,3)-β-D-glucan concentrations decrease significantly. However, although some patients had a recurrence after surgical and azole treatment, there was no correlation with (1,3)-β-D-glucan concentrations and risk of recurrence. (1,3)-β-D-glucan does not appear to be of use for the monitoring of therapeutic response and risk of recurrence in patients with eumycetoma.

AUTHOR CONTRIBUTIONS

Bertrand Nyuykonge: Investigation; data curation; methodology; writing – original draft; visualization; formal analysis; validation; writing – review and editing. **Emmanuel E. Siddig:** Investigation; methodology; validation; formal analysis; data curation; writing – review and editing. **Borna A. Nyaoke:** Funding acquisition; project administration; resources; data curation; writing – review and editing. **Eduard E. Zijlstra:** Funding acquisition; project administration; resources; data curation; writing – review and editing. **Annelies Verbon:** Writing – review and editing; supervision; resources. **Sahar M. Bakhiet:** Supervision; project administration; writing – review and editing. **Ahmed H. Fahal:** Resources; supervision; project administration; investigation. **Wendy W.J. van de Sande:** Conceptualization; investigation; writing – original draft; writing – review and editing; methodology; validation; visualization; software; formal analysis; project administration; supervision; resources; data curation.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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