Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

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SUPPLEMENTARY APPENDIX

Combination antifungal therapy reduces mortality in cryptococcal meningitis.

Jeremy N. Day, Tran T.H. Chau, Marcel Wolbers, Pham P. Mai, Nguyen T. Dung, Nguyen H. Mai, Nguyen H. Phu, Ho D. Nghia, Nguyen D. Phong, Cao Q. Thai, Le H. Thai, Ly V. Chuong, Dinh X. Sinh, Van A. Duong, Thu N. Hoang, Pham T. Diep, James I. Campbell, Tran P.M. Sieu, Stephen G. Baker, Nguyen V.V Chau, Tran T. Hien, David G. Lalloo, Jeremy J. Farrar.

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Methods

Cerebrospinal fluid examination and processing

CSF was examined from all patients at admission using standard methods including Gram's, Ziehl-Neelsen and India ink stains, and culture on blood, Sabouraud's and Lowenstein-Jensen media. Cerebrospinal fluid yeast quantitative counts were determined on all samples, Briefly, 100 uL aliquots of whole CSF were serially diluted in 0.9ml of sterile water under aseptic conditions to produce five 10 fold dilutions (maximum dilution 10^{-5}). 100uL of each dilution was spotted 20-25 times on each half of a Sabouraud-Dextrose agar plate. Whole CSF and each dilution were agitated with vortexing prior to aliquoting and spotting plates. Samples were processed within 4 hours of lumbar puncture. Plates were examined daily for one week and the number of colonies recorded. The quantitative count was calculated using the plate for each sample where the total number of colonies was between 30 and 130 colonies [1]. *Cryptococcus* antigen testing was performed using the Murex Cryptococcus Test (Remel, Lenexa, USA). HIV infection was confirmed or excluded through antibody and antigen testing (Determine HIV1/2, HIV AXSYM HIV1/2 gO, Abbott, Maidenhead, UK). CD4 lymphocyte counts were performed by flow cytometry (FACSCalibur, Becton Dickinson USA) as soon as possible after randomization.

Cryptococcus identification

All strains were confirmed as *Cryptococcus* species using classical mycological methods (see on-line appendix). , including colony morphology, microscopy, growth on bird seed

agar, urease production, sugar assimilation tests (API 32C, BioMerieux SA, Marcy l'Etoile, France), biotyping with canavanine-glycine bromothymol blue agar and speciation to the varietal level using *URA5* PCR-RFLP [2].

Drug Administration and monitoring

Patients received 1 liter normal saline infusions prior to amphotericin B administration [3]. Amphotericin B was infused over 4 hours. Flucytosine and fluconazole were administered orally or by nasogastric tube. Patients had weekly blood counts and blood chemistry for the first 4 weeks, at day 70 and 6 months. Plasma flucytosine levels were not monitored.

Randomisation

A computer-generated sequence of random numbers was used to assign treatment in blocks of 9 patients. The random allocations were placed in sealed opaque envelopes, which were opened by the study physician once each patient was enrolled into the trial after meeting the inclusion and exclusion criteria. Patients were enrolled in the order they presented and the sealed envelopes were opened in strict numerical sequence.

Adverse event recording

Adverse events were recorded during the 10 week treatment period. If a sign or symptom was present prior to the start of therapy, it was only recorded as an adverse event if there was a change in grade after treatment initiation and during the ten week study period.

Grading of adverse events was according to the National Cancer Institute Common Terminology Criteria (www.ctep.cancer.gov/).

Results

Table S1. Baseline characteristics of the study participants.

Characteristics	n	Amphotericin monotherapy (N=99, Arm I)	Amphotericin plus flucytosine (N=100, Arm II)	Amphotericin plus fluconazole (N=99, Arm III)
Age in years	297	28 (25, 31)	28 (25, 33)	27 (24, 31)
Male sex	298	81 (82%)	80 (80%)	84 (85%)
Intravenous drug use	281	51 (57%)	49 (52%)	53 (55%)
Duration of symptoms days	270	15 (7, 22)	14 (8, 18)	12 (7, 20)
New HIV diagnosis	282	49 (52%)	50 (54%)	39 (41%)
On ARV therapy	298	2 (2%)	5 (5%)	3 (3%)
Weight kg	292	46 (42, 50)	47 (40,50)	48 (44, 50)
Headache	295	95 (98%)	99 (100%)	98 (99%)
Fever	293	75 (77%)	75 (77%)	72 (73%)
Neck Stiffness	277	66 (73%)	64 (70%)	66 (69%)
Fits	290	9 (10%)	9 (9%)	2 (2%)
Glasgow Coma Score	294			
15		66 (68%)	67 (68%)	78 (80%)
11-14		21 (22%)	24 (24%)	15 (15%)
≤10		10 (10%)	8 (8%)	5 (5%)
Cranial Nerve Palsy	293	27 (28%)	22 (22%)	18 (18%)
Papilloedema	267	18 (21%)	19 (21%)	17 (18%)
Blind	250	0 (0%)	2 (2%)	0 (0%)
Blurred vision	250	20 (26%)	19 (22%)	16 (19%)
Hemiplegia	294	1 (1%)	7 (7%)	4 (4%)

Oral Thrush	293	60 (62%)	61 (63%)	60 (61%)
Investigations				
CSF Opening Pressure > 18cmCSF	244	56 (67%)	61 (76%)	55 (68%)
CSF White Cell Count cells/ml	264	33 (7, 76)	26(8, 61)	24 (7, 83)
CSF Glucose mmol/L	282	2.21 (1.50, 3.00)	2.30 (1.70, 2.98)	2.34 (1.70, 2.99)
Plasma Glucose mmol/L	276	5.69(4.84,6.50)	5.90(4.88,6.90)	5.43(4.80,6.20)
Log10 CSF Yeast Count CFUs/ml	236	5.91 (5.49, 6.48)	5.81 (4.74, 6.15)	5.74 (4.80, 6.34)
CSF Cryptococcal Antigen Titre	223	2048 (512, 8192)	2048 (256, 4096)	1024 (256, 2048)
Hepatitis B surface antigen positive	203	8 (12%)	4 (6%)	10 (15%)
CD4 count/Ul	218	18 (8.00, 37)	17 (9, 28)	14 (8, 41)

n refers to the number of patients with non-missing characteristic. Summary statistics are numbers (%) of patients with non-missing characteristic for categorical variables and median (interquartile range) for continuous variables.

Table S2. Summary of primary and key secondary outcomes of the study.

Outcome	Amphotericin monotherapy (N=99, Arm I)	Amphotericin plus flucytosine (N=100, Arm II)	Amphotericin plus fluconazole (N=99, Arm III)	Pairwise comparisons Estimate (95% CI); p-value
Deaths until day 14				Hazard ratios:
[co-primary outcome]				II vs. I: 0.57 (0.30, 1.08); p=0.08 #
- no deaths	25	15	20	III vs. I: 0.78 (0.44,1.41); p=0.42 #
- survival estimate (95% CI)	0.75 (0.67,0.84)	0.85 (0.78,0.92)	0.80 (0.73,0.88)	III vs. II: 1.38 (0.71,2.70); p=0.34
Deaths until day 70				Hazard ratios: $
[co-primary outcome]				II vs. I: 0.61 (0.39,0.97); p=0.04 #
- no deaths	44	30	33	III vs. I: 0.71 (0.45,1.11); p=0.13 #
- survival estimate (95% CI)	0.56 (0.47,0.66)	0.69 (0.61,0.79)	0.67 (0.58,0.77)	III vs. II: 1.15 (0.70,1.89); p=0.57
Deaths until day 70				Hazard ratios:
[per protocol population]				II vs. I: 0.60 (0.36,0.99); p=0.04
- no deaths	37/87	26/92	27/88	III vs. I: 0.68 (0.41,1.11); p=0.12
- survival estimate (95% CI)	0.58 (0.48,0.69)	0.71 (0.63,0.81)	0.69 (0.60,0.80)	III vs. II: 1.13 (0.66,1.94); p=0.65
Deaths until day 182				Hazard ratios: £
- no deaths	53	34	45	II vs. I: 0.56 (0.36,0.86); p=0.01
- survival estimate (95% CI)	0.46 (0.37,0.57)	0.65 (0.56,0.75)	0.54 (0.45,0.65)	III vs. I: 0.78 (0.53,1.16); p=0.23
				III vs. II: 1.39 (0.89,2.18); p=0.14
Disability on day 70				
- Good	14 (14%)	21 (21%)	19 (19%)	Odds ratios of status "good": §
- Intermediate	20 (20%)	25 (25%)	17 (17%)	II vs. I: 1.97 (0.92,4.21); p=0.08
- Severe disability	13 (13%)	7 (7%)	12 (12%)	III vs. I: 1.73 (0.81,3.69); p=0.15
- Death	44 (44%)	30 (30%)	33 (33%)	III vs. II:0.88 (0.43,1.79); p=0.72
- Patient not assessed	8 (8%)	17 (17%)	18 (18%)	
Disability on day 182				
- Good	17 (17%)	28 (28%)	17 (17%)	Odds ratios of status "good": §

- Intermediate	11 (11%)	7 (7%)	11 (11%)	II vs. I: 2.01 (1.04,3.88); p=0.04
- Severe disability	2 (2%)	8 (8%)	3 (3%)	III vs. I: 1.02 (0.50,2.05); p=0.96
- Death	53 (54%)	34 (34%)	45 (45%)	III vs. II:0.51 (0.26,0.997); p=0.049
- Patient not assessed	16 (16%)	23 (23%)	23 (23%)	
CSF fungal decline in first 14 days				Difference in estimated change:
- estimated change (95% CI) [log10 CFU/mL of CSF per day]	-0.31 (-0.34,-0.29)	-0.42 (-0.44,-0.40)	-0.32 (-0.34,-0.29)	II vs. I: -0.10 (-0.14,-0.07); p<0.0001 III vs. I: 0.00 (-0.04, 0.03); p=0.83 III vs. II: 0.10 (0.07, 0.14); p<0.0001
CSF fungal clearance				Hazard ratios:
- no of patients with documented fungal clearance	52	74	63	II vs. I: 3.18 (2.17,4.66); p<0.0001 III vs. I: 1.39 (0.94,2.07); p=0.10
- clearance rate (95% CI) [per person-weeks of follow up]	0.17 (0.13,0.23)	0.39 (0.31,0.50)	0.26 (0.20,0.34)	III vs. II:0.44 (0.30,0.63); p<0.0001

All reported results are on the intention to treat population, and not adjusted for baseline covariates or multiple comparisons unless mentioned otherwise (except for time to CSF fungal clearance which is adjusted for baseline fungal count).

Primary comparisons. Conservative Bonferroni multiplicity adjustment would require doubling the p-values (adjustment for multiple primary endpoints) or a four-fold increase (adjustment for multiple primary endpoints and for multiple comparisons of the combination therapies versus a common control group).

$ Hazard ratios adjusted for baseline covariates (as defined in the methods section): II vs I: 0.62 (0.38,0.996); p=0.048; III vs. I: 0.94 (0.58,1.51); p=0.80; III vs. II: 1.52 (0.91,2.55); p=0.11.

£ Hazard ratios adjusted for baseline covariates: II vs. I: 0.56 (0.36,0.87); p=0.01; III vs. I: 1.01 (0.66,1.53); p=0.97; III vs. II: 1.81 (1.14, 2.88); p=0.01.

§ Based on multiple imputation of missing outcomes for patients not assessed for disability. Multiple imputation estimated the proportion of patients with good disability status in the groups I-III as 16%, 27%, and 25%, respectively, on day 70 and 25%, 40%, and 25% on day 182.

Table S3. Summary of adverse events.

Type of adverse event	Amphotericin monotherapy (N=99, group I)	Amphotericin plus flucytosine (N=100, group II)	Amphotericin plus fluconazole (N=99, group III)	Overall comparison (p-value)
Any event				
- No of patients with at least one	82 (83%)	85 (85%)	85 (86%)	0.85
event	338	376	362	
- No of events				
Hypokalaemia				
- All	54 (55%)	56 (56%)	54 (55%)	0.98
- Grade 3 and 4	20 (20%)	22 (22%)	13 (13%)	0.24
Anaemia				
- All	62 (63%)	63 (63%)	57 (58%)	0.71
- Grade 3 and 4	46 (46%)	35 (35%)	29 (29%)	0.04
Neutropenia				
- All	19 (19%)	34 (34%)	32 (32%)	0.04
- Grade 3 and 4	2 (2%)	9 (9%)	9 (9%)	0.07
Thrombocytopenia				
- All	8 (8%)	15 (15%)	11 (11%)	0.32
- Grade 3 and 4	2 (2%)	4 (4%)	3 (3%)	0.91
Rigor	13 (13%)	7 (7%)	6 (6%)	0.18
Opportunistic infection*	32 (32%)	32 (32%)	28 (28%)	0.79
PCP	20 (20.2%)	22 (22%)	20(20.2%)	0.95
Pulmonary and extra-pulmonary TB	9 (9.09%)	12 (12%)	6 (6.06%)	0.36
Non-typhoidal salmonellosis	2 (2.02%)	1 (1%)	1 (1.01%)	0.85
CMV retinitis	1 (1.01%)	2 (2%)	0 (0%)	0.78
Zoster	1 (1.01%)	1 (1%)	1 (1%)	1.00
Cerebral lymphoma	0 (0%)	1 (1%)	0 (0%)	1.00

Penicilliosis	0 (0%)	0 (0%)	1 (1%)	0.66
Rash	5 (5%)	7 (7%)	5 (5%)	0.86
New neurological sign/symptom	11 (11%)	12 (12%)	10 (10%)	0.97
Fit	2 (2%)	0 (0%)	2 (2%)	0.4
Transaminitis				
All	38 (38%)	44 (44%)	42 (42%)	0.72
Grade 3 and 4	11 (11%)	6 (6%)	14 (14%)	0.14
Hyponatraemia				
All	28 (28%)	33 (33%)	33 (33%)	0.71
Grade 3 and 4	3 (3%)	8 (8%)	9 (9%)	0.19
Hyper creatininemia				
All	34 (34%)	41 (41%)	46 (46%)	0.22
Grade 3 and 4	2 (2%)	2 (2%)	2 (2%)	1.00
Other[$]	28 (28.28%)	23 (23%)	31 (31.31%)	0.41

Data are numbers (%) of patients, unless otherwise indicated.

*all opportunistic infections and all grade 3 and 4 events are classified as severe adverse events

[$]all occurred with frequency of 0.34% except nausea and vomiting (2.7%), sepsis (1.7%), thrombophlebitis, haematemesis, diarrhoea, urinary retention (each 0.7%).

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