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1 Short-form paper:

2 **Flow cytometry to assess CSF fungal burden in cryptococcal meningitis**

3 (*Running title:* Flow cytometry counting of cryptococci)

4

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33 **Abstract**

34 Fungal burden in the cerebrospinal fluid is an important determinant of mortality in
35 cryptococcal meningitis but its use to aid clinical decision-making is hampered by the time
36 involved to perform quantitative cultures. Here we demonstrate the potential of flow
37 cytometry as a novel and rapid technique to address this.

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38 **Text**

39 Cryptococcal meningitis (CM) remains one of the commonest causes of meningitis in sub-
40 Saharan Africa and a significant cause of death among persons with HIV-1 infection (1, 2).
41 Cerebrospinal fluid (CSF) fungal burden is an important determinant of mortality but requires
42 quantitative culture – a time consuming process taking several days limiting its usefulness as
43 a clinical decision aid (3). Recent *in vitro* work using broth dilutions of *Cryptococcus*
44 *neoformans* has demonstrated a very close association between the number of cryptococci
45 counted using a flow cytometer and quantitative culture (4). This raises the possibility that
46 flow cytometry might be a useful technique to rapidly assess fungal burden in patients with
47 cryptococcal meningitis. However, no studies have examined this technique on *ex vivo*
48 samples. We addressed this by performing flow cytometry counting of cryptococci in the
49 CSF of patients with HIV-1-associated CM and compared these counts with measurement of
50 fungal burden using quantitative CSF culture. This study formed part of a larger body of
51 work primarily aimed at examining the CSF immune response in CM.

52

53 A prospective cohort study was conducted in Cape Town, South Africa. All participants
54 provided written informed consent; surrogate consent was obtained from the next of kin for
55 patients with impaired consciousness. Ethical approval was obtained from the University of
56 Cape Town and Liverpool School of Tropical Medicine Research Ethics Committees. HIV-
57 infected persons aged ≥18 years with a first episode of cryptococcal meningitis (diagnosed by
58 antigen test or culture) were enrolled within 48 hours of diagnosis and lumbar puncture
59 performed to measure CSF opening pressure. CSF fungal burden was assessed with
60 quantitative culture (5) and cryptococcal antigen titre (CrAg® LFA, Immy, USA) (6) as
61 previously described. The volume of remaining CSF was measured and the cells pelleted
62 using centrifugation, this was incubated on ice with an amine viability dye (AQUA,

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63 Invitrogen) and anti-CD45-PECy5.5 (Biolegend), then at room temperature with FACS
64 lysing solution (BD Bioscience), protected from light at all times. Cells were fixed using 2%
65 paraformaldehyde and analyzed within 24 hours on a BD LSR Fortessa Flow Cytometer
66 using FACS Diva software (BD Biosciences). A forward scatter threshold of 5,000 was used
67 to avoid including any debris in counting; the sample was acquired in its entirety to allow
68 calculation of cell counts. Compensation was performed using species appropriate
69 compensation beads (BD Biosciences; Invitrogen). Data was analysed using Flow Jo version
70 9.5.3 (Tree Star software) [**Figure 1**]. Cryptococci were defined as CSF cells negative for the
71 pan-leukocyte marker CD45. Counts for the whole sample were divided by CSF volume to
72 obtain *Cryptococcus* counts per mL CSF. Statistical analyses were performed using Stata
73 version 12 (Stata Corp).
74
75 Sixty HIV-infected patients with cryptococcal meningitis were enrolled with a median CD4
76 count of 34 cells/µL. CSF samples were available for 58 participants, 36 of whom had not
77 received any amphotericin B prior to enrolment. The median CSF volume collected for flow
78 cytometry was 7 mL (interquartile range (IQR), 4.5-8). Median fungal burden was 4.74 $\log_{10}$
79 CFU/mL (IQR 3.5-5.75) measured by quantitative culture and 4.53 $\log_{10}$ *Cryptococcus* yeasts
80 per mL (IQR 3.33-5.21) measured by flow cytometry. Median CrAg® LFA titre was 1:8000
81 (IQR 2000-16000).
82
83 Cryptococcal counts measured by flow cytometry were strongly correlated with both
84 quantitative culture (Pearson's r=0.91, p<0.0001) [**Figure 2a**] and CrAg titre (Spearman
85 rho=0.75, p<0.0001). Linear regression showed that quantitative culture result could
86 accurately be predicted from flow cytometry counting ($\log_{10}$CFU/ml = 1.31 x $\log_{10}$ flow
87 count – 1.28; R^2=0.82, p<0.0001). The agreement between flow cytometry counting and

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88 quantitative culture was also assessed using a Bland-Altman plot [**Figure 2b**]. This showed
89 good agreement between these two measurements with a mean difference of -0.1 $\log_{10}$
90 CFU/mL and only 6.9% (4/58) of values outside the 95% limits of agreement. These outlying
91 values were mainly among participants with low fungal burdens (<500 CFU/mL) where flow
92 cytometry counting produced values that were approximately 1-2 $\log_{10}$ CFU/mL higher. A
93 similarly strong correlation and agreement between the two measurement techniques was also
94 noted when analysis was restricted to the 36 participants who had not received any anti-
95 fungal therapy prior to CSF sampling (Pearson's r=0.93, p<0.0001 [**Figure 2c**]; mean
96 difference -0.30 $\log_{10}$ CFU/mL, with 11.1% (4/36) of values outside the limits of agreement)
97 [**Figure 2d**].
98
99 These findings suggest that flow cytometry has the ability to provide a rapid and accurate
100 measurement of fungal burden in persons with HIV-associated cryptococcal meningitis. If
101 combined with a cryptococcal viability stain (as previously demonstrated *in vitro* (4)) flow
102 cytometry could also be used to assess the response to treatment.
103
104 Due to the well-recognized toxicity of amphotericin B (7), there is considerable interest in
105 short course regimens particularly in those patients with low fungal burden (8). Results from
106 quantitative culture are not available in a timely enough manner to inform clinical decision
107 making, but the rapidity of the result obtained from flow cytometric cryptococcal counting
108 could potentially overcome this problem. This could allow for the reduction of both drug
109 toxicity, cost and duration of hospitalization. Although this technique does require access to a
110 flow cytometer, suitable machines are available in many laboratories in sub-Saharan Africa
111 where they are used to measure CD4 count. In areas where they are not available, rapid
112 assessment of fungal burden might be possible using quantitative microscopy (4).

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113

114 There were a number of limitations to this study. No cryptococcal specific stain was used to
115 identify cryptococci, instead they were assumed to be any CD45 negative cell found in the
116 CSF. Given that all participants had laboratory confirmed cryptococcal meningitis, a lysis
117 buffer was used to ensure no erythrocytes remained in the CSF, and host leukocytes were
118 excluded using the pan-leukocyte marker CD45, we feel this is a reasonable assumption and
119 that our results are valid. However, to be a fully robust clinical assay an anti-cryptococcal
120 stain would ideally be incorporated into the panel and the assay validated on CSF samples
121 from patients who do not have CM. This would have the additional benefit of improving the
122 accuracy of flow cytometric counting at low fungal burdens where debris or miscellaneous
123 cells may have interfered with the counting process. In addition, we only examined the use of
124 flow cytometric counting to assess fungal burden at baseline and did not assess changes on
125 anti-fungal therapy. Future work should aim to incorporate a cryptococcal viability marker to
126 address this issue as previously assessed *in vitro* (4).

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147

148 **Conflict of Interest statement**

149 None of the authors have any conflict of interest to declare.

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155 **Figure 1.** CSF flow cytometry gating **(a1)** CSF cells (FSC-SCC) - poor definition of cell
156 subsets due to cryptococci; **(a2)** CD45 and live-dead stain are used to separate cells; **(b)**
157 FSC-SCC plot of cryptococci; **(c)** FSC-SCC plot CSF leukocytes; **(d)** FSC-SCC plot dead
158 CD45+ cells.
159
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161
162
163 **Figure 2. (a)** Scatterplot demonstrating association between flow cytometry counting and
164 quantitative culture; **(b)** Bland-Altman plot showing good agreement between flow counting
165 and quantitative culture (greyed area indicates the 95% limits of agreement); **(c)** Association
166 between flow counting and quantitative culture among participants who had not received
167 antifungal therapy before CSF sampling; **(d)** Bland-Altman plot showing agreement between
168 the two techniques limited to participants who had not received antifungal therapy before
169 CSF sampling (greyed area indicates the 95% limits of agreement).
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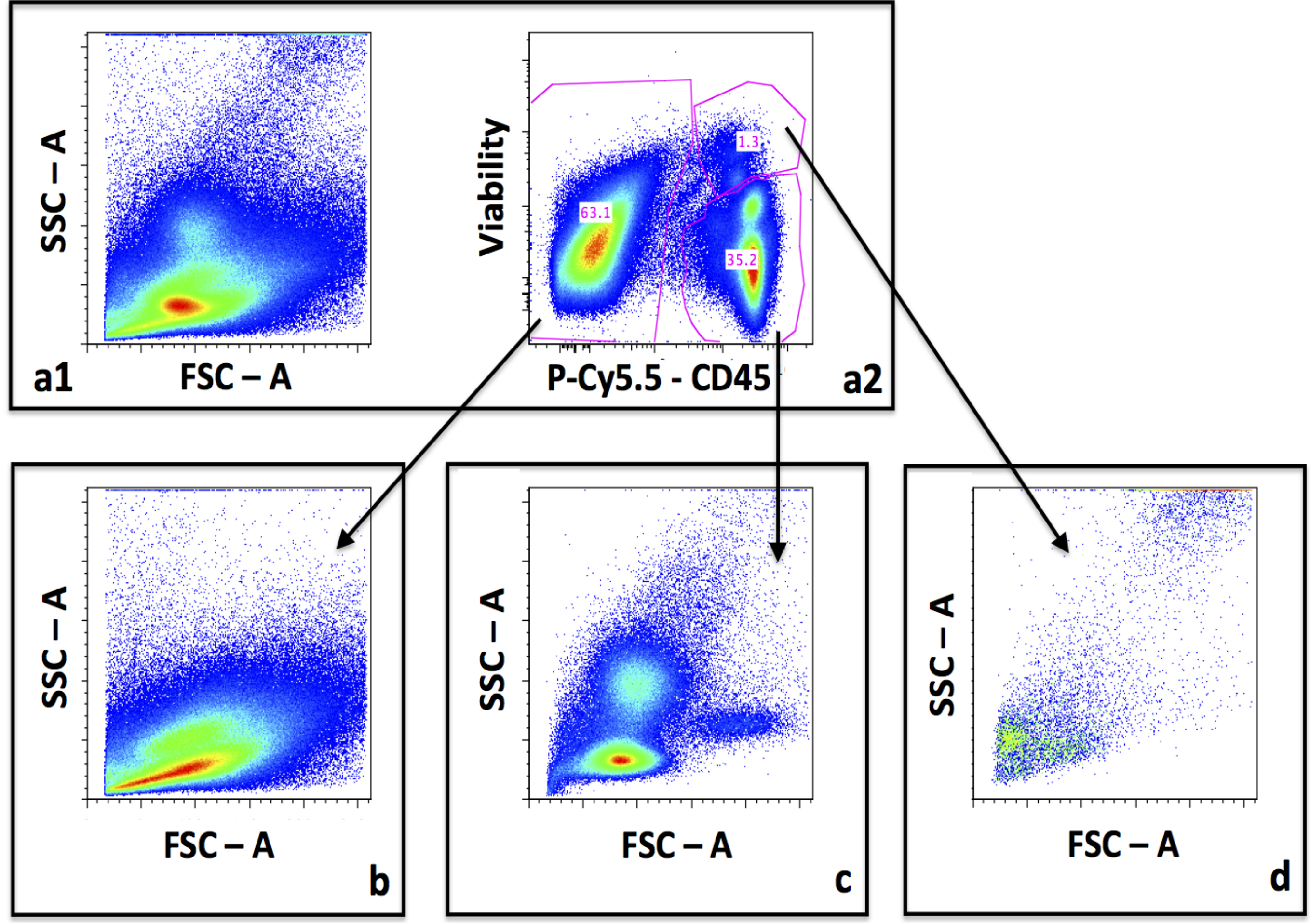

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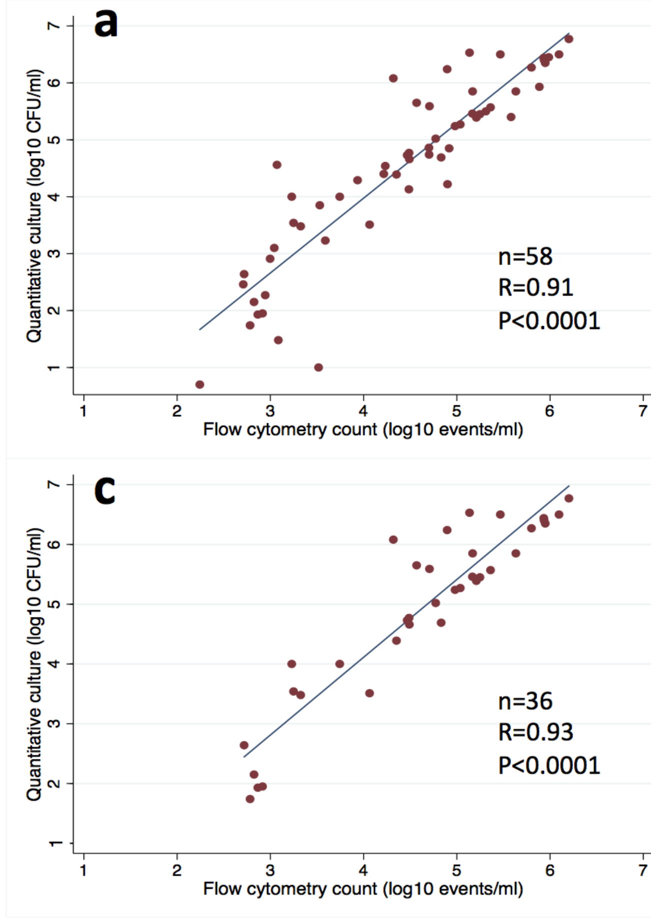

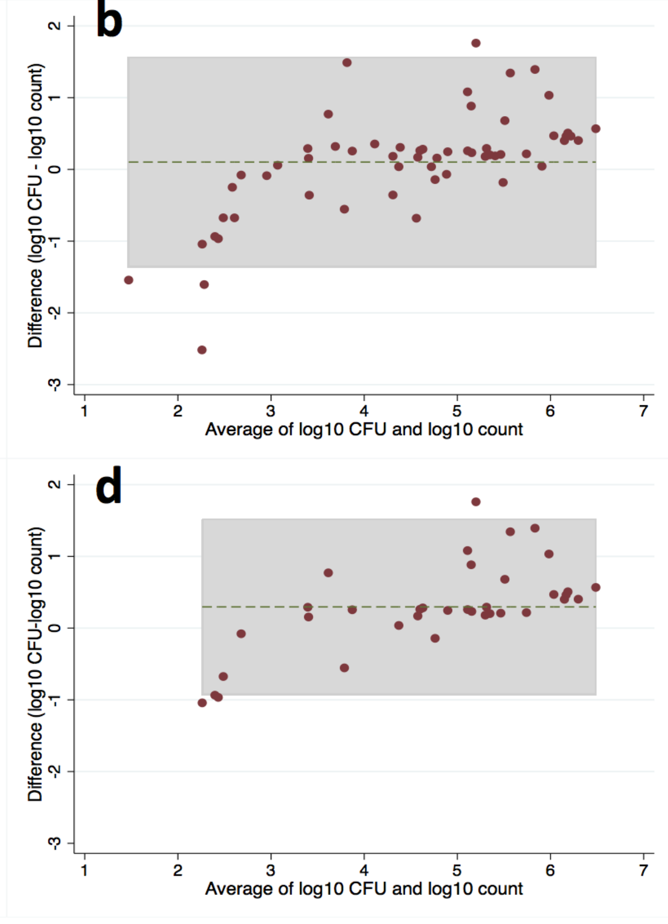

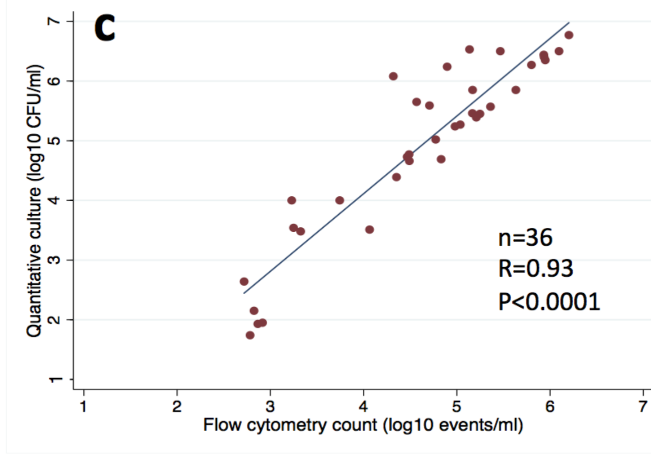

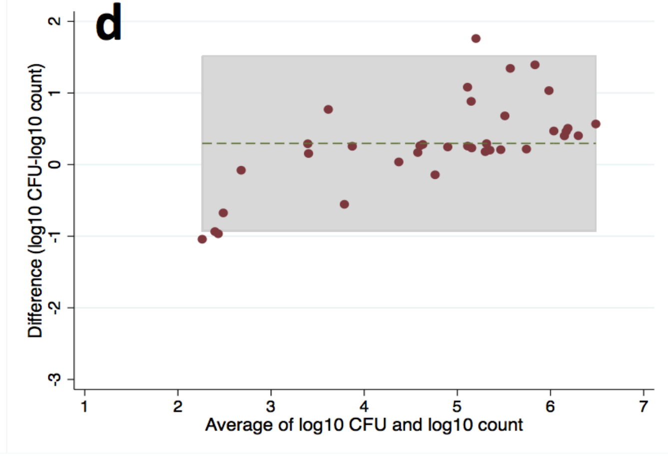

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