Cryptococcal infection in HIV-infected patients with CD4+ T-cell counts under 100/mL diagnosed in a high-income country: a multicentre cohort study

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abstract

Objectives: The World Health Organization recommends routinely screening HIV-infected patients with CD4+ T-cell counts **<**100/mL for cryptococcal infection to prevent cryptococcal meningitis (CM), based on studies in Sub-Saharan Africa where the prevalence of positive cryptococcal antigen (CrAg+) is 2: 3% in this subgroup. Data about such prevalence in Spain are unavailable and rare in other European countries. Thus, the Spanish AIDS Study Group guidelines do not recommend routinely screening. We aim to determine the prevalence and outcomes of cryptococcal infection in this subgroup of patients in Spain.

Methods: We determined CrAg using a lateral flow assay in banked plasma from participants in the cohort of the Spanish AIDS Research Network. Eligible patients had CD4+ T-cell counts≤100/mL at the time of plasma collection and a follow-up **>**4 weeks, unless they died.

Results: We included 576 patients from June 2004 to December 2017. Of these, 43 were CrAg+ for an overall prevalence of 7.5%. There were no differences depending on birthplace. The CrAg+ was independently associated with a higher mortality at eight weeks (hazard ratio (HR) 5.36, 95% confidence interval (CI) 1.46-19.56) and 6 months (HR 3.12, 95% CI 1.19e8.21). CM was reported in 10 of the 43 CrAg+ patients. There were no cases among negatives. Five patients had CM when the plasma was collected and five developed it during the follow-up. The number of subjects needed to screen to anticipate the diagnosis of one CM case was 114.

Conclusions: The CrAg+ prevalence among HIV-infected patients with CD4+ T-cell counts≤100/mL diagnosed in Spain, both immigrants and native-born Spanish, is **>**7%. Consequently, the Spanish AIDS Study Group guidelines have to be updated and recommend routine screening for cryptococcal infection in these patients. Future studies should explore whether this recommendation could be firmly applied to

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Introduction

Cryptococcal meningitis (CM) is due to the infection with encapsulated yeasts of the genus *Cryptococcus spp.* that are present in the environment with a worldwide distribution [1]. *C. neoformans* is the principal pathogenic species. CM can occur following primary lung infection or by reactivation and dissemination of latent pulmonary infection under circumstances of defective cell-mediated immunity [2] as in the late-stages of HIV-infection when CD4+ T-cell counts are <100/mL.

CM incidence has dropped with the widespread availability of antiretroviral treatment (ART) [3,4]. However, CM continues being a leading cause of mortality among HIV-infected patients and globally constitutes the second most common cause of AIDS-related mortality after tuberculosis [5].

Early diagnosis of cryptococcal infection (previous to central nervous system (CNS) involvement or when CM is still asymptomatic) is feasible through the detection of cryptococcal antigen (CrAg), present in blood around 4 weeks before the development of neurological symptoms [6e8]. Moreover, CrAg can be easily identified using a lateral-flow assay (LFA) that is simple and affordable [9]. Previous trials have demonstrated a reduction in CM incidence via pre-emptive therapy with oral fluconazole in patients with a positive CrAg (CrAg+) in blood and no CNS involvement [10]. Thus, the World Health Organization has recommended routine screening of HIV-infected patients with CD4+ T-cell counts <100/mL for cryptococcal infection since 2011 [11].

This recommendation is based on studies in Sub-Saharan Africa and Southeast Asia, where the CrAg+ prevalence is 2: 3% in this subgroup of HIV-infected population [5,12]. However, there are no available data about CrAg+ prevalence in patients with late-stages of HIV-infection in Spain and such datasets are rare in other European countries, limited to single-centre studies or cohorts from areas with a high proportion of African immigrants [13,14]. Consequently, the Spanish AIDS Study Group (GESIDA) does not recommend routine screening of Spanish patients because it is assumed that the prevalence is low [15]. Conversely, the European AIDS Clinical Society advises the clinicians to consider screening HIV-infected patients with CD4+ T-cell counts <100/mL for CrAg, but they remark on the different epidemiology of this infection between Europe and Africa [16].

We designed a multi-centre study to provide information about CrAg+ prevalence in HIV-infected patients with CD4+ T-cell counts ::;100/mL diagnosed in Spain. We further aimed to identify those patients with a higher risk of presenting CrAg+ and analysed the evolution in terms of morbidity/mortality in CrAg+ patients versus subjects negative for CrAg (CrAg-). This information could lead to an update of the recommendations.

Methods

Study material and population

Stored plasma samples from participants in the 'Cohort of adults with HIV-infection of the Spanish AIDS Research Network' (CoRIS) who fulfilled the inclusion criteria were used to determine the presence of CrAg. The CoRIS is an open, prospective, nationwide, multicentre cohort launched in January 2004 including HIV-infected adults, naïve to ART at entry, recruited in HIV-care units of the Spanish Public Health System. The information is subject to internal quality controls and audited by an external agency. The CoRIS includes >15,000 patients from 54 centres. Patients voluntarily provide plasma samples to be stored in the HIV-BioBank at the moment of inclusion in the CoRIS after giving written consent. Samples are processed following standard procedures and frozen immediately after their receipt. This programme has been approved by the Ethical Committees of the participating centres.

We included the CoRIS participants 2:18 years old, naïve to ART with CD4+ T-cell counts ≤100/mL when the plasma sample was collected. The minimum follow-up was 4 weeks unless the subject died. Patients were excluded if their sample was unavailable.

Laboratory testing

The capsular polysaccharide antigen of *C. neoformans* (glycuronoxylomannan) was detected in plasma using LFA-based immunochromatographic test CryptoPS-Biosynex® (Biosynexediagnostics, France). In comparison with the FDA-approved ImmyCrAg® LFA, the CryptoPS-Biosynex® has an agreement of 99.5% for plasma samples. Its sensitivity and specificity are 100% and 98%, respectively [17]. The test was performed according to the manufacturer's instructions. Briefly, 20 mL of plasma were transferred into the well of the test-cassette, followed by the addition of three drops of diluent in the same well. The result was available in 10 min. The test provides two bands: a qualitative T1-band and a semi-quantitative T2-band (only appears when CrAg-titres are elevated) [18]. Those positive samples were subsequently analysed using the latex-agglutination test Pastorex™CryptoPlus (BioRadeLaboratories, France) to titrate the quantity of antigen using two-fold serial dilutions [19].

Statistical analysis

Quantitative data were shown as the mean and standard deviation (SD) or median with interquartile range (IQR). Qualitative variables were expressed as absolute and relative frequencies. Categorical variables were compared using the chi-squared test or Fisher's exact test. Student's *t*-test or ManneWhitney *U*-test were used to compare continuous variables. Logistic regression was used to identify the effects of several variables on CrAg positivity. Associations were expressed as odds-ratios (ORs) and 95% confidence intervals (CIs). We determined the incidence of CM during the follow-up and the number needed to screen (NNS) to anticipate one CM case using the formula 1/(CM incidence) after excluding those with known CM at the moment of sample collection [20]. A receiver operating characteristic (ROC) curve was used to analyse the accuracy of CrAg-titres for CM identification. We used logarithms to the base 2 ($\log_2$) to express titration data because procedures involved two-fold serial dilutions. Survival probabilities were depicted graphically with the Kaplan-Meier curves and compared with a Log-rank test. Cox regression with a backward-stepwise selection method was used to evaluate the effects of different variables on the mortality. Associations were expressed as hazard-ratios (HRs) and 95% CIs. All significance tests were two-tailed. Statistical analysis was performed using SPSS v.22.0 (IBM Corporation®, Armonk, NY, USA).

Results

Study population

We identified 706 patients with CD4+ T-cell counts≤100/mL from June 2004 to December 2017. Of these, 130 (18.4%) were excluded: 98 (13.9%) due to sample unavailability and 32 (4.5%) because they were lost to follow-up in the first 4 weeks after sample collection without death documented. Thus, 576 patients were finally included (Supplementary Fig. S1), with a median follow-up of 1423 days (IQR=511e2474). The mean age of patients was 40.8 years (SD=10.9) and 460 (79.9%) were males. The mean CD4+ T-cell count was 40.77 cells/mL (SD=10.9). Most of the cohort was born in Spain (63.7%). Additional epidemiologic data are shown in Table 1. Characteristics of the excluded patients were similar to those included in terms of age, CD4+ T-cell count, birthplace, and AIDS-defining conditions; however, a higher rate of intravenous-drug users (IDUs) was observed among those excluded (20.0% vs 7.3%, p < 0.001) (Supplementary Table S1).

Cryptococcal infection

CrAg was positive in 43 patients, thus the overall CrAg+ prevalence among the whole cohort was 7.5% (95% CI=5.4e9.7%). There were no differences in prevalence depending on birthplace: Spain 7.4%, other European countries 5.9%, Latin-America 6.8%, Sub-Saharan Africa 9.8% (p=0.951). When comparing CrAg+ patients with those who were CrAg-, there were no differences in age, educational level or CD4+ T-cell counts. CrAg+ was more common among IDUs than in those with sexually transmitted HIV (17.9% vs 5.7%, p=0.003). This association remained significant when adjusting for CD4+ T-cell counts (OR=3.95, 95% CI=1.58e9.87) (Table 2). Considering that all IDUs were Spanish, we performed a sub-analysis excluding them. Again, there were no significant differences in CrAg+ prevalence depending on birthplace: Spain 6.1%, other European countries 6.6%, Latin-America 6.8%, Sub-Saharan Africa 10% (p=0.671).

Table 1
Characteristics of included population

Variable	*n* (%)
Age, years (mean ± SD) (*n*=576)	40.77 ± 10.85
Sex (*n*=576)	
Male	460 (79.9%)
Female	116 (20.1%)
Birthplace (*n*=573) Europe	
Spain	365 (63.7%)
Central and Eastern Europe	8 (1.4%)
Other European countries	9 (1.5%)
Africa	
Northern Africa	9 (1.5%)
Sub-Saharan Africa	41 (7.2%)
America	
North America	2 (0.4%)
Latin-America[a]	133 (23.2%)
Asia	
South Asia	2 (0.4%)
South Eastern Asia	4 (0.7%)
Educational level (*n*=482)	
No studies or only primary school	112 (23.2%)
High school	260 (53.9%)
University	110 (22.8%)
HIV routes of transmission (*n*=537)	
Blood-borne exposure	
IDUs	39 (7.3%)
Other	6 (1.1%)
Sexual	
Heterosexual	230 (42.8%)
MHM/bisexual	262 (48.8%)
HIV-viral load at inclusion, copies/mL (median, IQR) (*n*=558)	253,718 (IQR: 96,660e635,800)
CD4+/mL count at inclusion (mean ± SD) (*n*=576)	44.76 ± 28.38
Opportunistic disease[b] (*n*=576)	264 (45.8%)
AIDS-defining condition[b] (*n*=576)	297 (51.6%)

Values in parentheses indicate the number of patients for whom data were available. IDU, intravenous-drug user; IQR, interquartile range; MHM, men who have sex with men; SD; standard deviation.

[a] Includes Central and South America and the Caribbean Islands.

[b] Previous or within the first month after determining cryptococcal antigen (CrAg) (including cryptococcal meningitis).

Cryptococcal meningitis

CM was reported in 10 (23.25%) of the 43 CrAg+ patients, whereas there were no cases among CrAg- patients. CM was known at the moment of plasma collection in five patients. The remaining five developed it during the follow-up after a median-time of 35 days (IQR=12e61). All patients with CM received induction treatment with liposomal-amphotericin B alone or combined with flucytosine for at least 2 weeks, followed by consolidation therapy with fluconazole. The onset of highly active ART among CrAg+ patients with symptomatic CM at the moment of sample collection was significantly delayed versus those with CrAg+ who were asymptomatic (25 days, IQR=17-36 vs 7 days, IQR=1-18; p=0.044).

CrAg-titres were higher among those who had or developed symptomatic CM versus those who remained asymptomatic during the evolution (Table 3). The area under the ROC curve for the accuracy of CrAg-titres in the prediction or identification of symptomatic CM was 0.926 (Supplementary Fig. S2). Of CrAg+ patients, a cut-off titre of 1:32 was 90% sensitive and 84.8% specific for the presence or subsequent development of symptomatic CM.

Number needed to screen to anticipate the diagnosis of CM

To calculate the NNS to anticipate the diagnosis of one CM case, we excluded the five that had CM at the moment of sample collection because it could not have been prevented. After excluding them, the CrAg+ prevalence in the cohort was 6.7% and the CM incidence during the follow-up among those with CrAg+ was 13.2% with a CM incidence among the entire cohort of 0.88%. Thus, the NNS to anticipate the diagnosis of one CM case was 114 (95% CI=61e833).

Mortality

During the first 6 months after CrAg determination, five (11.6%) of the 43 CrAg+ patients died versus 24 (4.5%) of the 533 CrAg-. All except one of the deaths among CrAg+ patients occurred within the first 8 weeks of follow-up. The Kaplan-Meier curves showed a reduced survival for those with CrAg+ in the first 8 weeks (p=0.009, Fig. 1(a)) and first 6 months (p=0.031, Fig. 1(b)) of follow-up. The Cox-regression model revealed that CrAg+ was independently associated with a higher mortality at 8 weeks (HR=5.36, 95% CI=1.46-19.56) and 6 months (HR=3.12, 95% CI=1.19e8.21) (Table 4).

Discussion

This study shows a CrAg+ prevalence >7% in HIV-infected patients with CD4+ T-cell counts≤100/mL diagnosed in Spain. This prevalence is seen in immigrants from middle- and low-income

Table 2
Uni- and multivariate analysis of risk factors predicting the positivity of cryptococcal antigen (CrAg)

Variable	CrAg+ (*n*=43)	CrAg- (*n*=528)	p	Univariate analysis			Multivariate analysis		
				OR	95% CI	p	OR	95% CI	p
Age, years (mean ± SD)	41.95 ± 11.18	40.68 ± 10.83	0.791	1.011[b]	0.983-1.039	0.459			
Male gender (*n* (%))	38 (88.4%)	422 (79.2%)	0.148	1.999	0.769-5.198	0.155			
CD4+/mL (mean ± SD)	48.88 ± 28.22	44.43 ± 28.39	0.323	1.005[b]	0.995-1.016	0.323	1.009 [b]	0.997-1.021	0.152
Viral load copies/ml (mean ± SD)	547,762 ± 614,246	588,344 ±953,410	0.786						
Educational level									
Primary school or less (*n* (%))	12 (10.7%)	100 (89.3%)	0.241	1.53	0.600-3.902	0.373			
High school (*n* (%))	15 (5.8%)	245 (94.2%)		0.781	0.321-1.898	0.585			
University (*n* (%))	8 (7.3%)	102 (92.7%)		Reference					
HIV Transmission route									
IDUs (*n* (%))	7 (17.9%)	32 (82.1%)	0.003	3.625	1.47-8.937	0.005	3.952	1.582-9.871	0.003
Sexual (*n* (%))	28 (5.7%)	464 (94.3%)							
Other opportunistic diseases[a] (*n* (%))	14 (32.6%)	243 (45.6%)	0.098	0.576	0.298-1.115	0.102			
AIDS defining condition[a] (*n* (%))	17 (39.5%)	273 (51.2%)	0.140	0.623	0.330-1.174	0.143			
Birthplace									
Spain (*n* (%))	27 (7.4%)	338 (92.6%)	0.888	1.038	0.234-4.611	0.960			
Latin America (*n* (%))	9 (6.8%)	124 (93.2%)		0.944	0.193-4.624	0.943			
Sub Saharan Africa (*n* (%))	4 (9.8%)	37 (90.2%)		1.405	1.405-8.250	0.706			
Asia (*n* (%))	1 (16.7%)	5 (83.3%)		2.6	0.196-34.458	0.469			
Other (*n* (%))	2 (7.1%)	26 (92.9%)		Reference					

CI, confidence interval; IDU, intravenous-drug user; OR, odds ratio; SD, standard deviation. The bold in the tables is used to remark the statistically significant values.
[a] Previous or within the first month after determining CrAg (excluding cryptococcal meningitis).
[b] Per unitary increment.

countries as well as in native-born Spanish. Several studies on the ecology of *C. neoformans* revealed that this agent can be found in diverse environmental niches, like dried birds' excreta, rotten vegetation or tree fragments. The adaptation of pigeons to urban areas has contributed to the ubiquity of *C. neoformans*, which can be isolated from environmental sources such as household dust in regions with temperate climates but also in cold areas [21]. In fact, seroprevalence studies have suggested that the exposure to cryptococcal infection is widespread and asymptomatic colonization of the airways or latent infection of the lungs may be common [22,23]. Furthermore, a retrospective cohort study in the USA found a CrAg+ prevalence ≥3% among patients with advanced AIDS [24]. Thus, our findings in the autochthonous Spanish population are not totally unexpected.

To the best of our knowledge, this study is the first to identify intravenous-drug use as a risk factor for having CrAg+. Most previous studies were conducted in Sub-Saharan Africa where intravenous-drug use prevalence is unknown [25]; thus, this factor has not been fully examined. Only an Indonesian study analysed the history of intravenous-drug use according to CrAg status, but the authors did not find a significant higher CrAg+ prevalence among IDUs [26]. The higher CrAg+ proportion among IDUs in our cohort could be explained by the more frequent contact of this collective with environmental sources of *Cryptococcus spp.,* which may be linked to their socioeconomic marginalization that entails homelessness.

Even if IDUs have an increased morbidity/mortality due to co-infection with hepatitis viruses, elevated tuberculosis prevalence, or enhanced risk of endocarditis [27], the higher observed mortality among patients with CrAg+ was independent of the HIV-transmission route. Moreover, after adjusting for other potential confounders such as age, CD4+ T-cell count or other AIDS-defining conditions, CrAg+ remained an independent predictor of mortality similar to previous studies [6,8,28,29]. Nonetheless, the observed mortality among CrAg+ patients in our cohort was lower than that reported in Sub-Saharan African studies (23e55%) [6,8,18,28]. This difference may be explained by several factors. First, only two of the 10 patients who developed CM died of it despite the severity of the disease, probably due to better access to medical care in a high-income setting. Second, the CoRIS did not provide information about prophylactic treatments; thus, even if CrAg determination was not a standardized practice in our setting, it might be possible that it was performed in some cases and fluconazole administered.

Table 3
Distribution of cryptococcal antigen titres depending on the presence or subsequent development of crypotococcal meningitis (CM)

CrAg-titres[y]	CM at plasma sample collection (*n*=5)	CM during the evolution (*n*=5)	No CM (*n*=33)
1:1	0	0	20
1:2	0	1	2
1:8	0	0	5
1:32	0	0	1
1:64	0	2	3[a]
1:128	0	0	1
1:256	0	1	0
1:512	2	1	0
>1:1024	3	0	1[b]

CrAg, cryptococcal antigen.
[y] Titres have been determined with latex-agglutination test Pastorex™Crypto Plus. All the 43 samples shown a positive T1 band on Biosynex® CryptoPS-LFA. Eleven of them also showed a positive T2-band and their corresponding CrAg-titres measured with latex-agglutination were >1:256 in eight of the samples, 1:64 in two and 1:8 in the remaining one.
[a] One of the patients died at day þ26 due to *P. jirovecii* pneumonia evolving badly despite treatment.
[b] This patient died the same day of plasma collection due to cerebral toxoplasmosis.

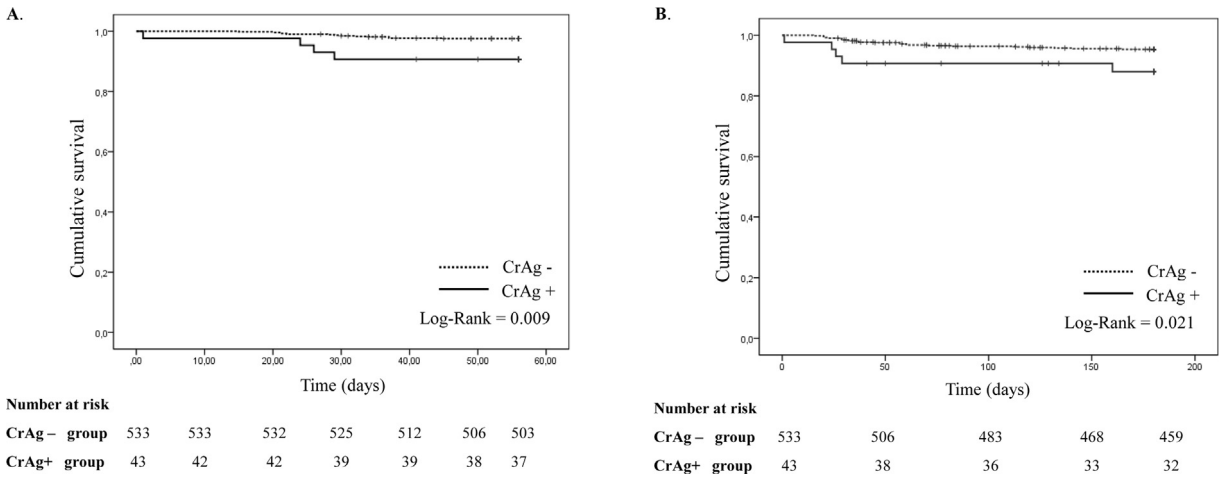

Fig. 1. Kaplan-Meier survival curves for HIV-infected patients with positivity and negativity of cryptococcal antigen (CrAg+ and CrAg-), (a) First 8 weeks after crytpococcal antigen (CrAg) determination. (b) First 6 months after cryptococcal antigen (CrAg) determination.

Finally, most of the CrAg+ patients had low CrAg-titres so maybe reconstitution of the immune system during highly active ART, initiated early after HIV diagnosis, could have led to effective clearance of asymptomatic infection [8,30]. In fact, we observed a direct relationship between higher CrAg-titres and symptomatic CM as reported by others [8,31].

In contrast with previous reports, we found a cut-off titre of 1:32 as optimal for predicting the presence or subsequent development of symptomatic CM while others have established this cut-off in 1:160 [7]. The reason for this difference is that we titrated the samples using a latex-agglutination technique rather than performing LFA on serially diluted samples. LFA has a higher analytical sensitivity than latex-agglutination. Thus, when directly comparing CrAg-titres by two-fold serial dilutions, LFA-titres are a median of five-fold higher than latex-agglutination titres [32]. Surprisingly, CM was not reported for one patient with CrAg-titres >1:1024. A detailed analysis showed that this patient died of cerebral toxoplasmosis on the day of plasma collection. We postulate that CM might have remained undiagnosed because neurological symptoms could have been attributed exclusively to toxoplasmosis. This fact raises the importance of systematically determining CrAg in HIV-infected patients with CD4+ T-cell counts≤100/mL to avoid the unrecognition of a treatable and potentially curable life-threatening disease.

A previous cost-effective analysis using South African data on CrAg prevalence, CM-incidence in ART programmes, CM-related mortality, and healthcare costs showed that a screen-and-treat strategy was more effective and less expensive than no screening [33]. Furthermore, the authors demonstrated that targeted pre-emptive treatment of all CrAg+ patients remained cost-effective even with a CrAg+ prevalence as low as 0.6%. Although we have found a CrAg+ prevalence as high as 7% in our cohort, both CM-incidence and CM-related mortality were lower than in those described in South Africa; thus, it is possible that these calculations are not fully applicable to our setting. Nevertheless, by screening the 114 patients required to anticipate the diagnosis of one symptomatic CM case, we would detect seven patients with asymptomatic CrAg+ whose management would be less expensive than

Table 4
Uni- and multivariate analyses of risk factors predicting all-cause mortality after cryptococcal antigen (CrAg) determination.

Variable	Univariate analysis			Multivariate analysis[a]		
	HR	95% CI	p	HR	95% CI	p
Within eight-weeks after CrAg determination[y]						
Age[b]	1.03	0.99-1.08	0.133			
CD4+ count[b]	0.97	0.95-0.99	0.016	0.97	0.95-0.99	0.025
CrAg+	3.99	1.30-12.24	0.016	5.36	1.46-19.64	0.011
Opportunistic disease[c]	2.30	0.85e6.23	0.100			
AIDS defining condition[c]	3.25	1.06e9.98	0.039	d	d	d
Transmission route (IDUs vs sexual)	3.91	1.08-14.22	0.038	d	d	d
Within six-months after CrAg determination[z]						
Age[b]	1.04	1.01-1.08	0.007	1.04	1.01-1.08	0.007
CD4+ count[b]	0.98	0.96-0.99	0.004	0.98	0.96-0.99	0.013
CrAg+	2.76	1.05e7.24	0.039	3.12	1.19e8.21	0.021
Opportunistic disease[c]	2.41	1.12e5.18	0.024			
AIDS defining condition[c]	3.18	1.36e7.45	0.008	2.54	1.06e6.09	0.037
Transmission route (IDUs vs sexual)	2.14	0.63e7.28	0.221			

CI, confidence interval; HR, hazard ratio; IDU, intravenous-drug user. The bold in the tables is used to remark the statistically significant values.

[y] Four deaths among 43 CrAg+ (cryptococcal meningitis in two cases, and cerebral toxoplasmosis and *P.jirovecii* pneumonia in one each) and 13 deaths among 533 CrAg-, within the first 8 weeks.

[z] Five deaths among 43 CrAg+ (cryptococcal meningitis in two cases, and cerebral toxoplasmosis, *P.jirovecii* pneumonia and sepsis in one each) and 24 deaths among 533 CrAg-, within the first 6 months.

[a] AIDS defining condition but not opportunistic disease was included in the model, due to the collinearity between both variables.

[b] Per unitary increment.

[c] Previous or within the first month after determining CrAg, excluding cryptococcal meningitis.

treating one symptomatic CM case without considering the morbidity/mortality associated with this entity [8,14,34].

This study has some limitations. First, we lost ~18% of the initially identified patients with CD4+ T-cell counts≤100/mL although there were no differences in age, CD4+ T-cell count, birthplace or AIDS-defining conditions between lost and included patients. A significantly higher proportion of IDUs was found among those excluded so we cannot rule out the possibility of an underestimation of the real cryptococcal infection prevalence considering that intravenous-drug use has been identified as a risk factor for CrAg+. Another handicap is the lack of information about prophylaxis use in the CoRIS as previously stated. Finally, the under-representation of patients from other high-income countries precludes us from generalizing our results to those settings.

In spite of these limitations, our analysis using samples from a large Spanish multi-centre cohort with a long prospective follow-up gives these results strong confidence. Our findings suggest that it is necessary to update GESIDA guidelines and recommend routine screening for CrAg in HIV-infected patients with CD4+ T-cell counts <100/mL diagnosed in Spain, including native-born Spanish. Future studies should explore if this recommendation could be firmly applied to this group of HIV-patients born in other European countries.

Transparency declaration

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Author contributions

M.A.P.J.A. proposed the study. M.A.P.J.A., O.B.P., R.R. and J.A.I. designed the analysis. All the authors contributed to data collection. A.P.R. was in charge of microbiological analysis. M.A.P.J.A. performed the analyses. M.A.P.J.A., O.B.P., R.R., J.A.I. and S.M. interpreted data. M.A.P.J.A. wrote the manuscript. All of the authors performed a critical review of the manuscript and approved the final version. M.A.P.J.A. had full access to the data and acts as guarantor for the report.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2020.09.053.

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