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Molecular diagnosis of toxoplasmosis in immunocompromised patients: a three-year multicenter retrospective study

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ABSTRACT. Toxoplasmosis is a life-threatening infection in immunocompromised patients (ICPs). The definite diagnosis relies on parasite DNA detection, but the incidence and burden of disease are ill-known in HIV-negative patients. A three-year retrospective study was conducted in 15 reference laboratories from the network of the French National Reference Center for Toxoplasmosis, to record the frequency of *Toxoplasma* DNA detection in ICPs, to review the molecular methods used for diagnosis and the prevention measures implemented in transplant patients. During the study period, of 31,640 PCR performed in ICPs, 610 were positive (323 patients). Blood (n=337), cerebro-spinal fluid (n=101) and aqueous humor (n=100) samples were more frequently positive. Chemoprophylaxis schemes in transplant patients differed between centers. PCR follow-up of allogeneic hematopoietic

stem-cell transplant (allo-HSCT) patients was implemented in 8/15 centers. Data from 180 patients (13 centers) were further analyzed regarding clinical setting and outcome. Only 68/180 (38%) patients were HIV+; the remaining 62% consisted in 72 HSCT, 14 solid-organ transplants, and 26 miscellaneous immunodeficiencies. Cerebral toxoplasmosis and disseminated toxoplasmosis were most frequently observed in HIV and transplant patients, respectively. Of 72 allo-HSCT patients with positive PCR, 23 were asymptomatic; all were diagnosed in centers performing systematic blood PCR follow-up and received specific treatment. Overall survival of allo-HSCT patients at two months was better in centers with PCR follow-up than in other centers ($p < 0.01$). This study provides actualized data on the frequency of toxoplasmosis in HIV-negative ICPs and suggests that regular PCR follow-up of allo-HSCT patients could guide pre-emptive treatment and improve outcome.

INTRODUCTION

Toxoplasmosis is a widespread parasitic infection, frequently asymptomatic in immunocompetent patients. However, this obligate intracellular protozoan parasite can evade the immune system (1, 2) and persist lifelong in its host as a cyst form, predominantly in brain, retina and muscles. Reactivation of latent cysts may occur when the immune system fails to maintain cytokinic pressure which mainly relies on IFN- γ (3). Cyst reactivation can lead to ocular toxoplasmosis, cerebral toxoplasmosis (CT) or disseminated toxoplasmosis frequently involving the lungs but potentially all organs. Failure of an efficient Th1 immune response mainly results from acquired immunosuppression, through HIV infection or immunosuppressive therapy. Both primary-acquired and reactivated infections are life-threatening in immunocompromised patients (ICPs). Definitive diagnosis can be obtained by the detection of parasites in blood, cerebrospinal fluid (CSF), broncho-alveolar lavage (BAL) or virtually any tissue by PCR, which is the most sensitive method (4).

Prevention of CT in HIV patients is rather consensual and guidelines recommend cotrimoxazole chemoprophylaxis in *Toxoplasma*-seropositive patients when CD4+ cell counts fall below 200 cells/ μ L (4), a prophylactic regimen which also protects patients from *Pneumocystis jirovecii* pneumonia. Nevertheless, toxoplasmosis remains the most prevalent cause of neurological opportunistic infection in Europe despite the use of highly active antiretroviral therapy (HAART) (5). Due to the mandatory reporting of AIDS cases in many countries, the estimation of the toxoplasmosis burden is relatively straightforward. In France, the last data from the Institut de Veille Sanitaire reported that cerebral toxoplasmosis was

associated to inaugural AIDS stage in 12% of patients and the annual number of cases was estimated to be about 160 in 2010 (6).

By contrast, the incidence of toxoplasmosis in solid organ transplant (SOT) patients or hematopoietic stem cell transplant (HSCT) patients is far less documented. It is supposed to parallel the seroprevalence in the general population, but it is not subjected to any reporting system, at least in France. Although occasional case reports have been published in the last 10 years (7-11), the incidence of toxoplasmosis in transplant patients is largely unknown. The risk for transplant patients differs according to the type of graft, and prevention measures may differ accordingly (reviewed in (12)). Reactivation of a chronic infection may occur in the recipient irrespective of the type of graft, and is closely related to the duration and degree of immunosuppression, with allogeneic HSCT (allo-HSCT) patients carrying the highest risk. In SOT patients, severe or disseminated toxoplasmosis can result from either reactivation of latent infection in the recipient or from organ-transmitted infection from a seropositive donor to a seronegative recipient (13), a situation where heart transplants patients are at high risk (12). Prevention measures rely on serologic screening of donor and/or recipient, and on chemoprophylaxis (14). Determination of the donor serostatus is mandatory in France and highly recommended for the recipient, but there are no guidelines regarding chemoprophylaxis, except in case of mismatch in heart transplant patients. Additionally, some authors advocated biological screening of allo-HSCT patients using PCR on whole blood on a regular basis, with the aim to detect early circulating parasites and start pre-emptive therapy, but the benefit of this practice have not been evaluated (15-17).

In the present multicenter study involving 15 reference laboratories from academic hospitals,

we investigated the molecular methods used for the diagnosis of toxoplasmosis in ICPs and the frequency of *Toxoplasma* DNA detection in these patients. The outcome of disease was examined in 180 patients, allowing to draw information about clinical picture and prevention practices.

PATIENTS AND METHODS

Data collection

A 3-year retrospective survey (2009-2011) was conducted through the French network of the Centre National de Référence de la Toxoplasmose (<http://cnrtoxoplasmose.chu-reims.fr/>). Fifteen centers participated in the study and responded to an extensive questionnaire including the following items: molecular method used for the diagnosis of toxoplasmosis, particular measures taken according to the sample type, total number of samples tested for *Toxoplasma* in ICPs, number of positive samples and sample types over the study period, number of transplantations in the center, type of transplant (heart, kidney, liver, HSCT), type of HSCT (allogeneic or autologous), duration and type of prophylaxis, implementation of iterative PCR follow-up after transplantation or allograft, and, if any, frequency and duration. More precise data on 180 patients with *Toxoplasma* DNA detection were obtained from 13 centers, including: immune background, type and number of positive samples, clinical setting, prophylaxis and outcome at two months. Data relative to diagnosis were extracted from laboratory databases or information systems; and data relative to patient management were obtained through local clinicians and/or information systems.

Data collection relative to routine diagnosis agreed with local ethical committee of each center, and the coordinator analyzed only aggregated data, thus no specific permission was required, in accordance with French rules.

Classification of cases

Patients were classified as “CT” when PCR was positive in CSF or blood sample with a compatible computerized tomography scan and/or neurological sign. Patients with parasite detection in BAL, bone marrow or any biopsy from a deep-seated organ (not considered as a classical sanctuary for *Toxoplasma* cysts), were considered as “disseminated toxoplasmosis” whatever the result of PCR on whole blood.

Patients with a positive PCR only in the blood, and no specific imaging signs and no symptoms except fever, were classified as “isolated fever”. Patients with a positive PCR in aqueous humor or blood and eye lesions were classified as “ocular toxoplasmosis”, provided that only ocular signs were present. Patients with a positive PCR in blood but no symptoms/signs at the time of sampling, were classified as “asymptomatic”. Survival was considered in the two months following the first positive PCR result.

Statistical analysis

Qualitative variables were expressed as numbers and percentage and quantitative variables as mean and standard error mean (SEM).

Statistical analysis was performed using GraphPad® Prism software. Chi-square test or Fisher exact test were used to compare qualitative variables between groups. Quantitative variables were compared using the Mann-Whitney test. A p-value <0.05 was considered significant.

RESULTS

Proficiency and validation of the molecular methods used in this study

Reference laboratories used a variety of methods for the molecular diagnosis of toxoplasmosis, as reported previously (18) (see details in Table S1). Briefly, DNA extraction was performed using manual commercial methods (Qiagen® or Roche Diagnostics®), according to the manufacturer's specifications, in 11/15 centers; two different manual 'in-house' methods were used in 1/15 centers (19, 20), and an automated method was used in 3/15 centers (Qiagen® or Roche Diagnostics®). The PCR devices used for amplification and the PCR probes used for detection of amplicons (FRET or hydrolysis probes) varied according to centers; but all centers used the same PCR target, i.e. the repetitive DNA sequence termed 'rep529' (Genbank AF146527) (Table S1). Internal controls and negative and positive controls were included in each PCR run. All assays had been previously validated by multicenter evaluations (21, 22) and are routinely evaluated through regular national external quality assessments (18)

The assays were the same as those used for the diagnosis of congenital toxoplasmosis in all centers, except for blood samples for which a

different extraction method was performed in 3 centers. Leukocyte fraction separation from 5-10 mL of whole blood was performed before blood DNA extraction in 5 centers (33%), whereas in the 10 remaining centers, DNA was extracted directly from whole blood.

Frequency of *Toxoplasma* DNA detection in immunocompromised patients.

Over a 3-year period (2009-2011), 610 positive PCR tests were observed from various samples obtained from 323 ICPs in the 15 University Hospitals participating in the study. Overall, the

mean frequency of a positive PCR result was about 3% of all samples analyzed per center (mean $2.9\% \pm 1.8$). The most frequently positive sample was blood (55% of positive samples), followed by CSF (17%), aqueous humor (16%), BAL (5%), and miscellaneous samples including various biopsies (7%)(Table 1). The frequency of *T. gondii* DNA detection in blood was not significantly different in centers performing DNA extraction on whole blood and in centers working on the leukocyte fraction (data not shown).

Table 1: PCR detection of *T. gondii* in 15 centers over the 3-year period (2009 to 2011).

Specimen type (no. of patients with positive PCR result)	No. of positive samples/total samples tested (%)	Mean SEM positive PCRs/center
Any specimen (323)	610/31,640 (1.9)	41 ± 33
Blood (152 s)	337/24,051 (1.4)	22 ± 23
CSF (93)	101/2,293 (4.4)	7 ± 5
BAL (25)	30/3,914 (0.8)	2 ± 1
Aqueous humor (95)	100/836 (12)	8 ± 8
Other specimen (30)	42/546 (na)	3.5 ± 3
Mean no. of positive PCR per patient	1.9 (na)	na

CSF; cerebro-spinal fluid, BAL; broncho-alveolar lavage, na; not applicable

Clinical picture and immune background in 180 patients with positive *Toxoplasma* PCR

The PCR data from 13 centers (180 patients) could be analyzed in more detail to determine the clinical significance of *Toxoplasma* DNA detection. Among these 180 patients, 68 (38%) were HIV+ patients, 72 (40%) HSCT patients, 14 (8%) SOT patients (4 with heart, 7 with kidney and 3 with liver transplant), and 26 (14%) had various causes for immunodeficiency: 16 (62%) had hematological malignancy including 5 chronic lymphoid leukemia and 7 lymphomas, five (19%) had a connective tissue disease treated with immunosuppressive drugs, two presented with an acute solid tumor (glioblastoma and breast cancer), two suffered from a congenital immunodeficiency (ataxia telangiectasia and IFN- γ receptor deficiency) and one presented with chronic alcoholism and acute pancreatitis. All HSCT patients were allograft recipients, except one who was an auto-HSCT patient. The type of allograft was a matched-related bone marrow donor in 11 cases (15%), a matched-unrelated (national file) donor in 37 cases (51%), cord blood in 13 cases (18%), or undetermined in 7 cases.

Overall, the incidence of a positive PCR result was higher in allo-HSCT patients (3.2%) than in other transplant patients (Table 2). PCR-positive (PCR+) samples were associated with clinical symptoms in 2.2% of allo-HSCT patients (Table 2). On the other hand, PCR was positive in 23 allo-HSCT patients who were asymptomatic (Table 2). Of these, 6 were receiving cotrimoxazole chemoprophylaxis, and a curative therapy was started in view of PCR results in the remaining 17. All survived. Clinical toxoplasmosis in SOT patients was acquired through the transplanted organ in three (one heart, one kidney and one liver transplant), due to *Toxoplasma* reactivation in seven, and to late primary infection (probable oral infection) in four of them. Actually, 7/14 (50%) patients with positive PCR were seronegative prior to transplantation; they consisted of 4 kidney, 2 liver and 1 heart transplant patients. Parasite DNA was also detected in three asymptomatic SOT patients (two heart transplant and one liver transplant) in one center who performed regular blood PCR follow-up. Of these three SOT patients, one was treated, one was not (both survived)

and one was lost to follow-up. HIV patients mostly presented with cerebral toxoplasmosis (65% of cases) or ocular toxoplasmosis (22%), whereas HSCT and SOT patients were more likely to have disseminated toxoplasmosis (41% and 43%, respectively, $p < 0.01$) (Table 2). The overall outcome did not differ statistically between the groups of patients (Table 2). However, when excluding the 23 HSCT patients detected through systematic PCR in the absence of clinical signs, survival proved to be significantly lower in allograft patients than in HIV-infected patients (67% versus 78%, $p < 0.05$) (Table 2). Similarly, when excluding ocular toxoplasmosis from the patient category with miscellaneous immunodeficiency background, the outcome appeared worse in these patients, than in HIV patients (survival of 53% and 78%, respectively, $p < 0.05$).

Heterogeneous prevention practices in transplant patients

Prevention practices were investigated in the 15 centers, who declared a transplantation activity of 3711 kidney transplants, 549 heart transplants, 2262 liver transplants, 2463 allo-HSCT and 3318 autologous HSCT (auto-HSCT), over the study period. Eight out of 13 allograft centers (61%) had implemented a regular follow-up using PCR on blood (Table 3). The frequency and duration of blood sampling after allo-HSCT were variable, ranging from twice a week to once a month, for 3-6 months or even lifelong in case of graft versus host disease. The iterative follow-up of heart transplant patients using blood PCR, as suggested by some authors in case of serologic mismatch (positive donor/negative recipient) (12), was performed in only 1 out of 10 centers. Chemoprophylaxis regimens also varied among centers. In fact, a prophylaxis regimen specifically targeting toxoplasmosis was rarely applied, except in heart transplant patients and in case of a serologic mismatch in other SOT patients. Most allo-HSCT patients usually benefited from *Pneumocystis jirovecii* prophylaxis guidelines using cotrimoxazole, which are applied in most centers. Overall, kidney, heart transplant and allo-HSCT patients were given cotrimoxazole in 14/14, 9/10, and 10/13 centers, respectively (Table 3). One additional center declared to use spiramycin in heart transplant patients, and another one used pyrimethamine-

sulfadoxine in allo-HSCT patients. The chemoprophylaxis was prescribed irrespective of the recipient serologic status, but the duration was longer or for life in heart transplant patients with serologic mismatch. Liver transplant patients usually were not given any chemoprophylaxis (7/12 centers) and auto-HSCT patients never were, except in one center. There seemed to be a consensus on the starting date for cotrimoxazole in HSCT patients, at about 20-30 days following allograft. Overall, a six-month duration was the most frequently used scheme, whatever the type of graft (51% of all responses) (Table 3).

Impact of PCR follow-up on the diagnosis and management of toxoplasmosis in HSCT patients

Data from the 72 PCR+ allo-HSCT patients were analyzed, taking into account the implementation or not of systematic PCR follow-up, with the aim of searching for a benefit for such screening. In centers where a systematic PCR follow-up was implemented, the mean annual number of PCR performed for HSCT patients was 16-fold higher than in other centers, and the mean number of PCR per allo-HSCT patient was 10 ± 2.8 (Table 4). The prevalence of patients with *Toxoplasma* DNA detection was estimated by dividing the number of PCR+ allo-HSCT patients by the total number of allograft patients in a given center during the study period. In centers with regular PCR screening, the percentage of PCR+ allograft patients was about 3-fold higher than in non-screening centers ($4.9 \pm 1.6\%$ compared to $1.7 \pm 0.6\%$, respectively) (Table 4). All PCR+ asymptomatic patients were detected in centers performing systematic PCR follow-up. The overall survival of allograft patients was better in centers with regular PCR screening than in other centers (86% versus 50% survival, respectively, $p < 0.01$). When asymptomatic patients, who all survived, were excluded for analysis, there remained a similar trend, with a better outcome for patients who benefited from PCR screening, compared to other patients (76% versus 50% survival) (Table 4).

Table 2: Characteristics of 180 immunocompromised patients with positive qPCR for *T. gondii* (13 centers)

Characteristics	HIV+ patients (n = 68)	HSCT patients (n = 72)	SOT patients ^a			Other ^b (n = 26)	p-value
			Heart transplant (n = 4)	Kidney transplant (n = 7)	Liver transplant (n = 3)		
Clinical setting, n (%)							<0.0001
Cerebral toxoplasmosis	44 (65)	12 (16.5)	-	4 (57)	-	6 (23)	<0.0001
Disseminated toxoplasmosis	8 (12)	30 (41.5)	2 (50)	2 (29)	2 (67)	9 (35)	0.007**
Ocular toxoplasmosis	15 (22)	2 (3)	-	-	-	9 (35)	<0.0001
Isolated fever	1 (1)	5 (7)	-	1 (14)	-	2 (7)	0.409 (ns)
Asymptomatic	-	23 (32)	2 (50)	-	1 (33)	-	<0.0001
No. of cases/No. grafts (%) ^c	Na	71/2220 ^d (3.2) 1/2940 ^e (0.03)	4/282 (1.4)	7/3180 (0.2)	3/1896 (0.16)	Na	-
No. of symptomatic cases/No. grafts (%)	Na	48/2220 ^d (2.2) 1/2940 ^e (0.03)	2/282 (0.7)	7/3180 (0.2)	2/1896 (0.11)	Na	-
Chemoprophylaxis, n (%)							Na
Yes	4 (6)	14 (19)	1 (25)	0	0	0	
No	17 (25)	37 (51)	3 (75)	6 (86)	2 (67)	13 (50)	
Unknown data	47 (69)	21 (29)	-	1 (14)	1 (33)	13 (50)	
Treatment, n (%)							0.0075**
Yes	64 (94)	54 (75)	2 (50)	7 (100)	2 (67)	18 (69)	
No	0	13 (18)	2 (50)	0	1 (33)	2 (8)	
Unknown data	4 (6)	5 (7)	0	-	-	6 (23)	
Outcome, n (%)							0.626 (ns) ^{c,d}
Survival (2 mo)	53 (78)	56 ^f (78)	4 (100)	4 (57)	2 (67)	18 ^g (69)	
Death	11 (16)	16 (22)	0	3 (43)	1 (33)	7 (27)	
Unknown data	4 (6)	-	-	-	-	1 (4)	

^a recipients were seropositive for *Toxoplasma* prior to transplantation in 7/14 cases, ^b consisting of hematological malignancies (16), connective tissue diseases receiving immunosuppressive drugs (5), solid tumors (2), congenital immunodeficiencies (2) and chronic alcoholism (1), ^c cumulative data from all centers on the study period, ^d allo-HSCT, ^e auto-HSCT, ^f survival was 67% when asymptomatic patients detected through systematic screening were excluded (p<0.05, compared to HIV+ patients), ^g survival was 53% when patients with ocular toxoplasmosis were excluded (p<0.05, compared to HIV+ patients). HSCT, hematopoietic stem cell transplant; SOT, solid organ transplant. Variables were compared using Chi-square or Fisher exact tests. Na, not applicable; **, p<0.01; ns, not significant.

Table 3: Prevention of toxoplasmosis and PCR follow-up practices in transplant patients (15 centers)

Characteristics	No. (mean per centre \pm SEM) or No./total (%)
No. of solid organ transplantation (2009-2011)	
Kidney (14 centres)	3711 (265 \pm 97)
Heart (10 centres)	549 (50 \pm 32)
Liver (12 centres)	2262 (174 \pm 86)
Haematopoietic stem cell transplantation (2009-2011)	
Allograft (13 centres)	2463 (189 \pm 79)
Autograft (14 centres)	3318 (237 \pm 101)
Systematic PCR follow-up, No. centers/total (%)	
Allograft patients	8/13 (61%)
Heart transplant patients	1/10 (10%)
Frequency of PCR follow-up in allo-HSCT patients, No. centers	
2 blood PCR/week	1/8 (12.5%)
1 blood PCR/week	4/8 (50%)
2 blood PCR/month	2/8 (25%)
1 blood PCR/month	1/8 (12.5%)
Chemoprophylaxis, No. centers/total	
Kidney transplant patients ^a	14/14 (100%)
Heart transplant patients ^b	10/10 (100%)
Liver transplant patients ^c	5/12 (42%)
Allograft transplant patients ^d	11/13 (85%)
Autograft transplant patients ^e	1/14 (7%)

^a variable duration: 4-6 wk (2 centers), 3 mo (4 centers), 6 mo (7 centers), unknown (1 center)

^b variable duration: 4-6 wk (1 center), 3 mo (1 center), 6 mo (6 centers), 1 yr (1 center), lifelong if mismatch (1 center)

^c variable duration: 6 mo (3 centers), variable (1 center), lifelong (1 center)

^d variable duration: 6 mo (4 centers), guided by CD4+ T cell count (4 centers), 1 yr (3 centers)

^e during 3 mo

Table 4: *Toxoplasma* DNA detection in allo-HSCT patients according to PCR follow-up policy (n = 72 patients from 11 centers)

Characteristics (2009-2011)	Centers with PCR follow-up N=6	Centers with no PCR follow-up N=5	p-value
Annual no. of allograft			
Annual no. of PCR, mean \pm SEM	796 \pm 624	48 \pm 26	0.0095**
No. of PCR/no. allo-HSCT, mean \pm SEM	10 \pm 2.8	0.6 \pm 0.2	0.0043**
No. of patients with PCR+ testing	56	16	na
No. of PCR+ patients/no. engrafted, mean % \pm SEM	4.9 \pm 1.6	1.7 \pm 0.6	0.177 (ns)
Asymptomatic patients with PCR+ testing:			
Total no.	23	0	0.0015**
No. detected/no. engrafted, mean % \pm SEM	2.1 \pm 0.8	na	
Type of positive sample	1.7 \pm 0.6	na	
No. of PCR+ results per patient, mean \pm SEM	8	na	
No. of patients under chemoprophylaxis	17 ^a (74%)	na	
No. treated, n (%)	23 (100%)	na	
Survival			
Symptomatic patients with PCR+:			
Total no.	33	16	

No. detected/no. engrafted, mean % ±	2.8 ± 0.9	1.7 ± 0.6	0.329 (ns)
SEM	21 (64%)	9 (56%)	0.756 (ns)
Disseminated toxoplasmosis, n (%)	8 (24%)	4 (25%)	1 (ns)
Cerebral toxoplasmosis, n (%)	3 (9%)	2 (13%)	0.672 (ns)
Isolated fever, n (%)	1 (3%)	1 (6%)	1 (ns)
Ocular toxoplasmosis, n (%)	25 (76%)	8 (50%)	0.106 (ns)
Survival, n (%)			
Overall survival (n=72), n (%)	48 (86%)	8 (50%)	0.005**

^a the 6 untreated patients were receiving chemoprophylaxis. na, not applicable; **, p<0.01; ns, not significant.

DISCUSSION

This study provides a global insight into the molecular diagnosis of toxoplasmosis in ICPs, and shows for the first time that the parasite is detected with a higher frequency in non-HIV patients than in HIV-infected patients (62% and 38% of cases, respectively). As for *Pneumocystis jirovecii* pneumonia (23), the present data show that toxoplasmosis is of increasing importance in non-HIV patients, who represent a growing at-risk population, due the wide use of immunosuppressive therapies and the increasing number of transplant patients. In HIV-infected patients, prevention measures are well codified, and the prevalence of the disease has remained stable since the use of HAART (6). By contrast, prevention of toxoplasmosis in non-HIV patients is not standardized, even though it often benefits from *Pneumocystis* pneumonia prevention in transplant patients, as shown here in heart and kidney transplant patients. However, it appeared that chemoprophylaxis regimens and durations vary among centers, underlining the need for guidelines according to the type of graft. Of great interest was the observation that 26 of 112 non-HIV patients (23%) were neither SOT nor HSCT patients, but had various causes of immunodeficiency, with a high proportion of hematological malignancies. This observation stresses the need for evaluating the risk factors for toxoplasmosis in more detail, which could lead to consider chemoprophylaxis in targeted patient populations. Indeed, in our study 7/26 patients died, of whom 6 had disseminated toxoplasmosis after delayed diagnosis.

Parasite was detected in 14 SOT patients with highly variable delay after transplantation, of whom 7 were not immunized before transplantation. In two cases (1 kidney, 1 heart) *Toxoplasma* seroconversion (and parasite DNA detection) occurred several years after

transplantation, making organ-transmitted infection unlikely. Toxoplasmosis occurred in 3 kidney transplant patients who should have benefited from chemoprophylaxis for 3 or 6 months, but the delay from transplantation to parasite DNA detection was not recorded so it was not possible to verify that toxoplasmosis occurred after chemoprophylaxis removal. The 2 remaining cases occurred in liver transplant patients from centers who declared not to use chemoprophylaxis. Overall, we observed in SOT patients a 2-fold higher mortality rate (29%) than that observed in a Spanish multicenter study (24), despite a similar incidence of symptomatic toxoplasmosis in these patients (0.14% in the study by Fernandez-Sabé et al. versus 0.2% in our study). Heart transplant patients have been early recognized to be more at risk for donor-related toxoplasmosis than other SOT patients, through multiple case reports or cohort studies (reviewed in (25)), and this is confirmed here. More rarely, *Toxoplasma* seroconversion has been described in SOT patients in the absence of clinical signs (26, 27), even in patients who did not receive prophylaxis (26). Here, we observed a positive blood PCR result in two heart transplant patients who were asymptomatic. Both were *Toxoplasma*-seropositive prior to transplantation. In the first patient, PCR was positive 6 weeks after transplantation, during the chemoprophylaxis period, thus it is likely that cotrimoxazole prevented full-blown toxoplasmosis. In the second case, blood PCR was positive twice, 22 years after transplantation; the patient was not under chemoprophylaxis anymore and the episode resolved spontaneously. These data clearly show that low circulating parasite levels can be detected in transplant patients, without any clinical impact, if they are given chemoprophylaxis or that they are mildly immunocompromised.

Not surprisingly, allo-HSCT patients accounted for the major part of toxoplasmosis cases in non HIV patients (64%), particularly patients engrafted with a matched-unrelated donor, as already described (28). The frequency of toxoplasmosis in these patients (2.2%) was similar to estimations made in areas of high seroprevalence (29), such as France. The overall mortality in allo-HSCT symptomatic patients (67%, Table 2) was lower here than in the study by Schmidt et al. (30) who included only cases of disseminated toxoplasmosis and found a mortality rate of 95%, but higher than in HIV patients, as observed in this same study (30). Routine PCR testing on blood has been proposed to monitor these patients in the months or even years following allo-HSCT, to allow early treatment and improve survival (15, 16, 31). We therefore analyzed separately the data from HSCT patients, considering whether such policy was implemented in the participating center or not. This analysis revealed that survival was indeed better in centers where PCR follow-up was implemented (86% versus 50%, $p < 0.01$).

The potential drawback of such systematic screening strategy is the possible detection of parasite circulating DNA in patients with no clinical signs (17), which raises the question of the necessity to start treatment or not. In the present study, 48% of allo-HSCT PCR+ patients detected in centers with systematic PCR follow-up were asymptomatic. This percentage was lower than that observed in two previous studies, which reported that 10/16 (62%) (16) and 9/13 (69%) (15) patients had a positive PCR result without clinical signs, yet it was similar to that reported by Meers et al. (8/18, 44%)(32). Overall, the incidence of PCR+ asymptomatic allo-HSCT patients detected through systematic PCR follow-up was also lower here (23 of 1220, 1.9%) than in other studies. Indeed, Martino et al. reported 10 asymptomatic PCR+ patients out of 106 allo-HSCT patients (16), Fricker-Hidalgo et al. found 9 out of 70 (12.8%)(15), and Edvinsson et al. observed 1 out of 12 (8%) allo-HSCT patients (17). Divergent attitudes were observed among the French centers regarding the management of these asymptomatic patients: 74% of them received a curative therapy, whereas the remaining 26% were left under cotrimoxazole chemoprophylaxis. No patient was left without any specific treatment, making it difficult to have

a clear view of the clinical significance of circulating DNA: early sign of toxoplasmosis reactivation or negligible event? In fact, for most patients, circulating *Toxoplasma* DNA was detected during the first six months following engraftment, thus could correspond to the early detection of *Toxoplasma* reactivation, which usually occurs during this time frame (32) (16), thus it can be hypothesized that reactivation did not evolve towards full-blown disease because the patients were either receiving chemoprophylaxis or pre-emptive treatment was started early. The significance of circulating *Toxoplasma* DNA was more debatable in 3 patients who were tested positive 18 months (1 patient) and 8 years (2 patients) after allograft—A technical false positive result can be ruled out in all instances, since these positive PCR tests were observed in centers who routinely use PCR decontamination measures. To circumvent the question of the significance of positive PCR results in asymptomatic patients, we excluded them for survival analysis. Interestingly, there remained a trend towards higher survival in symptomatic patients from centers with PCR follow-up when compared to centers with no PCR follow-up (76% versus 50%). This suggests again that symptomatic patients could have been detected at an early stage through regular PCR screening, thereby allowing pre-emptive therapy.

Limitations of the study

The main limitation of this study relies in the fact that it was a retrospective study. Accurate data about allo-HSCT patients' immune background were not recorded, and various confounding factors could interfere with the survival comparison. The date of transplantation was not always recorded, thus the delay of onset of toxoplasmosis could not always be determined. This is an important issue, which would be interesting to address, to evaluate whether the duration of chemoprophylaxis should be extended. Moreover, chemoprophylaxis data were inconstantly collected through medical charts, making difficult any inference about the compliance to, or efficacy of, chemoprophylaxis, and its effects on PCR positivity. Finally, some biological limitations could also be considered: i) the sensitivity of PCR on CSF was previously estimated to be only about 50% or less (33-35), therefore the number of cases with mild

cerebral toxoplasmosis may have been underestimated, although these data were obtained with less sensitive PCR methods than nowadays, and ii) cases of ocular toxoplasmosis may also have been underestimated since aqueous humor is not always collected for analysis when ocular lesions are typical, and the sensitivity of laboratory diagnosis does not exceed 80% (36, 37).

In conclusion, this study points to the need for standardization of prevention policies in transplant patients and for identification of new groups of at-risk patients who may benefit from chemoprophylaxis, such as patients with hematological malignancies, as recently underlined (38). Allo-HSCT clearly appears as the main risk factor, and the better outcome of these patients in centers who have implemented a regular biological follow-up using blood PCR follow-up opens an interesting perspective. This should be confirmed in a large prospective multicenter study considering the severity of the immune background of the patients and comorbidities.

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