Strategies for reducing the risk of transfusion-transmitted leishmaniasis in an area endemic for *Leishmania infantum*: a patient- and donor-targeted approach

Teresa Jimenez-Marco[1,2], Cristina Riera[3], Enrique Girona-Llobera[1,2], Carmen Guillen[3], Laura Iniesta[3], Magdalena Alcover[3], Diana Berenguer[3], Alba Pujol[3], Miriam Tomás-Pérez[3], Beatriz Cancino-Faure[3], Teresa Serra[2], Martín Mascaró[4], Joan Gascó[5], Roser Fisa[3]

[1]Blood and Tissue Bank Foundation of the Balearic Islands, Majorca; [2]University Institute for Research in Clinical Sciences (IUNICS), Majorca; [3]Parasitology Laboratory, Faculty of Pharmacology, University of Barcelona, Barcelona; [4]Son Llàtzer Hospital, Majorca; [5]Son Espases University Hospital, Majorca, Spain

Background. In the Balearic Islands, as in other areas of the Mediterranean basin, there is a significant proportion of asymptomatic *Leishmania* (*L.*) *infantum*-infected blood donors, who may represent an important threat to transfusion safety. The Balearic Islands blood bank, located in an area endemic for *L. infantum*, carried out a study of donors and patients to investigate the impact of this infectious disease on blood safety in the region.

Materials and methods. Twenty asymptomatic *Leishmania*-infected blood donors were followed-up between 2008 and 2011 to investigate the evolution of *Leishmania* infection in asymptomatic carriers. Their blood was periodically tested for anti-*Leishmania* antibodies by western blot and for *Leishmania* DNA by quantitative polymerase chain reaction (qPCR). Additionally, the prevalence of *L. infantum* infection was investigated in a group of 68 multiply transfused patients to ascertain the risk of transfusion-transmitted leishmaniasis (TTL) in the region, taking into account regular blood component production practices such as pre-storage leucodepletion and pathogen reduction technology.

Results. All 20 donors remained asymptomatic over the study period (2008-2011). Most donors had repeatedly positive qPCR results, either persistently or intermittently, but showed no symptoms of Leishmaniasis. Levels of parasitaemia were remarkably low in asymptomatic donors, with values ≤1 parasite/mL. Despite multiple transfusions received over 15 years, no transfused patient studied was infected with *L. infantum*.

Discussion. *L. infantum*-infected donors can remain asymptomatic for at least 3 years. In our region, no cases of TTL were detected, despite an active search in multiply transfused patients. This seems to be related to two independent variables: (i) a low concentration of the parasite in the peripheral blood of asymptomatic carriers and (ii) the application of methods with proven efficacy against TTL, such as leucodepletion and pathogen reduction technology.

Keywords: *Leishmania infantum,* leucodepletion, pathogen reduction technology.

Introduction

Leishmaniasis is a neglected vector-borne tropical infection prevalent in countries in Southeast Asia, East Africa and Latin America, but also in some Mediterranean countries. In Spain, as in other countries of the Mediterranean basin, *Leishmania infantum (L. infantum)* is endemic and responsible for zoonotic cutaneous and systemic diseases, with a reservoir in the domestic dog[1]. The clinical spectrum of this protozoan infection ranges from asymptomatic to fatal visceral leishmaniasis. Mainly spread through the bite of an infected female phlebotomine sandfly[2], the disease can also be transmitted by shared syringes among intravenous drug abusers[3], congenitally from mother to infant[4] and by blood transfusion[5-16]. Since most *L. infantum* infections are asymptomatic and resolve spontaneously in immunocompetent individuals, it is difficult to measure the blood transfusion transmission risk precisely. The concept of an asymptomatic carrier is defined as a parasite-carrying host without clinical manifestations. The existence of asymptomatic carriers is common in disease-endemic areas and may represent an important threat to transfusion safety.

In the Balearic Islands, a Mediterranean archipelago belonging to Spain, the prevalence of blood donors with asymptomatic *Leishmania* infection is high: in a screening of blood donors, 5.9% had *L. infantum* DNA and 3.1% had *L. infantum* antibodies in their blood[17]. These findings are consistent with those from other research studies performed in asymptomatic subjects from the Mediterranean basin[18-27] and Brazil[15,28].

This article describes a comprehensive study of the risk of transfusion-transmitted leishmaniasis (TTL),

taking a joint patient- and donor-targeted approach, based on our experience as a blood bank located in a *L. infantum* endemic area. First, *Leishmania* infection in donors was studied by investigating the natural evolution of the infection over 3 years in a selected group of asymptomatic blood donors living in the Balearic Islands. Secondly, the risk of TTL was addressed by studying the prevalence of *L. infantum* infection in patients in the region who had received multiple transfusions over a 15-year period.

Materials and methods

Donors

We studied 20 asymptomatic *L. infantum*-infected blood donors from the Balearic Islands, previously identified, in an epidemiological study carried out by our group[17], by the presence of anti-*Leishmania* antibodies and/or positive quantitative polymerase chain reaction (qPCR) results. At present, no routine screening test to detect *L. infantum* infection in blood donors is in place in our region and all donors identified as infected during the research study were excluded from regular blood donation. After obtaining written informed consent, peripheral blood samples were collected from 20 donors at different intervals, based on the convenience of blood drives during the study, which was carried out between 2008 and 2011. This study was approved by the Balearic Island Ethics Committee (Study identification number: PI 10/00533; protocol approval number IB 1129/09).

Patients

Sixty-eight chronic dialysis patients who received multiple transfusions over a 15-year period at the nephrology department of the "Son Llàtzer" Hospital in Majorca were studied in order to investigate *Leishmania* transfusion transmission. Informed consent was obtained from all participants.

Sample collection

Two peripheral blood samples, one in a tube containing EDTA and the other without anticoagulant, were collected from each study participant for serological and molecular studies.

Serological study

Anti-*Leishmania* antibodies were searched for by western blot (WB) using a whole *L. infantum* antigen (MHOM/FR/78/LEM 75 zymodeme MON-1) as previously described[17]. A single determination was performed for each serum sample. The WB assay was performed on 0.1% SDS-13% polyacrylamide gel on a Mini-gel System (Bio-Rad, Hercules, CA, USA). Sera diluted 1:50 were assayed and a protein A horseradish peroxidase conjugate (1:1,000 dilution) was used. We considered serum to be positive when immunoreactivity against the 14 and/or 16 kDa *L. infantum* antigen fraction was observed.

Real-time quantitative polymerase chain reaction

DNA was extracted from 200 µL of peripheral blood mononuclear cells in triplicate using a High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany). The presence of *Leishmania spp*. DNA was analysed by amplification of a kinetoplast DNA sequence by a real-time qPCR with a labelled Taq probe[29] (Bio-Rad). Positive controls (DNA from *L. infantum* MHOM/ES/04/BCN-61) and negative controls were included in each PCR analysis. A standard curve was constructed with 1:10 serial dilutions of total DNA extracted from *L. infantum* (1×10^5-0.1 parasite/mL). The parasitic load in parasite equivalents/mL of every sample was calculated using 7700 SDS 2.3 software (Applied Biosystems, Foster City, CA, USA). Each amplification was performed in triplicate in an ABI Prism 7700 system (Applied Biosystems). qPCR was considered positive for *Leishmania* when the threshold cycle (tC) was <45.

Results

Follow-up of asymptomatic *L. infantum*-infected blood donors from the Balearic Islands

The infectious status of 20 asymptomatic *L. infantum*-infected blood donors was studied in 2008-2011. The infections had been diagnosed in a previous research study by the presence of anti-*Leishmania* antibodies and/or positive qPCR results. Fourteen out of these 20 donors agreed to be monitored at different intervals. In the follow-up, peripheral blood samples were tested by WB and qPCR.

Thirteen out of 20 donors tested positive by WB, with bands of 14 and 16 kDa. Nine out of the 13 donors who were WB-positive were followed up over periods ranging from 2 months to 33 months. The WB test was persistently positive in seven out of nine during the follow-up period, whereas it was negative in two donors (donors 2 and 6) after 23 and 25 months of the follow-up period, respectively (Table I).

Fourteen out of 20 donors tested positive by qPCR. Half of them had a positive WB at least once during the study period. However, the remaining seven had negative WB results at all times in the study. Six out of 20 donors tested negative by qPCR but positive by WB (Table II).

The qPCR results were persistently positive in three out of the 14 donors, intermittently positive in eight, and three donors tested positive at least once during the follow-up period (Table III). In general, the quantification of *L. infantum* DNA in peripheral samples taken from blood donors was ≤1 parasite equivalent/mL, and the mean was 0.1 parasite equivalent/mL.

All the studied donors lived in rural areas of Majorca,

Table I - Results of the qPCR and WB assays in 20 asymptomatic *Leishmania infantum*-infected blood donors at different intervals during the study period.

Donors	Age (years)	Gender	Date	Western blot	Peripheral blood qPCR	Contact with animals
Donor 1	52	Male	October-08 November-10 March-11	POS 16 POS 16 POS 14, 16	POS* POS* POS*	Daily contact with dogs (a dog at home) Had a dog that died of leishmaniasis
Donor 2	44	Female	October-08 November-10	POS 14, 16 NEG	POS* POS*	Daily contact with dogs with leishmaniasis Works as a veterinarian
Donor 3	26	Male	October-08 March-10	NEG NEG	POS* NEG	Daily contact with dogs (a dog at home)
Donor 4	28	Male	November-08 March-10	NEG NEG	POS* NEG	Daily contact with dogs (a dog at home)
Donor 5	49	Male	January-09 March-10	NEG NEG	POS* NEG	Daily contact with dogs (two dogs at home) Had a dog that died of leishmaniasis
Donor 6	35	Female	February-09 June-09 November-10	NEG POS 14, 16 NEG	POS** NEG NEG	Daily contact with dogs (a dog at home) Works as a housekeeper in a country house with dogs
Donor 7	55	Male	February-09 March-10	NEG NEG	POS* NEG	No data
Donor 8	40	Male	February-09 May-09 November-10 March-11	NEG NEG NEG NEG	POS** NEG POS** POS**	Daily contact with dogs (a dog and a cat at home)
Donor 9	47	Male	June-09 March-10 October-10	POS 14, 16 POS 14, 16 POS 14, 16	POS* NEG POS*	Daily contact with dogs (a dog at home)
Donor 10	55	Male	October-09 January-10	POS 14, 16 POS 14, 16	NEG POS*	Currently no contact with dogs Owned a dog in the past
Donor 11	49	Male	August-09 October-09	POS 16 w POS 14	POS* NEG	Daily contact with dogs (a dog at home) Had a dog that died of leishmaniasis
Donor 12	60	Male	August-09 October-09	POS 14, 16 POS 14, 16	NEG NEG	No contact with dogs (a cat at home)
Donor 13	42	Female	August-09 October-09	POS 14, 16 POS 14, 16	NEG NEG	Daily contact with dogs (a dog at home) Had a dog that died of leishmaniasis
Donor 14	43	Male	October-11	NEG	POS*	No data
Donor 15	43	Male	September-11	NEG	POS*	No dog at home
Donor 16	55	Female	June-11	POS 14	POS*	Had three dogs that died of leishmaniasis
Donor 17	49	Male	October-08 July-11	POS 14, 16 POS 14, 16	NEG NEG	Occasional contact with dogs
Donor 18	33	Male	September-11	POS 14	NEG	No dog at home Family dog died of leishmaniasis
Donor 19	64	Male	October-11	POS 14	NEG	Daily contact with dogs (two dogs at home)
Donor 20	42	Female	October-11	POS 14, 16	NEG	Daily contact with dogs (two dogs at home)

qPCR: quantitative polymerase chain reaction; WB: western blot; POS*: ≤0.1 parasite equivalent/mL; POS**: 1 parasite equivalent/mL; POS 14, 16: WB positive with 14 and 16 kDa bands; POS 14: WB positive with a 14 kDa band; POS 16: WB positive with a 16 kDa band; POS 16 w: WB weakly positive with 16 kDa band.

Table II - General results of qPCR and WB tests of 20 asymptomatic *Leishmania infantum*-infected blood donors from Mallorca.

qPCR results	WB results		
	Positive	***Negative***	***Total***
Positive	7	7	14
Negative	6	0	6
Total	13	7	20

qPCR: quantitative polymerase chain reaction; WB: western blot.

and 16 of them had, or used to have, regular contact with dogs, either because they owned or worked with dogs. None of them had clinical symptoms of leishmaniasis, i.e., they remained asymptomatic throughout the follow-up period.

Prevalence of *L. infantum* infection in patients on chronic dialysis

Sera and peripheral whole blood from 68 patients from Majorca on chronic dialysis were collected for molecular and serological studies. Sixty patients were treated with haemodialysis and eight with peritoneal dialysis.

Forty patients (58.8%) were transfused whereas the other 28 patients (41.2%) did not receive any transfusions at all during their evolution. Twenty-six out of the 40 transfused patients (65%) were transfused after the implementation of universal leucodepletion in 2000 in our region. The remaining 14 patients (35%) received blood before 2000. Serology was positive in two patients who had not been transfused. Parasite DNA was not detected in any of the samples from either transfused or non-transfused patients. The prevalence of *L. infantum* antibodies was 0% (95% confidence interval [CI]: 0-10.4%) and 7.1% (95% CI: 0.9-23.7%) in the transfused and non-transfused patients, respectively. The total seroprevalence of *L. infantum* infection in haemodialysis patients was 2.9% (95% CI: 0.2-10.7%), which is in agreement with the finding of a previous study carried out by our group on blood donors in the Balearic Islands in which *L. infantum* antibodies were detected in 3.1% of blood donors tested (Table IV)[17].

Discussion

The risk of TTL associated with asymptomatic *Leishmania*-infected blood donors is a subject of controversy[30]. Most research on the prevalence of asymptomatic blood donors has been conducted in southern European countries, and the studies have found a wide range of values from 0 to 36.4%, depending on the assay used and the number of subjects tested[17-27].

The proportion of asymptomatic *Leishmania*-infected blood donors in the Balearic Islands has been reported previously by our group: *L. infantum* DNA was detected in the blood of 5.9% of tested blood donors and *L. infantum* antibodies were identified in 3.1%[17]. The percentages with positive PCR (10.5%) and *L. infantum* antibodies (6.8%) were higher among donors from rural areas in Majorca than the average value for the whole sample of donors. These results correlate with those of a recent study on asymptomatic *Leishmania* infection among blood donors in southeast Spain, in which PCR and enzyme-linked immunosorbent assay (ELISA) tests were positive in 8 and 2%, respectively, of the study sample. Notably, the percentage of positive PCR results was also much higher in rural areas (18%) than in larger communities (3%), and was strongly related to dog ownership[27].

From a strict point of view, only direct methods, such as microscopic examination, culture or PCR, which show *L. infantum* parasites can be used for establishing asymptomatic carriership[30]. PCR assay can be considered as a true direct method for detecting the parasite, as recent investigations demonstrated that *Leishmania* nucleic acids detected by PCR came from living parasites and were quickly degraded after the parasites' death[31]. However, indirect methods for detecting asymptomatic carriers have also been used, such as the lesihmanin skin test and antibody detection by various techniques including ELISA and WB. Several studies have demonstrated that the detection of antibodies against 14-16 kDa *L. infantum* antigens by WB is generally more sensitive and specific than ELISA with crude antigens[19,22,32]. The sporadic but repeatedly positive WB results observed over time in some individuals living in endemic areas could be explained by an asymptomatic infection rather than by cycles of infection followed by total parasite clearing[18,32].

All 20 blood donors who tested positive by qPCR and/or WB for *Leishmania* were living in rural areas of Majorca. Sixteen of them either had, or used to have, contact with dogs, mostly as owners or at work. Seven out of the 16 had contact with a dog that had died of leishmaniasis, which is not surprising since Majorca is

Table III - Follow-up peripheral blood qPCR results.

	Number of positive results			qPCR negative
	Persistently positive	*Intermittently positive*	*At least 1 qPCR positive*	
Peripheral blood qPCR	3	8	3	6

qPCR: quantitative polymerase chain reaction.

Table IV - *Leishmania infantum* infection status in chronic dialysis patients.

	Transfused patients (n.)	Non-transfused patients (n.)	Total (n.)
Patients	40	28	68
WB positive	0	2	2
qPCR positive	0	0	0
Prevalence	0%	7.1%	2.9%

qPCR: quantitative polymerase chain reaction; WB: western blot.

an area in which canine leishmaniasis is endemic and in some rural areas the prevalence of *Leishmania* infection in dogs, calculated from the detection of anti-*Leishmania* antibodies and/or by PCR, is between 58 and 67%. The prevalence of infection in dogs is much higher than the prevalence of overt *Leishmania*-related disease[33,34].

More than 50% of infected dogs, the main reservoir hosts of *L. infantum*, are asymptomatic carriers and can easily infect sandflies. However, people co-infected with this parasite and human immunodeficiency virus are also highly infectious to sandflies and may play a role in the transmission of the parasitic infection in some areas[2], so the impact of asymptomatically infected carriers on the natural transmission cycle is currently not known.

All 20 blood donors remained asymptomatic over the 3-year study period, and most of them repeatedly tested positive by qPCR, either persistently or intermittently. It is unclear whether they became re-infected every year during the active phase of the phlebotomine sandfly cycle, or whether they had persistent infections that were being controlled by their immune system. The levels of parasitaemia were remarkably low in asymptomatic blood donor carriers (mean: 0.1 parasite equivalent/mL) compared to those in patients with visceral leishmaniasis (50 parasites/mL)[35].

The control mechanisms by which *L. infantum* carriers with subclinical infection manage to restrain their infection are unknown. It has been suggested that interleukin-17 could play an essential role in blocking parasite replication, thus avoiding the development of visceral leishmaniasis in the infected individual[36,37].

Interestingly, it has been reported that there is a lack of correlation between qPCR findings and anti-*Leishmania* antibody detection in asymptomatic *L. infantum* carriers. Riera *et al*. previously showed that serology tests can undervalue the prevalence of *Leishmania* infection in blood donors[17], which would be more accurately determined by PCR. Additionally, compared to PCR, culture techniques have demonstrated low sensitivity in asymptomatic carriers[17]. The absence of an antibody response during an asymptomatic infection, despite the presence of the parasite, has also been observed in healthy subjects with cryptic *L. infantum* infection in other endemic regions[28,38-40]. Currently, no routine screening test is used to detect *Leishmania* infection in blood donors, but should one be established, the most convenient assay would certainly be PCR-based.

A recent review of the risk of TTL[41] using a classification scheme based on the criteria of the National Healthcare Safety Network of the Centers for Disease Control and Prevention[42], pointed out that only ten individual reports in the available literature describe suspected transfusion-related transmission of Leishmania spp. in a total of 14 patients[5-14]; in addition, a seroprevalence study in Brazil investigated 32 cases of visceral leishmaniasis diagnosed out of 82 patients undergoing haemodialysis and multiple transfusions[15].

Although four possibly infected donors were identified in the ten reported cases, it was impossible to prove the presence of *Leishmania spp.* in any of them, either in the donors' peripheral blood or the transfused blood components. Moreover, no information on the recipients' *Leishmania* infection status prior to transfusion was provided in any of the reports. Given the lack of scientific evidence, all the cases were classified as possible, and none of them as definitive cases of TTL[41].

The unambiguous identification of cases of TTL is hindered by several factors. First of all, detecting transfusion-related transmission is very challenging, especially in endemic regions in which the infection is mostly transmitted via the bite of phlebotomine sandflies. Secondly, the lack of clinical manifestations in infected immunocompetent subjects impedes or delays the diagnosis[16]. A prompt diagnosis of TTL is also prevented by the absence of data on the *Leishmania* infectious status of the patient before transfusion, the donor or the transfused blood component[42].

The reporting of negative results regarding TTL is also essential, especially in endemic regions such as the Balearic Islands where measures to prevent TTL, including leucodepletion, have been in place for some time. Thus, in our study of chronic dialysed patients, no transfused patients were found to be infected. In two patients who had *L. infantum* antibodies and yet tested negative by PCR, blood transfusion could be ruled out as the cause of *L. infantum* infection since neither had received any transfusions.

Our results differ from those of a study carried out in Brazil, another endemic area, which investigated the prevalence of anti-*Leishmania* antibodies among blood donors and multiply transfused haemodialysis patients[15]. In the Brazilian study, the seroprevalence was approximately 9-25% in blood donors and there was a high rate of positivity (37%) in dialysis patients who had received multiple transfusions. Although the authors stressed the need to consider blood transfusion as a possible route of *Leishmania* transmission in their region, no evidence was provided that blood transfusion was the real cause of the *Leishmania* infection in patients undergoing dialysis. There were no data about *Leishmania* infection status of patients prior to transfusion, which is essential in an endemic area, or about the donors or transfused blood components. It was also not stated in the aforementioned study whether the transfused blood components were leucodepleted or not. This information is crucial, since it has been proven that filtration using a whole-blood and red blood cell leucodepletion system is an effective method for removing *Leishmania* parasites from blood given by asymptomatic *L. infantum*-infected

donors[17,26]. Our negative results in multiply transfused patients can be explained by the implementation of red blood cell leucodepletion at the bedside in the Balearic Islands more than 20 years ago and, as in most blood banks in Spain, pre-storage universal leucodepletion for all blood components, which was introduced 15 years ago. Consequently, all blood components -red blood cells, platelets and plasma- transfused to patients in the Balearic Islands have been pre-storage leucodepleted since 2000.

In order for *Leishmania* to be transmitted through a transfusion, amastigotes must be present in the blood, either within monocytes or free in the plasma, and they must survive processing and storage conditions. Several pathogen reduction technologies to decrease the risk of TTL, besides leucodepletion, have been tested. These are mostly based on damaging nucleic acids, thereby preventing replication of the pathogen's genomes, and include the use of riboflavin and ultraviolet light (Mirasol® Pathogen Reduction Technology System; Terumo BCT, Lakewood, CO, USA) in plasma and platelets[43,44], photochemical treatment by amotosalen plus ultraviolet light (INTERCEPT® Blood System for Platelets; Cerus, BV, Amersfoot, the Netherlands) in plasma and platelets[45,46] as well as by thiopyrylium in red blood cell suspensions[47].

In the Balearic Islands, pathogen reduction technology based on amotosalen plus ultraviolet light for platelets was introduced in 2008. More recently, in 2012, pathogen reduction technology based on riboflavin and ultraviolet light was established in our blood bank for treating platelets and plasma. Until now, despite an active and thorough investigation in patients receiving multiple blood components, no cases of TTL have been reported in our region. Both prevention strategies, leucodepletion and pathogen reduction technologies, seem to provide a sufficient level of protection against *Leishmania* infection transmitted by transfusion. A recent study on *Leishmania donovani* has clearly shown the very high efficacy of treating whole blood with pathogen reduction technology based on riboflavin and ultraviolet light to eliminate *Leishmania*[48].

Conclusions

When assessing the possible transmission of *Leishmania* by blood transfusion, two key factors stand out: first, a significant proportion of asymptomatic blood donors in some endemic regions have transient and low parasitaemia; secondly, scientific evidence regarding the few TTL cases reported in the literature is scarce and as a result they have been classified as *possible* rather than *definitive* cases[41].

It is difficult to establish a case of transfusion-transmitted *Leishmania* beyond any reasonable doubt. Not only can asymptomatic *L. infantum* infection in adults go unnoticed, but the real level of transmission risk may be low. This is related to the extremely low parasitaemia in asymptomatic carriers, along with the gradual implementation of different blood processing methods to improve blood safety, such as pre-storage leucodepletion and pathogen reduction technologies.

Indeed, pathogen inactivation technology using riboflavin and ultraviolet light to eliminate *Leishmania*, among other germs, in whole blood[48] appears to be very promising, from both logistic and economic points of view. It is particularly useful in endemic regions in which whole blood is not usually fractioned into components, and in areas receiving immigrants from endemic regions.

Acknowledgements

We would like to thank the donors and patients who participated in this study. We are grateful to Dr. Josep Muncunill for his assistance in the study design and Carmen Serret for her help with sample handling and transportation. Finally, we would like to thank Martin Hadley-Adams and Lucy Brzoska for their invaluable advice on the preparation of the manuscript.

Funding and support

This work is part of a research study supported by the National R&D+i Plan 2008-2011 and ISC III - *Subdirección General de Evaluación y Fomento de la Investigación* (PI 10/00533) and is also part of the *Generalitat de Catalunya* 2014 SGR 1241 programme.

Authorship contributions

TJ-M, CR and RF designed and performed the research, analysed the results and wrote the manuscript, contributing equally to this work; CG, LI, MA, DB, AP, MT-P, and BC-F performed the experiments. EG-Ll, TS, MM, and JG contributed with essential tools and assisted with preparation of the manuscript. TJ-M, CR, and RF contributed equally to this work.

The Authors declare no conflicts of interest.

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Arrived: 20 July 2016 - Revision accepted: 16 January 2017
Correspondence: Teresa Jimenez-Marco
Fundació Banc de Sang i Teixits de les Illes Balears
C/ Rosselló i Caçador, 20
07004 Palma de Mallorca, Balearic Islands, Spain
e-mail: tjimenez@fbstib.org