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Abbreviations – Glossary of terms

AE	Adverse event
AESI	Adverse Event of Special Interest
ALT	Alanine aminotransferase (SGPT)
AST	Aspartate aminotransferase
BID	Twice a day
BMI	Body mass index
CBC	Complete blood count
CI	Confidence Interval
CIOMS	Council for International Organizations of Medical Sciences
CRE	Creatinine
CRF	Case report form
CTCAE	Common Terminology Criteria for Adverse Events
DNDi	Drugs for Neglected Diseases initiative
DSMB	Data Safety Monitoring Board
EMA	European Medicines Agency
EOT/EOS	End of treatment/End of Study
FDA	Food and Drug Administration
FPI	First patient in
ICH	International Council on Harmonization
IEC	Independent Ethics Committee
IMP	Investigational Medicinal Product
GCP	Good Clinical Practice
HIV	Human immunodeficiency virus
ISC	Indian Sub-continent
ITT	Intent to Treat
IUCD	Intra uterine contraceptive device
IV	Intravenous
LC-MS/MS	Liquid chromatography mass spectrometry
LPO	Last patient out
MedRA	Medical Dictionary for Regulatory Activities
MSF	Médecins Sans Frontières (Doctors without Borders)
PCR	Polymerase chain reaction
PD	Pharmacodynamic
PI	Principal investigator
PK	Pharmacokinetics
PKDL	Post-Kala-Azar Dermal Leishmaniasis
PM	Paromomycin
PP	Per protocol
SAE	Serious adverse event
SD	Standard deviation
SOC	System, organ and class
SSG	Sodium Stibogluconate
TB	Tuberculosis
TBIL	Total bilirubin
TID	Three times a day

VL	Visceral Leishmaniasis
WBC	White blood cell
WHO	World Health Organization

PROTOCOL SYNOPSIS

Protocol Title	An Open label, Randomized, Clinical Trial of Two Regimens to Assess the Safety and Efficacy for Treatment of PKDL Patients in the Indian subcontinent
Phase	Phase II
Indication	PKDL patients in India and Bangladesh
Protocol Number	DNDi-MILT/COMB-01-PKDL
Background Information and Trial Rationale	Post-Kala-Azar Dermal Leishmaniasis (PKDL) commonly follows visceral leishmaniasis (VL) caused by *L. donovani*. It is characterized by skin lesions in which parasites can be identified, in a patient who is otherwise fully recovered from VL or exposed to *L. donovani* infection. It occurs in the Indian subcontinent (mainly India and Bangladesh) as well as in Africa (mainly Sudan), where *L. donovani* is the causative parasite. There are major differences in epidemiology, clinical presentation and treatment between these regions. The reasons for these differences are not well understood. Factors related to the parasite and the host may play a role. **<u>Epidemiology</u>** In Bangladesh, VL is most common in Fulbaria and Trishal sub-district of district Mymensingh. In a cross-sectional study an increase in estimated PKDL incidence was observed from 1 case to 21 cases per 10,000 person-years in 2002-2004 and 2007, respectively. In another cross-sectional study (2007) the point prevalence of PKDL was estimated in 2007 at 3.8 – 7.3/10,000, with PKDL rates of 13.9-16% among patients who had a VL episode; 60 % of patients presented PKDL within 24 months, with 20% presenting within 6 months after VL treatment. In a series of cross-sectional surveys between 2002 - 2010, the cumulative incidence of PKDL was estimated at 17% by 5 years. Most patients had been treated with SSG (95%); the mean interval between VL and PKDL was 19 months. In Trishal sub-district, in a cross-sectional study, the prevalence of probable PKDL was 6.2/10,000 people in 2010. The median time between VL and PKDL was 36 months (IQR, 24-48 months). All had been treated for VL with Sodium Stibogluconate (SSG). A recent longitudinal study in Fulbaria (2015) of 1918 VL patients with active follow-up showed PKDL rates of 10.2% at 36 months (mean 17.6 months;

range 4-43 months) after VL treated with 3 x 5 mg/kg Ambisome®.

In all above studies, diagnosis was based on clinical judgement and not based on microscopy or PCR report.

In India the only recent cross-sectional study from 2012 estimates the prevalence of PKDL in 16 VL-endemic communities in Bihar as 4.4/10,000 for confirmed cases and 7.8/10,000 when probable cases were included. Cases had been treated for VL with SSG, paromomycin or amphotericin B.

Also in Bihar, in an observational, retrospective cohort study with passive case-finding of PKDL in 8311 VL patients treated for VL with Ambisome® 20 mg/kg, PKDL was diagnosed in 0.3%. The interval after VL was longer in a small group treated with SSG (2.9 years in the SSG group vs. 1.2 years for the Ambisome® group). The interval between VL and onset of PKDL was 1.5 ±0.9 years (range 0.5-3.4); the interval between VL and PKDL diagnosis was longer: 2.4 years (range 1.0-4.4).

In Africa, PKDL is even more common but there are important intraregional differences. In Sudan it is most common: up to 50-60% of VL cases develop PKDL, usually within 6 months after VL episode, and virtually all cases develop within 12 months (mean 4.5 months). In Ethiopia, Kenya and Uganda PKDL is less common for reasons that are not well understood. Factors relating to the host, the parasite and treatment given for VL may all play a role.

PKDL in patients co-infected with HIV is more common and more severe; in Ethiopia in areas where 40% of VL patients are HIV infected, PKDL is more common (28% vs. 14%, in HIV positive and HIV negative patients, respectively) and more severe. PKDL is also occasionally seen in VL caused by *L. infantum* in particular in HIV-VL co-infected patients.

Clinical presentation

The clinical presentation varies in each region: in Bangladesh >90% of patients have a macular rash. While the face is often involved, macules may occur on all parts of the body, without a clear pattern.

In India the rash is often polymorphic with a mixture of macules, papules and nodules. It should be noted that most reported cases are from tertiary referral centres and may reflect chronic PKDL cases of long duration, thus presenting with more advanced disease.

The PKDL patients do not have other associated signs or symptoms, and apart from the skin lesions they are generally healthy individuals.

In Africa (Sudan) the rash is usually papular or maculopapular; in severe cases these coalesce to form nodules or plaques. A minority of patients present with a macular rash only. The rash usually affects the face and may spread to arms and trunk and further caudally to involve the whole body in severe cases. The clinical distribution is thus described in 3 grades of severity taking into account the degree of spread.

Pathogenesis and natural history

PKDL often occurs in the sun-exposed parts of the body suggesting the influence of UV light. It has been shown that PKDL occurs when the immune response shifts from a Th2 response in active VL to a mixed Th1 / Th2 response after treatment for VL with persistence of IL-10. This means that while systemically there is a predominantly Th1 response as a result of VL treatment and cure (i.e. with systemic clearance of parasites), in the skin a Th2 response persists with demonstrable parasites which leads to the clinical presentation described above.

There is little information on the natural history of PKDL in Bangladesh and India. While apparently the interval between VL and PKDL is documented as long (median 2 years), there is limited information that this may be shorter when patients are actively followed after VL. Indeed, some PKDL cases may present during VL or shortly after VL treatment. Self-healing has been described in Bangladesh in two reports by retrospective history (self-reported by the patient, 8% resolution after 1-year) and a few early cases not well characterized. This finding has not been confirmed by direct observation of patients. In practice, in Asia, as most patients are diagnosed late, it is assumed all patients are chronic and self-healing is not expected. All PKDL cases are treated, irrespective of the extent of the rash.

Studies from Sudan show that most PKDL patients (85%) self-heal within 12 months, majority within 6 months of PKDL onset. Only chronic patients are treated and those who present with severe disease (grade 3).

Skin smears and biopsies from lesions may demonstrate the presence of parasites and it is thought that PKDL patients play a role in transmission (anthroponotic cycle) as (limited) studies have found that sand flies feeding from PKDL patients become infected with Leishmania parasites.

There is therefore an important public health concern that these patients may, for the duration of their rash, be infectious to sand flies and thus play a role in transmission.

Treatment

In the Indian Subcontinent, all patients are treated.

In a cohort study of 26 patients treated with miltefosine (2011), 23 of 24 patients who completed the study were cured (96%), with no relapses after 12-months follow-up. Of these, 13 received miltefosine 150mg (50mg tid) for 60 days, 3 patients received miltefosine 150mg (50mg tid) for 75-90 days, and other 7 patients had the dose reduced to 100 mg daily (50mg bid) due to gastrointestinal complaints, and were treated for 90 days. The one patient who failed had received miltefosine 150mg (50mg tid) for 90 days.

In another study from India (2013), miltefosine 100 mg/day for either 12 or 8 weeks was evaluated. The ITT and per-protocol cure rates after 12 months of follow-up for patients receiving 12 weeks were 78% (14/18 patients, 95% CI

	= 61–88%) and 93% (14/15 patients, 95% CI = 71–95%), respectively, whilst the ITT and per-protocol cure rates for patients receiving 8 weeks were 76% (13/17 patients, 95% CI = 53–90%) and 81% (13/16 patients, 95% CI = 57–93%), respectively. In a study from India (2015) of 33 patients treated with miltefosine 100 mg (50mg bid) for 12 weeks, the cure rate was 97% (28/29 patients that could be evaluated) after 1-year follow-up. In 1 patient treatment was stopped because of adverse effect. It should be noted that in a recent descriptive study (2015) from India (non-randomized, non-controlled, unblinded observational study), decline of clinical efficacy of miltefosine was reported; 11/73 (15%) of PKDL patients relapsed by 18-months follow-up and this was highest (31%) in those with shorter duration treatment (50 mg tid for 60 days) as compared with longer duration (50 mg bid for 90 days) of which 10.5% relapsed. Parasite load at baseline was significantly higher in relapse patients as compared to cured cases. Previously similar decrease in response to miltefosine in treatment of VL has been reported. Treatment of 60 PKDL cases in India with SSG 10 mg/kg for 90 days, resulted in improvement with disappearance of parasites but the difficulty in assessing cure was emphasized, in particular in macular lesions. In imprint smears, the mononuclear infiltrate decreased (papular lesions) or persisted (macular lesions). In a recent field experience by MSF on routine patient management in Bangladesh (Fulbaria), 1400 patients were treated with Ambisome® 6 x 5 mg/kg given over 2 weeks. Results were satisfactory, with 86.5% of patients presenting total disappearance of all nodular and papular lesions and complete or very significant re-pigmentation of macular lesions after one year of follow-up. Among the 13.5% of cases who did not have significant re-pigmentation of macular lesions at 12 months, improvement was observed in subsequent visits, and only 2% of the patients required a second course of treatment. This experience was based on clinical assessment in the field, and not done systematically by standardized procedures. In order to better characterize the Ambisome treatment for PKDL patients in Bangladesh, MSF conducted a clinical trial where patients were again treated with Ambisome® 30mg/kg (n=110). In this trial, treatment outcome was standardised, including photographs and evaluated independently by clinicians. A total of 88 patients completed 12-month follow up, out of which 80% had satisfactory response (67% patients had complete resolution of lesions and 13% had substantial improvement (>75%) of lesions). In this study however, severe side-effects were noted as rhabdomyolysis was reported in 6 patients, 3 of which had severe hypokalemia that was ascribed to Ambisome® therapy. In an on-going second study, patients are treated with 5 x 3 mg/kg (2 injections per week) with potassium and magnesium supplementation and intensive monitoring. Preliminary reports do not suggest severe hypokalemia occurring

during this regimen, and patients are responding to treatment satisfactorily.

In addition, expert opinion in Bangladesh is that patients with severe PKDL in Bangladesh had to be treated with high total doses of Ambisome (up to 60mg/Kg total dose) and careful monitoring of treatment help to prevent severe hypokalemia or rhabdomyolisis (Dr. Mondal, personal communication). Perhaps severe PKDL leads to significant destruction of the skin and this in addition to higher dose of AmBisome facilitate better penetration of the drug to the skin. This emphasizes the need of skin PK analysis of anti-PKDL drugs and to correlate it with response to treatment.

In Africa (Sudan) only chronic PKDL patients, i.e. showing persistence of lesions after 6 months of disease onset, and those with severe PKDL are treated. There are no conclusive studies on treatment of PKDL in Sudan; current practice is treatment with SSG 20 mg/kg for 40-60 days. There is limited experience with Ambisome®, one study showed cure rate of 83% (10/12 patients) with Ambisome® given intravenously at 2.5 mg/kg per day for 20 days, without detectable adverse effects.

Recent data showed good cure rates of combined SSG 20 mg/kg and paromomycin 11 mg/kg for 30-40 days in 8 of 9 patients treated.

As the pathogenesis of PKDL implies an important role for the immune response, there is a rationale for immune modulation. In one study, combined immunochemotherapy with alum-precipitated autoclaved Leishmania major (Alum/ALM) vaccine + Bacille Calmette-Guérin (BCG) and SSG showed better cure rates than SSG alone (87% vs. 53%). Newer immunomodulatory agents are under development but are not expected to enter clinical studies until 2018. Currently there are no immune markers that may guide the duration of treatment or indicate outcome.

Rationale for the study and selection of treatments to be tested

In the Indian Sub-continent (ISC) all patients with PKDL are treated irrespective of interval after VL, previous VL treatment, duration of the rash, or clinical presentation. While treatment may be indicated for clinical reasons, it is thought that PKDL patients may play an important role in transmission of VL and chronic PKDL patients have been implicated in major VL outbreaks in the past. Obviously, this is of crucial importance in the current VL elimination efforts in the ISC.

This study aims primarily to improve current treatment options. In addition, this will be the first study ever in PKDL in which outcome will be described in clinical, parasitological and immunological terms in relation to pharmacokinetics thus providing the rationale and focus for subsequent studies.

Currently there are no satisfactory treatments for any forms of PKDL. Available treatments include pentavalent antimonials: sodium stibogluconate (Pentostam, Stibonate) and meglumine antimoniate (Glucantime) which have been used since the 1940s and in some regions,

	such as Sudan remain as the main first-line drugs. Conventional amphotericin B has been used in India for prolonged periods (60 infusions) but this is impractical and requires careful clinical and biochemical monitoring. Both miltefosine and Ambisome® as monotherapy have shown to be effective. However, with the current recommended schemes there are some drawbacks such as the length of the treatment with miltefosine alone (8-12 weeks); the costs and recently observed toxicity of a high dose Ambisome® (total dose of 30 mg/kg). There is also the potential risk for development of resistance with miltefosine as monotherapy. In addition, there is an issue of access, as currently there is only one manufacturer for MF and situations of drug stock rupture have already occurred at some occasions, leaving no drugs in filed for treatment of PKDL patients. The current scenario does not offer many alternatives given that no new drugs are available or in late stage of development. Miltefosine, despite its teratogenic problems, is currently the only oral drug available with the advantages that this route of administration offers. Rather than dismissing the use of miltefosine due to its teratogenic effects, efforts to optimize its use by combining it with other drugs aiming to provide a safe, efficacious and shorter treatment with a high compliance should be explored. In terms of numbers, PKDL cases occur less frequently compared to VL cases which makes it possible to monitor contraception properly; indeed, no large scale PKDL treatment programs are expected and treatment is focussed on individual PKDL patients. Combination treatment is well accepted in the treatment of VL, paromomycin (PM) combined with miltefosine in Asia; or SSG combined with PM for example in East Africa and may offer shorter duration of treatment thus preventing prolonged hospitalization. Another possible advantage is prevention of development of resistance and reduced cost. These principles also apply to PKDL, where the need for an ambulatory treatment with a highly safe, efficacious and user-friendly regimen is even more pressing as patients are not ill, except for the rash. While ideally the choice of study regimens would be based on drug-penetration of PK levels in the skin and the plasma, this would require studying a large number of patients with drugs in regimens that may or may not be appropriate as future PKDL treatment in the ISC. Therefore, we have chosen to design a study with 2 treatment arms that reasonably could be effective and safe for PKDL treatment and integrate the PK study so as to guide future treatment studies also depending on outcome. Except for the presence of localized skin lesions, subjects with PKDL are otherwise healthy, so we may assume that the pharmacokinetics of Ambisome® and miltefosine in these subjects could be similar to that in healthy volunteers. However, there is no information about the levels of Ambisome® and miltefosine in skin of human or animal studies and how it correlates with plasma concentrations. This study aims to determine if accumulated Ambisome® and miltefosine levels are detectable in the skin of

	PKDL patients at the end of treatment, and if so how these skin levels relate to the individual systemic concentrations of the drugs and their correlation to the in vitro drug susceptibility of the Leishmania parasites. Miltefosine concentrations keep accumulating until the end of the 28-day treatment period in patients. Therefore, we expect that a single tissue sample at the end of treatment will indicate the extent of miltefosine accumulation in skin tissue, even when assuming a delayed tissue distribution. In contrast to the fungal diseases, in which the free amphotericin B fraction probably represents the active component of Ambisome®, in leishmaniasis it is most probably the liposomal fraction of the drug, as this is the fraction that is preferably phagocytosed by the host's macrophages in which the Leishmania parasites reside. The liposomal fraction of Ambisome® also represents the largest fraction of the drug in circulation after administration in human. Given the fact that logistic difficulties will preclude us from differentiating between free, tissue-bound, protein-bound and liposomal amphotericin B in this field trial, total amphotericin B levels will be measured in the skin and plasma and used for pharmacokinetic assessments. This exploratory study proposes to determine the efficacy and safety of Ambisome® monotherapy regimen (5 x 4 mg/kg, given twice per week, total dose of 20 mg/kg) and Ambisome® (5 x 4 mg/kg, given twice per week, total dose of 20 mg/kg) in combination with miltefosine daily for three weeks (allometric dosing), for PKDL patients in the ISC. The limitation of Ambisome® at the total dose of 20mg/Kg is due to the safety concerns, mainly the cases of rhabdomyolysis observed in Bangladesh with the dose of 30mg/Kg. It is assumed that by decreasing the dose to 20mg/Kg safety will be improved with no significant loss of efficacy. The Ambisome® will be given in 5 administrations over 2 ½ weeks to reduce safety risks. In addition, PD data from previous DNDi trials on VL have shown that parasite clearance is faster when Ambisome® was administered in multiple injections as compared to single administration. Although in this protocol we are not evaluating VL, but PKDL, splitting the dose over time may be beneficial for parasite dynamics, which will be monitored over time. Miltefosine has a long half-life (7 days) and accumulates to eventually reach a steady state after 4 weeks of treatment. A minimum of 3-weeks treatment was considered necessary to reach sufficient exposure for the activity of miltefosine in PKDL patients. Still, this is a significant reduction as compared to the current 8-12 weeks of monotherapy regimen. If the proposed scheme shows to be safe and efficacious, it may be possible to consider regimens to treat PKDL which have shorter duration of treatment thus increasing compliance. It has long been known that the underlying mechanism for controlling Leishmania infection and self-healing of dermal lesions is the cellular immune response, mediated by antigen-specific γ-interferon producing T-cells. Additional information to better understand the status and role of the host

	immune response and how it interacts when different treatment modalities are provided is needed. Similarly, studies to demonstrate that appropriate concentration of the drug is present in the right compartment, and its correlation with the clinical outcome are crucial when optimizing a treatment modality. Lastly, data on parasite clearance during treatment of PKDL and follow-up may be correlated to immunological parameters and clinical outcome and thus may contribute to identify the best parameter to indicate cure in PKDL.
Trial Objectives	General Objectives: The overall objective of this study is to assess the safety and efficacy of two treatment modalities for PKDL patients in ISC. **Primary Objective:** To measure the safety and efficacy of Ambisome® monotherapy regimen (5 x 4 mg/kg IV, twice per week, total dose of 20 mg/kg) and Ambisome® (5 x 4 mg/kg IV, twice per week, total dose of 20 mg/kg) in combination with miltefosine allometric dose orally for three weeks, for PKDL patients in the ISC. **Secondary Objectives:** • To assess skin and plasma concentrations of total Ambisome® and miltefosine. • To evaluate the host immune response in each treatment arm before, during and after treatment. • To evaluate parasite clearance in each arm as indicated by direct microscopy and qPCR • To assess relationship between clinical, parasitological and immunological responses to identify a potential biomarker for cure. • To assess relationship between pharmacokinetic parameters with clinical outcome and parasite clearance. **Exploratory Objective:** • To assess non-invasive tape disc method of skin sample collection for molecular parasitological diagnosis.

Trial Endpoints	**Primary Endpoints:** Efficacy To measure the efficacy (definitive cure at 12 months) of two treatment regimens in subjects with PKDL according to clinical criteria: complete resolution of papular and nodular lesions (flattening of 100% of lesions) and significant improvement (> 80% re-pigmentation) of macular lesions by 12 months after the end of treatment. Safety: To assess the safety of the two regimens (SAEs. AESI and frequency and severity of AEs) from the start of treatment through the 12-month follow-up period and 24 months study visit. **Secondary Endpoints:** Efficacy • To measure the efficacy of the two treatment regimens in subjects with PKDL according to clinical criteria: complete resolution of papular and nodular lesions (flattening of 100% of lesions) and significant improvement (> 80% re-pigmentation) of macular lesions by 24 months after the end of treatment. • To assess the overall assessment of the clinical presentation of PKDL lesions by the investigator at 12 and at 24 months according to the following categories: • Category 1: All lesions have improved, and the majority of lesions have resolved. Any remaining lesions are only faintly visible as re-pigmentation is almost complete. • Category 2: The majority of lesions show significant improvement some lesions have resolved. • Category 3: No new lesions, but the majority of lesions show no or slight improvement. • Category 4: New lesions have appeared and there is no or little improvement of other lesions Pharmacokinetics • To assess the maximal accumulation of total amphotericin B and miltefosine in the skin at the end of treatment and correlate these with plasma levels. Immune Response • To assess the change in immune response during and after end of treatment as compared to baseline by measuring cytokines profiles level in the peripheral blood.

	Parasitology • To assess the clearance of parasites by microscopy and qPCR (samples collected from skin, blood and tape disc) at various time-points before, during and after treatment and during follow-up.
Trial Design	This is a non-comparative, open label, randomized phase II clinical trial to assess the safety and efficacy of Ambisome® monotherapy (5 x 4 mg/kg IV given twice per week at a total dose of 20 mg/kg given) and a combination of Ambisome® (5 x 4 mg/kg IV given twice per week at a total dose of 20 mg/kg) plus miltefosine allometric dose orally for three weeks for PKDL patients in the ISC. Strategies to reach PKDL patients will be adapted according to the site context. Activities may include active case detection, targeted active case detection based on VL registry at the site, referral network from dermatologists, etc. Subjects will be hospitalized during the administration of Ambisome®; although hospital-day care at the dates of Ambisome® injection will also be acceptable. The miltefosine treatment will start at the same time as Ambisome® treatment, and it will be continued until completion of 3 weeks on an out-patient basis. During hospitalization compliance to treatment is assured. For patients randomized to the combination arm, the patient or parent/guardian (in case of children) will be educated on the administration of miltefosine, and how to manage possible gastro-intestinal events. Patient is discharged with clear instructions on how to continue miltefosine treatment, and when to return for clinical assessment. Study assessments will be performed at baseline, D1, D8, D15, D22 (only for subjects allocated to combination arm), and D30 (+/- 3 days) during treatment phase. All subjects will have follow-up visits at 3 months (+/- 7 days), 6 months (+/- 7 days), 12 months (+/- 14 days), and 24 months (- 1 month/+ 3 months) after the onset of treatment to assess efficacy and safety. A strategy will be put in place according to the context of each trial site to assure patients comply with the schedule of scheduled study visits. This can include active tracking of the patients through telephone contact or domiciliary visits. At any time-point during the study, if the patient does not respond to treatment, or lesions are worsening, or if after the healing period, the lesions reappear/reactivate, rescue treatment can be initiated at the discretion of the study investigator. A subject who receive rescue treatment at any time during the trial will be considered a treatment failure and will be withdrawn from the study. **<u>Sample collection</u>** PK samples from the skin (biopsy) will be collected at the end of treatment i.e. at day 15 for patients allocated in the Ambisome® arm and day 22 for

	patients allocated in the Ambisome® + miltefosine arm. For patients allocated to Ambisome+miltefosine arm, miltefosine concentration will be measured in EDTA blood plasma collected at D8, D15, D22, D30 and 3 months visits. In addition, miltefosine concentration will be measured in the skin biopsy collected at D22 from patients allocated to combination arm. For a sub-sample of 30 patients per arm, total amphotericin B concentration will be measured in EDTA blood plasma collected at D1 (at the end of infusion, and at 2, 4, 8, and 22 hrs after the end of infusion), D15 (at prior to infusion, at the end of infusion and at 2, 6, and 22 hrs after the end of infusion) and at D22 (single sample for combination arm only) or D30 (single sample for monotherapy arm only). In addition, total amphotericin B concentration will be measured in the skin biopsy collected at D15 from patients allocated in the Ambisome monotherapy arm, and at D22 from patients allocated to combination arm. As much as possible, a single blood draw should be performed per study visit to avoid multiple vein punctures. Sample for PK will be a 2mL EDTA whole blood sample/time point. Blood samples for immunological studies will be collected at baseline, D15, D30 and at 3, and 12-months follow-up visits. Blood samples for qPCR will be collected at baseline, Day 30 (EOT) and at 3 and 12 months follow up visits. Skin snip samples for parasitological examination by microscopy and qPCR will be collected at day 0, D30 (EOT), 3-months, 12-months and 24 months follow-up. In addition, as an exploratory objective, skin sample will be collected at the same time-points, with the exception of 24 months, using non-invasive tape disc method, from where DNA will be extracted for qPCR. Miltefosine and total amphotericin B (free + protein-bound + liposomal) concentrations in plasma and skin biopsy samples will be determined using validated bioanalytical methods employing liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Parasite loads will be measured by quantitative real-time PCR (qRT-PCR) based on the detection of Leishmania kDNA target. Cytokines and cell count: IL-2, IFNY, TNFα, IL-10 and Granzyme B (and possibly other biomarkers, after a preliminary screening) will be measured in the stimulated whole blood supernatant by BD™ Cytometric Bead Array Human Th1/Th2 Cytokine CBA. Lymphocyte subsets will be identified by FACS analysis.
Main Entry Criteria Inclusion	Patients who fulfil all inclusion criteria and do not present any of the exclusion criteria will be eligible to be enrolled in this study.

Exclusion	**Inclusion criteria:** • Confirmed PKDL case by clinical presentation and demonstration of parasites by microscopy in a skin smear or biopsy or by PCR, with stable or progressive disease for at least 4 months • Male or Female patients aged 6 to 60 years • Written voluntarily informed consent from adult patient and from parent / guardian in case children <18 years old. In the case of minors, assent from the children will be obtained according to country regulations. **Exclusion criteria:** • Patients who had prior treatment of PKDL within last 2 years • Pregnant and lactating women and women of childbearing age (12 to 55 years) who, before randomization, cannot be assured contraceptive cover during treatment and 5 months thereafter • Patients with signs and symptoms of severe diseases: defined as suffering from a concomitant severe infection such as TB or any other serious known underlying disease (cardiac, renal, hepatic), • Severe malnutrition defined by BMI for age WHO reference curves for gender, Z score < -3 for subjects 6 - 19 years; BMI < 16 for subjects > 19 years old. • Patients with haemoglobin < 5g/dL • Patients with abnormal liver function (ALT and AST) tests of more than three times the normal range. • Patients with total bilirubin levels >1.5 times the upper normal range • Patients with serum creatinine above the upper normal range • Patients with serum potassium < 3.5 mmol/L • Patients with a positive HIV test, as applicable • Patients / guardian not willing to participate • Patients with history of allergy or hypersensitivity to the relevant study drug • Patients on immunomodulators

Study Duration	6 months are expected to be needed to obtain all ethical and official clearances. The recruitment period will be 12 months with possible extension up to 18 months depending on recruitment capacity and the follow-up period will be 24 months. Therefore, the overall trial duration will be 36-42 months (FPI to LPO).
Test Drugs	• Ambisome® monotherapy (5 x 4 mg/kg IV twice per week, for 2 ½ weeks, at D1, D4, D8, D11, D15) • Combination of Ambisome® (5 x 4 mg/kg IV, twice per week, for 2 ½ weeks, D1, D4, D8, D11, D15) plus miltefosine allometric dose orally BID administration for 3 weeks. An easy-to-use table with dosing scheme by weight, height, and gender, will be provided to the investigators to define the exact daily miltefosine dose to be administered.
Statistics	**Sample size** We estimated that a minimum sample size of 50 subjects per treatment arm would provide a precision estimate of 10% with 95% CI, based on an anticipated cure rate at 12 months in 85% of patients. Accounting for 10% subjects lost during follow up and 15% subjects with treatment discontinuation 13 more subjects were added per arm, resulting an increase of the sample size to 63 for each regimen. The overall sample size for the two regimens is 126 subjects. The anticipated cure rate was selected based on the criteria set on the cure rates previously reported with the use of Miltefosine or Ambisome® alone. A computer generated, randomization code will be used for patient treatment allocation to one of the two treatment arms indicated. Eligible patients who fulfil all the inclusion criteria and have none of the exclusion criteria, and from whom informed consent has been obtained, will be randomized to one of the treatment regimens. **Summary of analysis** Descriptive statistics will be used to present study data. Continuous variables will be presented as number of observations (n), mean, standard deviation (SD), median, minimum and maximum values. Categorical variables will be presented as counts and percentages. Median time to the development of any AE will be determined using life-table analysis. All data will be presented separately by treatment group, local and systemic AEs. AEs and medical history will be coded using the most recent version of the Medical Dictionary of Regulatory Activities (MedDRA) preferred terms and will be grouped by system, organ, and class (SOC)

	designation. Summary statistics will be provided by group including the frequency, severity, seriousness and relationship of AEs to study drug organized by preferred term by SOC grouping. Efficacy endpoints will be presented as summary statistics by treatment group and will be conducted once all subjects complete the 12-months study visit. The number of subjects who met the criteria for cure divided by the total number of subjects in that group will be presented by study group. In addition, secondary efficacy endpoints will be described for the 24 months follow-up. Subjects who are lost to follow-up will be compared to those completing the study with respect to study site, demographic, and clinical factors. If lost to follow-up exceeds 20% of the sample, attempts will be made to seek out these dropouts (or their proxies) to ascertain final outcomes. The attributes of people that are found through this process will be compared to those not lost to follow up. For the pharmacokinetic data analysis, a population-based approach will be used employing non-linear mixed effects modelling (using NONMEM), to take into account within- and between-subject variability in all pharmacokinetic parameters, to optimally identify potential covariates such as body size descriptors (age, lean body mass, total body weight, etc) and other demographic parameters, but also interaction effects such as treatment-arm related differences in pharmacokinetics parameter estimates. The objective function value (equals -2 log-likelihood, derived from NONMEM) will be used to assess statistical significance of evaluated covariates and hierarchical structural models. The individual blood plasma:skin distribution ratio will be estimated using the developed pharmacokinetic model. Individual blood plasma:skin ratios of drug concentrations will be contrasted with the IC90 values previously obtained for L. donovani parasites.

1 Background and Study Rationale

Post-Kala-Azar Dermal Leishmaniasis (PKDL) commonly follows visceral leishmaniasis (VL) caused by *L. donovani*. It is characterized by skin lesions in which parasites can be identified, in a patient who is otherwise fully recovered from VL or exposed to *L. donovani* infection. It occurs in the Indian subcontinent (mainly India and Bangladesh) as well as in Africa (mainly Sudan), where *L. donovani* is the causative parasite. There are major differences in epidemiology, clinical presentation and treatment between these regions.[1] The reasons for these differences are not well understood. Factors related to the parasite and the host may play a role.

1.1 Epidemiology

In Bangladesh, VL is most common in Fulbaria and Trishal sub-district of district Mymensingh.

In a cross-sectional study an increase in estimated PKDL incidence was observed from 1 case to 21 cases per 10,000 person-years in 2002-2004 and 2007, respectively.[2]

In another cross-sectional study (2007) the point prevalence of PKDL was estimated in 2007 at 3.8 – 7.3/10,000, with PKDL rates of 13.9-16%; 60 % of patients presented PKDL within 24 months, with 20% presenting within 6 months.[3]

In a series of cross-sectional surveys between 2002-2010, the cumulative incidence of PKDL was estimated at 17% by 5 years. Most patients had been treated with SSG (95%); the mean interval between VL and PKDL was 19 months.[4]

In Trishal subdistrict, in a cross-sectional study, the prevalence of probable PKDL was 6.2/10,000 people in 2010. The median time between VL and PKDL was 36 months (IQR, 24-48 months). All had been treated for VL with SSG.[5]

A recent longitudinal study in Fulbaria (2015) of 1918 VL patients with active follow-up showed PKDL rates of 10.2% at 36 months (mean 17.6 months; range 4-43 months) after VL treated with 6 x 5 mg/kg Ambisome® (Koert Ritmeijer, MSF, personal communication).

In all above studies, diagnosis was based on clinical judgement and not based on microscopy or PCR report.

In India the only recent cross-sectional study from 2012 estimates the prevalence of PKDL in 16 VL-endemic communities in Bihar as 4.4/10,000 for confirmed cases and 7.8/10,000 when probable cases were included. Cases had been treated for VL with SSG, paromomycin or amphotericin B.[6]

Also in Bihar, in an observational, retrospective cohort study with passive case-finding of PKDL in 8311 VL patients treated for VL with Ambisome® 20 mg/kg, PKDL was diagnosed in 0.3%. The interval after VL was longer in a small group treated with SSG (2.9 years in the SSG group vs. 1.2 years for the Ambisome® group). The interval between VL and onset of PKDL was 1.5 ±0.9 years (range 0.5-3.4); the interval between VL and PKDL diagnosis was longer: 2.4 years (range 1.0-4.4).[7]

In Africa, PKDL is even more common but there are important intraregional differences; in Sudan it is most common: up to 50-60% of VL cases develop PKDL, usually within 6 months and virtually all cases develop within 12 months (mean 4.5 months). In Ethiopia, Kenya and Uganda PKDL is less common for reasons that are not well understood. Factors relating to the

host, the parasite and treatment given for VL may all play a role.[1,8]

PKDL in patients co-infected with HIV is more common and more severe; in Ethiopia where 40% of VL patients are HIV infected, PKDL is more common (28% vs. 14%, in HIV positive and HIV negative patients, respectively) and more severe. PKDL is also occasionally seen in VL caused by *L. infantum* in particular in HIV-VL co-infected patients.[9-11]

1.2 Clinical presentation

The clinical presentation varies in each region: in Bangladesh >90% of patients have a macular rash. While the face is often involved, macules may occur on all parts of the body, without a clear pattern.[12]

In India the rash is often polymorphic with a mixture of macules, papules and nodules. It should be noted that most reported cases are from tertiary referral centres and may reflect chronic PKDL cases of long duration, thus presenting with more advanced disease.[13-15]

The PKDL patients do not have other associated signs or symptoms, and apart from the skin lesions they are generally healthy individuals.

In Africa (Sudan) the rash is usually papular or maculopapular; in severe cases these coalesce to form nodules or plaques. A minority of patients present with a macular rash only. The rash usually affects the face and may spread to arms and trunk and further caudally to involve the whole body in severe cases. The clinical distribution is thus described in 3 grades of severity taking into account the degree of spread.[1,16]

1.3 Pathogenesis and natural history

PKDL often occurs in the sun-exposed parts of the body suggesting the influence of UV light. It has been shown that PKDL occurs when the immune response shifts from a Th2 response in active VL to a mixed Th1 / Th2 response after treatment for VL with persistence of IL-10. This means that while systemically there is a predominantly Th1 response as a result of VL treatment and cure (i.e. with systemic clearance of parasites), in the skin a Th2 response persists with demonstrable parasites which leads to the clinical presentation described above.[17,18]

There is little information on the natural history of PKDL in Bangladesh and India. While apparently the interval between VL and PKDL is documented as long (median 2 years), there is limited information that this may be shorter when patients are actively followed after VL. Indeed, PKDL may present during VL or shortly after VL treatment. Self-healing has been described in Bangladesh in two reports by retrospective history (self-reported by the patient, 8% resolution after 1-year) and a few early cases not well characterized[4]. This finding has not been confirmed by direct observation of patients. In practice, in Asia, as most patients are diagnosed late, it is assumed all patients are chronic and self-healing is not expected and all are treated, irrespective of the extent of the rash.

Studies from Sudan show that most PKDL patients (85%) self-heal within 12 months, majority within 6 months of PKDL onset. Only chronic patients are treated and those who present with severe disease (grade 3).[19]

Skin smears and biopsies from lesions may demonstrate the presence of parasites and it is thought that PKDL patients play a role in transmission (anthroponotic cycle) as studies have found that sand flies feeding from PKDL patients become infected with Leishmania parasites.[1]

There is therefore an important public health concern that these patients may, for the duration of their rash, be infectious to sand flies and thus play a role in transmission.

Currently it is unclear whether all PKDL patients should be treated given the risk-benefit between giving prolonged courses of potentially toxic and expensive drugs, and spontaneous cure occurring only after several months.

1.4 Treatment

In the Indian Subcontinent, all patients are treated.

In a cohort study of 26 patients (2011), 23 of 24 patients who completed the study were cured (96%), with no relapses after 12-months follow-up. Of these, 13 received miltefosine 150mg (50mg tid) for 60 days, 3 patients received miltefosine 150mg (50mg tid) for 75-90 days, and other 7 patients had the dose reduced to 100 mg daily (50mg bd) due to gastrointestinal complaints, and were treated for 90 days. The one patient who failed had received miltefosine 150mg (50mg tid) for 90 days.[20]

In another study from India (2013), miltefosine 100 mg/day for either 12 or 8 weeks was evaluated. The ITT and per-protocol cure rates after 12 months of follow-up for patients receiving 12 weeks were 14 of 18 patients (78%,95% CI = 61–88%) and 14 of 15 patients (93%, 95% CI = 71–95%), respectively, whilst the ITT and per-protocol cure rates for patients receiving 8 weeks were 76% (95% CI = 53–90%) and 81% (95% CI = 57–93%), respectively.[21]

In a study from India (2015) of 33 patients treated with miltefosine 100 mg (50mg bid) for 12 weeks, the cure rate was 97% (28 of 29 patients that could be evaluated) after 1-year follow-up. In 1 patient treatment was stopped because of adverse effect.[22]

It should be noted that in a recent descriptive study (2015) from India, decline of clinical efficacy of miltefosine was reported; 11/73 (15%) of PKDL patients relapsed by 18-months follow-up and this was highest (31%) in those with shorter duration treatment (50 mg tid for 60 days) as compared with longer duration (50 mg bid for 90 days) of which 10.5% relapsed.[23] This data has to be analysed with caution, as this was not a randomized clinical trial, but more a description of cases treated in routine care. Previously, similar decrease in response to miltefosine in treatment of VL has been reported.[24]

Treatment of 60 PKDL cases in India with SSG 10 mg/kg for 90 days, resulted in improvement with disappearance of parasites but the difficulty in assessing cure was emphasized, in particular in macular lesions. In imprint smears, the mononuclear infiltrate decreased (papular lesions) or persisted (macular lesions). Prolonging miltefosine administration to 16 weeks has been proposed.

In a recent field experience by MSF on routine patient management in Bangladesh (Fulbaria), 1400 patients were treated with Ambisome® 6 x 5 mg/kg given over 2 weeks. Results were satisfactory, with 86.5% of patients presenting total disappearance of all nodular and papular lesions and complete or very significant re-pigmentation of macular lesions after one year of follow-up. Among the 13.5% of cases who did not have significant re-pigmentation of macular lesions at 12 months, improvement was observed in subsequent visits, and only 2% of the patients required a second course of treatment. This experience was based on clinical assessment in the field, and not done systematically by standardized procedures. In order to better characterize the Ambisome treatment for PKDL patients in Bangladesh, MSF conducted a clinical trial where patients were again treated with Ambisome® 30mg/kg (n=110). In this

trial, treatment outcome was standardised, including photographs and evaluated independently by clinicians. A total of 88 patients completed 12 months follow up, out of which 80% responded satisfactorily to treatment (67% patients had complete resolution of lesions and 13% had substantial improvement (>75%) of lesions). In this study however, severe side-effects were noted as rhabdomyolysis was reported in 6 patients, 3 of which had severe hypokalemia that was ascribed to Ambisome® therapy. In an on-going second study, patients are treated with 5 x 3 mg/kg (2 injections per week) with potassium and magnesium supplementation and intensive monitoring. Preliminary reports do not suggest severe hypokalemia occurring during this regimen, and patients are responding to treatment satisfactorily.

In addition, expert opinion in Bangladesh is that patients with severe PKDL in Bangladesh had to be treated with high total doses of Ambisome (up to 60mg/Kg total dose) and careful monitoring of treatment help to prevent severe hypokalemia or rhabdomyolisis (Dr. Mondal, personal communication). Perhaps severe PKDL leads to significant destruction of the skin and this, in addition to higher dose of AmBisome, facilitate better penetration of the drug to the skin. This emphasizes the need of skin PK analysis of anti-PKDL drugs and to correlate it with response to treatment.

In Africa (Sudan) only chronic PKDL patients, i.e. showing persistence of lesions after 6 months of disease onset, and those with severe PKDL are treated. There are no conclusive studies on treatment of PKDL in Sudan; current practice is treatment with SSG 20 mg/kg for 40-60 days. There is limited experience with Ambisome®; one study showed cure rate of 83% (10/12 patients) with Ambisome®, given intravenously at 2.5 mg/kg per day for 20 days, without detectable adverse effects.[26]

Recent data showed good cure rates of combined SSG 20 mg/kg and paromomycin 11 mg/kg for 30-40 days in 8 of 9 patients treated.[27]

As the pathogenesis of PKDL implies an important role for the immune response, there is a rationale for immune modulation. In one study, combined immunochemotherapy with alum-precipitated autoclaved Leishmania major (Alum/ALM) vaccine+Bacille Calmette-Guérin (BCG) and SSG showed better cure rates than SSG alone (87% vs. 53%).[28] Newer immunomodulatory agents are under development but are not expected to enter clinical studies until 2016. Currently there are no immune markers that may guide the duration of treatment or indicate outcome.

1.5 Rationale for the study and selection of treatments to be tested

In the Indian Sub-continent (ISC) all patients with PKDL are treated irrespective of interval after VL, previous VL treatment, duration of the rash, or clinical presentation. While treatment may be indicated for clinical reasons, it is thought that PKDL patients may play an important role in transmission of VL and chronic PKDL patients have been implicated in major VL outbreaks in the past. Obviously, this is of crucial importance in the current VL elimination efforts in the ISC.

This study aims primarily to improve current treatment options. In addition, this will be the first study ever in PKDL in which outcome will be described in clinical, parasitological and immunological terms in relation to pharmacokinetics thus providing the rationale and focus for subsequent studies.

Currently there are no satisfactory treatments for any forms of PKDL. Available treatments include pentavalent antimonials: sodium stibogluconate (Pentostam, Stibonate) and meglumine antimoniate (Glucantime) which have been used since the 1940s and in some regions such as Sudan remain as the main first-line drugs. Conventional amphotericin B has been used in India for prolonged periods (60 infusions) but this is impractical and requires careful clinical and biochemical monitoring.[29] Both miltefosine and Ambisome® as monotherapy have shown to be effective. However, with the current recommended schemes there are some drawbacks such as the length of the treatment with miltefosine alone (8-12 weeks); the costs and recently observed toxicity of a high dose Ambisome® (total dose of 30 mg/kg). There is also the potential risk for development of resistance with miltefosine as monotherapy. In addition, there is an issue of access, as currently there is only one manufacturer for MF and situations of drug stock rupture have already occurred at some occasions, leaving no drugs in filed for treatment of PKDL patients.

The current scenario does not offer many alternatives given that no new drugs are available or in late stage of development. Paromomycin has been shown not to reach the skin in significant levels. Miltefosine, despite its teratogenic problems, is currently the only oral drug available with the advantages that this route of administration offers. Rather than dismissing the use of miltefosine due to its teratogenic effects, efforts to optimize its use by combining it with other drugs aiming to provide a safe, efficacious and shorter treatment with a high compliance should be explored. In terms of numbers, PKDL cases occur less frequently compared to VL cases which makes it possible to monitor contraception properly; indeed, no large scale PKDL treatment programs are expected and treatment is focussed on individual PKDL patients.

Combination treatment is well accepted in the treatment of VL for example in East Africa (SSG and paromomycin) and may offer shorter duration of treatment thus preventing prolonged hospitalization. Another possible advantage is prevention of development of resistance and reduced cost. These principles also apply to PKDL, where the need for an ambulatory treatment with a highly safe, efficacious and user-friendly regimen is even more pressing as patients are not ill, except for the rash.

While ideally the choice of study regimens would be based on drug- penetration of PK levels in the skin and the plasma, this would require studying a large number of patients with drugs in regimens that may or may not be appropriate as future PKDL treatment in the ISC. Therefore, we have chosen to design a study with 2 treatment arms that reasonably could be effective and safe for PKDL treatment and integrate the PK study so as to guide future treatment studies also depending on outcome.

Except for the presence of localized skin lesions, subjects with PKDL are otherwise healthy, so we may assume that the pharmacokinetics of Ambisome® and miltefosine in these subjects could be similar to that in healthy volunteers. However, there is no information about the levels of Ambisome® and miltefosine in skin of human or animal studies and how it correlates with plasma concentrations. This study aims to determine if accumulated Ambisome® and miltefosine levels are detectable in the skin of PKDL patients at the end of treatment, and if so how these skin levels relate to the individual systemic concentrations of the drugs and their correlation to the in vitro drug susceptibility of the Leishmania parasites.

Miltefosine concentrations keep accumulating until the end of the 28-day treatment period in patients.[30,31] Therefore, we expect that a single tissue sample at the end of treatment will

indicate the extent of miltefosine accumulation in skin tissue, even when assuming a delayed tissue distribution.

In contrast to the fungal diseases, in which the free amphotericin B fraction probably represents the active component of Ambisome®, in leishmaniasis it is most probably the liposomal fraction of the drug, as this is the fraction that is preferably phagocytosed by the host's macrophages in which the Leishmania parasites reside.[32] The liposomal fraction of Ambisome® also represents the largest fraction of the drug in circulation after administration in human.[33] Given the fact that logistic difficulties will preclude us from differentiating between free, tissue-bound, protein-bound and liposomal amphotericin B in this field trial, total amphotericin B levels will be measured in the skin and plasma and used for pharmacokinetic assessments.

This exploratory study proposes to determine the efficacy and safety of Ambisome® monotherapy regimen (5 x 4 mg/kg, given twice per week, total dose of 20 mg/kg) and Ambisome® (5 x 4 mg/kg, given twice per week, total dose of 20 mg/kg) in combination with miltefosine daily for three weeks (allometric dosing), for PKDL patients in the ISC. The limitation of Ambisome® at the total dose of 20mg/Kg is due to the safety concerns, mainly the cases of rhabdomyolysis observed in Bangladesh with the dose of 30mg/Kg. It is assumed that by decreasing the dose to 20mg/Kg safety will be improved with no significant loss of efficacy.

The Ambisome® will be given in 5 administrations over 2 ½ weeks to reduce safety risks. In addition, PD data from previous DNDi trials on VL have shown that parasite clearance is faster when Ambisome® was administered in multiple injections as compared to single administration. Although in this protocol we are not evaluating VL, but PKDL, splitting the dose over time may be beneficial for parasite dynamics, which will be monitored over time.

Miltefosine has a long half-life (> 6 days) and accumulates to eventually reach a steady state after 4 weeks of treatment. A minimum of 3-weeks treatment was considered necessary to reach sufficient exposure for the activity of miltefosine in PKDL patients. Still, this is a significant reduction as compared to the current 8-12 weeks of monotherapy regimen. If the proposed scheme shows to be safe and efficacious, it may be possible to consider regimens to treat PKDL which have shorter duration of treatment thus increasing compliance.

It has long been known that the underlying mechanism for controlling Leishmania infection and self-healing of dermal lesions is the cellular immune response, mediated by antigen-specific γ-interferon producing T-cells. Additional information to better understand the status and role of the host immune response and how it interacts when different treatment modalities are provided is needed. Similarly, studies to demonstrate that appropriate concentration of the drug is present in the right compartment, as well as to understand the drug to drug interaction and its correlation with the clinical outcome are crucial when optimizing a treatment modality. Lastly, data on parasite clearance during treatment of PKDL and follow-up may be correlated to immunological parameters and clinical outcome and thus may contribute to identify the best parameter to indicate cure in PKDL.

2 Study Objectives and Endpoints

2.1 Objectives

General Objectives: The overall objective of this study is to assess the safety and efficacy of two treatment modalities for PKDL patients in ISC.

2.1.1 Primary Objective

To measure the safety and efficacy of Ambisome® monotherapy regimen (5 x 4 mg/kg IV, twice per week, total dose of 20 mg/kg) and Ambisome® (5 x 4 mg/kg IV, twice per week, total dose of 20 mg/kg) in combination with miltefosine orally daily for three weeks (allometric dosing), for PKDL patients in the ISC.

2.1.2 Secondary Objectives

- To assess skin and plasma concentrations of total Ambisome® and miltefosine.
- To evaluate the host immune response in each treatment arm before, during and after treatment.
- To evaluate parasite clearance in each arm as indicated by direct microscopy and qPCR
- To assess relationship between clinical, parasitological and immunological responses to identify a potential biomarker for cure
- To assess relationship between pharmacokinetic parameters with clinical outcome and parasite clearance

2.1.3 Exploratory Objectives:

- To assess non-invasive tape disc method of skin sample collection for molecular parasitological diagnosis.

2.2 Study Endpoints

2.2.1 Primary Endpoint

Efficacy:

- To measure the efficacy (definitive cure at 12 months) of two treatment regimens in subjects with PKDL according to clinical criteria: complete resolution of papular and nodular lesions (flattening of 100% of lesions) and significant improvement (> 80% re-pigmentation) of macular lesions by 12 months after the end of treatment.

Safety:

- To assess the safety of the two regimens (SAEs, AESI and frequency and severity of AEs) from the start of treatment through the 12--month follow-up period and 24 months study visit.

2.2.2 Secondary Endpoint(s)

Efficacy

- To measure the efficacy of the two treatment regimens in subjects with PKDL

according to clinical criteria: complete resolution of papular and nodular lesions (flattening of 100% of lesions) and significant improvement (> 80% re-pigmentation) of macular lesions at 24 months after the end of treatment.

- To assess the overall assessment of the clinical presentation of PKDL lesions by the investigator at 12 and at 24 months according to the following categories [34]:
  - Category 1: All lesions have improved and the majority of lesions have resolved. Any remaining lesions are only faintly visible as re-pigmentation is almost complete.
  - Category 2: The majority of lesions show significant improvement some lesions have resolved.
  - Category 3: No new lesions, but the majority of lesions show no or slight improvement.
  - Category 4: New lesions have appeared and there is no or little improvement of other lesions

Pharmacokinetics

- To assess the maximal accumulation of total amphotericin B and miltefosine in the skin at the end of treatment and correlate these with plasma levels.

Immune Response

- To assess the change in immune response during and after end of treatment as compared to baseline by measuring cytokines profiles level in the peripheral blood.

Parasitology

- To assess the clearance of parasites by microscopy and qPCR (samples collected from skin, blood and tape disc) at various time-points before, during and after treatment and during follow-up.

3 Study design and study design rationale

3.1 Study design

This is a non-comparative, open label, randomized phase II clinical trial to assess the safety and efficacy of Ambisome® monotherapy (5 x 4 mg/kg IV given twice per week at a total dose of 20 mg/kg given) and a combination of Ambisome® (5 x 4 mg/kg IV given twice per week at a total dose of 20 mg/kg) plus miltefosine orally daily (allometric dosing BID) for three weeks for PKDL patients in India and Bangladesh.

In order to have representatives of PKDL patients from different countries and to increase recruitment capacity, three sites will participate in this study; 2 sites in India – KMRC, Muzzafarpur and RMRI, Patna and one site in Bangladesh – icddr,b (Surya Kant Kala Azar Research Centre as satellite site of icddr,b).

3.2 Study duration and duration of subject participation

Six months are expected to be needed to obtain all ethical and official clearances. With 3 active sites, recruitment period shall last 12 months, with possible extension up to 18 months depending on recruitment capacity. The follow-up period will be 12 months. In addition,

patients will be further assessed for their clinical and parasitological outcome at 24 months follow-up visit.

Therefore, the overall study duration is expected to be 36-42 months from first patient in (FPI) to last patient out (LPO). Taking into account the analysis and reporting period, the study shall last 42-48 months.

3.3 Rationale of study design

Combination treatment is well accepted in the treatment of VL, paromomycin (PM) combined with miltefosine in Asia; or SSG combined with PM for example in East Africa and may offer shorter duration of treatment thus preventing prolonged hospitalization. Another possible advantage is prevention of development of resistance and reduced cost. These principles also apply to PKDL, where the need for an ambulatory treatment with a highly safe, efficacious and user-friendly regimen is even more pressing as patients are not ill, except for the rash.

This study aims primarily to improve current treatment options. In addition, this will be the first study ever in PKDL in which outcome will be described in clinical, parasitological and immunological terms in relation to pharmacokinetics thus providing the rationale and focus for subsequent studies.

The present protocol will have estimation of pharmacokinetics levels and drug levels in skin after administration of treatment. Pharmacodynamic parameters of repeated qPCR measurements in blood and in tissue at baseline, EOT, and at the 3, 12 months follow-up visits, and skin qPCR at 24 month follow up visit will be assessed to allow for a simultaneous population PK-PD analysis to investigate in detail the drug exposure-treatment response relationship.

4 Selection of Subjects

A total of 126 patients will be included in this study. Patients will be recruited at following sites: Surya Kanta Kala Azar Research Centre (SKKRC), Mymensingh in Bangladesh; and two sites in India at KAMRC Muzzafarpur and RMRI, Patna. Sites will be notified when the maximum number of patients to be recruited is reached in the study.

The average number of PKDL patients treated at each site are as follows: KMRC-4-5 patients/month, RMRI-10-15 patients/month and Mymensingh-4-5 patients/month.

Considering a potential of 20 PKDL confirmed cases per month and a conservative estimation of 50% of these cases meeting inclusion and exclusion criteria for the study, there will be 10 patients enrolled/month and recruitment will be completed in 10-12 months. As mentioned above, this is based on historical data, and recruitment may take longer, up to 18 months.

The following eligibility criteria were designed to select subjects for whom the protocol treatment is considered appropriate. All relevant medical and non-medical conditions should be taken into consideration when deciding whether this protocol is suitable for a particular subject. Eligibility criteria may not be waived by the investigator. Any questions regarding a subject's eligibility should be discussed with the DNDi Clinical Manager prior to subject's enrolment.

4.1 Inclusion criteria

Subjects must meet all of the following inclusion criteria to be eligible for enrolment into the study:

- Confirmed PKDL case by clinical presentation and demonstration of parasites by microscopy in a skin smear or biopsy or by PCR, with stable or progressive disease for at least 4 months
- Male or Female patients aged 6 to 60 years
- Written voluntarily informed consent from adult patient and from parent / guardian in case of children <18 years old. In the case of minors, assent from the children will also be obtained according to country regulations.

4.2 Exclusion criteria

The presence of any of the following will exclude a subject from study enrolment:

- Patients who had prior treatment of PKDL within last 2 years.
- Pregnant and lactating women and women of childbearing age (12 to 55 years) who, before randomization, cannot be assured contraceptive cover during treatment and 5 months thereafter
- Patients with sign and symptoms of severe diseases: defined as suffering from a concomitant severe infection such as TB or any other serious known underlying disease (cardiac, renal, hepatic)
- Severe malnutrition defined by BMI for age WHO reference curves for gender, Z score < -3 for subjects 6 - 19 years; BMI < 16 for subjects > 19-years old
- Patients with haemoglobin < 5g/dL
- Patients with abnormal liver function (ALT and AST) tests of more than three times the normal range.
- Patients with total bilirubin levels >1.5 times the upper normal range
- Patients with serum creatinine above the upper normal range
- Patients with serum potassium < 3.5 mmol/L
- Patients with a positive HIV test as applicable
- Patients / guardian not willing to participate
- Patients with history of allergy or hypersensitivity to the relevant study drug
- Patients on immunomodulators

Women who are sexually active and not using an assured method of contraception i.e. either IUCD or medroxyprogesterone acetate (Depo-Provera) but who wish to participate in the trial must sign an additional consent form and agree to receive a long-acting assured form of contraception medroxyprogesterone acetate (Depo-Provera). One injection (which is effective for 3 months) will be administered at baseline, and a 2nd injection will be administered at 3 months visit. This is needed to ensure adequate coverage taking into account the long half-life of approximately 7 days of Miltefosine. Oral contraceptives are not considered adequate

in this context because of the high prevalence of vomiting and diarrhea associated with miltefosine treatment.

Note: Patients not suitable for inclusion in the trial but with confirmed PKDL cases will be treated at the trial sites following National Program treatment policy for PKDL cases.

5 Schedule of events

Protocol activities	Screening	Treatment period (+/- 1 day)				EOT	Follow-up period			
	D-28 to D0	D1	D8	D15	D22[a]	D30 (+/- 3 days)	3M (+/- 7 days)	6M (+/- 7 days)	12M (+/-14 days)	24M [f] / EOS (-1month/+3 months)
Consent form	x									x
Inclusion and Exclusion criteria	x									
Vital signs and physical exam	x	x	x	X	x	x	x	x	x	x
Demographic data and Medical history	x									
Pregnancy test	x									
HIV test	x									
rK 39 test	x									
CBC, ALT / AST, Creatinine, Potassium, bilirubin	x		x	X	x	x	x[b]	x[b]	x[b]	
Skin snip for microscopy and qPCR	x					x	x		x	x
Blood PD (qPCR)	x					x	x		x	
Tape disc skin sample for qPCR	x					x	x		x	
Skin biopsy for PK				x[c]	x[a]					
Plasma sample for Miltefosine PK [a]			x	X	x	x	x			
Plasma sample for Ampho B PK[d]		x		X	x	x				
Blood sample for immunological parameters	x			X		x	x		x	
PKDL evolution assessment, including photographs	x					x	x	x	x	x
Ambisome monotherapy treatment		Amb injections: D1, D4, D8, D11, D15								
Ambisome + miltefosine treatment		Amb injections: D1, D4, D8, D11, D15 + Milt allometric D1-D21								
Safety assessment	SAEs and study related AEs	---------- SAEs and AEs monitoring ----------							----------SAEs and AEs related to study drugs or procedures----	

a) Assessments to be performed only for the Ambisome® + Miltefosine arm
b) Only if abnormal at the previous assessment in a scheduled or unscheduled visit
c) Day 15 skin biopsy sample only for the Ambisome® monotherapy arm
d) A total of 30 subjects per arm will be included in the Ampho B PK assessment. Day 1 sampling to be performed at the end of infusion and at 2, 4, 8 and 22 hrs after the end of infusion. Day 15 sampling to be performed prior to infusion, at the end of infusion and at 2, 6 and 22 hrs after the end of infusion. One single sample to be collected at D22 for the Ambisome+miltefosine combination arm. One single sample to be collected at D30 for the Ambisome monotherapy arm.
f. If the patient has completed 24 months /EOS before the approval of Protocol Amendment V3.0, 25 Feb 2020, the patient can be called as soon as possible for the final visit, this won't be considered a protocol deviation.

6 Enrolment procedures

A computer generated, randomization code will be used for patient treatment allocation to one of the two treatment arms. One set of individuals, opaque, sealed and sequentially numbered envelopes will be provided to the treatment sites, one envelope per patient. Eligible patients who fulfil all the inclusion criteria and have none of the exclusion criteria, and from whom informed consent has been obtained, will be randomized to one of the treatment regimens using the sealed envelopes.

The patient receives the treatment written on the card inside. The card must then be placed back in the envelope and retained for drug accountability and monitoring purposes. This method of treatment concealment will minimise selection bias as the investigator will not know the allocation of treatment for a specific patient until the envelope is opened.

7 Study treatments

7.1 DNDi study treatment: Investigational Products

All investigational products will be supplied by the DNDi from commercially available stocks.

7.1.1 Name: Liposomal Amphotericin B (Ambisome®)

Class: a macrocyclic, polyene antifungal antibiotic produced by Streptomyces nodosus.

Mechanism of action: affects sterol biosynthesis, disrupting the parasite membrane.

Commercial source: Gilead.

Product appearance: Ambisome® comes as a sterile lyophilised powder in a 15ml sterile Type 1 clear glass vial containing a yellow powder with the active ingredient amphotericin B 50mg encapsulated in liposomes. The closure consists of a butyl rubber stopper and aluminium ring seal with a removable plastic cap. Vials are packed in cartons of 10, with 10 filters provided.

The total dose of Ambisome® treatment will be 20mg/Kg, given as 4mg/Kg/day over 5 administrations at D1, D4, D8, D11 and D15.

Administration:

• Add 12mL of water for injection to each Ambisome® vial, to yield a preparation containing 4mg/mL (do not use saline or any bacteriostatic agents).

Ambisome® is incompatible with saline and should not be mixed with other drug or electrolyte solutions during administration.

• Immediately after the addition of water, shake the vials vigorously for 30 seconds to completely disperse the Ambisome®. Visually inspect the vials for particulate matter and continue shaking until complete dispersion is obtained.

• The infusion providing from 2.00 to 0.20mg of amphotericin per mL is obtained by dilution with 1 to 19 parts respectively by volume of 5% dextrose injection.

• Calculate the volume needed based on the dose of 4mg/Kg, according to patient's weight. Withdraw the calculated volume of reconstituted Ambisome® into a sterile syringe.

• Using the 5 micron filter provided, instil the Ambisome® preparation into a sterile container with the correct volume of dextrose injection.

7.1.2 Name : Miltefosine (Impavido®)

Class: Phosphocholine analogue.

Mechanism of action: interferes with the synthesis and metabolism of phospholipids.

It may also interfere with the parasite's membrane signal transduction, and glycosylphosphatidylinositol anchor biosynthesis.

Commercial source: Knight Therapeutics

Product appearance: comes as 10mg and 50mg capsules as a pack of 28 or 56 capsules sealed in 4 or 8 aluminium blister stripes, each containing 7 capsules.

Administration: oral, with food. For miltefosine, there are no known interactions with other commonly used medications, though this cannot be excluded.

7.2 Doses and treatment regimens

Ambisome® monotherapy 5 x 4 mg/kg IV at D1, D4, D8, D11 and D15.

Combination of Ambisome® (5 x 4 mg/kg IV at D1, D4, D8, D11 and D15) plus miltefosine orally BID administration (allometric dosing) for 3 weeks.

Table 1 below describes the miltefosine regimen by an allometric scale, to be used for patients up to 30Kg.

Patients with weight > 30Kg, there is no difference between the allometric scale and conventional dose of 2.5mg/Kg/day. For these patients, the miltefosine regimen will be:

Patients > 30Kg and < 45Kg: 100mg of miltefosine per day, administered as 1 capsule of 50mg in the morning and 1 capsule of 50mg in the afternoon, with food, for 21 days.

Patients > 45Kg: 150mg of miltefosine per day, administered as 2 capsules of 50mg in the morning and 1 capsule of 50mg in the afternoon, with food, for 21 days.

An easy-to-use table (Table 2) with dosing scheme by weight and height will be provided to the investigators to define the exact daily miltefosine dose to be administered.

Table 1 Daily allometric miltefosine dose for female and male children based on fat-free mass

FEMALE									
HT (cm)	80-89	90-99	100-109	110-119	120-129	130-139	140-149	150-159	160
WT (kg)									
7									
8	30								
9	30								
10	30	40							
11	40	40	40						
12	40	40	40						
13	40	40	40						
14	40	40	40	50					
15	40	40	50	50					
16	40	50	50	50					
17	40	50	50	50	50				
18	50	50	50	50	60				
19	50	50	50	60	60				
20	50	50	50	60	60	60			
21	50	50	60	60	60	60			
22	50	50	60	60	60	60			
23	50	50	60	60	60	70	70		
24	50	50	60	60	60	70	70		
25	50	60	60	60	70	70	70		
26	50	60	60	60	70	70	70	70	
27	50	60	60	70	70	70	70	80	
28	50	60	60	70	70	70	80	80	
29	50	60	60	70	70	70	80	80	
30	50	60	60	70	70	80	80	80	80

RISK OF SEVERE MALNUTRITION

REFER TO THE WHO REFERENCE CURVES

MALE									
HT (cm)	80-89	90-99	100-109	110-119	120-129	130-139	140-149	150-159	160
WT (kg)									
7									
8	40								
9	40								
10	40	40							
11	40	40							
12	40	50							
13	50	50	50						
14	50	50	50						
15	50	50	60	60					
16	50	50	60	60					
17	50	60	60	60					
18	50	60	60	60	70				
19	50	60	60	70	70				
20	50	60	60	70	70				
21	60	60	60	70	70	70			
22	60	60	70	70	70	80			
23	60	60	70	70	80	80			
24	60	60	70	70	80	80	80		
25	60	60	70	70	80	80	80		
26	60	70	70	80	80	80	100		
27	60	70	70	80	80	80	100		
28	60	70	70	80	80	100	100	100	
29	60	70	70	80	80	100	100	100	
30	60	70	80	80	100	100	100	100	

RISK OF SEVERE MALNUTRITION

REFER TO THE WHO REFERENCE CURVES

Administration of the daily dosage must be divided in 2 administrations, during or after a meal, to avoid direct gastrointestinal adverse effects of miltefosine, as follows:

Table 2 Morning and afternoon doses

Total daily dose, divided:	30mg	40mg	50mg	60mg	70mg	80mg	90mg	100mg
Morning (breakfast)	2x 10mg	2x 10mg	1x 50mg	1x 50mg	1x 50mg	1x 50mg	1x 50mg	1x 50mg
Afternoon (dinner)	1x 10mg	2x 10mg	-----	1x 10mg	2x 10mg	3x 10mg	1x 50mg	1x 50mg

With this scheme, a maximum of 3 capsules of 10mg will be administered per intake.

All administrations of Ambisome® will be directly observed by the study nurse in the hospital to assure compliance. Subjects will be admitted for whole duration of Ambisome® administration. In case of combination arm of Ambisome® and miltefosine, miltefosine administration will be directly observed by the study nurse for the time subject is admitted in the hospital. After each drug intake all subjects will be observed for vomiting for 30 minutes. All vomiting episodes will be recorded. In case of vomiting 30 minutes after miltefosine intake, the dose will be re-administered. If a repeated vomiting occurs (vomiting after re-dose) the treatment will not be re-administered.

Patients allocated in the combination arm may be discharged on day 16 and given the remaining doses of Miltefosine to be taken at home. Each subject will be contacted by telephone by a health worker on a daily basis until completion of the Miltefosine treatment. It is also permissible for a subject to be hospitalized for the entire duration of treatment for those in the combination arm.

Compliance will be assessed by total dose taken by the patient. Full compliance will be considered if 90-110% of the prescribed dose is administered.

If the patient vomits a dose within 30min of drug intake and is re-dosed (without vomiting this 2nd re-dose), the re-dose will be considered for compliance. However, if the patient is not re-dosed within 30min or if s/he vomits the re-dose, this dose will not be accounted for compliance.

In the case of a patient vomiting after 30min of miltefosine intake, the dose will be accounted for compliance.

In the Miltefosine regimen, treatment may be temporarily interrupted (due to AEs or at the discretion of the investigator) and resumed if this interval is no longer than 72 hours. Once resumed, the subject will complete the 21 days of treatment by prolonging the treatment up to Day 24 (missing doses will be compensated). In this case, the total administered dose will still be within the 90% range of full compliance, therefore it will not be considered a compliance deviation.

If treatment needs to be interrupted for > 72 hours due to an AE, rescue treatment will be considered, at the discretion of the investigator.

7.3 Study treatments labelling, packaging

Commercially available Ambisome® and miltefosine will be used. Trial specific labelling will be applied prior to use.

7.4 Accountability

All study medications must be kept in a locked room that can be accessed only by the appropriate study personnel designated by the investigator. The study medications must not be used for purposes other than this protocol. Under no circumstances may the investigator or site staff supply study medications to other investigators or sites or allow the medications to be used other than as directed by this protocol without prior authorization from the sponsor or delegated responsible party.

Specific study forms for drug accountability will be designed to allow for adequate records on receipt, use, return, loss, or other disposition of medication. These accountability forms must be maintained in a locked cabinet by the study pharmacist or designated personnel. Since the

same drugs are used in routine practice, SOPs for labelling the boxes to be used in the trial will be developed, and the drugs used in the trial will be trial specifically labelled and stored completely separated from the routine drugs.

In case of Miltefosine treatment given to patient to be taken at home, the subjects will be instructed to return the empty blisters in order for site staff to perform full accountability. The compliance is assessed and reported in the CRF.

7.5 Storage

Study drug must be kept in a locked cabinet and/or in a room with restricted access, under the control of the study pharmacist.

Temperature of the storage location will be monitored daily and recorded in a temperature log. In the event of temperature excursions below or above the allowable range site staff must inform the study monitor.

Ambisome®: The product should be stored at 25°C or below, not frozen or exposed to light. The shelf life is 3 to 4 years. Once reconstituted, chemical and physical in-use stability has been demonstrated for 24 hours at 25+/-2°C in vials exposed to ambient light. This is increased to 7 days if stored at 2-8°C. However, to avoid contamination, once reconstituted, the product should be stored at 2-8°C and be used within 24 hours. Vials will be exclusive to each patient. Storage to these requirements will be ensured throughout the study.

Miltefosine: The product should be stored in the original package and protected from moisture at 20-25°C, with excursions permitted at 15-30°C. The shelf life is 5 years. Packaging should be undamaged prior to use.

7.6 Blinding and procedures for unblinding

Not applicable. This is an open label study.

7.7 Concomitant treatments

All medications required for concomitant conditions should be postponed until after the end of treatment for PKDL, unless warranted by immediate medical need. Common conditions such as malaria and respiratory tract infections should be treated prior to starting PKDL treatment.

There are no known interactions with other commonly used medications, though this cannot be excluded. All concomitant medications used during the study up to 24 months visit shall be recorded in the patients' chart and in the CRF.

7.8 Rescue and Escape treatments

At any time-point during the study, if the patient does not respond to treatment, or lesions are worsening, or if after the healing period, the lesions reappear/reactivate, rescue treatment can be initiated at the discretion of the study investigator. A subject who receive rescue treatment at any time during the trial will be considered a treatment failure and will be withdrawn from the study. Rescue treatment will be provided to the patient as per standard recommendations by National Program with Miltefosine monotherapy for 12 weeks.

Patients will be given rescue treatment if:

- Progression of the disease is documented at any point in time during the study treatment or FU, and rescue treatment is indicated by the investigator
- They fail to tolerate trial medication or if occurrence of adverse event(s) during receipt of test drug dose (refer to the Ambisome® pack insert for withdrawing the patients) requires permanent interruption of treatment, and rescue treatment is indicated by the investigator
- If after healing period lesions reappear/reactivate

8 Study Assessments

8.1 Timing of Assessments:

Study assessments will be performed at baseline, D1, D8, D15, D22 (only for subjects allocated to combination arm), and D30 (EOT, +/- 3 days) during treatment phase.

All subjects will have follow-up visits at 3 months (+/- 7 days), 6 months (+/- 7 days) and 12 months (+/- 14 days) after the onset of treatment to assess efficacy and safety. A strategy will be put in place according to the context of each trial site to assure patients comply with the schedule of scheduled study visits. This will include active tracking of the patients through telephone contact or domiciliary visits.

In addition, a follow-up visit will be performed at 24 months (-1 month/+3 months) to assess the long-term response to treatment and safety. All patients will be invited to present to the 24 months follow-up visit, except those who have been withdrawn from the trial [e.g. for safety reasons (AE leading to treatment discontinuation and rescue treatment received), patients who withdrew consent, and cases of death, or patients who were treatment failure (received rescue treatment at any time during the 12 months follow-up period, including at 12 months visit).

8.2 Baseline Assessments

The following baseline assessments will be done during screening (-28 days to day 0) and after having obtained Informed Consent in order to confirm PKDL diagnosis and verify inclusion and exclusion criteria.

- Demographic data including age and gender
- Medical history including relevant past history, previous VL episode, previous history of PKDL, concomitant medications
- Clinical examination: Physical examination including clinical signs, skin lesions, PKDL assessment, body weight, height, vital signs (Temperature, Heart rate and Blood pressure), spleen and liver size
- Clinical safety laboratory evaluations:
    - Haematology: CBC, including total WBC with differential, platelets
    - Biochemistry: Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), total bilirubin (TBIL), Creatinine (CRE), Potassium
    - Pregnancy test

- HIV test – routine counselling for HIV test will be given for patients or patients' parents in case of minors, as part of the study informed consent procedure interview or a separate consent will be requested, according to the local requirements. Testing will be done according to national guidelines and undergo a single rapid diagnostic test for the purposes of the clinical trial. If the RDT for HIV is positive, the patient must be excluded from participating in the clinical trial and receive current standard treatment for PKDL and further assessment and treatment for HIV according to the national HIV treatment Guidelines.
- rK 39 test
- Skin snip sample for parasitological examination by microscopy and pharmacodynamics assessment (qPCR)
- Blood sample for pharmacodynamics assessment (qPCR)
- Blood sample for immunological parameters assessment (cytokines IL-2, IFNY, TNFα, IL-10, and Granzyme B)
- Photograph of the patient as per SOP (front and back side with lesions)

8.3 Assessment of Efficacy

Primary efficacy (definitive cure at 12 months) of the two treatment regimens in subjects with PKDL will be measured clinically according to following criteria: complete resolution of papular and nodular lesions (flattening of 100% of lesions) and significant improvement (> 80% re-pigmentation) of macular lesions by 12 months after the end of treatment.

Secondary efficacy variables will be assessed by clearance of parasites by microscopy and qPCR before, during and after treatment during 12-month of follow up.

Secondary efficacy clinical assessment of PKDL lesions will also be done by the investigator at 12-months visit as per categories defined below:

- Category 1: All lesions have improved, and the majority of lesions have resolved. Any remaining lesions are only faintly visible as re-pigmentation is almost complete.
- Category 2: The majority of lesions show significant improvement some lesions have resolved.
- Category 3: No new lesions, but the majority of lesions show no or slight improvement.
- Category 4: New lesions have appeared and there is no or little improvement of other lesions

In addition, secondary efficacy variables will be assessed clinically (as per cure and categorical assessments described above) and by clearance of parasites by microscopy and qPCR at 24-months follow-up visit.

8.3.1 Clinical assessment and photo documentation of lesions

Clinical Assessment:

Clinical evolution will be recorded systematically by the Investigator using a scoring system (see below) and photographs at screening, Day 30, 3 months, 6 months and 12 months after

treatment onset. Definitive cure will be assessed at 12 months visit.

The same clinical assessment will be recorded at the 24 months follow-up visit.

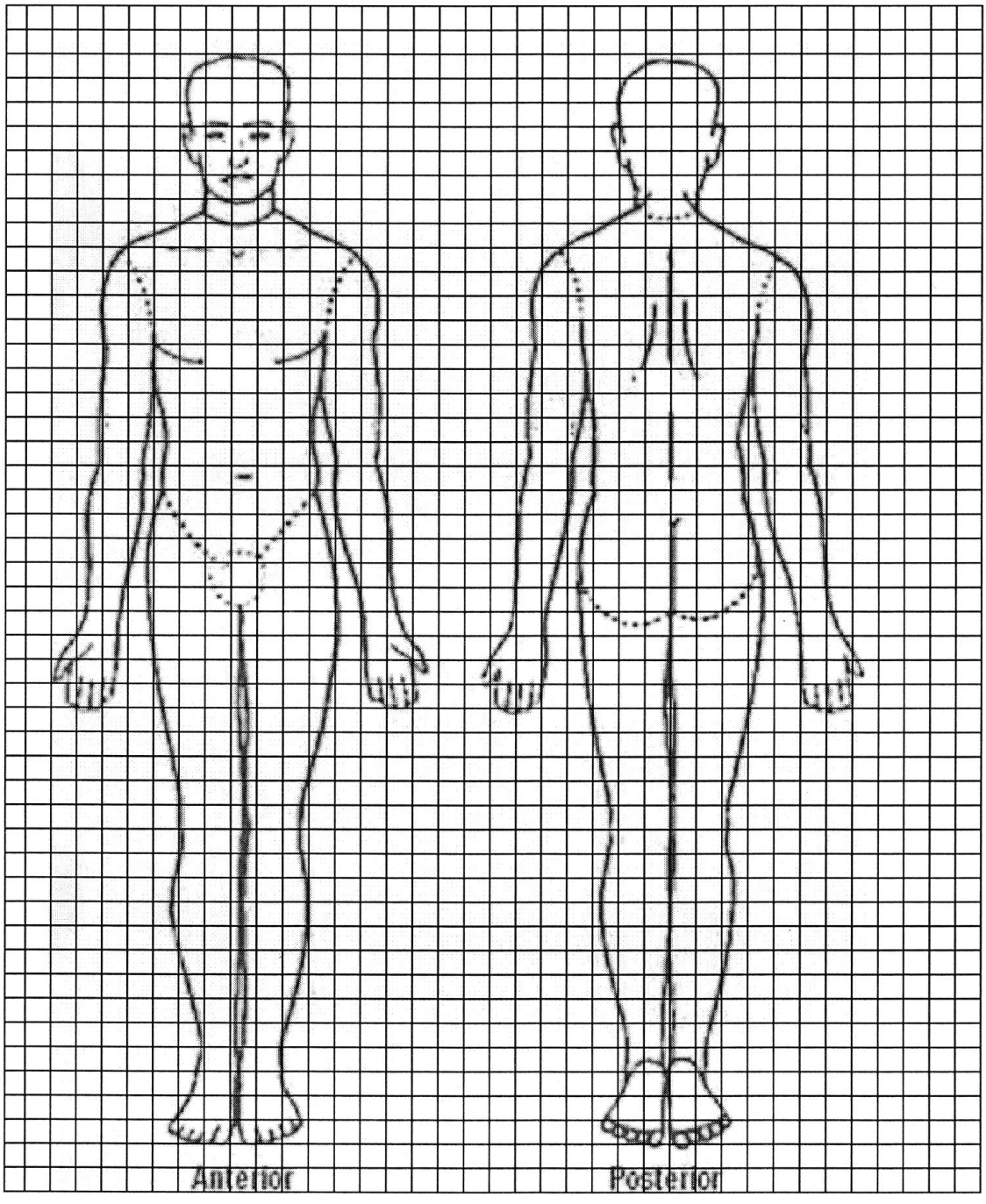
PKDL Scoring:

To date, there was no standardized scoring method to objectively measure the extension of affected skin area in patients with PKDL. In order to improve PKDL evolution assessment, a scoring system was developed and validated by Dr. Mondal and his team in Bangladesh. Using the diagram below, skin lesions will be plotted in squares corresponding to the affected skin areas of the body. The total number of squares containing lesions will be counted before (screening) and after treatment at Day 30, 3, 6, 12 and 24-months follow-up. The percentage of skin lesions cured by the treatment will be calculated as follows: total number of squares affected by the lesions at Visit 'X' ×100/total number of squares affected by the lesions at baseline. Indian sites will be trained and qualified to use this scoring system. Training records will be available in the Investigator file/TMF.

Figure 1: Scoring System

Patient's Initial: Patient's ID: Time: before / after treatment

Score (total number of squares affected by lesions):



Name of Physician: Signature: Date:

Photographs:

All lesions will be serially photographed under standardized conditions described in a photograph SOP (distance, light, camera, etc) at screening, Day 30 (EOT), 3-month, 6-month, 12-month, and at 24-month follow up visits.

Photographs taken during patient evaluation will be retained as source documents in the hospital files and in the Investigator's File. In addition, the electronic files with the photographs will be transferred to an independent external expert for remote blinded review of the photographs. The assessment of the investigator and the independent blinded reviewer will be compared. Cases of discrepancy will be assessed by a 3rd blind reviewer.

8.3.2 Parasitological assessment

Parasitological assessment will be done at baseline and at Day 30 (EOT), 3-month, 12- month and 24-month after treatment onset through microscopy examination of skin snip imprint smear sample and qPCR. In addition, skin sample will be collected from 20 subjects at the same time-points using non-invasive tape disc method (with the exception of 24 months), from where DNA will be extracted for qPCR. Results from the qPCR using skin snip sample will be compared with tape disc method.

The parasite load will be assessed by high power field (10x eyepiece and 100x objective). All slides will be coded and will be stored for reassessment.

The parasite load will be graded as follows:

Score	No. of parasites
0	0 parasites per 1000 fields
1+	1-10 parasites per 1000 fields
2+	1-10 parasites per 100 fields
3+	1-10 parasites per 10 fields
4+	1-10 parasites per field
5+	>10 parasites per field

8.4 Pharmacokinetics and Pharmacodynamics Assessments

8.4.1 Pharmacokinetic Assessments

PK assessment from the skin (biopsy) will be collected at the end of treatment i.e. at day 15 for patients allocated in the Ambisome® arm and day 22 for patients allocated in the Ambisome® + miltefosine arm. PK samples will be collected after administration of the drug dose. Adherence records, tissue weight of the samples, time of drug administration and time of PK samples collection have to be recorded systematically.

For patients allocated to Ambisome+miltefosine arm, miltefosine concentration will be measured in EDTA blood plasma collected at D8, D15, D22, D30 and 3 months visits. In addition, miltefosine concentration will be measured in the skin biopsy collected at D22 from patients allocated to combination arm.

For a sub-set of 30 patients per arm, total amphotericin B concentration will be measured in EDTA blood plasma collected at D1 (at the end of infusion, and at +2h, +4h, +8h, +22 hrs after the end of infusion), D15 (at prior to infusion, at the end of infusion and at +2h, +6h, +22hrs

after the end of infusion), and D22 (single sample for patients allocated to combination arm) or D30 (single sample for patients allocated to Ambisome monotherapy arm). In addition, total amphotericin B concentration will be measured in the skin biopsy collected at D15 from patients allocated in the Ambisome® monotherapy arm, and at D22 from patients allocated to combination arm.

As much as possible, a single blood draw should be performed per study visit to avoid multiple vein punctures. Sample for PK will be a 2mL EDTA whole blood sample/time point.

Samples will be stored on site in dark sample containers at minimally -20 ^{o} C and regularly shipped minimally at -20^{o} C conditions for analysis. Samples will be analysed for their amphotericn B and miltefosine content in plasma and skin following a procedure using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) validated according to FDA and EMA guidelines. The development and validation of the miltefosine plasma assay has previously been published (T. P. Dorlo et al., 2008), validation documents concerning the amphotericin B plasma assay are on record.

8.4.2 Pharmacodynamic Assessments

Blood samples for qPCR will be collected at baseline, Day 30 (EOT) and at 3 and 12 months follow up visits.

Skin snip samples for parasitological examination by microscopy and qPCR will be collected at baseline, D30 (EOT), 3 month, 12 month and 24-month follow-up.

Parasite load in blood and tissue aspirate: PD assessment will be based on real time quantitative polymerase chain reaction (qPCR) for *L. donovani* in EDTA blood or tissue aspirate, based on the amplification of kinetoplastid DNA (kDNA), to assess the parasite clearance over time during the treatment with miltefosine.

Approximately 1 mL EDTA whole blood will be immediately separated from blood EDTA samples taken for the qPCR analysis. qPCR will be performed according to SOP and standardised across the study sites. The qPCR will be performed at each respective hospital.

8.4.3 Immunological Assessment

Blood samples for immunological studies (3mL in heparin tube) will be collected at baseline, D15, D30 and at 3, and 12 months follow-up visits.

An aliquot of the whole blood (500 µL) will be placed in 3 separate tubes: one containing 10 µg/ml of soluble Leishmania antigen (SLA), one containing phytohaemagglutinin (PHA) as positive control, and one negative control; which will be subsequently incubated at 37ºC for 24h. They are then centrifuged and supernatant is separated for analysis.

Cytokines IL-2, IFNY, TNFα, IL-10, and Granzyme B (and possibly other biomarkers after a preliminary screening) will be measured in the stimulated whole blood supernatant by BD™ Cytometric Bead Array (CBA) Human Th1/Th2 Cytokine CBA. Lymphocyte subsets will be identified by FACS analysis.

Although whole blood assay with CBA and FACS would be the preferable method for cytokine analysis, ELISA reading of the whole blood supernatant will also be considered appropriate.

8.5 Assessment of Safety

Safety of the treatments will be assessed through routine monitoring of adverse events. At each study visit, the patients will be enquired about current adverse events or any events observed during the period previous to the visit. In addition, evaluation of hematology and blood chemistry parameters, regular measurement of vital signs and physical examinations will be made at D1, D8, D15, D22, D30 and each scheduled follow-up visit.

The frequency, severity, seriousness and causality assessments of AEs will be described, as well as frequency of SAEs or AEs that lead to treatment discontinuation.

Laboratory examinations

Hematology parameters CBC, WBC with differential and platelets will be analysed at screening, on days 8, 15, 22 (only for Ambisome® and miltefosine combination arm), and day 30 (EOT).

Biochemistry parameters creatinine, Potassium, AST, ALT and bilirubin will be analyzed at screening, on days 8, 15, 22 (only for Ambisome® and miltefosine combination arm), and day 30 (EOT).

Hematology and Biochemistry will be repeated at 3, 6 or 12-month follow-up visits only if abnormal at the previous assessment in a scheduled or unscheduled visit.

Samples will be analysed at the local laboratory using standardized equipment.

Approximately 4 mL of blood will be collected at each visit: 2mL in EDTA for the hematology and 2mL in dried tube for the biochemistry.

Lab parameters will be graded according to CTCAE v4.03 and abnormal values for clinical significance.

8.6 Adverse event definitions and reporting

8.6.1 Adverse Event definition

An adverse event will be defined as any untoward medical occurrence in a patient or clinical trial subject administered an investigational treatment and which does not necessarily have a causal relationship with this treatment.

It can therefore be any unfavourable and unintended sign (including an abnormal laboratory finding, for example), symptom, or disease temporally associated with the use of an investigational treatment, whether or not considered related to the investigational treatment.

Definition of an AE includes worsening (in severity and frequency) of pre-existing conditions/abnormalities ("Medical history") before first Investigational Medicinal Product (IMP) administration and abnormalities of procedures (i.e. ECG, X-ray…) or laboratory results which are assessed as "clinically significant" (see details in section 8.6.2).

What is not an AE?

Medical conditions present at the screening or initial study visit that do not worsen in severity or frequency during the study are NOT considered as AE.

Symptoms, exacerbation or worsening of the studied disease will NOT to be considered as AE nor captured on the AE page of the CRF if consistent with the anticipated natural progression

of the disease (overall and for this given subject).

Lack of efficacy of the IMP is NOT considered as AE.

8.6.2 Assessment of laboratory abnormalities

For every laboratory assessment, the investigator will evaluate if the lab test is normal or abnormal. If abnormal, the investigator will assess if this finding is clinically significant or not. If a lab parameter is abnormal and clinically significant, it should be reported as an adverse event (AE).

Please also note that an abnormal test must be compared with the previous value. An AE is a new event after the administration of the 1st dose of the study drug or a worsening in the condition (in the case of lab tests, increase in severity by CTCAE v4.03 which is judged clinically significant by the investigator).

Laboratory abnormalities should be assessed as "clinically significant" (and therefore have to be reported as an AE) only if they meet AT LEAST ONE of the following conditions:

- The abnormality suggests a disease and/or organ toxicity **AND** this abnormality was not present at the screening visit or is assessed as having evolved since the screening visit
- The abnormality results in discontinuation of the study drug
- The abnormality requires medical intervention or concomitant therapy

Lab parameters which are classified with regards of severity as CTCAE v4.03 Grade 3 or Grade 4 must be carefully assessed, as they are likely to require medical intervention and/or be life-threatening in terms of severity.

For the purpose of this protocol, all **clinically significant** Grade 3 and Grade 4 lab abnormalities must be reported as an AE.

Furthermore, lab abnormalities associated with clinical signs and symptoms will also be considered clinically significant. When reporting an abnormal lab as an adverse event, a clinical diagnosis should be recorded rather than the abnormal value itself, if available. However, in these cases, the adverse event should be recorded as the syndromic clinical diagnosis (ex. acute pancreatitis instead of each finding separately: high levels of amylase, high levels of lipase, abdominal pain and vomiting; Ex "hypokalemia" rather than "decreased potassium levels").

8.6.3 Serious Adverse Event

An adverse event will be defined as serious if it results in one or more of the following:

- results in death,

  i.e. causes or contributes to the death.

- is life-threatening,

  in this context refers to an AE/AR in which the patient was at risk of death at the time of the AE/AR; it does not refer to an AE/AR that hypothetically might have caused death if more severe.

- requires in-patient hospitalization or prolongs existing hospitalization,

i.e. the AE requires at least an overnight admission or prolongs a hospitalisation beyond the expected length of stay.

- Hospital admissions or prolongation of hospitalisation for an elective surgery planned before study entry, for social reasons and/or financial reasons, for any elective surgery (i.e. plastic surgery) or for normal disease management (including treatment adjustment) are NOT to be considered as a SAE according to this criterion (i.e. if the protocol or the standard management of the disease under study requires planned hospitalisation).
- results in persistent or significant disability or incapacity,
- is a congenital anomaly/birth defect,

  i.e. an adverse event outcome in a child or foetus of a subject exposed to the Investigational Medicinal Product (or marketed medicinal product) before conception or during pregnancy.
- is an important medical event, i.e. medically significant.

  Medical and scientific judgment should be exercised in deciding whether other situations should be considered serious events/reactions, such as important medical events that might not be immediately life threatening or does not directly result in death or hospitalization, but which might jeopardize the patient or might require intervention to prevent one of the other outcomes listed above.

What is not an SAE?

In this study, the patient may be hospitalized for receiving Ambisome® treatment, with discharge expected on D16. This hospitalization for receiving treatment to participate in the study will NOT be considered as a SAE.

Similarly, hospitalization or prolongation of hospitalization for standard PKDL disease management will NOT be considered as a SAE.

Any prolongation of the hospitalization that is not related to a medical event but related to any social and/or financial reasons and/or an elective planned surgery, will NOT be considered as an SAE.

SAE onset date:

SAE onset date is the SAE start date or date a pre-existing AE becomes serious.

8.6.4 Adverse Event of Special Interest

Adverse event of special interest (AESI) is an adverse event (serious or non-serious) of scientific and medical concern by the Sponsor, for which monitoring and rapid communication by the investigator to the Sponsor may be appropriate.

For this study, allergic reactions to Ambisome® or infusion related reactions occurring during test dose or during study drug administration resulting in treatment discontinuation will be considered AESI.

Severe Test Dose Reaction: If during administration of test dose, severe anaphylactic reaction occurs, then Ambisome® infusion should be immediately discontinued and patients should not receive further infusions of Ambisome®. These severe anaphylactic reactions should be

reported as serious Adverse Event of Special Interest (AESI).

8.6.5 Eliciting Adverse Event information

The investigator is required to report all directly observed adverse events and all adverse events spontaneously reported by the trial subject using concise medical terminology. In addition, each trial subject will be questioned about the occurrence of adverse events at each study visit (scheduled and non-scheduled) since the time of the last study visit, as well as follow-up from previously recorded adverse event up to 24 months.

8.6.6 Adverse Event reporting period

All adverse events that occur during the adverse event reporting period specified below must be reported to DNDi.

The adverse events reporting period for this trial will be as follows:

Non-serious adverse events: All AEs (related or unrelated) will be reported upon administration of the first dose of trial medication until the 6-months follow-up visit. Furthermore, AEs judged related to study procedures that occur during the screening period (from signature of the informed consent) will also be reported.

Non-serious AEs that occur between 6 months and 24 months visit that are judged as related to the study drug or study-related will be reported.

Serious adverse events: All SAEs must be reported upon subject enrolment in the trial (signature of the informed consent) until the end of the subject participation in the trial (including post-treatment follow-up period 24 Month).

For 24 months follow-up visit: for patients who have already completed the 12 months visit and consented for the 24 months follow-up visit, the complete history of signs and symptoms since the 12 months visit will be recorded, including medications that could affect the treatment outcome (*e.g.* immunomodulators), any other medications and adverse events that may have occurred during this period. Adverse events that fulfil the criteria of seriousness will be reported within 24hs of knowledge of the event, following the reporting requirements as described below and AEs assessed as related to the previous study treatment or study-related will be collected too.

8.6.7 Adverse Event reporting requirements

Information on adverse events must be evaluated by a physician. Each adverse event is to be classified by the investigator as serious or non-serious (based on the criteria mentioned in 8.7.3). This classification will determine the reporting procedure for the event to DNDi and Health Authorities/Ethics Committees (as per local regulatory requirements).

All serious adverse events (SAE) are to be reported immediately (within 24 hours of occurrence of SAE by the investigator) to DNDi (on SAEPKDLStudyAsia@dndi.org), regulatory authorities, and Institutional Ethics Committee, as per local regulations, using the SAE report form. This includes a description of the event, onset date and seriousness criteria, duration, severity, relationship to study drugs, outcome, measures taken and all other relevant clinical and laboratory data. The initial report is to be followed by submission of additional information (follow-up SAE form) as it becomes available within required timelines as per local regulations to regulatory authorities, to Ethics Committee where the trial has been conducted.

In the situation where an AESI fulfils the criteria of a serious AE, this information will be captured in the AESI form and reported to the DNDi clinical team as per instructions below. There is no need to complete an SAE form because the AESI form contains all necessary information pertaining to the SAE and additional AESI sections.

The PI and sponsor must ensure that all Sponsor reviewed SAEs (CIOMS report) are reported to the regulatory authorities and Institutional Ethics Committee within the allocated timeframe as per current regulatory guidelines, as described above.

Serious adverse events should also be reported on the clinical trial adverse event case report form (CRF). It should be noted that the form for reporting of SAE (SAE form) is not the same as the adverse event section of the CRF. Where the same data are collected, the two forms must be completed in a consistent manner, and the same medical terminology should be used.

All AESI are to be reported immediately (within 24 hours of awareness of the AESI by the investigator) to the DNDi clinical team (on SAEPKDLStudyAsia@dndi.org), using the AESI report form. This includes a description of the event, onset date and type, duration, severity, relationship to study drug, outcome, measures taken and all other relevant clinical and laboratory data. The initial report is to be followed by submission of additional information (follow-up AESI form) as it becomes available. Any follow-up reports should be submitted as soon as possible and if possible within 5 working days. AESI will be transmitted to the primary PV contact (and back-up) and the PV Team Leader via email.

If serious, these AESI reports will be transmitted by the clinical team to DNDi PV team (as any other SAE at pharmacovigilance@dndi.org). The Investigator should follow the regular process of reporting SAE to the local authorities and Ethics Committee.

AESI are also to be reported in the clinical trial adverse event case report form (CRF).

Non-serious AESIs **do not** need expedite submission to the Ethics Committee. These will be reported to the DSMB and in the regular periodic report, as per local regulations.

Non-serious adverse events are to be reported on the CRF, including description of the event, onset date, duration, severity, seriousness, relationship to study drugs, actions taken and outcome. In the CRF, a given adverse event will be recorded only one time per patient, and the severity recorded will be the maximum level reached. If several distinct episodes of the same condition occur, their number will be recorded in the CRF.

8.6.8 Grading of Adverse Event severity

Severity is a clinical determination of the intensity of an AE. The severity for an AE should be graded using the National Cancer Institute's Common Terminology Criteria for Adverse Events (CTCAE, version 4.03).

In case of AEs that are not described in the CTCAE v 4.03, the investigator will use the terminology MILD, MODERATE, SEVERE or LIFE-THREATENING to describe the maximum severity of the adverse event as follows:

- MILD (grade 1) The subject is aware of the event or symptom but the event or symptom is easily tolerated (i.e. no reduction in daily activities of the subject)
- MODERATE (grade 2) The subject experiences sufficient discomfort to interfere with or reduces his or her usual level of activity.

- SEVERE (grade 3) Significant impairment of functioning: the subject is unable to carry out usual activities and/or the subject's life is at risk from the event.
- LIFE-THREATENING (grade 4) The subject experiences immediate life-threatening consequences which requires urgent intervention.

This information on AE grading will be entered in the adverse event CRF.

It is to be noted the distinction between severity (intensity) and seriousness of adverse events. A severe adverse event is not necessarily a serious adverse event.

8.6.9 Adverse Event causality assessment

For each serious and non-serious adverse events, the investigator is required to assess if there is a causal relationship between the adverse event and the study drugs, i.e. to determine whether **there is at least a reasonable possibility** that one of both study drugs caused or contributed to the adverse event. This means that there are facts and arguments to suggest a causal relationship.

To help investigators with the decision binary tree in the evaluation of causality, the CIOMS VI group recommends that investigators be asked to consider the following before reaching a decision:

- Medical history
- Lack of efficacy/worsening of existing condition
- Study medications
- Other medications (concomitant or previous)
- Withdrawal of study medication, especially following trial discontinuation / end of study medication
- Erroneous treatment with study medication (or concomitant)
- Protocol related procedure

Using this information, the investigator will classify each adverse event as:

Not related:	There is no reasonable possibility of causal relationship between an AE and study drug
Related:	There is **<u>at least a reasonable possibility</u>** of a causal relationship between an AE and a study drug. This means that there are **facts (evidence) or arguments** to suggest a causal relationship.

The decision to suspend, and resume treatment or to permanently interrupt treatment due to an adverse event will be left to the clinician in charge.

8.6.10 Exposure in utero

<u>All pregnant women are excluded from the trial.</u> This is specifically due to the teratogenic potential of miltefosine (demonstrated in rats). The manufacturer and Sponsor recommend that effective contraception should be used during and up to 5 months after treatment of miltefosine.

If any trial subject becomes or is found to be pregnant within the initial 6 months of her participation in this trial the investigator must submit the event on the Pregnancy Report form. This must be done irrespective of whether an adverse event has occurred. The information submitted should include the anticipated date of delivery.

The investigator will follow the subject until completion of the pregnancy or until pregnancy termination (i.e., induced / spontaneous abortion). The investigator will provide pregnancy outcome information in the Pregnancy Outcome report form.

A pregnancy is not an SAE.

In the case of a live birth, a pediatrician should assess the infant at the time of birth and submit a Child report. An SAE should be declared in the case of unfavourable pregnancy outcome (abortion, still birth) or congenital abnormality. In case of *in utero* exposure, the parents will be proposed a follow-up of the new-born up to the age of 2 years old.

8.6.11 Adverse event follow up

All adverse events should be followed until they are resolved or the investigator assesses them as chronic or stable or the subject participation in the trial ends (i.e., until a final report is completed for that subject).

In addition, all serious adverse events and those non-serious events assessed by the investigator as possibly related to the investigational drug must continue to be followed even after the subject participation in the trial is over. Such events should be followed until they resolve or until the investigator assesses them as “chronic” or “stable.” Resolution of such events is to be documented on the CRF and SAE form.

9 Withdrawal criteria

A subject should be withdrawn from the study treatment if, in the opinion of the investigator, it is medically necessary, or if it is the wish of the subject.

If a subject does not return for a scheduled visit, every effort should be made to contact the subject. In any circumstance, every effort should be made to document subject outcome, if possible.

If the subject withdraws consent, no further evaluations should be performed and no attempts should be made to collect additional data, with the exception of safety data, which should be collected if possible and in accordance with patient consent. If a subject withdraws from the study, the reason must be noted on the CRF.

If a subject is withdrawn from the study because of a treatment limiting adverse event, thorough efforts should be made to clearly document the outcome of AE.

9.1 Rules in case of treatment suspension or interruption

In the event that treatment is interrupted (e.g. due to an adverse event), the decision to resume treatment will be taken by the site Principal Investigator. If miltefosine treatment is interrupted for more than 3 days and requires additional PKDL treatment (other than that described in the protocol) they will be considered a treatment failure for the purposes of the ITT analysis and excluded from the per protocol analysis. If interruption was for 3 or less days,

treatment will start again till the complete dose is administered.

Note that for the Ambisome® treatments, delay for >1 week of the administration of a dose which requires additional PKDL treatment (other than that described in the protocol), they will be considered a treatment failure for purposes of ITT analysis and excluded from the per protocol analysis. If interruption was for 6 or less days, treatment may start again at discretion of the investigator, until the complete dose is administered.

9.2 Rules for permanently interrupting study treatment

If a subject is withdrawn from the study before the full course of the treatment is completed, the physician must make all necessary arrangements to ensure that the subject receives the appropriate treatment for the relevant medical condition (e.g. with drug/s currently recommended by the national policy).

9.3 Subject withdrawal from the study and subject replacement

Subjects withdrawn from the study will not be replaced.

10 Data Analysis and Statistical Methods

10.1 Sample size determination

We estimated that a minimum sample size of 50 subjects per treatment arm would provide a precision estimate of 10% with 95% CI, based on an anticipated cure rate at 12 months in 85% of patients. Accounting for 10% subjects lost during follow up and 15% subjects with treatment discontinuation, 13 more subjects were added per arm resulting an increase of the sample size to 63 for each regimen. The overall sample size for the two regimens is 126 subjects.

The anticipated cure rate was selected based on the criteria set on the cure rates previously reported with the use of Miltefosine or Ambisome® alone.

A computer generated, randomization code will be used for patient treatment allocation to one of the two treatment arms indicated.

Eligible patients who fulfil all the inclusion criteria and have none of the exclusion criteria, and from whom informed consent has been obtained, will be randomized to one of the treatment regimens.

10.2 Summary of analyses

Descriptive statistics will be used to present study data. Continuous variables will be presented as number of observations (n), mean, standard deviation (SD), median, minimum and maximum values. Categorical variables will be presented as counts and percentages.

Median time to the development of any AE will be determined using life-table analysis. All data will be presented separately by treatment group, local and systemic AEs. AEs and medical history will be coded using the most recent version of the Medical Dictionary of Regulatory Activities (MedDRA) preferred terms and will be grouped by system, organ, and class (SOC) designation.

Concomitant medications will be coded using the most recent version of WHO drug Dictionary.

Summary statistics will be provided by group including the frequency, severity, seriousness and relationship of AEs to each study drug organized by preferred term by SOC grouping.

Efficacy endpoints will be presented as summary statistics by treatment group and will be conducted once all subjects complete their initial cure assessment visit and the 12-months visit. The number of subjects who met the criteria for cure divided by the total number of subjects in that group will be presented by study group, and the correspondent 95% confidence interval. In addition, secondary efficacy endpoints will be described for the 24-months follow-up visit.

Subjects who are lost to follow-up will be compared to those completing the study with respect to study site, demographic, and clinical factors. If lost to follow-up exceeds 20% of the sample, attempts will be made to seek out these dropouts (or their proxies) to ascertain final outcomes. The attributes of people that are found through this process will be compared to those not lost to follow up.

For the pharmacokinetic data analysis, a population-based approach will be used employing non-linear mixed effects modelling (using NONMEM), to take into account within- and between-subject variability in all pharmacokinetic parameters, to optimally identify potential covariates such as body size descriptors (age, lean body mass, total body weight, etc) and other demographic parameters, but also interaction effects such as treatment-arm related differences in pharmacokinetics parameter estimates. The objective function value (equals -2 log-likelihood, derived from NONMEM) will be used to assess statistical significance of evaluated covariates and hierarchical structural models. The individual blood plasma:skin distribution ratio will be estimated using the developed pharmacokinetic model. Individual blood plasma:skin ratios of drug concentrations will be contrasted with the IC90 values previously obtained for L. donovani parasites.

10.3 Definition of study populations included in the analysis

For the primary analysis, an intention to treat (mITT) and per protocol (PP) analysis will be performed.

- Modified ITT population (mITT): all randomized patients receiving at least one dose of treatment. In case of error of treatment allocation, the actual treatment received will be used in the analysis. Modified ITT set of patients will be used in the primary and secondary efficacy analysis and in safety analysis.
- Per protocol population (PP): subset of patients belonging to the modified ITT and who did not have major protocol violations. Major protocol violations are any violation that may bias the result of the study, which will be defined a priori in the statistical analysis plan.

To take account of missing data, each of the above analysis sets (mITT and PP), the efficacy endpoints will be calculated in two ways: worst case scenario (missing outcome is defined as failure) and complete case analysis (missing outcome is excluded from the analysis, population is defined as set of completers).

- Set of completers: subset of patients belonging to the mITT or PP, who attended the 12 months follow-up visit. This set will be used in a sensitivity analysis of the primary

efficacy analysis.

A sensitivity analysis will also be performed for the secondary efficacy endpoints with the subset of patients who attended the 12 and 24-months follow-up visits.

Missing outcome data on the efficacy endpoints could arise if a patient withdraws consent, is lost to follow-up, or has an exam omitted by investigator oversight in patients who are not confirmed as cured.

10.4 Subject Disposition

The number of patients who were screened, failed screening, randomized, completed the treatment period, completed the follow-up, early terminations and reasons for early termination of study will be summarized by number of patients (n) and percentages (%).

10.5 Baseline

The continuous variables of the baseline and demographic characteristics will be summarized using number of patients (n), mean, SD, median, minimum and maximum. The categorical variable gender will be summarized using number of patients (n) and percentage. (%).

10.6 Efficacy Analysis

Efficacy of the treatment will be assessed at 12 and at 24-month follow-up visit. The efficacy estimate will be reported as proportion of cured with its 95% confidence interval.

The data will be analysed as proportion, according to either mITT or PP populations.

Definitions

- **Cure**: Complete resolution of papular and nodular lesions (flattening of 100% of lesions) and significant improvement (> 80% re-pigmentation, as per scoring system, section 8.3.1) of macular lesions at 12 or at 24 months follow-up visits.
- **Treatment Failure:**

  A patient will be considered a treatment failure:

  - If rescue treatment is indicated by the investigator due to:
    - Non-response to treatment or worsening of PKDL lesions anytime during the treatment phase of the study
    - Occurrence of adverse events that leads to treatment discontinuation and require rescue treatment
    - Relapse: patient presented clinical response/improvement of lesions, but in subsequent follow-up visits the investigator observes appearance of new lesions and/or worsening of PKDL lesions, and indicates rescue treatment at any-time during follow-up period, including 24 months visit.

  Or

  - Patient did not fulfil the criteria for cure at the 12 or 24-months follow-up: presenting persistence of papular or nodular lesions and/or not significant improvement (< 80% re-pigmentation, as per section 8.3.1) of macular lesions.

- **Lost to Follow Up**: Patient did not turn up for 24 month follow up assessment and could not be contacted by health educators.

In addition, the overall clinical presentation of the patient will be assessed by the Investigator using the following categories [34]:

  - Category 1: All lesions have improved and the majority of lesions have resolved. Any remaining lesions are only faintly visible as re-pigmentation is almost complete.
  - Category 2: The majority of lesions show significant improvement, some lesions have resolved
  - Category 3: No new lesions, but the majority of lesions show no or slight improvement.
  - Category 4: New lesions have appeared and there is no or little improvement of other lesions

The proportion of PKDL patients in category 1, 2, 3 and 4 with its 95% confidence interval will be summarized per study arm, at 12 and at 24 months follow-up visits.

The proportion of PKDL patients in categories 1 and 2 who did not require rescue treatment up to and including 24 months visit will be summarized for all patients included in the study and per study arm.

The proportion of PKDL patients in categories 1 and 2 with negative parasitology by microscopy at 12 and 24 months will be summarized for all patients included in the study and per study arm

The proportion of PKDL patients in categories 1 and 2 with negative parasitology by qPCR at 12 and 24 months will be summarized for all patients included in the study and per study arm

10.7 Safety Analysis

All patients enrolled and who have been started on study medication will be included in the safety analysis (treatment-emergent AEs). The AE and medical history data will be coded using MedDRA dictionary.

AE's will be tabulated by System Organ Class (SOC), severity (based on the CTCAE v4.03 see section 8.7.7), seriousness and by causal relationship to study drugs.

The proportion of patients with SAE, AESI and/or AEs leading to treatment discontinuation will be described per System Organ Class (using preferred terms defined by MedDRA) and summarised according to relationship to treatment and severity (CTCAE, version 4.03). In addition, a narrative for each of the SAE, AESI and AE leading to treatment discontinuation will be developed describing all aspects of the medical event.

The proportion of patients presenting at least one treatment emergent AE (TEAE) will be presented. All TEAEs will be described per study arm using the same classification as presented above. AEs will be tabulated by severity (CTCAE V4.03) and causality.

Abnormalities of laboratory/procedures results will be tabulated with respect to clinical significance. In addition, shift tables and graphs will be generated to describe the laboratory parameter values over time, per treatment arm.

10.8 PK Analysis

For the pharmacokinetic data analysis, a population-based approach will be used employing non-linear mixed effects modelling (using NONMEM), to take into account within- and between-subject variability in all pharmacokinetic parameters, to optimally identify potential covariates such as body size descriptors (age, lean body mass, total body weight, etc) and other demographic parameters, but also interaction effects such as treatment-arm related differences in pharmacokinetics parameter estimates. Separate PK models will be developed for miltefosine in plasma and for total amphotericin B in plasma. The objective function value (equals -2 log-likelihood, derived from NONMEM) will be used to assess statistical significance of evaluated covariates and hierarchical structural models. The individual blood plasma:skin distribution ratio will be assessed based on the end of treatment samples. Exposure estimates in the skin that follow from the pharmacokinetic analysis will be compared to the IC90 values previously obtained for *L. donovani* parasites.

10.9 PD Analysis

Quantitative qPCR data of parasite numbers will be used to estimate the time till negativity in skin snip, blood and tape disc samples and perform a survival curve analysis. Appropriate covariates, such as respective drug exposures and drug plasma:skin penetration ratio, will be evaluated to estimate the influence of these covariates on the parasite clearance in patients. If the quantitative data allow for it, other regression methods will be used to estimate the dynamics of parasite clearance in patients, which will be detailed in a separate post-hoc analysis plan.

10.10 Immunological parameters Analysis

The cytokines IL-2, IFNY, TNFα, IL-10, and Granzyme B (and possibly other biomarkers, after a preliminary screening) are secreted by T- cells after stimulation and will be measured in the stimulated whole blood supernatant by BD™ Cytometric Bead Array Human Th1/Th2 Cytokine CBA.

Results for each cytokine will be expressed as the difference between the SLA stimulated concentration and the negative control plasma concentration. Levels of cytokines will be described over the different time-points at baseline, treatment and follow-up phases for the overall population, by treatment arm and by treatment outcome (cure vs failure at D210). Sub-group analysis for cytokines will be detailed in the Statistical Analysis Plan.

11 Data Safety Monitoring Board

A Data Safety Monitoring Board (DSMB), composed of at least 3 members independent of the investigator and sponsors, will be set up prior to study initiation. The DSMB will review the study data at pre-determined intervals and issue recommendations about the study. The data and intervals will be agreed prior to the study initiation and documented in the DSMB Charter.

12 Quality Assurance and Quality Control Procedures

12.1 Investigator's file

The investigator must maintain adequate and accurate records to enable the conduct of the study to be fully documented and the study data to be subsequently verified. These documents include Investigator's Site File, subject clinical source documents and screening / enrolment logs. The Investigator's Site File will contain the protocol/protocol amendments, CRF and query forms, IEC and regulatory approval with correspondence, sample informed consent, drug accountability records, staff curriculum vitae and authorization forms and other appropriate documents/correspondence etc.

12.2 Case report forms (CRFs)

Data will be collected by laboratory technicians, medical doctors, clinical officers and nurses authorized by the investigator. It will be supervised by the Investigator and signed by the investigator or by an authorised staff member. Study-specific information will be entered into the Case Report Form (CRF). Data that are derived should be consistent with the source documents or the discrepancies should be explained. All CRF data should be anonymised, i.e. identified by study patient number only.

The investigator at each trial site should ensure the accuracy, completeness, legibility, and timelines of all data reported to the sponsor in the CRFs and any other additional information that is required. The investigator is responsible for keeping all consent forms, screening forms, CRF and the completed subject identification code list in a secure location.

In case of electronic data capture (EDC) software used in the study; all requested information must be entered on the eCRFs in the areas provided in a timely manner. When changes or corrections are made in the eCRF, the EDC system will maintain a complete audit trail of the person making the changes, the date and time of the change and the reason for the change. Only individuals listed on the Delegation of Responsibilities Log with responsibility for eCRF completion may make entries on the eCRFs. Usernames and passwords will be provided to each authorized user to allow access to the training module. Access to additional features and functions will not be enabled until the user has successfully completed the training.

The investigator or physician sub-investigator (delegated by Investigator) must electronically sign and date each subject's eCRF. Individuals who will be providing electronic signatures must first submit documentation with a handwritten signature acknowledging that their electronic signature is a legally binding equivalent to their handwritten signature.

12.3 Source documents

The verification of the CRF data must be by direct inspection of source documents. Source documents include subject hospital/clinic records, physician's and nurse's notes, appointment book, original laboratory reports, ECG, EEG, X-ray, pathology and special assessment reports, signed informed consent forms, consultant letters, and subject screening and enrolment logs.

The investigator must maintain source documents such as laboratory and consultation reports, history and physical examination reports, etc., for possible review and/or audit by DNDi and/or Regulatory Authorities. The Investigator / designee will record the date of each subject's visit together with a summary of their status and progress in the study.

12.4 Record Retention

The investigator must keep all study documents on file for at least 25 years after completion or discontinuation of the study. After that period of time the documents may be destroyed with prior permission from DNDi, subject to local regulations.

Should the investigator wish to assign the study records to another party or move them to another location, DNDi must be notified in advance.

12.5 Monitoring, audits and inspections

Monitoring visits to the trial site will be made periodically by DNDi representatives or designated clinical monitors to ensure that GCPs and all aspects of the protocol are followed. Source documents and SAE forms/Data clarification forms (DCFs; sent to DNDi pharmacovigilance) will be reviewed for verification of consistency with data on CRFs.

The investigator will ensure direct access to source documents by DNDi or designated representatives. It is important that the investigators and their relevant personnel are available during the monitoring visits.

The investigators will permit representatives of DNDi and/or designated clinical monitors to inspect all CRFs/SAE forms/DCFs, medical records, laboratory work sheets and to assess the status of drug storage, dispensing and retrieval at anytime during the study. The corresponding source documents for each subject will be made available provided that subject confidentiality is maintained in accord with local regulations. The inspections are for the purpose of verifying the adherence to the protocol and to ensure the study is conducted according to GCP. It is important that the investigators and other trial site staff are available at these visits.

The monitoring visits provide DNDi with the opportunity to evaluate the progress of the study, verify the accuracy and completeness of CRFs/SAE/AESI forms/DCFs, resolve any inconsistencies in the study records, as well as to ensure that all protocol requirements, applicable regulations, and investigator's obligations are being fulfilled. Four visit types are planned: pre-study, study start, during the study, and study end. Visits may also be performed by regulatory authorities.

It will be the clinical monitor’s responsibility to inspect the CRF/SAE/AESI forms/DCFs at regular intervals throughout the study, to verify the adherence to the protocol and the completeness, consistency and accuracy of the data being entered on them. The investigator agrees to cooperate with the clinical monitor to ensure that any problems detected in the course of these monitoring visits are resolved.

12.6 Audits and inspections

The trial site may also be subject to quality assurance audits by DNDi or designated representatives and/or to inspection by regulatory authorities or Institutional Ethics Committees (IEC). It is important that the investigators and their relevant personnel are available for possible audits or inspections.

12.7 Data Management

Data management team (CRO) will be responsible for the activities associated with the data

management of this study, including the production of CRF/eCRF, setting up a relevant database, along with appropriate validation of data and resolution of queries. The trial data will be stored in a computer database maintaining confidentiality in accordance with national data legislation. In order to ensure data quality, a uniform e-CRF will be designed for use at all the sites. Data will then be checked by CRO for data cleaning. The monitor and data manager will be responsible for checking the completeness and the quality of the data and clarifying any inconsistent information with the site. The data manager will be responsible for sending the data for periodic analysis.

12.8 Confidentiality of trial documents and subject's records

The investigator must assure that subjects' anonymity will be maintained and that their identities are protected from unauthorized parties. On CRFs or other documents submitted to the sponsor, subjects should not be identified by their names, but exclusively by an identification code. The investigator should keep a subject enrolment list showing codes, names, and addresses. The investigator should maintain documents for submission to sponsor authorized representative, and subject's signed written consent forms, in strict confidence.

13 Protocol Amendments

The Principal investigator will ensure that the study protocol is strictly adhered to throughout, and that all data are collected and recorded correctly on the CRF. The Principal investigator may contact the DNDi study coordinator for a protocol waiver for minor deviations from the protocol, e.g. patient unable to attend during visit window.

All protocol modifications must be documented in writing. Any protocol amendment must be approved and signed by the sponsor and the Principal investigator and is to be submitted to the appropriate IEC for information and approval in accordance with local requirements, and to regulatory agencies if required. Approval by IEC (and Regulatory Authority, if applicable) must be awaited before any changes can be implemented, except for changes necessary to eliminate an immediate hazard to trial subjects, or when the change involves only logistical or administrative aspects of the trial [e.g. change in clinical monitor[s], change of telephone number[s].

The protocol amendment can be initiated by either sponsor or by any Principal investigator. The investigator will provide in writing the reasons for the proposed amendment and will discuss with the medical coordinator and sponsor.

14 Early Termination of the Study

Both the sponsor and the investigator reserve the right to terminate the study at any time prior to inclusion of the intended number of subjects, but they intend to exercise this right only for valid scientific or administrative reasons. Should this be necessary, both parties will arrange the procedures on an individual study basis after review and consultation. In terminating the study, the sponsor and the investigator will assure that adequate consideration is given to the protection of the subject's interest.

Reasons for early termination by the sponsor(s) may include but not be limited to:

- Too low enrolment rate
- Protocol violations
- Inaccurate or incomplete data
- Unsafe or unethical practices
- Questionable safety of the test article
- Suspected lack of efficacy of the test article
- Following the recommendation of IEC or regulatory authority
- Administrative decision

Reasons for early termination by the investigator may be:

- Insufficient time or resource to conduct the study
- Lack of eligible patients

In the event that a study is early terminated either by the sponsor or by the investigator, the investigator has to:

- Complete all CRFs to the greater extent possible
- Return all test articles and related study materials to the sponsor who provided them
- Answer all questions of the sponsors or their representatives related to data of subjects enrolled at the site prior to study termination
- Ensure that subjects enrolled in the study who had not yet reached a follow up time point are followed up with the necessary medical care.
- Provide in writing the reasons for his decision to the national health authority and the sponsor.

15 Ethics

The protocol for this study has been designed in accordance with the general ethical principles outlined in the Declaration of Helsinki and ICH guidelines for Good Clinical Practice (International Council for Harmonization). DNDi assures that it will comply with all applicable state, local and national laws for protecting the rights and welfare of human subjects. This protocol and any protocol amendments will be reviewed / approved by an IEC before its implementation.

Written approval from all involved IECs must be obtained before implementation of any protocol-specified intervention /investigation provided to the subject [such as subject information sheets or descriptions of the study].

Any modifications made to the protocol after receipt of the IEC approval must also be submitted by the investigator in writing to the IEC in accordance with local procedures and regulatory requirements.

15.1 Informed consent process

Inclusion in the study will occur only if the subject (for adults) or the parent/guardian (for children) gives written informed consent. It is the responsibility of the investigator / designee to obtain written informed consent from each individual participating in this study, after adequate presentation of aims, methods, anticipated benefits, and potential hazards of the study. The written informed consent document will be translated into the local language or a language understood by the subject(s). If needed, the person will be given time to discuss the information received with members of the community or family before deciding to consent. The subject or parent/guardian will be asked to provide written and signed consent.

If the subject is illiterate or unable to write, a literate witness must sign (this person should have no connection to the research team and the sponsor, and, if possible, should be selected by the participant). The investigator should also obtain the assent of children (if appropriate), but their assent must be completed by the permission of a parent or guardian.

An additional follow-up visit will be performed at 24 months. Patients will be re-contacted and an addendum to the ICF will be used for patients to voluntarily consent to attend the 24 months visit, explaining the reasons why this visit is important and procedures that will need to be performed.

If new safety information results in significant changes in the risk/benefit assessment, the consent form should be reviewed and updated if necessary. All subjects (including those already being treated) should be informed of the new information, given a copy of the revised form and give their consent to continue in the study.

15.2 Ethical aspects of subject inclusion and study procedures

Combination treatment is well accepted in the treatment of VL, paromomycin (PM) combined with miltefosine in Asia; or SSG combined with PM for example in East Africa, and may offer shorter duration of treatment thus preventing prolonged hospitalization. Another possible advantage is prevention of development of resistance and reduced cost. These principles also apply to PKDL, where the need for an ambulatory treatment with a highly safe, efficacious and user-friendly regimen is even more pressing as patients are not ill, except for the rash.

Patients will experience some pain during skin snip smear procedure for parasitology and while blood is drawn during venipuncture. Local anaesthetic according to the routine practice for the management of patients will be used for skin smear procedure.

15.3 Ethical aspects of study treatments

Ambisome® is given as first line of treatment for Visceral Leishmaniasis by National Program of India and Bangladesh. Both drugs marketed for Visceral Leishmaniasis indication and has been extensively used as primary treatment for VL in Asia, with very good safety profile.

15.4 HIV status and VCT

All patients will be offered counselling and screening for HIV (voluntary counselling and testing programme (VCT). This may either be done at the same time as consent is obtained for inclusion in the trial.

Patients who are found to be HIV positive will not be eligible to participate in this trial but will

receive appropriate treatment, according to national treatment guidelines. Additionally, they will be referred onwards for treatment, surveillance and follow up according to the national protocol for HIV positive patients.

15.5 Patient costs

Patients will be reimbursed for travel to and from the study site but will not receive any payment for trial participation. Any medication that is required during the trial period will be provided free of charge to the patient. Food during the in-patient treatment phase will also be provided free of charge to the patient. This is seen as an essential part of the patient care plan.

16 Insurance and Liability

DNDi is insured to indemnify the investigator against any claim for damages brought by a research subject who suffers from a research related injury during the performance of the trial according to the protocol.

17 Reporting and publication

All clinical trials will be registered with a recognised clinical trial registry such as www.clinicaltrials.gov.

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