Prediction of visceral leishmaniasis development in a highly exposed HIV cohort in Ethiopia based on *Leishmania* infection markers: results from the PreLeisH study

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Summary

Background Targeted preventive strategies in persons living with HIV (PLWH) require markers to predict visceral leishmaniasis (VL). We conducted a longitudinal study in a HIV-cohort in VL-endemic North-West Ethiopia to 1) describe the pattern of *Leishmania* markers preceding VL; 2) identify *Leishmania* markers predictive of VL; 3) develop a clinical management algorithm according to predicted VL risk levels.

Methods The PreLeisH study followed 490 adult PLWH free of VL at enrolment for up to two years (2017–2021). Blood RT-PCR targeting *Leishmania* kDNA, *Leishmania* serology and *Leishmania* urine antigen test (KAtex) were performed every 3–6 months. We calculated the sensitivity/specificity of the *Leishmania* markers for predicting VL and developed an algorithm for distinct clinical management strategies, with VL risk categories defined based on VL history, CD4 count and *Leishmania* markers (rK39 RDT & RT-PCR).

Findings At enrolment, 485 (99%) study participants were on antiretroviral treatment; 360/490 (73.5%) were male; the median baseline CD4 count was 392 (IQR 259–586) cells/μL; 135 (27.5%) had previous VL. Incident VL was diagnosed in 34 (6.9%), with 32 (94%) displaying positive *Leishmania* markers before VL. In those without VL history, baseline rK39 RDT had 60% sensitivity and 84% specificity to predict VL; in patients with previous VL, RT-PCR had 71% sensitivity and 95% specificity. The algorithm defined 442 (92.3%) individuals at low VL risk (routine follow-up), 31 (6.5%) as moderate risk (secondary prophylaxis) and six (1.2%) as high risk (early treatment).

Interpretation *Leishmania* infection markers can predict VL risk in PLWH. Interventional studies targeting those at high risk are needed.

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Keywords: Visceral leishmaniasis; Kala-azar; HIV; Prediction; Asymptomatic infection

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Research in context

Evidence before this study

HIV-visceral leishmaniasis (VL) coinfection is most prevalent in North-West Ethiopia, where up to 20–30% of patients with VL are coinfected with HIV. In East-Africa, VL is caused by *Leishmania donovani*. Given the poor prognosis in persons living with HIV (PLWH) once *Leishmania* infection has evolved to VL, tackling *L. donovani* infection before disease onset would be preferable. As overt VL is thought to be preceded by a period of asymptomatic infection, detectable with different markers of *Leishmania* infection, this period constitutes a window of opportunity for screening strategies integrating markers of *Leishmania* infection to capture those at high risk of VL. To inform such a strategy, studies are needed that describe the pattern of *Leishmania* markers preceding VL and identify which *Leishmania* markers are predictive of VL. Based on this, clinical management algorithms should then be developed, to predict VL risk levels and target PLWH to distinct management strategies. To summarize the available evidence on this topic prior to this study (before January 1, 2017), we conducted a Pubmed search (February 14, 2024). Search terms included "visceral leishmaniasis", "kala-azar", "primary", "relapse", "HIV", "*Leishmania*", "(bio)markers", "prediction", "risk", "serological test", "molecular tests", "urine antigen", "PCR". There were no language restrictions. The asymptomatic phase has been studied to some extent in immunocompetent individuals across the globe, mainly from an epidemiological perspective, but detailed clinical studies in PLWH are scarce. Several of these studies indicated that quantitative PCR and perhaps also *Leishmania* urine antigen tests or *Leishmania* culture could be useful to predict VL relapse. No single study aimed at predicting primary VL in PLWH and none used a comprehensive panel of *Leishmania* markers to study the asymptomatic phase prior to VL development in PLWH, including both individuals with or without a history of VL. None developed a comprehensive clinical algorithm to guide clinical management.

Added value of this study

We conducted a longitudinal study (the PreLeisH study) in a large HIV cohort in a VL-endemic region in North-West Ethiopia to 1) describe the pattern of *Leishmania* markers prior to VL onset; 2) identify *Leishmania* markers that can predict VL and 3) develop a clinical management algorithm according to predicted VL risk levels. A total of 490 individuals enrolled in HIV care in a VL endemic area in North-West Ethiopia were included, including 135 with a history of VL. Overall, 34 developed a VL episode during follow-up. The vast majority (32/34 (94%)) displayed positive *Leishmania* markers at (several) visits during the months prior to the development of VL, indicating that progression of VL from the asymptomatic stage takes several months. In those with a history of VL, a positive blood reverse transcriptase (RT) PCR test was typically the first signal of imminent VL, followed by a positive *Leishmania* urine antigen test. In those without history of VL, no uniform pattern of *Leishmania* markers could be seen prior to VL. Baseline *Leishmania* markers had the highest predictive value, detecting 29 out of the 32 with detectable *Leishmania* markers before VL. Monitoring *Leishmania* markers during follow-up yielded only limited benefit, identifying three additional incident cases with VL. Overall, testing at baseline with the rK39 rapid test/rK39 ELISA/RT-PCR for those without VL history, and with RT-PCR & the KAtex *Leishmania* urine antigen test for those with a VL history would detect 29/34 incident cases with VL (sensitivity of 85.3%), with a specificity of 76.2% and a number needed to test of 15. As such a strategy using four different *Leishmania* tests would be costly and not practical to apply in practice, we developed more simplified predictive algorithms for use in routine care. Three clinically relevant risk categories (low, moderate, high) were defined, based on differential clinical management. Study participants were separated in risk groups, based on VL history and CD4 count, with targeted testing with *Leishmania* markers (rK39 RDT & RT-PCR). Such an algorithm would restrict laboratory marker testing to 97 individuals (20%; 13% with rK39 rapid diagnostic test and 7% with RT-PCR), and would direct individuals to distinct clinical management strategies, according to their predicted risk of VL. The predicted VL risk was 2.0% in the low risk category, 45% in the moderate risk category and 100% in the high risk category.

Implications of all the available evidence

Our study shows that the vast majority of PLWH in Ethiopia display positive *Leishmania* markers at (several) visits during the months prior to the development of VL, indicating that progression of VL from the asymptomatic stage takes several months, and can be picked up during routine HIV clinical visits. This is in line with small, older studies from Europe, studying one or few *Leishmania* markers to predict VL relapse. This period provides a window of opportunity for interventions to prevent VL in those at highest risk that could be economically identified by applying a simplified algorithm combing clinical parameters for pre-selecting those to be subsequently tested on *Leishmania* infection markers. Besides validation of the proposed algorithm, intervention studies are now needed to assess the efficacy of preventive interventions in those at high risk of VL. Immunological studies are needed to better understand the host–pathogen interaction and the pathophysiological processes predicting VL, to define the factors determining immune control or progression to disease (VL) after *Leishmania* infection.

Introduction

Visceral leishmaniasis (VL) is a vector-borne disseminated protozoan infection transmitted by sandflies and caused by the *Leishmania donovani* complex.[1] Globally, around 30,000 cases occur each year.[2] In Latin America and the Mediterranean region, VL is caused by *L infantum;* transmission is zoonotic, with dogs as main reservoir. In the Indian subcontinent and East-Africa, predominantly in Sudan, South Sudan, Kenya and Ethiopia, VL is caused by *L. donovani* and transmission is primarily anthroponotic.[1] Clinical manifestations of VL include chronic fever, hepatosplenomegaly and pancytopenia. Whereas most infections remain asymptomatic, overt VL is lethal without treatment.

HIV is one of the major challenges for VL control. HIV-VL coinfection is now most prevalent in North-West Ethiopia, where up to 20–30% of patients with VL are coinfected with HIV.[3] This region attracts high numbers of seasonal migrant labourers during the harvest season, most of them originating from the (VL-non-endemic) highlands.[3,4] Persons living with HIV (PLWH) have a much higher risk of progression to VL after *Leishmania* infection, and face a poor prognosis once VL develops.[5] Many patients with VL/HIV coinfection (up to 50%) fail to clear parasites from infected tissues and/or suffer from recurrent relapses.[3,6–9] Mortality is multifold higher, reaching 14% in some studies.[3,9]

Given this poor prognosis in PLWH once *Leishmania* infection has evolved to VL, tackling *L. donovani* infection before disease onset—during the asymptomatic phase–would be preferable. There are several successful evidence-based examples of World Health Organisation (WHO) recommended preventive strategies for other opportunistic infections.[10,11] This includes a screen and treat strategy recommended for cryptococcal infection, with pre-emptive treatment using fluconazole given to asymptomatic individuals screening positive for early cryptococcal infection. Similarly, isoniazid prophylactic therapy is used to prevent tuberculosis in those with latent infection.[10,11]

As overt VL is usually preceded by a prolonged period of asymptomatic infection, detectable with different markers of *Leishmania* infection,[12,13] this period constitutes a window of opportunity for screening strategies integrating markers of *Leishmania* infection to capture and manage those at high risk of VL. Such a strategy could be useful not only to predict the onset of a first VL episode, but also to predict subsequent VL relapse, as a history of VL is one of the strongest risk factors for VL.[8]

We conducted a longitudinal study (the PreLeisH study) in a large HIV patient cohort in a VL-endemic region in North-West Ethiopia to describe the pattern of *Leishmania* markers prior to VL onset and to identify *Leishmania* markers that can predict VL. Based on this information, we developed a clinical case management algorithm to direct individuals to distinct management strategies, according to the predicted VL risk level.

Methods

Study setting

This study builds on a scientific collaboration between the University of Gondar, Médecins sans Frontières (MSF)-Amsterdam and the Institute of Tropical Medicine, Antwerp (ITM-A) that started in 2010. MSF is supporting VL care delivery at the Abdurafi health centre, located in one of the main VL-endemic areas in Ethiopia bordering the Tigray region, where war erupted in November 2020. At the University of Gondar, the study was conducted at the Leishmaniasis Research and Training Centre (LRTC).

For this study, patients were recruited at the antiretroviral treatment (ART) clinic in the Abdurafi health centre, where basic study-related laboratory analyses were performed. After sample shipment, LRTC conducted the specialized laboratory analyses and provided support and training for the conduct of the study. The ITM-A Clinical Trials Unit provided support in the set-up, implementation, monitoring of the study, and statistical analysis. The ITM-A Clinical Reference Laboratory provided support for all laboratory procedures, including the set-up of the assays, establishment of the laboratory analytical plan, quality control and training on site.

Study design, population and recruitment

The PreLeisH study–registered on ClinicalTrials.gov (identifier NCT03013673)[14]–is a prospective cohort study implemented at the Abdurafi health centre between October 2017 and May 2021. All adult (>18 years old) PLWH enrolled in the Abdurafi HIV care program and not intending to move out of the region within the near future were screened for inclusion in the study. Those found with VL at baseline were not eligible, and individuals lacking *Leishmania* testing at baseline or without any further follow-up were excluded during analysis. Whereas the majority of PLWH in the Abdurafi HIV program are female, the vast majority of cases with VL occur in men.[3] For this reason, we decided to aim for a male/female balance of 70–80%/20–30% in the included study population (see Supplementary Information), to have a higher number of males included—as most at risk of VL and hence expected to contribute most study outcome events—while also ensuring that some data would be collected on females, as they could possibly also benefit from a VL prediction strategy. The study was stopped in May 2021, as the follow-up of study participants had become impossible due to the military conflict in the study area (see Supplementary Information).

Study procedures

Study visits were planned along with the routine HIV care visits, which depended on patient characteristics and ranged between every three to six months. Unplanned study visits were also recorded for any patients presenting at the health centre with VL symptoms in

between study visits to check for incident VL. Patients were followed-up for two years or until study closure.

At each visit, a full clinical examination and anamnesis was done. Information was collected related to ART, adherence, prophylaxis of opportunistic infections), concurrent conditions and medication prescribed. ART adherence was assessed by self-reporting the number of missed doses over the previous month and categorized as 0, 1–2, 3–9 and >9 missed doses. At each visit, blood, stool and urine were collected. Study participants were categorized as stable residents of the study area if they had been living there for at least a year and further categorized as native or non-native, depending on whether they had been living there since birth or not. Temporary residents were those who had moved to the region only recently.

Patients were evaluated for VL at each study visit and, in case of clinical suspicion, referred for diagnostic work-up according to the national guidelines.[15] VL diagnosis was based on microscopy of spleen or bone marrow aspiration, and grading of the parasite load was done as reported before.[16] Exceptionally, *e.g.* in case of contra-indications for tissue aspiration, VL diagnosis could also be based on clinical suspicion and the rK39 rapid diagnostic test (RDT) results. Treatment consisted of one or two full courses of AmBisome 30 mg/kg (6 × 5 mg/kg IV) combined with miltefosine 100–150 mg/day for 28 days.[17]

Study-related information was collected using a clinical and a laboratory case report form. Data were collected electronically using the GCP compliant software MACRO. Data review was done throughout the study and before database lock in June 2022.

Laboratory assays and quality control

The following tests were done systematically at each study visit: complete blood count (CBC) on the Humacount TS30 analyzer (Human, Wiesbaden, Germany), stool wet mount microscopy, malaria RDT (SD Bioline, Pan/Pf RDT, Standard Diagnostics, Ingbert, Germany; ref nr. SD-05FK60), *Leishmania* serological tests (direct agglutination test (DAT, Institute of Tropical Medicine, Antwerp, Belgium), rK39 RDT (IT-LEISH, Biorad, USA; ref nr. 710124), rK39 ELISA (Serion Leishmania IgG ELISA, Serion Diagnostics, Wurzburg, Germany; ref nr. VS-ESR147G), a *Leishmania* urine antigen test (KAtex, Kalon Biological Ltd, Guilford England UK; ref nr. L3-040)[18] and real-time PCR (RT-PCR) targeting kinetoplast DNA (kDNA) on whole blood as described before.[19] Briefly, DNA was extracted from 300 µL whole blood using the Maxwell 16 LEV Blood DNA extraction kit (Promega, Leiden, The Netherlands; ref nr. AS1290) with the automated Maxwell 16 Instrument (AS1000, Promega), including a negative extraction control (NEC) for every 15 samples. The kDNA PCR was run on a Rotor-Gene Q instrument (Qiagen, Venlo, The Netherlands), including two positive (*L. donovani* DNA) and negative PCR controls and the NECs in duplicate. CD4 count (BD FACSCount Cell Analyzer, BD Biosciences, USA), and HIV-1 viral load (Cobas® 4800 system, Roche Molecular Systems, Switzerland) were done every six months.

CD4 count, CBC, stool microscopy, malaria RDT, rK39 RDT and microscopy of tissue aspirates were performed onsite at the Abdurafi health centre. Samples for DAT, rK39 ELISA, KAtex and RT-PCR were transported in cold chain boxes with temperature monitoring to LRTC in Gondar for further analysis in batch. All tests were conducted following test-specific standard operating procedures (SOPs) developed in accordance to the manufacturer's guidelines. Interpretation of test results was done as following: rK39 ELISA: values < 10 U/mL were negative, 10–15 U/mL were borderline positive, and ≥15 U/mL were positive, a DAT titer ≥1/1600 was used to define positive tests, the KAtex agglutination reaction was scored as 1+: weakly positive; 2+: moderately positive; 3+: strongly positive. RT-PCR results were expressed in cycle threshold (Ct) values. For all samples with Ct values above 35, a repeated extraction and RT-PCR was done. If the repeat test was positive again, the Ct of the first run was used and the sample was called positive. In case this result was not confirmed in the second run, the sample was called weakly positive and the Ct value of the first run was used for the analyses.

Statistical analysis

Continuous variables were summarized using medians (interquartile range (IQR)). Categorical variables were summarized using frequencies and proportions. For each *Leishmania* marker, we calculated the sensitivity, specificity, the area under the receiver operating curve (AUROC) for predicting the occurrence of subsequent VL, the positive and negative likelihood ratios, the diagnostic odds ratio and the number needed to test (NNT) to predict one case of VL.

The strategy to construct clinical management algorithms that could be applied in routine practice to predict VL is explained in the results section, as the development was based on the data.

Ethics

The study was approved by the institutional review board of the ITM-A (1091/16) and the ethics review committee of the University of Antwerp (Belgium; 16/24/253), the University of Gondar (Ethiopia; V/P/RCS/05/708/2017), MSF (1632), the Amhara Regional Health Bureau (Ethiopia; H/D/T/Sh/1/449/09) and the national research ethics review committee in Ethiopia (310/169/2016). The study was conducted in accordance with the protocol (see Study Protocol). Written informed consent was obtained from all study participants.

Role of funders

The funders had no role in study design, in the collection, analysis and interpretation of data, in the writing of the report and in the decision to submit the paper for publication.

Results

Baseline characteristics

Of the 571 PLWH enrolled in the study, 490 were retained in analysis (Fig. 1, Table S1 and Supplementary Information). The vast majority (360, 73.5%) were male, and the median age was 38 years (IQR 32–45) (Table 1). The majority were daily labourers (44.5%) or farmers (21.8%). Around 25% had moved to the study area from elsewhere.

Almost all (n = 485, 99%) were on ART at enrolment, for a median of 5.6 (IQR 2.3–8.9) years. Five individuals

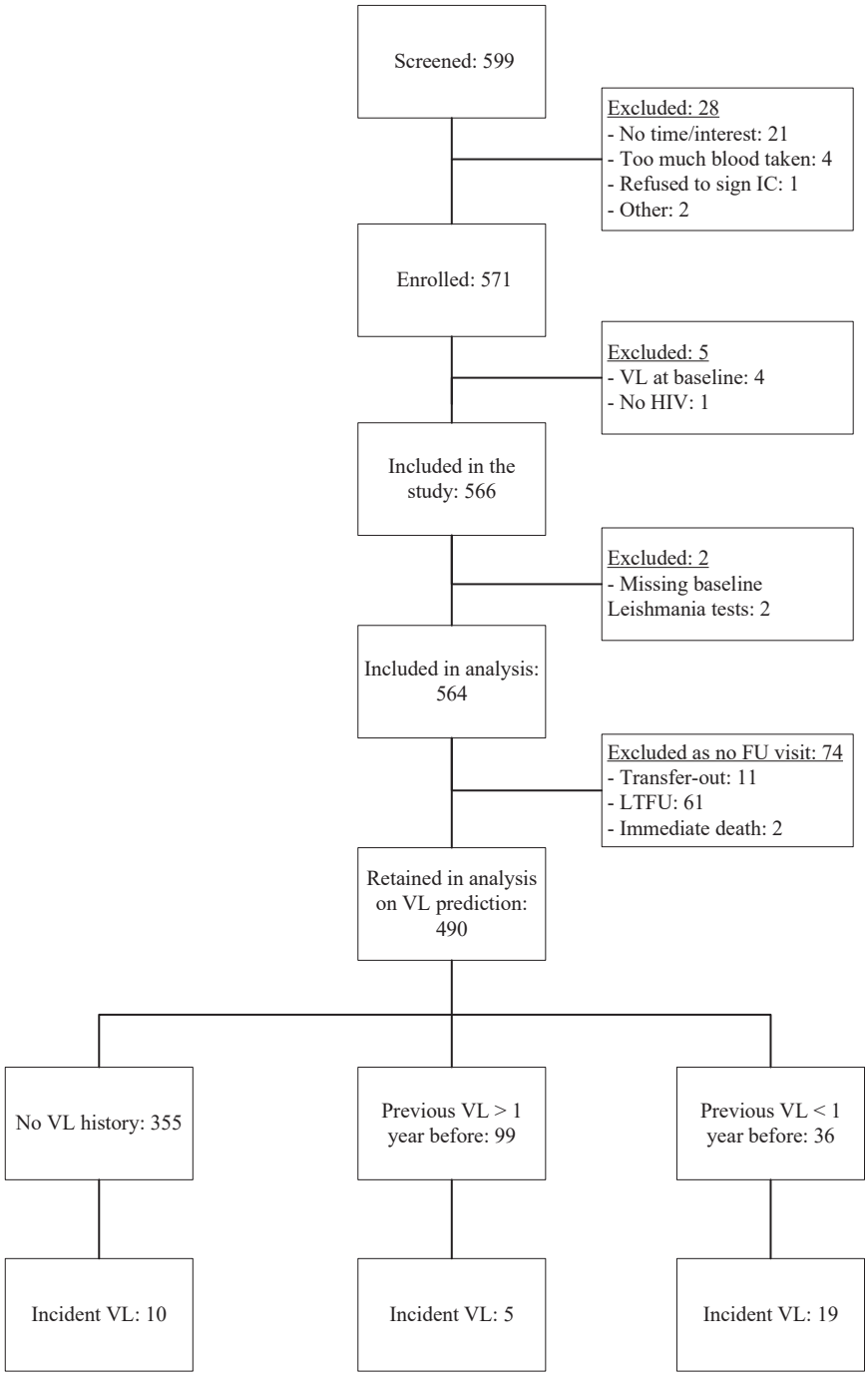

Fig. 1: **Flow chart describing the number of persons living with HIV screened and retained in analysis in the PreLeisH study, North–Ethiopia (2017–2021).** VL: visceral leishmaniasis; LTFU: lost to follow-up; IC: informed consent. Past VL: VL episode >1 year before enrolment. Recent VL: VL episode ≤ 1 year before enrolment.

	Total	No VL history	Past VL	Recent VL
Total	490	355	99	36
Male sex	360 (73.5)	228 (64.2)	96 (97.0)	36 (100)
Median age, years (IQR)	38 (32–45)	38 (32–45)	40 (35–46)	35 (30–40)
Daily labourer/farmer	325 (66.3)	213 (60.1)	81 (81.7)	31 (86.1)
Residence in study area				
Stable residents				
Native[b]	368 (75.1)	273 (76.9)	79 (79.8)	16 (44.4)
Non-native residents	101 (20.6)	68 (19.1)	19 (19.2)	14 (38.9)
Temporary residents	21 (4.3)	14 (3.9)	1 (1.0)	6 (16.7)
Years since HIV diagnosis, median (IQR)	6.8 (2.7–9.5)	6.2 (2.2–9.4)	8.6 (6.4–10.5)	3.7 (0.9–7.3)
On ART at enrolment	485 (98.9)	352 (99.1)	98 (99.0)	35 (97.2)
Years on ART, median (IQR)	5.6 (2.3–8.9)	5.0 (1.7–8.5)	7.9 (4.8–9.8)	3.3 (0.6–5.9)
Perfect ART adherence over last month (n = 477)	443 (92.9)	322 (93.3)	88 (90.7)	33 (94.3)
Median CD4 count, cells/μl (IQR) (n = 479)	392 (259–586)	417 (284–630)	391 (269–493)	218 (90–313)
HIV viral load (n = 408)				
Undetectable	254 (62.2)	192 (63.8)	54 (64.3)	8 (34.8)
<1000	84 (20.6)	64 (21.3)	14 (16.7)	6 (26.1)
1000–100.000	49 (12.0)	34 (11.3)	12 (14.3)	3 (13.0)
>100.000	21 (5.1)	11 (3.6)	4 (4.8)	6 (26.1)
Number of previous VL episodes, range	0 (0–1)	NA	1 (1–1) (1–5)	2 (1–3) (1–12)
BMI (kg/m^2), median (IQR)	18.7 (17.2–20.4)	19.0 (17.3–20.6)	18.2 (16.7–19.5)	18.0 (16.6–19.3)
Splenomegaly	72 (14.7)	37 (10.4)	20 (20.2)	15 (41.7)
Hemoglobine (g/dL), median (IQR) (n = 486)	13.6 (12.1–14.8)	13.7 (12.5–14.9)	13.8 (12.0–15.0)	10.1 (8.8–12.5)
White blood cells/μL, median (IQR) (n = 483)	4.4 (3.3–5.8)	4.6 (3.6–5.9)	4.0 (2.9–5.1)	3.2 (2.2–5.0)
Platelets/μL, median (IQR) (n = 484)	216 (161–270)	227 (169–276)	183 (145–225)	183 (130–233)

ART: antiretroviral treatment; IQR: interquartile range; VL: visceral leishmaniasis; BMI: body mass index; Past VL (previous VL episode >1 year before enrolment); Recent VL (previous VL episode ≤1 year before enrolment); NA: not applicable. [a]Data are n (%) unless otherwise indicated. [b]Born in study area.

Table 1: **Key characteristics of persons living with HIV stratified by VL history, North-Ethiopia (2017–2021).**[a]

were not on ART at study enrolment, including one with a history of recent VL and one with past VL. The median CD4 count at baseline was 392 (IQR 259–586) and 77 (16.1%) had a baseline CD4 count below 200 cells/μL. Of 408 with available viral load results, 254 (62.1%) had an undetectable viral load. The median time on ART for those with a detectable viral load was 5.5 years (IQR 2.5–8.6). Women were less likely to be daily labourers or farmers, temporary residents, have a history of VL and had higher CD4 cell counts at baseline (Table S2).

Overall, 355 (72.5%) were never diagnosed with VL before (no VL history), 99 (20.2%) had experienced VL more than a year before enrolment (past VL) and 36 (7.3%) within one year before enrolment (recent VL). Baseline characteristics stratified by VL history are displayed in Table 1. Those with recent VL had the lowest baseline CD4 count (median of 218 cells/μL) and the highest proportion (65%) with a detectable HIV viral load at baseline.

Leishmania markers at baseline

At enrolment (baseline), serological markers were positive in up to 20.7% of those without VL history, up to 66.7% of those with past VL and in up to 94.4% of those with recent VL (Table 2). RT-PCR positivity in blood ranged from 4.0% in those without VL history to 44.4% in those with recent VL. All *Leishmania* markers were clearly more often positive in males compared to females (Table S2). The 23.9% overall positivity rate of *Leishmania* markers in those without VL history suggests a substantial exposure of the study population to *Leishmania*.

Incident VL and pattern of *Leishmania* markers prior to VL diagnosis

Incident VL was diagnosed in 34 (6.9%) of the 490 individuals included in the analysis, with an incidence of 2.8% (10/355) in those without VL history, 5.0% (5/99) in those with past VL and 52.8% (19/36) in those with recent VL (Fig. 1). This included one of the five individuals not on ART at study enrolment. Incident VL occurred at a median of 20 weeks (IQR 12–33) after enrolment.

Table S3 provides an overview of the different patterns of *Leishmania* markers at baseline and during follow-up for each of the 34 cases with incident VL, by history of VL. There were 17 individuals with one visit before overt VL, ten with two, five with three, one with five and one with seven visits prior to VL. Examples of the observed pattern of *Leishmania* markers prior to onset of VL are displayed in Table 3 (for individuals

	No VL history	Past VL	Recent VL
Total	355	99	36
rK39 RDT (n = 489)			
Negative	292 (82.5)	33 (33.3)	3 (8.3)
Positive	62 (17.5)	66 (66.7)	33 (91.7)
rK39 ELISA (n = 488)			
Neg (<10 U/ml)	257 (72.8)	34 (34.3)	2 (5.6)
Borderline (10–14.9 U/ml)	23 (6.5)	5 (5.0)	0 (0)
Positive (≥15 U/ml)	73 (20.7)	60 (60.6)	34 (94.4)
DAT (n = 488)			
Negative	325 (92.1)	43 (43.4)	6 (16.7)
Positive (≥1/1600)	28 (7.9)	56 (56.6)	30 (83.3)
RT-PCR (n = 487)			
Negative	323 (91.8)	89 (89.9)	18 (50.0)
Weakly	15 (4.3)	4 (4.0)	2 (5.6)
Positive	14 (4.0)	6 (6.1)	16 (44.4)
KAtex (n = 474)			
Negative	328 (95.9)	94 (96.9)	23 (65.7)
1+	5 (1.5)	1 (1.0)	2 (5.7)
2+	8 (2.3)	2 (2.1)	2 (5.7)
3+	1 (0.3)	0 (0)	8 (22.9)
Any marker positive	85 (23.9)	74 (74.5)	36 (100)

RDT: rapid diagnostic test; ELISA: Enzyme-Linked Immuno Sorbent Assay; DAT: direct agglutination test; RT-PCR: real-time polymerase chain reaction; KAtex: Leishmania urine antigen test; VL: visceral leishmaniasis; Past VL (previous VL episode ≥1 year before enrolment); Recent VL (previous VL episode <1 year before enrolment).

***Table 2:* Leishmania markers at baseline in persons living with HIV stratified by VL history, North-Ethiopia (2017–2021).**

without a history of VL) and Table 4 (for individuals with a history of VL). For those with a history of VL, only direct markers (RT-PCR or KAtex) were considered relevant as "signals", as serological test can generally be expected to be positive in such patients.

"Signals" of imminent VL within the next few months displayed by the *Leishmania* markers were present in nine out of ten incident cases with VL in those without a history of VL. In one case, no positive markers were observed prior to VL, albeit no follow-up visit occurred in the six months prior to VL (case 7 in Table 3). A diverse range of patterns was observed, with blood RT-PCR, rK39 ELISA and rK39 RDT as the most common positive signals. At the last visit preceding VL, nine out of ten had at least one positive serological marker, five were RT-PCR positive and two had a positive KAtex test (Table S3).

In the 24 participants with a history of VL, signals (RT-PCR or KAtex) were observed prior to VL in 23 (95.8%) individuals. In one case (case 14, Table 4), no positive markers were detected in the single visit prior to the VL diagnosis. While for some RT-PCR was positive before KAtex (*e.g.* case 8, 10 and 11 in Table 4), others (such as case 9, 12 and 13) were positive on both markers from the onset, but the lack of data from earlier visits did not allow to determine which marker turned positive first.

Ignoring serological markers for those with a history of VL, 32 (94.1%) of the 34 incident cases had at least one positive *Leishmania* marker during the follow-up period preceding the VL diagnosis. In 29 (85.3%) of these, markers were already positive at baseline (baseline markers) and three others had only positive markers during follow-up (incident markers) prior to the VL diagnosis, all in individuals with a history of VL.

The association between baseline *Leishmania* markers and the risk of subsequent VL

The sensitivity and specificity of the different *Leishmania* markers at baseline to predict subsequent VL is displayed in Table 5 for those without VL history and in Table 6 for those with a VL history. For those without VL history, rK39 RDT & ELISA had a fair sensitivity (60–70%) and specificity (81–84%). For direct markers, specificity was generally above 95% but sensitivity was lower, at 50–60% for RT-PCR and 10% for KAtex. RT-PCR had the highest diagnostic odds ratio (21–37). By combining rK39 RDT or ELISA with RT-PCR, a sensitivity of 80% and a specificity of close to 80% was obtained. In those with two of these markers positive, the risk of subsequent VL was clearly raised, increasing from a pre-test probability of 2.8% to a post-test probability of 50%. Combining RT-PCR, rK39 RDT and ELISA yielded a sensitivity of 90% and a specificity of 71%.

For all tests but KAtex, the number of individuals NNT to predict one VL case was between 50 and 118, with slightly lower values when combining two or three tests. While obviously missing some cases (4 out of 10), restricting testing to individuals with CD4 counts below 200 cells/μL was more efficient (Table S4).

For those with past VL, the sensitivity was 80% for baseline RT-PCR and 20% for KAtex (Table 6). Specificity was 98% for both. For those with recent VL, sensitivity was 68% for baseline RT-PCR and 58% for KAtex. RT-PCR had the highest diagnostic odds ratio (58.7–184). For all those with a history of VL, the combination of RT-PCR and KAtex had a sensitivity of 83% and a specificity of 93%. The risk of VL ranged from 3.8% in those with both markers negative to 100% in those with both markers positive. For individuals with a history of VL, the NNT to predict one VL case was between 15 and 27.

The association between incident *Leishmania* markers and the risk of subsequent VL

Amongst individuals with negative baseline markers and with follow-up visits prior to VL development, the incidence of a positive marker ranged from 7.4% for RT-PCR to 8.3% for KAtex and 17.5% for rK39 ELISA. The sensitivity of positive incident markers to predict subsequent VL ranged from 33.3% for rK39 ELISA to 43%

	Before VL diagnosis/treatment			VL treatment	
Case 1	**-7M**	**-4M**	**-1M**	**T0**	**EOT**
rK39 RDT	-	-	+	+	+
rK39 ELISA	-	-	39	40	36
DAT	-	1/6400	-	-	1/1280
KAtex	-		3	3	3
RT-PCR Ct	30	19	20	20	32
Case 2			**-3M**	**T0**	**EOT**
rK39 RDT			-	+	+
rK39 ELISA			17	63	19
DAT			-	1/6400	1/6400
KAtex			-	-	-
RT-PCR Ct			31	40	-
Case 3		**-5M**	**-2M**	**T0**	**EOT**
rK39 RDT		+	+	+	+
rK39 ELISA		117	101	90	95
DAT		1/204800	1/1600	1/1600	1/3200
KAtex		-	-	-	-
RT-PCR Ct		44	-	40	-
Case 4		**-6M**	**-3M**	**T0**	**EOT**
rK39 RDT		+		+	+
rK39 ELISA		-		-	23,0
DAT		-		-	-
KAtex		-		-	-
RT-PCR Ct		-		39	-
Case 5	**-9M**	**-6M**	**-3M**	**T0**	**EOT**
rK39 RDT	-	-	-		
rK39 ELISA	27	29	40		
DAT	-	-	-		
KAtex	-	-	-		
RT-PCR Ct	-	-	-		
Case 6		**-4M**	**-1M**	**T0**	**EOT**
rK39 RDT		+	+	+	+
rK39 ELISA		371	>800	>800	>800
DAT		1/12800	1/51200	-	1/51200
KAtex		-	-	-	-
RT-PCR Ct		28	33	34	-
Case 7	**-9M**	**-6M**	**-3M**	**T0**	**EOT**
rK39 RDT	-	-		+	-
rK39 ELISA	-	-		26	17
DAT	-	-		1/1600	1/6400
KAtex	-	-		3	1
RT-PCR Ct	-	-		25	-

RDT: rapid diagnostic test; ELISA: Enzyme-Linked Immuno Sorbent Assay; DAT: direct agglutination test; RT-PCR: real-time polymerase chain reaction; KAtex: *Leishmania* urine antigen test; M: month; T0: start of treatment; EOT: end of treatment; (+): positive; (–): negative. **Orange** areas indicate time points with VL treatment; **Green** areas indicate time points without VL treatment; **Grey** areas indicate that no visit occurred; **Red** areas indicate time-points prior to VL with positive *Leishmania* markers as potential predictors of VL. Positive KAtex results are graded as 1+/2+/3+; for positive PCR results, the cycle threshold (Ct) value is given. For positive DAT results, the titer is given. For positive ELISA results, U/mL values are shown.

Table 3: **Illustration of patterns of *Leishmania* markers prior to the onset of visceral leishmaniasis (VL) in persons living with HIV without a history of VL in North-Ethiopia (2017–2021).**

	Before VL diagnosis/treatment					VL treatment	
Case 8				**-M4**	**-M1**	**T0**	**EOT**
rK39 RDT				+	+	+	+
rK39 ELISA				160	509	317	556
DAT				1/204800	1/204800	1/51200	1/204800
KAtex				-	1	3	3
RT-PCR Ct				29	26	26	28
Case 9			**-M7**	**-M4**	**-M1**	**T0**	**EOT**
rK39 RDT			+	+	+	+	+
rK39 ELISA			333	>800	>800	448	>800
DAT			1/204800	1/204800	1/204800	1/204800	1/204800
KAtex			2	1	3	3	3
RT-PCR Ct			21	23	21	24	-
Case 10	**-M19**	**-M16**	**-M7**	**-M4**	**-M1**	**T0**	**EOT**
rK39 RDT	+	+	-	+	-	+	+
rK39 ELISA	>800	>800	>800	>800	>800	>800	558
DAT	1/51200	1/3200	-	1/51200	-	1/3200	1/25600
KAtex	-	-	-	-	2	3	1
RT-PCR Ct	-	-	-	31	24	23	32
Case 11			**-M5**	**-M3**	**-M1**	**T0**	**EOT**
rK39 RDT			+	+	+	+	
rK39 ELISA			26	16	16	-	
DAT			-	-	1/6400	1/6400	
KAtex			-	-	2	2	2
RT-PCR Ct			35	35	31	30	
Case 12			**-M6**	**-M5**	**-M3**	**T0**	**EOT**
rK39 RDT			-	+	+	+	+
rK39 ELISA			292	359	160	465	123
DAT			1/204800	1/204800	1/204800	1/204800	1/3200
KAtex			3	3	3	3	3
RT-PCR Ct			24	23	21	21	25
Case 13					**-M3**	**T0**	**EOT**
rK39 RDT					+	+	+
rK39 ELISA					18	507	642
DAT					-	1/51200	1/51200
KAtex					2	3	3
RT-PCR Ct					30	22	30
Case 14					**-M3**	**T0**	**EOT**
rK39 RDT					+	+	+
rK39 ELISA					>800	657	799
DAT					-	1/204800	1/6400
KAtex					-	3	3
RT-PCR Ct					-	19	41

RDT: rapid diagnostic test; ELISA: Enzyme-Linked Immuno Sorbent Assay; DAT: direct agglutination test; RT-PCR: real-time polymerase chain reaction; KAtex: *Leishmania* urine antigen test; M: month; T0: start of treatment; EOT: end of treatment; (+): positive; (–): negative. **Orange** areas indicate time points with VL treatment; **Green** areas indicate time points without VL treatment; **Red** areas indicate time-points prior to VL with positive *Leishmania* markers as potential predictors of VL; for those with a history VL, only direct parasite markers (KAtex/PCR) are considered relevant. Grey areas indicate the serological markers. Positive KAtex results are graded as 1+/2+/3+; for positive PCR results, the cycle threshold (Ct) value is given. For positive DAT results, the titer is given. For positive ELISA results, U/mL values are shown.

Table 4: **Illustration of patterns of *Leishmania* markers prior to the onset of visceral leishmaniasis (VL) in persons living with HIV with a history of VL in North–Ethiopia (2017–2021).**

	VL	No VL	Total	% VL	Sens/spec	LHR	OR	NNT
rK39 RDT								
Positive	6	56	62	9.7	60.0/83.7	3.7	7.7	71
Negative	4	288	292	1.4		0.5		
rK39 ELISA								
Positive	7	66	73	9.6	70.0/80.8	3.6	9.8	50
Negative	3	277	280	1.1		0.4		
DAT								
Positive	3	25	28	10.7	30.0/92.7	4.1	5.4	118
Negative	7	318	325	2.1		0.7		
KAtex								
Positive	1	13	14	7.1	10.0/96.1	2.6	2.7	342
Negative	9	319	328	2.7		0.9		
RT-PCR1								
Positive	5	9	14	35.7	50.0/97.4	19	37	70
Negative	5	333	338	1.5		0.5		
RT-PCR2								
Positive	6	23	29	20.7	60.0/93.3	8.9	20.8	59
Negative	4	319	323	1.2		0.4		
RT-PCR1 & rK39 ELISA								
Both negative	2	271	273	0.7	80.0/79.2	0.2	–	44
One positive	4	67	71	5.6	–	2.0		
Both positive	4	4	8	50	–	34.2		
RT-PCR1 & rK39 RDT								
Both negative	2	280	282	0.7	80.0/81.9[a]	0.2	–	44
One positive	5	59	64	7.8	–	2.9		
Both positive	3	3	6	50	–	34.1		
rK39 RDT & ELISA								
Both negative	2	249	251	0.8	80.0/72.6[a]	0.1	–	61
One positive	3	66	69	4.3		0.8		
Both positive	5	28	33	15.1		3.8		
rK39 RDT & ELISA & RT-PCR1								
Positive	9	99	108	8.3	90.0/71.0[a]	3.1	22.1	39
Negative	1	243	244	0.4		0.1		

RDT: rapid diagnostic test; ELISA: Enzyme-Linked Immuno Sorbent Assay; DAT: direct agglutination test; RT-PCR: real-time polymerase chain reaction; KAtex: Leishmania urine antigen test; VL: visceral leishmaniasis; sens: sensitivity; spec: specificity; LHR: likelihood ratio; OR: diagnostic odds ratio; NNT: number needed to screen (to predict one incident VL case); RT-PCR1: weakly positive results considered as negative; RT-PCR2: weakly positive results considered as positive. [a]Sensitivity and specificity calculated taking either one of the two tests as positive.

Table 5: **Association between *Leishmania* markers at baseline and incident visceral leishmaniasis in HIV-infected individuals without a history of VL in North–Ethiopia (2017–2021).**

for RT-PCR (Table 7). Specificities ranged from 82.6% to 93.1%. However, the NNT to predict one incident VL case ranged from 72 for KAtex to 319 for rK39 ELISA (Table 7).

Overall, testing at baseline with rK39 RDT/rK39 ELISA/RT-PCR for those without VL history, and with RT-PCR & KAtex for those with a VL history would detect 29/34 (85.3%) cases with incident VL with a NNT of 15.5 (see above & Table S5). Further testing during follow-up for those with VL history and negative baseline markers would detect an additional three cases with incident VL (see above), but would require follow-up testing in 100 individuals. As such a strategy would be costly and not practical to apply in practice, more simplified predictive algorithms would be needed for routine care.

Development of (simplified) algorithms for VL prediction

The strategy to construct a clinical algorithm that could be applied in routine practice is explained in Box 1. First, three clinically relevant risk categories (low, moderate, high) were defined by three VL experts (JvG, KR, ED), based on differential clinical management. For the low risk category (risk of VL < 25%), routine follow-up was considered to be sufficient. For the category with moderate VL risk (risk of VL 25–75%), pentamidine secondary prophylaxis would be indicated, in line with recently revised WHO guidelines.[17] For the high risk category (risk >75%), management is less defined but could entail pre-emptive therapy/early initiation of VL treatment.

Second, we reasoned that VL history (particularly recent VL) and CD4 count should constitute the backbone of the algorithm, as these are the strongest well-established risk factors for VL and are routinely available. As those with recent VL had a clearly higher risk of VL (52.8%), and the risk for those without (2.8%) and with past VL (5.0%) was comparable, two groups were defined: those with or without recent VL.

To define CD4 count cut-offs, we plotted the risk of VL according to CD4 count (Figure S1), and defined the CD4 count cut-off by the Youden index (maximal sensitivity + specificity) for those with or without recent VL separately, with rounding to commonly used clinical cut-offs. This resulted in a cut-off <100 cells/μL for those with recent VL and <200 cells/μL for those without recent VL. Together, this provided four patient groups: no recent VL with CD4 counts below or above 200 cells/μL; recent VL with CD4 counts below or above 100 cells/μL. Each of these groups had a different predicted risk of VL: 1% for those without recent VL & CD4 counts ≥200 cells/μL; 16% for those without recent VL & CD4 counts <200 cells/μL; 27% for those with recent VL & CD4 counts ≥100 cells/μL; 88% for those with recent VL & CD4 counts <100 cells/μL.

Third, for each of the four groups, we assessed whether additional testing with *Leishmania* markers led to reclassification across risk categories (*e.g.* from <25% to 25–75%). If there was no reclassification after testing, testing was not deemed clinically relevant. For instance, for those without recent VL and CD4 counts above 200 cells/μL, the risk of VL was 1% before testing, and would be 0.4% for those with a negative rK39 RDT and 2.8% for those with a positive rK39 RDT result. As both test results did not change the risk category, this step was removed from the algorithm. In a first scenario, we used the rK39 RDT as the preferential test (routinely available) and restricted RT-PCR testing to those with recent VL (as generally rK39 RDT-positive). In a second scenario, RT-PCR was replaced by KAtex (cheaper and

easier to implement). A one year time window was taken, to predict the risk of VL within one year after testing.

Fig. 2 represents an algorithm with baseline CD4 count and VL history as backbone and targeted testing with *Leishmania* markers (rK39 RDT & RT-PCR) restricted to those groups where testing would lead to reclassification across the three risk categories. No further testing would be done for those without recent VL and CD4 counts ≥200 cells/μL, rK39 RDT testing would be done for those without recent VL and CD4 counts <200 cells/μL and RT-PCR testing for those with recent VL. Such an algorithm would restrict laboratory marker testing to 97 individuals (20%; 13% with rK39 RDT and 7% with RT-PCR), and would direct individuals to distinct clinical management strategies, according to their predicted risk of VL. The predicted VL risk was 2.0% in the low risk category, 45% in the moderate risk category and 100% in the high risk category. The version using KAtex instead of RT-PCR is shown in Figure S2.

Discussion

To the best of our knowledge, this is the largest study in PLWH longitudinally analysing the pattern and evolution of *Leishmania* infection markers preceding the onset of VL. The vast majority of PLWH displayed positive *Leishmania* markers at (several) visits during the months prior to the development of VL, indicating that progression of VL from the asymptomatic stage takes several months, and can be picked up during routine HIV clinical visits. This provides a window of opportunity for interventions to prevent VL in those at highest risk that could be economically identified by applying a simplified algorithm combing clinical parameters for pre-selecting those to be subsequently tested on *Leishmania* infection markers.

Other studies have looked into predictors of relapse in PLWH, predominantly in Europe.[20–29] Several studies found *Leishmania* PCR,[21–25,28,29] and some found that KAtex[26,27] measured during follow-up could be useful to predict relapse. One study from Ethiopia found three immunological markers predictive of VL relapse.[22] However, we are not aware of another study besides PreLeisH that used a comprehensive panel of *Leishmania* markers to study the asymptomatic phase prior to VL development, also included individuals without a history of VL to predict primary VL, and developed a clinical algorithm to guide clinical management.

First, we described the pattern of *Leishmania* markers prior to VL onset. For those with a VL history, the vast majority were blood RT-PCR positive, and most were KAtex positive at the visit prior to VL diagnosis. The first signal upon reactivation of parasite replication seems to be a positive blood RT-PCR test. A positive urine antigen test likely concurs with substantial parasite replication and spread, also reflected by decreasing blood RT-PCR Ct values (hence higher parasite load).

In those without a history of VL, a variety of patterns of *Leishmania* markers was seen prior to VL. There was no single marker that consistently constituted the first positive signal preceding VL, with the majority of individuals positive on several markers at baseline. The visit before the diagnosis of VL was typically characterised by positive serological markers, a positive blood RT-PCR for half and rarely a positive urine antigen test. The lower proportion of RT-PCR and KAtex positives in primary cases of VL compared to those with VL relapse could potentially indicate lower levels of parasite replication, and/or less spread from the spleen and other organs to the blood.

	VL	No VL	Total	% VL	Sens/spec	LHR	OR	NNT
Past VL (>1 year)								
KAtex								
Positive	1	2	3	33.3	20.0/97.8	9.2	11.3	97
Negative	4	90	94	4.3		0.8		
RT-PCR1								
Positive	4	2	6	66.7	80.0/97.9	37.6	184	25
Negative	1	92	93	1.1		0.2		
RT-PCR2								
Positive	4	6	10	40.0	80.0/93.6	12.5	58.7	25
Negative	1	88	89	1.1		0.2		
Recent VL (≤1 year)								
KAtex								
Positive	11	1	23	91.7	57.9/93.8	9.3	20.6	3
Negative	8	15	11	34.8		0.4		
RT-PCR1								
Positive	13	3	16	81.2	68.4/82.4	3.9	10.1	3
Negative	6	14	20	30.0		0.4		
RT-PCR2								
Positive	15	3	18	83.3	78.9/82.4	4.5	17.5	3
Negative	4	14	18	22.2		0.2		
Past & recent VL								
RT-PCR1 & KAtex								
Both negative	4	100	104	3.8	83.4/92.6[a]	0.2	–	6.6
One positive	11	8	19	57.9		6.2		
Both positive	9	0	9	100		–		
RT-PCR1								
Positive	17	5	22	77.3	70.8/95.5	15.7	51.5	8
Negative/Indeterminate	7	106	113	6.2		0.3		
RT-PCR2								
Positive/Indeterminate	19	9	28	67.9	79.2/91.9	9.8	43.1	7
Negative	5	102	107	4.7		0.2		

Past VL: VL episode >1 year before enrolment. Recent VL: VL episode ≤ 1 year before enrolment. RT-PCR: real-time polymerase chain reaction; KAtex: *Leishmania* urine antigen test; VL: visceral leishmaniasis; sens: sensitivity; spec: specificity; LHR: likelihood ratio; OR: diagnostic odds ratio; NNT: number needed to screen (to predict one incident VL case); RT-PCR1: weakly positive results considered as negative; RT-PCR2: weakly positive results considered as positive. [a]Sensitivity and specificity calculated taking either one of the two tests as positive.

Table 6: **Association between *Leishmania* markers at baseline and incident visceral leishmaniasis in persons living with HIV with a history of VL in North-Ethiopia (2017–2021).**

Marker	VL	No VL	Total	% VL	Sens/spec	NNT (indiv)	Total test	NNT (test)
RT-PCR1								
Baseline marker (+)	22	14	36					
Baseline marker (−)	12	439	451					
With follow-up	7	438	445					
Incident marker (+)	3	30	33 (7.4)	9.1	42.9	148	2149	716
Incident marker (−)	4	408	412 (83.6)	1.0	93.1			
RT-PCR2								
Baseline marker (+)	25	32						
Baseline marker (−)	9	421	430					
With follow-up	5	420	425					
Incident marker (+)	3	41	44 (6.8)	6.8	60.0	142	2042	681
Incident marker (−)	2	379	381 (0.5)	0.5	90.2			
KAtex								
Baseline marker (+)								
Baseline marker (−)	21	424	445					
With follow-up	12	423	435					
Incident marker (+)	6	30	36 (8.3)	16.7	50.0	72.5	2070	345
Incident marker (−)	6	393	399 (91.7)	1.5	92.9			
rK39 ELISA								
Baseline marker (+)	30	137	167					
Baseline marker (−)	4	317	321					
With follow-up	3	316	319					
Incident marker (+)	1	55	56 (17.5)	1.8	33.3	319	1530	1530
Incident marker (−)	2	261	263 (82.5)	0.8	82.6			
rK39 RDT								
Baseline marker (+)	27	134						
Baseline marker (−)	7	321	328					
With follow-up	6	321	327					
Incident marker (+)	2	30	32 (9.8)	6.2	33.3	163.5	1653	826.5
Incident marker (−)	4	291	295 (90.2)	1.4	90.6			

RDT: rapid diagnostic test; ELISA: Enzyme-Linked Immuno Sorbent Assay; DAT: direct agglutination test; RT-PCR: real-time polymerase chain reaction; KAtex: Leishmania urine antigen test; VL: visceral leishmaniasis; sens: sensitivity; spec: specificity; LHR: likelihood ratio; OR: diagnostic odds ratio; NNT: number needed to screen (to predict one incident VL case); RT-PCR1: indeterminate results considered as negative; RT-PCR2: indeterminate results considered as positive.

***Table 7*: Association between *Leishmania* markers during follow-up (incident markers) and incident visceral leishmaniasis in persons living with HIV in North–Ethiopia (2017–2021).**

Subsequently, we assessed the predictive value of the different *Leishmania* markers, and found baseline markers to be most useful, with fair NNTs. In those without VL history, rK39 RDT & ELISA markers generally had moderate sensitivity but better specificity. In those with or without VL history, RT-PCR had moderate sensitivity but clearly higher specificity. Using a combination of four different markers yielded a sensitivity of 85.3%, a specificity of 76.2% and a NNT of 15.5. Such NNT is comparable to screening strategies used for tuberculosis, such as with the Gene Xpert.[30]

As using all *Leishmania* markers would not be feasible in routine care, simplified predictive algorithms are needed. From a clinical perspective, restricting testing to those in which testing leads to reclassification across risk categories with distinct clinical management strategies would make sense. From an operational perspective, using only two tests at one time-point would be much more feasible. The algorithm we developed integrates two important VL risk factors (recent VL history and CD4 count) with restricted testing using rK39 RDT and RT-PCR. Such an algorithm would require testing with *Leishmania* markers in only a fifth of the population, while identifying three risk categories predicting the risk over the subsequent year. It could for instance be applied every year to HIV-infected individuals in HIV care. While RT-PCR is currently not available in most health facilities in East Africa, the loop-mediated isothermal amplification (LAMP) assay has high potential as a molecular point of care test and was found accurate and simple for VL diagnosis in several studies in East-Africa.[31,32]

Because the disease is highly T-cell dependent, we are currently evaluating if markers of the patient's general or *Leishmania*-specific cellular immunity such as whole blood interferon-gamma release assays (IGRA)

Box 1.

Strategy for the development of a clinical algorithm for targeted testing using *Leishmania* markers to predict visceral leishmaniasis (VL)

Definition of VL risk categories

Based on clinical consensus: risk of VL[a]

- Low risk (<25%): routine follow-up
- Moderate risk (25–75%): secondary prophylaxis
- High risk (>75%): early/pre-emptive treatment

VL history and CD4 count as backbone of the algorithm

CD4 count and VL history strongest well-established risk factors for VL and routinely available

- Recent VL versus no recent VL
  - Risk of VL high in those with recent VL: 52.8%
  - Risk of VL lower and comparable in no VL history (2.8%) or past VL group (5%)
  - Hence categorized as recent vs no recent VL
- CD4 count cut-off defined by Youden index (maximal sensitivity + specificity) for those with or without recent VL separately
  - Recent VL: cut-off of 100 cells/μL; no recent VL: cut-off of 200 cells/μL
- This resulted in four different patient groups:
  - No recent VL with CD4 counts above or below 200 cells/μL
  - Recent VL with CD4 counts above or below 100 cells/μL

Inclusion of *Leishmania* markers: different scenario's

rK39 RDT/PCR:

- rK39 RDT preferred test as routinely available
- RT-PCR more complex and costly hence restricted to those with recent VL (as rK39 RDT +)

rK39 RDT/KAtex:

- RT-PCR replaced by KAtex as more easy and cheaper to implement

Inclusion of *Leishmania* markers: clinical value of testing

For each of four different groups defined by combination of VL history and CD4 count category

- The predicted risk of VL was calculated
- Evaluation whether additional testing with *Leishmania* markers led to reclassification across the three risk categories (<25%, 25–75; >75%)
- If no reclassification, testing without clinical implications hence removed from algorithm
  A one year time window was taken, to predict the risk of VL within one year after testing

VL: visceral leishmaniasis; RT-PCR: real-time polymerase chain reaction; RDT: rapid diagnostic test. KAtex: *Leishmania* urine antigen test. Past VL: VL episode >1 year before enrolment. Recent VL: VL episode ≤ 1 year before enrolment. [a]Prior to the development of the clinical algorithm, three experienced VL clinicians were asked at which probability of risk of developing VL, they would recommend secondary prophylaxis. For instance, at 2% there was consensus that this was not indicated, at for instance 50% there was consensus that this was indicated, and at 25% there was doubt (perhaps yes, perhaps no) or "equipoise". This is how the 25% cut-off was defined. The same process was used to define the cut-off of 75% above which there was consensus secondary prophylaxis might not be sufficient and a more "aggressive" approach might be indicated (for instance early/pre-emptive therapy).

could add more discriminatory power to classify patients or in VL prediction in addition to antibody or antigen based infection markers. In line, we showed that those with recurrent VL in this cohort exhibited a pronounced T cell anergy that underlined their chronicity, and associated markers could have predictive value in VL development and relapse prediction.[33] Building on the potential value as alternative test-of-cure in individuals with VL-HIV coinfection, detailed proteomic and transcriptomic blood screenings may identify newly predictive signatures of disease progression.[34] Nevertheless, decentralisation to lower levels of the health care systems would require simpler tests, such as (more sensitive) urine antigen tests, and biomarker discovery efforts should solve constraints in cross platform implementation to successfully integrate findings in future diagnostic settings. Similarly, HIV-1 viral load measurements were not used in the clinical algorithm, as in routine settings it often takes many months before the results are available. However, if point of care viral load testing would become increasingly available, this would be worth exploring.

For individuals at risk of VL relapse, secondary prophylaxis has recently been recommended by WHO.[17] The targeted use of *Leishmania* markers can help to better define those at higher risk. For some, a risk of VL of 100% was predicted in our study. Whether in this situation prophylaxis would be effective or carries a substantial risk of emergence of drug-resistance when there could be active parasite replication, requires due consideration. For such individuals, a short treatment course (pre-emptive treatment) could be of value—as done for cryptococcosis–but this requires further study.[10]

While precluding the development of more complex predictive models, it is encouraging (from the patient perspective) to see that the incidence of VL in HIV-infected individuals stably enrolled in HIV care was found to be lower than expected in this study. This is likely related to the high baseline CD4 counts and ART

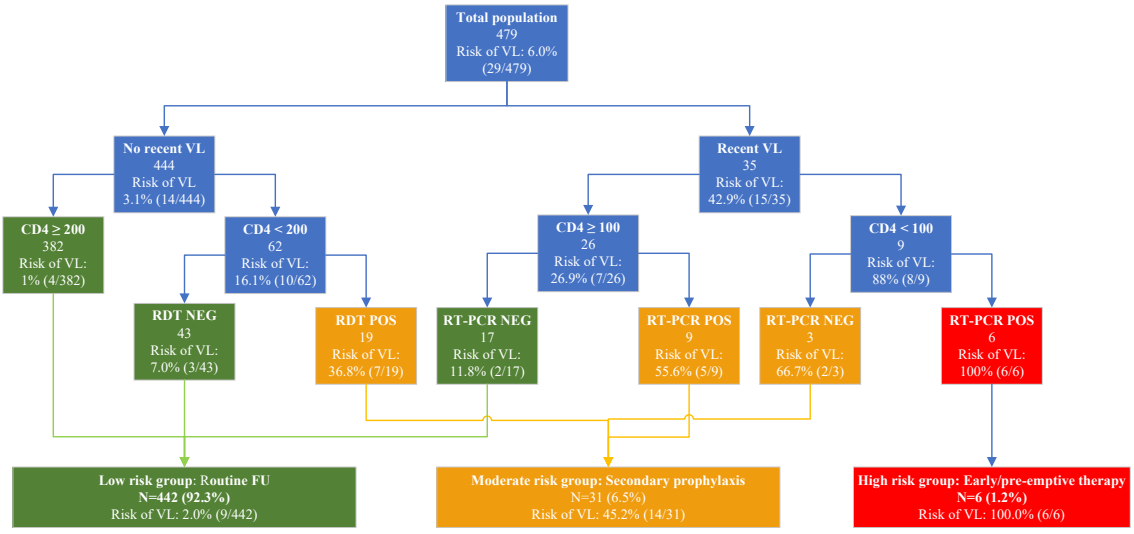

Fig. 2: **Algorithm using targeting testing with the rK39 RDT and RT-PCR to define VL risk categories.** VL: visceral leishmaniasis; RDT: rapid diagnostic test; RT-PCR: real-time polymerase chain reaction. *Leishmania* testing is done in 97 (20%) individuals; 62 (12.9%) with rK39 RDT and 35 (7.3%) with RT-PCR. Recent VL: VL episode ≤ 1 year before enrolment. The algorithm excludes individuals with missing data; a one year time window was taken, to predict the risk of VL within one year after testing.

use at enrolment, probably reflecting the implementation of the WHO recommended HIV test and treat strategy.[35] However, the reported VL incidence can probably not be generalized to the wider HIV population at risk. Individuals excluded from analysis due to the lack of follow-up data, appeared to be at higher risk of VL based on a clearly higher prevalence of *Leishmania* markers at baseline. ART adherence was also lower. Moreover, many highly mobile PLWH such as seasonal workers might not attend the health care facility and/or enrol into HIV care in the VL endemic area.[4,36] When returning to their VL non-endemic villages in the highland, a VL diagnosis is likely to be missed.[4,36–38] Additional studies and approaches are required for highly mobile PLWH only temporarily residing in VL endemic areas.[36]

Not a single woman enrolled in the study developed VL. Although cases with VL are occasionally detected amongst women at this and other study sites in Ethiopia,[6,39–41] this seems to suggest that the risk of VL—at least for PLWH enrolled in HIV care–is very low. Probably this is partly linked to a lower exposure to the parasite, as women were clearly less likely in our and other studies to be migrant workers or farmers and hence likely less exposed to *Leishmania*.[19,41–43]

Strengths of the study include the fact that, while done in a remote area, it is was conducted according to high research standards, adhering to GCP and GCLP and with support from a well-established clinical trials unit. One of the limitations is that the frequency of the clinical visits was not the same for all study participants, as we followed routine clinical practice to mimic programmatic settings. Moreover, a substantial number of study participants were lost to follow-up or had irregular clinical visits. Since the number of incident cases with VL was lower than planned, this precluded the development of more refined predictive models (see Statistical Analysis Plan). VL often occurred rapidly after enrolment in the study (for some rapidly after a previous VL episode), hence few time points were available to study the entire pattern of *Leishmania* markers before VL onset. As we only included one health centre in this study, and had a substantial amount of lost to follow-up, our findings might not be generalizable. Additionally, several *Leishmania* markers used in our algorithm are currently not routinely available in Ethiopia. Finally, the proposed algorithm requires validation, and more evidence is needed on the most appropriate clinical management strategies for the different risk categories. The value of such an algorithm should also be assessed in other countries where VL-HIV coinfection is common, such as in India.

Conclusions

The vast majority of PLWH displayed positive *Leishmania* markers prior to the development of VL, providing proof of concept for a screen and prevent/treat strategy. A simple algorithm with targeted screening allowed the definition of three risk categories, which could constitute the basis for secondary prophylaxis or pre-emptive treatment. The overall risk of VL was rather low, suggesting that with good access to ART,

the risk of VL can be reduced. Studies amongst HIV-infected individuals should also be conducted in more mobile populations not stably enrolling in HIV care.

Contributors

All authors read and approved the final version of the manuscript. Conceptualisation: JvG, ED, KR, SvH, FV, WA; Data curation: CB, HL, TB; Formal analysis: JvG, ED; both directly accessed and verified the underlying data; Funding acquisition: JvG, ED, KR; Investigation: MK, AK, HB, DM, KS, HS, HE, FK, RM, AY, MP, SA, DVdB; Methodology: JvG, SvH, FV, WA, JvG, ED, KR, MP; Project administration: AdH, BS, SvH, FV, WA, ED, RM, KR, JvG, MP; Resources: NA; Software: CB; Supervision: JvG, ED, RM, KR; Validation: DvdB, SA, MK, SA, DVdB, MP, HL, TB; Visualisation: JvG; Writing—original draft: JvG, ED, KR; Writing—review & editing: all.

Data sharing statement

Deidentified data from the PreLeisH study are available on reasonable request to the authors, considering scientific and ethical aspects. Please contact J van Griensven, jvangriensven@itg.be.

Declaration of interests

None to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2024.105474.

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