Protocol for an observational study on the clinical features, immunological interactions and household determinants of visceral leishmaniasis and malaria co-infections in West Pokot, Kenya

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1　**Protocol for an observational study on the clinical features,**

2　**immunological interactions and household determinants of visceral**

3　**leishmaniasis and malaria co-infections in West Pokot, Kenya**

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Abstract

Introduction: Visceral leishmaniasis (VL) and malaria are two deadly parasitic diseases that co-exist in West Pokot County, Kenya. The local population is at considerable risk of co-infection with VL and malaria, however, few studies have described the clinical implications of this co-morbidity. Questions remain regarding the immune responses responsible for possible predisposing or protective effects. Moreover, characterisation of environmental and household risk factors for co-acquiring VL and malaria is warranted to increase awareness and guide implementation of targeted control strategies. This protocol intends to address these knowledge gaps concerning VL-malaria co-infections.

Methods and analysis: this observational research project will have a multimethod approach, starting with a cross-sectional study at Kacheliba Sub-County Hospital in West Pokot, Kenya. Patients with laboratory confirmation of a VL and/or malaria infection will be clinically assessed and symptomatology of mono- and co-infections will be compared. Secondly, a questionnaire will be addressed to all included patients and to healthy controls in local communities. This case-control study will aim to describe household and environmental determinants associated with VL-malaria co-infection. Lastly, blood samples will be collected from a small cohort of VL and malaria mono- and co-infected patients during treatment of their infection(s), and from healthy controls and asymptomatic VL and malaria cases recruited in local communities. These specimens will be used for serum cytokine measurements and molecular quantitation of *Plasmodium* and *Leishmania*. In this way, the immune response and parasite dynamics during VL-malaria co-infection will be characterised longitudinally and compared to those observed in clinical and asymptomatic mono-infections.

Ethics and Dissemination: Ethical approval was obtained from the Ethics and Scientific Research Committee of Amref Health Africa. The study findings will be presented at international conferences and published in open-access, peer-reviewed journals.

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44 **Study Registration:** This study protocol has been registered at the ISRCTN registry (ID:

45 ISRCTN15023306).

46 **Key Words:** Visceral Leishmaniasis, Malaria, Coinfection, Study Protocol

Strengths and limitations of this study

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48 • Through a tripartite design, this research project will address clinical, immunological and

49 epidemiological knowledge gaps concerning VL-malaria co-infections.

50 • This will be the first study to investigate individual and household risk factors for VL-malaria

51 co-infections.

52 • Longitudinal characterisation of cytokine profiles in VL-malaria co-infections and comparison

53 with both symptomatic and asymptomatic mono-infections will offer the opportunity to study

54 associations between the immune response, parasite densities and clinical presentation.

55 • Given the lack of recent data on VL-malaria co-infection rates in West Pokot, the number of

56 co-infected cases recruited in this study could potentially be low.

57 • Measuring cytokine levels will not reflect the full extent of the immune response induced by a

58 VL-malaria co-infection.

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INTRODUCTION

Visceral leishmaniasis (VL) and malaria (caused by *Leishmania* and *Plasmodium* species, respectively) are two vector-borne protozoan parasite infections that cause high morbidity and mortality, particularly in remote regions of low income countries. In East Africa, Kenya is one of the countries most affected by VL.[1] Here, an important focus endemic VL transmission is located in West Pokot County, which is part of the Pokot territory situated at the border region between Kenya and Uganda.[2-4] Apart from being endemic for VL, this area is also characterised by recurrent outbreaks of seasonal malaria.[5] Due to the overlapping epidemiology of VL and malaria in the Pokot region, the local population is at risk of being infected with both diseases concurrently. Indeed, it appears that co-infections with *Leishmania donovani* and *Plasmodium falciparum* are not uncommon: studies among VL patients attending the regional VL treatment hospitals of Kacheliba (Kenya) and Amudat (Uganda) have reported rates of concomitant malaria ranging from 3.8% to 34.4%.[3, 4, 6] Despite these apparently high numbers of VL-malaria co-infections, the condition is still understudied in terms of risk factors, clinical presentation and immunology.

The overlap of VL and malaria transmission in West Pokot relies on the presence of favourable environmental conditions for their insect vectors, and subsequently, human exposure to these vectors. The local malaria mosquito vectors, *Anopheles arabiensis* and *An. funestus*, have a preference for dry savannah habitats where they lay eggs in small, temporary freshwater pools.[7, 8] As such, malaria incidence in West Pokot often peaks during and after seasonal rainfall. The individual malaria risk may vary from person to person due to household factors: house structure aspects have been associated with indoor *Anopheles* abundance in neighbouring Baringo County.[9] It is unknown whether these results are also applicable in the context of West Pokot. Like malaria, the endemicity of VL in West Pokot is partly attributable to its semi-arid climate. However, the ecology of the local VL vector is substantially different, as the sandfly *Phlebotomus martini* is believed to lay its eggs in the ventilation shafts of termite mounds.[10-13] Some studies have therefore found living close to these mounds to

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85   be associated with VL infection risk.[14, 15] Considering the differences in VL and malaria vector

86   ecology in West Pokot, a very specific combination of human behavioural, environmental and

87   household conditions may predispose for concomitant infections with both parasites. Better

88   understanding of this VL-malaria co-infection risk profile is crucial for increasing awareness among

89   exposed populations, and could also guide policy makers in drafting more focused VL and malaria

90   vector control strategies.

91   Despite the potentially deadly outcome of VL and malaria mono-infections, much remains unknown

92   about the clinical consequences when both these infections occur in one individual. Only a handful of

93   case reports have described the symptomatology of VL-malaria co-infections, and larger scale studies

94   have shown contradictory results.[6, 16-26] A case-control analysis of hospitalised VL patients in

95   Amudat Hospital found that co-occurring malaria did not clearly exacerbate the clinical picture of VL,

96   and correlated with a lower frequency of anaemia.[6] On the other hand, a study in Sudan in patients

97   with VL-malaria co-infection revealed an increased frequency of anaemia, emaciation and jaundice,

98   compared with their VL mono-infected counterparts.[23] As neither of these studies included a control

99   group of malaria mono-infected patients, it was not studied how malaria symptomatology is affected

100   by a co-occurring VL infection. Hence, additional research into the clinical interactions seen in VL-

101   malaria co-infections is warranted to improve recognition and management of this condition.

102   Beneath the clinical features of a VL-malaria co-infection lie the pathophysiological processes and

103   immune responses elicited by the infecting *Leishmania* and *Plasmodium* parasites, which have both

104   developed mechanisms to evade host immunity and alter it to their advantage.[27-29] During a VL-

105   malaria co-infection, *Leishmania* and *Plasmodium* parasites will simultaneously modulate the host

106   immune response, which may have an effect on the control or progression of the concomitant disease.

107   Such mechanisms are well known for people living with HIV, but have also been described for

108   conditions of polyparasitism, such as helminth co-infections in malaria and *Leishmania* patients.[30-

109   35] So far, there has been limited research into the parasitological and immunological dynamics during

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110 VL-malaria co-infections. Results from animal models have shown both aggravating and mitigating

111 effects of the two diseases upon each other.[36-40] To date, there has only been a single study looking

112 at the immunology of VL-malaria co-infections in humans: Van den Bogaart *et al.* compared the

113 cytokine profiles of VL and malaria mono- and co-infected patients in Sudan and found that the

114 immune response during a co-infection was mainly characterised by the release of pro-inflammatory

115 cytokines and reflected features of the responses seen in both mono-infections.[41] Interestingly, high

116 levels of the pro-inflammatory cytokine IL-17A distinguished co-infected patients from both mono-

117 infected groups, suggesting a synergistic interaction of the two diseases. The same study also found a

118 significantly lower *Plasmodium* parasitaemia in VL-malaria co-infected patients compared to malaria

119 mono-infections. As the interpretation of these study results is limited by their cross-sectional nature,

120 longitudinal assessment of patients with VL-malaria co-infections and comparison with mono-infected

121 patients (both clinical and asymptomatic) is required to unravel the associations between the immune

122 response, parasite loads and clinical features.

123 To address the knowledge gaps in our understanding of VL-malaria co-infections, this paper describes

124 the protocol of an observational research project aimed at characterising VL-malaria co-infections in

125 West Pokot on three different levels: symptomatology, epidemiology and immunology. These aspects

126 will be studied respectively by means of a cross-sectional study, a case-control study and a cohort

127 study. The research project will be conducted at Kacheliba Sub-County Hospital in West Pokot through

128 a collaboration between the Amsterdam University Medical Centres, Amref Health Africa and the

129 Kenya Ministry of Health (MoH).

Study objectives

131 The following study objectives have been formulated for this research project:

132 - To determine the prevalence of VL and malaria co-infections among patients suspected with

133 either infection attending Kacheliba Sub-County Hospital, West Pokot, Kenya.

134   -   To identify and compare clinical features and parasitaemias of patients with malaria and VL

135   mono- and co-infections and establish whether a co-infection exacerbates or alleviates

136   symptoms of both diseases.

137   -   To identify individual and household level determinants of VL-malaria co-infections in West

138   Pokot, Kenya.

139   -   To examine and compare the cytokine response in patients with VL and malaria mono- and co-

140   infections (both clinical and asymptomatic), before, during and after treatment, and determine

141   whether these cytokine responses can be related to the (sub)clinical presentation of the

142   infection(s).

METHODS

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Study design

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145   To address the different study objectives, this research project will consist of three components: a

146   prospective, hospital-based cross-sectional study among patients; a case-control study among hospital

147   patients and healthy volunteers in local communities; and a prospective cohort study among hospital

148   patients and healthy and asymptomatically infected household members of these patients.

149   For the prospective cross-sectional study, patients attending Kacheliba Sub-County Hospital in West

150   Pokot, Kenya, with clinical suspicion of malaria and/or VL infection will be asked to participate.

151   Laboratory diagnostic tests will be performed for both malaria and VL in consenting patients. A patient

152   will be included in the study if positive for one or both infections. Clinical and parasitological data will

153   be collected from these study subjects and compared between VL-malaria co-infected cases and

154   patients with VL and malaria mono-infections.

155   Participants of the cross-sectional study will also serve as cases in the case-control study, to whom a

156   structured household questionnaire will be administered. Exposure to certain individual and

157 household factors will be compared between mono- and co-infected patients. Additionally, per VL-

158 infected case, two age- and sex-matched healthy controls living in the same village as the case will be

159 recruited and administered the questionnaire as well.

160 Lastly, a small cohort of subjects of the cross-sectional study with confirmed VL and/or malaria

161 infection will be followed up during standard treatment. This cohort study will entail repeated

162 collection of venous blood samples from participating patients, to characterise their immunological

163 profiles over time. Additionally, blood samples will also be collected from healthy individuals and

164 asymptomatic VL/malaria cases, who will be actively recruited in the households of the patient cohort.

165 The healthy individuals will provide immunological baseline data, whereas the immunological profiles

166 of asymptomatic VL/malaria cases will be compared to those of patients with active clinical disease.

167 Healthy and asymptomatic subjects will be sampled once upon inclusion into the study. In case

168 asymptomatic cases require treatment for their VL and/or malaria infection, they will also be sampled

169 several times during this treatment.

Study site and timing

171 The research will be performed in West Pokot County in Kenya, an area that is endemic for VL year-

172 round and has seasonal transmission of malaria. Previous studies have reported that VL-malaria co-

173 infections occur in the Pokot region.[3, 4, 6] Participants will be recruited from the catchment area of

174 the Kacheliba Sub-County Hospital, which is a government hospital located about 40 km northwest of

175 West Pokot's county capital, Kapenguria. It is an important regional reference centre for VL diagnosis

176 and treatment, supported by Drugs for Neglected Diseases Initiative (DNDi).[42] The study will be

177 conducted in October and November 2022. This two-month period coincides with the short rainy

178 season (October – December) during which malaria incidence often peaks.[43]

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Study population

180 The population of the cross-sectional study will comprise individuals who attend the Kacheliba Sub-

181 County Hospital and have a laboratory confirmed infection with VL and/or malaria. The study

182 participants will be grouped according to their VL and malaria diagnosis, as determined by routine

183 diagnostic procedures:

184   -   Newly diagnosed patients with active primary VL, defined as patients with clinical symptoms

185   such as prolonged fever (>2 weeks), splenomegaly, weakness or wasting, with either a positive

186   rk39 rapid diagnostic test (RDT), positive Direct Agglutination Test (DAT titre ≥ 1:3200) and/or

187   microscopy-positive spleen aspirate.

188   -   Patients with uncomplicated malaria, defined as patients with fever or history of fever within

189   the last 48 hours (with or without other symptoms) and a positive thick and thin blood film for

190   *Plasmodium*, with a parasite count <250,000/µL of blood.

191   -   Patients co-infected with malaria and primary VL (actively for one or both infections) defined

192   as patients with symptoms of VL and/or malaria, with a positive *Plasmodium* blood film

193   (parasite count <250,000/µL of blood) and positive VL diagnostic test (rk39 RDT, DAT, spleen

194   aspirate).

195 All subjects of the cross-sectional study will also be included as cases in the case-control study. Two

196 age- and sex-matched healthy controls per VL-infected case (including those co-infected with malaria)

197 will be recruited in the case's village of residence and will be defined as individuals without current

198 signs or symptoms of VL or malaria, no history of VL, no malaria in the preceding two weeks, and with

199 a negative rk39 RDT and negative malaria RDT. The individual should have lived in their current house

200 for at least one year.

201 Clinical subjects of the cohort study will be recruited among the mono- and co-infected participants of

202 the cross-sectional study. Only malaria infections with *P. falciparum* will be eligible, and the patient

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203   must be aged between 6 and 30 years old. These age limits are set to exclude infants and children

204   whose immune system has not yet fully developed, and patients above 30 years who are more likely

205   to have developed a significant level of acquired immunity to malaria.[6, 44] The cohort study will also

206   recruit healthy controls and asymptomatic cases in the households of the clinically ill participants. This

207   recruitment strategy will minimise the variability of environmental confounders between the different

208   study groups. Moreover, the likelihood of finding asymptomatic VL and malaria infections will be

209   higher in households of symptomatic patients.[45-49] The healthy and asymptomatic cohorts are

210   defined as follows:

211   -   Healthy endemic controls, defined as individuals above the age of 6 years, without current

212   signs or symptoms of VL or malaria, with no self-reported history of VL, no malaria in the

213   preceding two weeks, with a negative DAT test (DAT titre ≤ 1:200) and negative malaria blood

214   films.

215   -   Patients with asymptomatic VL, defined as individuals above the age of 6 years, without VL-

216   associated symptoms for at least 15 days before study inclusion and no self-reported history

217   of active VL, with a positive DAT (DAT titre ≥ 1:3200).

218   -   Patients with asymptomatic malaria, defined as individuals above the age of 6 years, with no

219   symptoms suggestive of malaria at the time of inclusion and with no history of malaria in the

220   preceding two weeks, with a positive thick and thin blood film for *P. falciparum.*

221   Sample size

222   The cross-sectional study aims to include 244 malaria infected patients, allowing to detect an odds

223   ratio (OR) of 1.8 at a confidence level of 95% (two-sided), with an expected 20% of exposure

224   among VL-infected cases and a power of 80%.[50] Within the two-month time frame of the study,

225   all patients at the study hospital with a confirmed VL infection will be included. Considering that 350

226   VL cases were reported at Kacheliba Sub-County Hospital in the first five months of 2022 (personal

227   communication with David Kiptanui, clinical officer at Kacheliba Sub-County Hospital, 2022), this

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228   pragmatic approach is expected to recruit approximately 140 VL patients. Among these subjects,

229   approximately 5 to 30 cases are expected to be co-infected with malaria, based on previously reported

230   co-infection rates in Kacheliba Hospital ranging from 3.8% to 21.4%.[4]

231   All participants of the cross-sectional study will also be included in the case-control study. Additionally,

232   two healthy controls will be recruited per VL-infected patient, meaning that the study will aim to

233   include approximately 280 healthy controls.

234   Given the explorative nature of the immunological cohort study, the population size per study group

235   will be set at 20 to 30 subjects, depending on their availability. Based on the results of Van den Bogaart

236   *et al.* about cytokine levels in VL-malaria co-infected patients, this group size should be sufficient to

237   detect significant differences in immunological parameters with 80% power at 5% level of statistical

238   significance.[41]

Clinical sample and data collection

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Cross-sectional study

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241   Patients presenting at the study hospital with clinical signs and/or symptoms indicating a potential

242   malaria and/or VL infection will be asked to participate in the study if they meet the inclusion criteria.

243   After giving their informed consent, their clinical features and medical history will be recorded on a

244   case report form (CRF). Patients not willing to participate in the research will be excluded from the

245   study and will be referred to the clinician for usual diagnosis and treatment. Included participants will

246   be tested at the hospital for both malaria and VL according to routine procedures: a finger prick blood

247   sample will be collected to prepare a thick and thin blood film for microscopic detection of malaria. VL

248   diagnosis will be done by means of an rk39 RDT for detection of VL antibodies in finger prick blood. In

249   case of a negative rk39 test, a direct agglutination test (DAT) will be performed to confirm or rule out

250   VL. If the DAT result is borderline, a spleen aspirate will be taken for parasitological diagnosis by

251   microscopy. All test outcomes, data on malaria parasitaemia (as determined by microscopy) and DAT

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252   titre will be recorded. Patients who test negative for both malaria and VL will be excluded from the

253   study.

254   The finger prick blood of each participant will also be used to measure the haemoglobin (Hb) level and

255   to prepare dried blood spots (DBS) on a filter paper card. These will be sent to the Amref Health

256   Laboratories in Nairobi, where they will be used for DNA extraction and confirmation of malaria and/or

257   VL diagnosis using real-time quantitative PCR (qPCR). This will allow for detection of low density

258   malaria/VL infections that might be missed by the point-of-care diagnostics, and for quantitation of

259   parasite densities.

260   According to the diagnosis outcome at the clinic, participants will be treated for their infection(s)

261   through the Kenyan national treatment programme for VL and malaria.[2, 51] Before treatment is

262   initiated, some patients will be asked to participate in the cohort study as well.

263   Case-control study

264   Directly after the participants of the cross-sectional study have received the first treatment for their

265   infection, a trained interviewer will administer a structured household questionnaire. Information

266   collected will include place of residence, housing conditions, house environment, occupation, sleeping

267   habits, night time activities and travel history. Participants below the age of 15 years may be assisted

268   in answering questions by their parent or legal guardian.

269   For each VL-infected patient case, two healthy controls will be recruited at the case's village of

270   residence. At the central point of the village, a household will be randomly selected by spinning a pen.

271   In the selected household, an individual, age- and sex-matched with the VL-infected case and meeting

272   the eligibility criteria, will be asked to participate. If multiple household members are eligible, one will

273   be selected by rolling a die. After providing informed consent, finger prick blood from the household

274   member will be tested with a malaria RDT and a VL rk39 RDT to exclude both infections. In case both

275   tests are negative, the structured household questionnaire will be administered to the healthy control,

276   or parent/legal guardian in case of children <15 years. Afterwards, a pen will be spun at the doorstep

277   of the house to select the next household where the second matched control will be recruited. All

278   procedures will be repeated until two healthy controls per VL-infected case have been recruited.

279   Cohort study

280   Subjects in the cross-sectional study with a laboratory confirmed infection with VL, *P. falciparum*

281   malaria or both will be asked to participate in the cohort study as well. After giving informed consent,

282   they will be monitored during the treatment of their infection(s). Treatment will be according to the

283   national treatment guidelines for both infections: for VL, this is sodium stibogluconate (SSG) injections

284   (20 mg/kg body weight/day) and paromomycin (PM) injections (15 mg/kg body weight/day) for 17

285   days;[2] for uncomplicated *P. falciparum* malaria, oral doses of 20 mg artemether and 120 mg

286   lumefantrine tablets, twice per day for three days (dosing adjusted by weight and age).[51] In case of

287   VL-malaria co-infection, malaria is treated first before initiation of VL treatment. From each

288   participant, 10 mL of peripheral venous blood (5 mL in a serum isolation tube, 5 mL in an EDTA

289   anticoagulation tube) will be collected prior to treatment initiation (day 0) and on the following time

290   points during their treatment:

291   -   In VL mono-infected patients, on day 7 of VL treatment and day 17 (end of VL treatment);

292   -   In malaria mono-infected patients, on day 1 of malaria treatment and day 3 (end of malaria

293   treatment);

294   -   In VL-malaria co-infected patients, on day 1 and day 3 of malaria treatment, and day 7 and day

295   17 of VL treatment.

296   At each follow-up time point, the patients' clinical features will be recorded on their CRF.

297   Healthy individuals and cases with asymptomatic VL or malaria will be recruited by a study team visiting

298   the households of the clinically ill participants of the cohort study. When a household member has no

299   history of VL or recent malaria and shows no symptoms of either disease, a finger prick blood sample

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300   will be taken for microscopic detection of malaria and for VL testing with DAT at Kacheliba Sub-County

301   Hospital. Based on the results of these tests, participants will be grouped either in the healthy control

302   cohort, the asymptomatic malaria cohort or the asymptomatic VL cohort. The study team will return

303   to the local communities to share the results with the respective participants. Participants that

304   complain of symptoms suggestive of VL and/or malaria at this stage will be referred for further

305   management and excluded from the study. If still without symptoms, participants will be physically

306   examined and 10 mL of venous blood (5 mL in a silicone-coated tube for separating the serum, 5 mL in

307   EDTA anticoagulation tube) will be collected. Healthy controls will only be sampled at this time.

308   Asymptomatically infected patients will be referred to Kacheliba Hospital for further management. If

309   placed on treatment, the asymptomatic patients will be sampled during their treatment, following the

310   same scheme as the clinically ill patients of the cohort study.

311   All collected venous blood samples will be processed at the Kacheliba Sub-County Hospital for isolation

312   of serum, white blood cell (WBC) counting using an automated blood cell counter and preparation of

313   DBS. DBS cards will be shipped to the Amref Laboratories, where they will be used for DNA isolation

314   and subsequent qPCR for *Plasmodium* and *Leishmania* detection and quantitation. Isolated serum

315   samples will be sent to the Amsterdam UMC and used in a Luminex-based assay, to measure levels of

316   pro- and anti-inflammatory cytokines that have been shown to play an important role in the immune

317   response against VL and/or malaria: TNF-α, IFN-γ, IL-1β, IL-2, IL-4, IL-10, IL-12p70, IL-13, IL-17A and IL-

318   22.[41, 52-58]

319   ## Statistical analysis

320   Cross-sectional study

321   All data collected from the cross-sectional study will be compared between VL mono-infected cases,

322   malaria mono-infected cases and VL-malaria co-infected cases. In a univariate analysis, the association

323   between a VL-malaria co-infection and measured characteristics will be explored using the Pearson

324   Chi-square test or the Fisher Exact Probability test. Continuous variables will be categorized into

325   predefined groups. Found associations will be quantified as prevalence odds ratios with 95%

326   confidence intervals, determined at the 5% level. To identify independent characteristics associated

327   with VL-malaria co-infection and adjust for confounding, a multivariate logistic regression model will

328   be made in a backward stepwise manner with variables that have a p-value <0.10 in the univariate

329   analyses. Only variables with a p-value <0.05 will be retained in the final model.

330   Case-control study

331   Data collected with the structured questionnaire will be used to identify household and environmental

332   risk factors associated with VL and malaria (co-)infections in West Pokot. VL and malaria infections will

333   be considered as two separate response variables, for which individual univariate logistic regression

334   analyses will be applied to evaluate associations (expressed as odds ratios) with the questionnaire

335   variables. Per predefined thematic section of the household questionnaire, variables with a p-value of

336   <0.2 in the univariate analysis will be included in a multivariate regression model. The same variables

337   will also be used as input for multivariate multiple response regression models, which will identify

338   predictors that jointly contribute to both VL and malaria infections and as such, co-infections. Both the

339   separate disease models and multiple response models of each section will be optimised through

340   stepwise backward elimination of variables with p>0.2. The retained significant variables of each

341   thematic section will then be merged into final multivariate regression models for VL, malaria and VL-

342   malaria co-infections, in which only significant (p<0.05) variables will be kept.

343   Cohort study

344   Cytokine levels and clinical characteristics measured at baseline (day 0) in VL-malaria co-infected

345   patients will be compared to those of VL or malaria mono-infected patients, either actively or

346   asymptomatically, and of healthy controls, who will provide immunological reference data.

347   Longitudinal comparison of cytokine levels will be performed within the separate groups that are

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348 followed up during treatment. For both baseline and longitudinal comparisons, standard parametrical

349 statistical tests will be used for normally distributed numeric data. Non-normal data will be analysed

350 using non-parametric tests. Comparison of nominal data will be done with the Chi-square test or

351 Fisher's Exact Probability test. For all statistical analyses, significance will be determined at the 5% level

352 (p-value<0.05). Correlations between the levels of individual cytokines will be investigated with

353 Spearman's rank correlation analysis.

Ethics and dissemination

355 The protocol of this study received ethical approval from the Amref Health Africa Ethics and Scientific

356 Review Committee (ref. ESRC P1196/2022). The ESRC is accredited by the Kenyan National Commission

357 for Science, Technology and Innovation (NACOSTI). A NACOSTI research license was obtained before

358 study initiation (ref. 791964).

359 Written informed consent will be collected from all participants, or their parents/legal guardians, for

360 study participation, export of clinical samples for analysis at the Amsterdam UMC and future use of

361 study data and samples. All collected data and clinical specimens will be anonymized and stored at the

362 Amsterdam UMC for at least 5 years after completion of the study. Dataset will be available upon

363 reasonable request to the corresponding author. None of the results of the study will be published

364 with individual name identification or with identifiers of patients.

365 All study findings will be communicated to the national health authorities of Kenya. The research team

366 will write scientific papers on the study results, which will be submitted to open-access, peer-reviewed

367 international scientific journals and presented at national and international scientific meetings.

Patient and public involvement

369 Due to the remote setting in which this study will be conducted, it was not possible to involve the local

370 public of West Pokot in the design phase of the study. However, during study implementation,

371 awareness among local communities will be achieved by involvement of community leaders and the

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372   patients recruited at the hospital. Community health workers will be approached to assist with the

373   recruitment of asymptomatic patients and healthy controls in local villages. Patients, local health care

374   staff and the public will be consulted to select an appropriate method for dissemination of the study

375   findings among the community.

376   # DISCUSSION

377   With a cross-sectional study, a case-control study and a cohort study, this observational research

378   project will apply a multifaceted approach to address important knowledge gaps concerning the

379   clinical implications, environmental risk factors and immunology of VL-malaria co-infections in West

380   Pokot. The significance of these studies is underlined by the fact that concomitant VL and malaria

381   infections are still largely neglected, despite the apparently high rates of this condition in West

382   Pokot.[3, 4, 6] This research will contribute to increased awareness among the local population of West

383   Pokot, its healthcare workers and disease control policy makers. This may lead to more timely

384   detection and treatment of VL-malaria co-infections, thereby reducing associated morbidity and

385   mortality.

386   This will be the first study to describe the parasite dynamics and cytokine responses of VL-malaria co-

387   infection in a longitudinal fashion. This approach will allow investigating associations of the

388   immunological profile of a VL-malaria co-infection with its clinical picture. Although cytokine levels

389   only partly reflect the full scope of the immune response mounted against a VL-malaria co-infection,

390   findings from this explorative study will generate an evidential basis to direct future research into co-

391   infection immunology. Eventually, better understanding of the immunology of VL-malaria co-infections

392   will help improve clinical management and support the development of official treatment guidelines.

393   The questionnaire study will generate critical data on individual, household and environmental factors

394   that may increase the risk of co-acquiring VL and malaria. In this way, the results of the case-control

395   study can guide a more targeted approach to control and elimination of both infections in the Pokot

396 area. This translational step will be facilitated by the involvement of the Kenyan MoH in the research

397 project. Although this study is focusing on VL-malaria co-infections in West Pokot, its results may

398 provide valuable insights for other co-endemic areas as well.

AUTHOR CONTRIBUTIONS

400 NvD was responsible for the instigation of this research project, developed the protocol and drafted

401 the manuscript. JC, PM and HS contributed to the study design and protocol development, and

402 critically read the manuscript. WO provided national surveillance and healthcare data on VL and

403 malaria in Kenya and critically read the protocol and manuscript. All authors approved the final version

404 of the manuscript.

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COMPETING INTERESTS

409 The authors declare that they have no competing interests.

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412 current incidence and clinical epidemiology of VL, malaria and co-infections in the catchment area of

413 Kacheliba Hospital.

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26

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation	Page No
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	6
Methods			
Study design	4	Present key elements of study design early in the paper	7
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	8
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	9
		Case-control study—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls	9
		Cross-sectional study—Give the eligibility criteria, and the sources and methods of selection of participants	8
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed	9
		Case-control study—For matched studies, give matching criteria and the number of controls per case	9
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	11-14
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	11-14
Bias	9	Describe any efforts to address potential sources of bias	9-10
Study size	10	Explain how the study size was arrived at	10
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	14-16
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	14-16
		(*b*) Describe any methods used to examine subgroups and interactions	14-16
		(*c*) Explain how missing data were addressed	N.A.
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed	N.A.
		Case-control study—If applicable, explain how matching of cases and controls was addressed	12
		Cross-sectional study—If applicable, describe analytical methods taking account of sampling strategy	14
		(*e*) Describe any sensitivity analyses	N.A.

Continued on next page

Results

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	N.A.
		(b) Give reasons for non-participation at each stage	N.A.
		(c) Consider use of a flow diagram	N.A.
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	N.A.
		(b) Indicate number of participants with missing data for each variable of interest	N.A.
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	N.A.
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	N.A.
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	N.A.
		Cross-sectional study—Report numbers of outcome events or summary measures	N.A.
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	N.A.
		(*b*) Report category boundaries when continuous variables were categorized	N.A.
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	N.A.
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	N.A.

Discussion

Key results	18	Summarise key results with reference to study objectives	17
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	17
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	N.A.
Generalisability	21	Discuss the generalisability (external validity) of the study results	17

Other information

Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	18

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

BMJ Open

Protocol for an observational study on the clinical features, immunological interactions and household determinants of visceral leishmaniasis and malaria co-infections in West Pokot, Kenya

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Primary Subject Heading:	Infectious diseases
Secondary Subject Heading:	Infectious diseases, Immunology (including allergy), Epidemiology
Keywords:	PARASITOLOGY, MICROBIOLOGY, Epidemiology < INFECTIOUS DISEASES, Immunology < TROPICAL MEDICINE

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Protocol for an observational study on the clinical features,

immunological interactions and household determinants of visceral

leishmaniasis and malaria co-infections in West Pokot, Kenya

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1

Abstract

Introduction: Visceral leishmaniasis (VL) and malaria are two deadly parasitic diseases that co-exist in West Pokot County, Kenya. The local population is at considerable risk of co-infection with VL and malaria, however, few studies have described the clinical implications of this co-morbidity. Questions remain regarding the immune responses responsible for possible predisposing or protective effects. Moreover, characterisation of environmental and household risk factors for co-acquiring VL and malaria is warranted to increase awareness and guide implementation of targeted control strategies. This protocol intends to address these knowledge gaps concerning VL-malaria co-infections.

Methods and analysis: this observational research project will have a multimethod approach, starting with a cross-sectional study at Kacheliba Sub-County Hospital in West Pokot, Kenya. Patients with laboratory confirmation of a VL and/or malaria infection will be clinically assessed and symptomatology of mono- and co-infections will be compared. Secondly, a questionnaire will be addressed to all included patients and to healthy controls in local communities. This case-control study will aim to describe household and environmental determinants associated with VL-malaria co-infection. Lastly, blood samples will be collected from a small cohort of VL and malaria mono- and co-infected patients during treatment of their infection(s), and from healthy controls and asymptomatic VL and malaria cases recruited in local communities. These specimens will be used for serum cytokine measurements and molecular quantitation of *Plasmodium* and *Leishmania*. In this way, the immune response and parasite dynamics during VL-malaria co-infection will be characterised longitudinally and compared to those observed in clinical and asymptomatic mono-infections.

Ethics and Dissemination: Ethical approval was obtained from the Ethics and Scientific Research Committee of Amref Health Africa. The study findings will be presented at international conferences and published in open-access, peer-reviewed journals.

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44   **Study Registration:** This study protocol has been registered at the ISRCTN registry (ID:

45   ISRCTN15023306).

46   **Key Words:** Visceral Leishmaniasis, Malaria, Coinfection, Study Protocol

Strengths and limitations of this study

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48   • Through a tripartite design, this research project will address clinical, immunological and

49   epidemiological knowledge gaps concerning VL-malaria co-infections.

50   • This will be the first study to investigate individual and household risk factors for VL-malaria

51   co-infections in West Pokot, Kenya.

52   • Longitudinal characterisation of cytokine profiles in VL-malaria co-infections and comparison

53   with both symptomatic and asymptomatic mono-infections will offer the opportunity to study

54   associations between the immune response, parasite densities and clinical presentation.

55   • Given the lack of recent data on VL-malaria co-infection rates in West Pokot, the number of

56   co-infected cases recruited in this study could potentially be low.

57   • Measuring cytokine levels will not reflect the full extent of the immune response induced by a

58   VL-malaria co-infection.

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INTRODUCTION

Visceral leishmaniasis (VL) and malaria (caused by *Leishmania* and *Plasmodium* species, respectively) are two vector-borne protozoan parasite infections that cause high morbidity and mortality, particularly in remote regions of low income countries. In East Africa, Kenya is one of the countries most affected by VL.[1] Here, an important focus endemic VL transmission is located in West Pokot County, which is part of the Pokot territory situated at the border region between Kenya and Uganda.[2-4] Between 2018 and 2021, annual numbers of reported VL cases in West Pokot varied from 250 to 450, which is likely to be an underestimation of the actual incidence of this neglected disease. (unpublished data, patient records from Kacheliba Sub-County Hospital, West Pokot County, Kenya) Apart from being endemic for VL, this area is also characterised by recurrent outbreaks of seasonal malaria, with 40,000 confirmed *Plasmodium falciparum* cases in 2020 (data from Kenya Ministry of Health, 2023).[5] Due to the overlapping epidemiology of VL and malaria in the Pokot region, the local population is at risk of being infected with both diseases concurrently. Indeed, it appears that co-infections with *Leishmania donovani* and *P. falciparum* are not uncommon: studies among VL patients attending the regional VL treatment hospitals of Kacheliba (Kenya) and Amudat (Uganda) have reported rates of concomitant malaria ranging from 3.8% to 34.4%.[3, 4, 6] Despite these apparently high numbers of VL-malaria co-infections, the condition is still understudied in terms of risk factors, clinical presentation and immunology.

The overlap of VL and malaria transmission in West Pokot relies on the presence of favourable environmental conditions for their insect vectors, and subsequently, human exposure to these vectors. The local malaria mosquito vectors, *Anopheles arabiensis* and *An. funestus*, have a preference for dry savannah habitats where they lay eggs in small, temporary freshwater pools.[7, 8] As such, malaria incidence in West Pokot often peaks during and after seasonal rainfall. The individual malaria risk may vary from person to person due to household factors: house structure aspects have been associated with indoor *Anopheles* abundance in neighbouring Baringo County.[9] It is unknown whether these

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85   results are also applicable in the context of West Pokot. Like malaria, the endemicity of VL in West

86   Pokot is partly attributable to its semi-arid climate. However, the ecology of the local VL vector is

87   substantially different, as the sandfly *Phlebotomus martini* is believed to lay its eggs in the ventilation

88   shafts of termite mounds.[10-13] Some studies have therefore found living close to these mounds to

89   be associated with VL infection risk.[14, 15] Considering the differences in VL and malaria vector

90   ecology in West Pokot, a very specific combination of human behavioural, environmental and

91   household conditions may predispose for concomitant infections with both parasites. Better

92   understanding of this VL-malaria co-infection risk profile is crucial for increasing awareness among

93   exposed populations, and could also guide policy makers in drafting more focused VL and malaria

94   vector control strategies.

95   Despite the potentially deadly outcome of VL and malaria mono-infections, much remains unknown

96   about the clinical consequences when both these infections occur in one individual. Only a handful of

97   case reports have described the symptomatology of VL-malaria co-infections, and larger scale studies

98   have shown contradictory results.[6, 16-26] A case-control analysis of hospitalised VL patients in

99   Amudat Hospital found that co-occurring malaria did not clearly exacerbate the clinical picture of VL,

100   and correlated with a lower frequency of anaemia.[6] On the other hand, a study in Sudan in patients

101   with VL-malaria co-infection revealed an increased frequency of anaemia, emaciation and jaundice,

102   compared with their VL mono-infected counterparts.[23] As neither of these studies included a control

103   group of malaria mono-infected patients, it was not studied how malaria symptomatology is affected

104   by a co-occurring VL infection. Hence, additional research into the clinical interactions seen in VL-

105   malaria co-infections is warranted to improve recognition and management of this condition.

106   Beneath the clinical features of a VL-malaria co-infection lie the pathophysiological processes and

107   immune responses elicited by the infecting *Leishmania* and *Plasmodium* parasites, which have both

108   developed mechanisms to evade host immunity and alter it to their advantage.[27-29] During a VL-

109   malaria co-infection, *Leishmania* and *Plasmodium* parasites will simultaneously modulate the host

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110 immune response, which may have an effect on the control or progression of the concomitant disease.

111 Such mechanisms are well known for people living with HIV, but have also been described for

112 conditions of polyparasitism, such as helminth co-infections in malaria and *Leishmania* patients.[30-

113 35] So far, there has been limited research into the parasitological and immunological dynamics during

114 VL-malaria co-infections. Results from animal models have shown both aggravating and mitigating

115 effects of the two diseases upon each other.[36-40] To date, there has only been a single study looking

116 at the immunology of VL-malaria co-infections in humans: Van den Bogaart *et al.* compared the

117 cytokine profiles of VL and malaria mono- and co-infected patients in Sudan and found that the

118 immune response during a co-infection was mainly characterised by the release of pro-inflammatory

119 cytokines and reflected features of the responses seen in both mono-infections.[41] Interestingly, high

120 levels of the pro-inflammatory cytokine IL-17A distinguished co-infected patients from both mono-

121 infected groups, suggesting a synergistic interaction of the two diseases. The same study also found a

122 significantly lower *Plasmodium* parasitaemia in VL-malaria co-infected patients compared to malaria

123 mono-infections. As the interpretation of these study results is limited by their cross-sectional nature,

124 longitudinal assessment of patients with VL-malaria co-infections and comparison with mono-infected

125 patients (both clinical and asymptomatic) is required to unravel the associations between the immune

126 response, parasite loads and clinical features.

127 To address the knowledge gaps in our understanding of VL-malaria co-infections, this paper describes

128 the protocol of an observational research project aimed at characterising VL-malaria co-infections in

129 West Pokot on three different levels: symptomatology, epidemiology and immunology. These aspects

130 will be studied respectively by means of a cross-sectional study, a case-control study and a cohort

131 study. The research project will be conducted at Kacheliba Sub-County Hospital in West Pokot through

132 a collaboration between the Amsterdam University Medical Centres, Amref Health Africa and the

133 Kenya Ministry of Health (MoH).

134   ## Study objectives

135   The following study objectives have been formulated for this research project:

136   -   To determine the prevalence of VL and malaria co-infections among patients suspected with

137   either infection attending Kacheliba Sub-County Hospital, West Pokot, Kenya.

138   -   To identify and compare clinical features and parasitaemias of patients with malaria and VL

139   mono- and co-infections and establish whether a co-infection exacerbates or alleviates

140   symptoms of both diseases.

141   -   To identify individual and household level determinants of VL-malaria co-infections in West

142   Pokot, Kenya.

143   -   To examine and compare the cytokine response in patients with VL and malaria mono- and co-

144   infections (both clinical and asymptomatic), before, during and after treatment, and determine

145   whether these cytokine responses can be related to the (sub)clinical presentation of the

146   infection(s).

147   # METHODS

148   ## Study design

149   To address the different study objectives, this research project will consist of three components: a

150   prospective, hospital-based cross-sectional study among patients; a case-control study among hospital

151   patients and healthy volunteers in local communities; and a prospective cohort study among hospital

152   patients and healthy and asymptomatically infected household members of these patients.

153   For the prospective cross-sectional study, patients attending Kacheliba Sub-County Hospital in West

154   Pokot, Kenya, with clinical suspicion of malaria and/or VL infection will be asked to participate.

155   Laboratory diagnostic tests will be performed for both malaria and VL in consenting patients. A patient

156   will remain included in the study if positive for one or both infections. Clinical and parasitological data

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157 will be collected from these study subjects and compared between VL-malaria co-infected cases and

158 patients with VL and malaria mono-infections.

159 Participants of the cross-sectional study will also serve as cases in the case-control study, to whom a

160 structured household questionnaire will be administered. Exposure to certain individual and

161 household factors will be compared between mono- and co-infected patients. Additionally, per VL-

162 infected case, two age- and sex-matched healthy controls living in the same village as the case will be

163 recruited and administered the questionnaire as well.

164 Lastly, a small cohort of subjects of the cross-sectional study with confirmed VL and/or malaria

165 infection will be followed up during standard treatment. This cohort study will entail repeated

166 collection of venous blood samples from participating patients, to characterise their immunological

167 profiles over time. Additionally, blood samples will also be collected from healthy individuals and

168 asymptomatic VL/malaria cases, who will be actively recruited in the households of the patient cohort.

169 The healthy individuals will provide immunological baseline data, whereas the immunological profiles

170 of asymptomatic VL/malaria cases will be compared to those of patients with active clinical disease.

171 Healthy and asymptomatic subjects will be sampled once upon inclusion into the study. In case

172 asymptomatic cases require treatment for their VL and/or malaria infection, they will also be sampled

173 several times during this treatment.

Study site and timing

175 The research will be performed in West Pokot County in Kenya (Figure 1), an area that is endemic for

176 VL year-round and has seasonal transmission of malaria. Previous studies have reported that VL-

177 malaria co-infections occur in the Pokot region.[3, 4, 6] Participants will be recruited from the

178 catchment area of the Kacheliba Sub-County Hospital, which is a government hospital located about

179 30 km northwest of West Pokot's county capital, Kapenguria. It is an important regional reference

180 centre for VL diagnosis and treatment, supported by Drugs for Neglected Diseases Initiative (DNDi).[42]

181 The study will be conducted in October and November 2022. This two-month period coincides with

182 the short rainy season (October – December) during which malaria incidence often peaks.[43]

Study population

184 The population of the cross-sectional study will comprise individuals who attend the Kacheliba Sub-

185 County Hospital and are clinically suspected of an infection with VL and/or malaria. The study

186 participants will be grouped according to their VL and malaria diagnosis, as determined by routine

187 diagnostic procedures:

188   - Newly diagnosed patients with active primary VL, defined as patients with clinical symptoms

189   such as prolonged fever (>2 weeks), splenomegaly, weakness or wasting, with either a positive

190   rk39 rapid diagnostic test (RDT), positive Direct Agglutination Test (DAT titre ≥ 1:3200) and/or

191   microscopy-positive spleen aspirate.

192   - Patients with uncomplicated malaria, defined as patients with fever or history of fever within

193   the last 48 hours (with or without other symptoms) and a positive thick and thin blood film for

194   *Plasmodium*, with a parasite count <250,000/µL of blood.

195   - Patients co-infected with malaria and primary VL (actively for one or both infections) defined

196   as patients with symptoms of VL and/or malaria, with a positive *Plasmodium* blood film

197   (parasite count <250,000/µL of blood) and positive VL diagnostic test (rk39 RDT, DAT, spleen

198   aspirate).

199 All subjects of the cross-sectional study with laboratory-confirmed VL and/or malaria infection will also

200 be included as cases in the case-control study. Two age- and sex-matched healthy controls per VL-

201 infected case (including those co-infected with malaria) will be recruited in the case's village of

202 residence and will be defined as individuals without current signs or symptoms of VL or malaria, no

203 history of VL, no malaria in the preceding two weeks, and with a negative rk39 RDT and negative

204 malaria RDT. The individual should have lived in their current house for at least one year.

205   Clinical subjects of the cohort study will be recruited among the mono- and co-infected participants of

206   the cross-sectional study. Only malaria infections with *P. falciparum* will be eligible, and the patient

207   must be aged between 6 and 30 years old. These age limits are set to exclude infants and children

208   whose immune system has not yet fully developed, and patients above 30 years who are more likely

209   to have developed a significant level of acquired immunity to malaria.[6, 44] The cohort study will also

210   recruit healthy controls and asymptomatic cases in the households of the clinically ill participants. This

211   recruitment strategy will minimise the variability of environmental confounders between the different

212   study groups. Moreover, the likelihood of finding asymptomatic VL and malaria infections will be

213   higher in households of symptomatic patients.[45-49] The healthy and asymptomatic cohorts are

214   defined as follows:

215   -   Healthy endemic controls, defined as individuals above the age of 6 years, without current

216   signs or symptoms of VL or malaria, with no self-reported history of VL, no malaria in the

217   preceding two weeks, with a negative DAT test (DAT titre ≤ 1:200) and negative malaria blood

218   films.

219   -   Patients with asymptomatic VL, defined as individuals above the age of 6 years, without VL-

220   associated symptoms for at least 15 days before study inclusion and no self-reported history

221   of active VL, with a positive DAT (DAT titre ≥ 1:3200).

222   -   Patients with asymptomatic malaria, defined as individuals above the age of 6 years, with no

223   symptoms suggestive of malaria at the time of inclusion and with no history of malaria in the

224   preceding two weeks, with a positive thick and thin blood film for *P. falciparum.*

225   A complete overview of all eligibility criteria for the different study components can be found in

226   Supplemental table 1.

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Sample size

228 The cross-sectional study aims to include 244 malaria infected patients, allowing to detect an odds

229 ratio (OR) of 1.8 at a confidence level of 95% (two-sided), with an expected 20% of exposure

230 among VL-infected cases and a power of 80%.[50] Within the two-month time frame of the study,

231 all patients at the study hospital with a confirmed VL infection will be included. Considering that 350

232 VL cases were reported at Kacheliba Sub-County Hospital in the first five months of 2022 (personal

233 communication with David Kiptanui, clinical officer at Kacheliba Sub-County Hospital, 2022), this

234 pragmatic approach is expected to recruit approximately 140 VL patients. Among these subjects,

235 approximately 5 to 30 cases are expected to be co-infected with malaria, based on previously reported

236 co-infection rates in Kacheliba Hospital ranging from 3.8% to 21.4%.[4]

237 All participants of the cross-sectional study will also be included in the case-control study. Additionally,

238 two healthy controls will be recruited per VL-infected patient, meaning that the study will aim to

239 include approximately 280 healthy controls.

240 Given the explorative nature of the immunological cohort study, the population size per study group

241 will be set at 20 to 30 subjects, depending on their availability. Based on the results of Van den Bogaart

242 *et al.* about cytokine levels in VL-malaria co-infected patients, this group size should be sufficient to

243 detect significant differences in immunological parameters with 80% power at 5% level of statistical

244 significance.[41]

Clinical sample and data collection

Cross-sectional study

247 Patients presenting at the study hospital with clinical signs and/or symptoms indicating a potential

248 malaria and/or VL infection will be asked to participate in the study if they meet the inclusion criteria

249 (Supplemental table 1). Patients not willing to participate in the research will be excluded from the

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250 study and will be referred to the clinician for usual diagnosis and treatment. Patients that give their

251 informed consent will be included and tested at the hospital for both malaria and VL according to

252 routine procedures: a finger prick blood sample will be collected to prepare a thick and thin blood film

253 for microscopic detection of malaria. VL diagnosis will be done by means of an rk39 RDT for detection

254 of VL antibodies in finger prick blood. In case of a negative rk39 test, a direct agglutination test (DAT)

255 will be performed to confirm or rule out VL. If the DAT result is borderline, a spleen aspirate will be

256 taken for parasitological diagnosis by microscopy. All test outcomes, data on malaria parasitaemia (as

257 determined by microscopy) and DAT titre will be recorded on a case report from (CRF).

258 Blood from the diagnostic finger prick of each VL and/or malaria suspected participant will also be used

259 to measure the haemoglobin (Hb) level and to prepare dried blood spots (DBS) on a filter paper card.

260 These will later be sent to the Amref Health Laboratories in Nairobi, where they will be used for DNA

261 extraction with QIAamp DNA Mini Kit (Qiagen, Hilden, DE). DNA isolates will be tested with real-time

262 quantitative PCR (qPCR) for malaria and VL, using an 18s rRNA gene target for *P. falciparum* and

263 kinetoplast DNA (kDNA) target for *Leishmania,* respectively.[51, 52] This will allow for detection of low

264 density malaria/VL infections that might be missed by the point-of-care diagnostics, and for

265 quantitation of parasite densities.

266 For patients with a confirmed VL and/or malaria infection, according to the diagnostic testing at the

267 recruitment hospital in Kacheliba, clinical features and medical history will be recorded on their CRF,

268 while those who test negative for both malaria and VL will be excluded from further study procedures.

269 According to the diagnosis outcome at the clinic, participants will be treated for their infection(s)

270 through the Kenyan national treatment programme for VL and malaria.[2, 53] Before treatment is

271 initiated, some patients will be asked to participate in the cohort study as well.

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Case-control study

272   Case-control study

273   Directly after the participants of the cross-sectional study have received the first treatment for their

274   infection, a trained interviewer will administer a structured household questionnaire. Information

275   collected will include place of residence, housing conditions, house environment, occupation, sleeping

276   habits, night time activities and travel history. Participants below the age of 15 years may be assisted

277   in answering questions by their parent or legal guardian.

278   For each VL-infected patient case, two healthy controls will be recruited at the case's village of

279   residence. At the central point of the village, a household will be randomly selected by spinning a pen.

280   In the selected household, an individual, age- and sex-matched with the VL-infected case and meeting

281   the eligibility criteria (Supplemental table 1), will be asked to participate. If multiple household

282   members are eligible, one will be selected by rolling a die. After providing informed consent, finger

283   prick blood from the household member will be tested with a malaria RDT and a VL rk39 RDT to exclude

284   both infections. In case both tests are negative, the structured household questionnaire will be

285   administered to the healthy control, or parent/legal guardian in case of children <15 years. Afterwards,

286   a pen will be spun at the doorstep of the house to select the next household where the second matched

287   control will be recruited. All procedures will be repeated until two healthy controls per VL-infected

288   case have been recruited.

Cohort study

289   Cohort study

290   Subjects in the cross-sectional study with a laboratory confirmed infection with VL, *P. falciparum*

291   malaria or both, and meeting all eligibility criteria (Supplemental table 1), will be asked to participate

292   in the cohort study as well. After giving informed consent, they will be monitored during the treatment

293   of their infection(s). Treatment will be according to the national treatment guidelines for both

294   infections: for VL, this is sodium stibogluconate (SSG) injections (20 mg/kg body weight/day) and

295   paromomycin (PM) injections (15 mg/kg body weight/day) for 17 days;[2] for uncomplicated *P.*

296 *falciparum* malaria, oral doses of 20 mg artemether and 120 mg lumefantrine tablets, twice per day

297 for three days (dosing adjusted by weight and age).[53] In case of VL-malaria co-infection, malaria is

298 treated first before initiation of VL treatment. From each participant, 10 mL of peripheral venous blood

299 (5 mL in a serum isolation tube, 5 mL in an EDTA anticoagulation tube) will be collected prior to

300 treatment initiation (day 0) and on the following time points during their treatment:

301 - In VL mono-infected patients, on day 7 of VL treatment and day 17 (end of VL treatment);

302 - In malaria mono-infected patients, on day 1 of malaria treatment and day 3 (end of malaria

303   treatment);

304 - In VL-malaria co-infected patients, on day 1 and day 3 of malaria treatment, and day 7 and day

305   17 of VL treatment.

306 At each follow-up time point, the patients' clinical features will be recorded on their CRF.

307 Healthy individuals and cases with asymptomatic VL or malaria will be recruited by a study team visiting

308 the households of the clinically ill participants of the cohort study. When a household member has no

309 history of VL or recent malaria and shows no symptoms of either disease (see eligibility criteria in

310 Supplemental table 1), a finger prick blood sample will be taken for microscopic detection of malaria

311 and for VL testing with DAT at Kacheliba Sub-County Hospital. Based on the results of these tests,

312 participants will be grouped either in the healthy control cohort, the asymptomatic malaria cohort or

313 the asymptomatic VL cohort. The study team will return to the local communities to share the results

314 with the respective participants. Participants that complain of symptoms suggestive of VL and/or

315 malaria at this stage will be referred for further management and excluded from the study. If still

316 without symptoms, participants will be physically examined and 10 mL of venous blood (5 mL in a

317 silicone-coated tube for separating the serum, 5 mL in EDTA anticoagulation tube) will be collected.

318 Healthy controls will only be sampled at this time. Asymptomatically infected patients will be referred

319 to Kacheliba Hospital for further management. If placed on treatment, the asymptomatic patients will

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320 be sampled during their treatment, following the same scheme as the clinically ill patients of the cohort

321 study.

322 All collected venous blood samples will be processed at the Kacheliba Sub-County Hospital for isolation

323 of serum, white blood cell (WBC) counting using an Ac-T diff Hematology Analyzer (Beckman Coulter,

324 Brea, CA) and preparation of DBS. DBS cards will be shipped to the Amref Laboratories, where they will

325 be used for nucleic acid isolation and subsequent *Leishmania* and *P. falciparum* detection and

326 quantitation, using qPCR for *Leishmania* kDNA, and real-time quantitative nucleic acid sequence-based

327 amplification (QT-NASBA) for *P. falciparum* 18s rRNA .[52, 54] Isolated serum samples will be sent to

328 the Amsterdam UMC and used in a Luminex-based assay, to measure levels of pro- and anti-

329 inflammatory cytokines that have been shown to play an important role in the immune response

330 against VL and/or malaria: TNF-α, IFN-γ, IL-1β, IL-2, IL-4, IL-10, IL-12p70, IL-13, IL-17A and IL-22.[41, 55-

331 61]

Statistical analysis

Cross-sectional study

334 All data collected from the cross-sectional study will be compared between VL mono-infected cases,

335 malaria mono-infected cases and VL-malaria co-infected cases. In a univariate analysis, the association

336 between a VL-malaria co-infection and measured characteristics will be explored using the Pearson

337 Chi-square test or the Fisher Exact Probability test. Continuous variables will be categorized into

338 predefined groups. Found associations will be quantified as prevalence odds ratios with 95%

339 confidence intervals, determined at the 5% level. To identify independent characteristics associated

340 with VL-malaria co-infection and adjust for confounding, a multivariate logistic regression model will

341 be made in a backward stepwise manner with variables that have a p-value <0.10 in the univariate

342 analyses. Only variables with a p-value <0.05 will be retained in the final model.

Case-control study

344 Data collected with the structured questionnaire will be used to identify household and environmental

345 risk factors associated with VL and malaria (co-)infections in West Pokot. VL and malaria infections will

346 be considered as two separate response variables, for which individual univariate logistic regression

347 analyses will be applied to evaluate associations (expressed as odds ratios) with the questionnaire

348 variables. Per predefined thematic section of the household questionnaire, variables with a p-value of

349 <0.2 in the univariate analysis will be included in a multivariate regression model. The same variables

350 will also be used as input for multivariate multiple response regression models, which will identify

351 predictors that jointly contribute to both VL and malaria infections and as such, co-infections. Both the

352 separate disease models and multiple response models of each section will be optimised through

353 stepwise backward elimination of variables with $p > 0.2$. The retained significant variables of each

354 thematic section will then be merged into final multivariate regression models for VL, malaria and VL-

355 malaria co-infections, in which only significant ($p < 0.05$) variables will be kept.

Cohort study

357 Cytokine levels and clinical characteristics measured at baseline (day 0) in VL-malaria co-infected

358 patients will be compared to those of VL or malaria mono-infected patients, either actively or

359 asymptomatically, and of healthy controls, who will provide immunological reference data.

360 Longitudinal comparison of cytokine levels will be performed within the separate groups that are

361 followed up during treatment. For both baseline and longitudinal comparisons, standard parametrical

362 statistical tests will be used for normally distributed numeric data. Non-normal data will be analysed

363 using non-parametric tests. Comparison of nominal data will be done with the Chi-square test or

364 Fisher's Exact Probability test. For all statistical analyses, significance will be determined at the 5% level

365 (p-value<0.05). Correlations between the levels of individual cytokines will be investigated with

366 Spearman's rank correlation analysis.

Ethics and dissemination

The protocol of this study received ethical approval from the Amref Health Africa Ethics and Scientific Review Committee (ref. ESRC P1196/2022). The ESRC is accredited by the Kenyan National Commission for Science, Technology and Innovation (NACOSTI). A NACOSTI research license was obtained before study initiation (ref. 791964).

Written informed consent will be collected from all participants, or their parents/legal guardians, for study participation, export of clinical samples for analysis at the Amsterdam UMC and future use of study data and samples. All collected data and clinical specimens will be anonymized and stored at the Amsterdam UMC for at least 5 years after completion of the study. Dataset will be available upon reasonable request to the corresponding author. None of the results of the study will be published with individual name identification or with identifiers of patients.

All study findings will be communicated to the national health authorities of Kenya. The research team will write scientific papers on the study results, which will be submitted to open-access, peer-reviewed international scientific journals and presented at national and international scientific meetings.

Patient and public involvement

Due to the remote setting in which this study will be conducted, it was not possible to involve the local public of West Pokot in the design phase of the study. However, during study implementation, awareness among local communities will be achieved by involvement of community leaders and the patients recruited at the hospital. Community health workers will be approached to assist with the recruitment of asymptomatic patients and healthy controls in local villages. Patients, local health care staff and the public will be consulted to select an appropriate method for dissemination of the study findings among the community.

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DISCUSSION

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390 With a cross-sectional study, a case-control study and a cohort study, this observational research

391 project will apply a multifaceted approach to address important knowledge gaps concerning the

392 clinical implications, environmental risk factors and immunology of VL-malaria co-infections in West

393 Pokot. The significance of these studies is underlined by the fact that concomitant VL and malaria

394 infections are still largely neglected, despite the apparently high rates of this condition in West

395 Pokot.[3, 4, 6] This research will contribute to increased awareness among the local population of West

396 Pokot, its healthcare workers and disease control policy makers. This may lead to more timely

397 detection and treatment of VL-malaria co-infections, thereby reducing associated morbidity and

398 mortality.

399 This will be the first study to describe the parasite dynamics and cytokine responses of VL-malaria co-

400 infection in a longitudinal fashion. This approach will allow investigating associations of the

401 immunological profile of a VL-malaria co-infection with its clinical picture. It should be mentioned that

402 the design of the immunological cohort study is restricted by the limited available resources in the

403 remote setting of the study hospital. For example, participants will not be screened for other

404 underlying (infectious) conditions, such as HIV or helminthiasis, which are known to significantly

405 impact the host's immune response. Furthermore, this study will not isolate patient peripheral blood

406 mononuclear cells to investigate leukocyte dynamics underlying the observed cytokine responses.

407 Nevertheless, findings from this explorative study will generate an evidential basis to direct future

408 research into co-infection immunology. Eventually, better understanding of the immunology of VL-

409 malaria co-infections will help improve clinical management and support the development of official

410 treatment guidelines.

411 The questionnaire study will generate critical data on individual, household and environmental factors

412 that may increase the risk of co-acquiring VL and malaria. In this way, the results of the case-control

413   study can guide a more targeted approach to control and elimination of both infections in the Pokot

414   area. This translational step will be facilitated by the involvement of the Kenyan MoH in the research

415   project. Although this study is focusing on VL-malaria co-infections in West Pokot, its results may

416   provide valuable insights for other co-endemic areas as well.

417   # LIST OF ABBREVIATIONS

418   **Amsterdam UMC**: Amsterdam University Medical Centres

419   **CRF**: Case report form

420   **DAT**: Direct agglutination test

421   **DBS:** Dried blood spot

422   **DNA**: Deoxyribonucleic acid

423   **DNDi**: Drugs for Neglected Diseases initiative

424   **EDTA**: Ethylenediaminetetraacetic acid

425   **ESRC**: Ethics and Scientific Review Committee

426   **Hb**: Haemoglobin

427   **HIV**: Human immunodeficiency virus

428   **IFN**: Interferon

429   **IL**: Interleukin

430   **kDNA**: Kinetoplast DNA

431   **MoH**: Ministry of Health

432   **NACOSTI**: National Commission for Science, Technology and Innovation

433   **OR**: Odds ratio

434   **PCR**: Polymerase chain reaction

435   **PM**: Paromomycin

436   **qPCR**: Real-time quantitative PCR

437   **QT-NASBA**: Quantitative nucleic acid sequence-based amplification

438   **RDT**: Rapid diagnostic test

439   **rRNA**: Ribosomal ribonucleic acid

440   **SSG**: Sodium stibogluconate

19

441 **TNF**: Tumour necrosis factor

442 **VL**: Visceral leishmaniasis

443 **WBC**: White blood cell

444

AUTHOR CONTRIBUTIONS

446 NvD was responsible for the instigation of this research project, developed the protocol and drafted

447 the manuscript. JC, PM and HS contributed to the study design and protocol development, and

448 critically read the manuscript. WO provided national surveillance and healthcare data on VL and

449 malaria in Kenya and critically read the protocol and manuscript. All authors approved the final version

450 of the manuscript.

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COMPETING INTERESTS

455 The authors declare that they have no competing interests.

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648   ## FIGURE LEGENDS

649   **Figure 1: Map of West Pokot County, Kenya, indicating the study site in Kacheliba and the county**
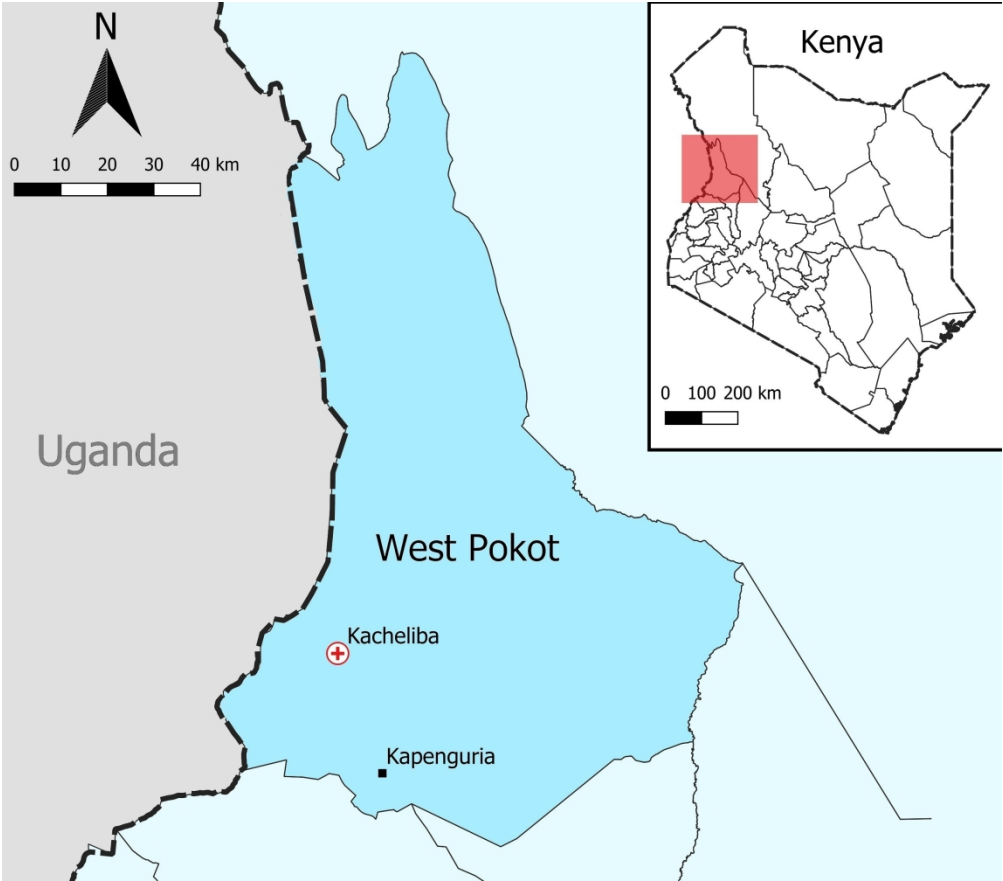
650   **capital Kapenguria.**

Figure 1: Map of West Pokot County, Kenya, indicating the study site in Kacheliba and the county capital Kapenguria.

597x524mm (120 x 120 DPI)

Supplemental table 1: Eligibility criteria for the different study components

Cross-sectional study
Inclusion criteria - Showing symptoms suggestive of malaria and/or VL - Living in the catchment area of the study hospital *Exclusion criteria* - Already under drug treatment for malaria and/or VL - Having a positive VL diagnosis in their medical history - Pregnant
Case-control study: patient cases
Inclusion criteria - Included in the cross-sectional study - Having a laboratory-confirmed VL diagnosis and/or malaria diagnosis *Exclusion criteria* None
Case-control study: healthy controls
Inclusion criteria - Living in the village of residence of a VL-infected participant of the cross-sectional study - Living in the current house for at least 1 year - Not showing symptoms suggestive of malaria and/or VL - Negative for malaria with malaria RDT - Negative for VL with rk39 RDT *Exclusion criteria* - A positive VL diagnosis in their medical history - A history of clinical malaria in the preceding 2 weeks - Already under drug treatment for malaria and/or VL - Pregnant
Cohort study: clinically ill patients
Inclusion criteria - Included in the cross-sectional study - Between 6 and 30 years of age - Having a laboratory-confirmed VL diagnosis and/or malaria diagnosis with *P. falciparum* (parasite counts between 1000 and 250,000 /μL of blood only) - Eligible for first-line treatment for VL and/or malaria as stated in the national treatment guidelines *Exclusion criteria* - Having a haemoglobin level of ≤5 g/dL - Being diagnosed with malaria caused by a *Plasmodium* species different than *P. falciparum* - Suffering from any other infectious disease, acute or chronic, different from malaria and/or VL, of which the patient has knowledge - Suffering from any immune system disorder, acute or chronic, of which the patient has knowledge

1

- Being under antimicrobial and/or anti-inflammatory treatment
- Being under immune-suppressive or immune-stimulatory treatment

Cohort study: healthy and asymptomatic household members

Inclusion criteria
- Living in the household of one of the clinically ill participants of the cohort study
- 6 years old and above
- Not showing any major symptoms suggestive of malaria and VL
- Having a body temperature below 37.5°C

Exclusion criteria
- A positive VL diagnosis in their medical history
- A history of clinical malaria in the preceding 2 weeks
- Already under drug treatment for malaria and/or VL
- Pregnant
- Being diagnosed with malaria caused by a *Plasmodium* species different than *P. falciparum*
- Suffering from any other infectious disease, acute or chronic, different from malaria and/or VL, of which the patient has knowledge
- Suffering from any immune system disorder, acute or chronic, of which the patient has knowledge
- Being under antimicrobial and/or anti-inflammatory treatment
- Being under immune-suppressive or immune-stimulatory treatment

2

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation	Page No
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	7
Methods			
Study design	4	Present key elements of study design early in the paper	7
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	8
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	10
		Case-control study—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls	9
		Cross-sectional study—Give the eligibility criteria, and the sources and methods of selection of participants	9
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed	10
		Case-control study—For matched studies, give matching criteria and the number of controls per case	9
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	11-15
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	11-15
Bias	9	Describe any efforts to address potential sources of bias	9-10
Study size	10	Explain how the study size was arrived at	10-11
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	15-16
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	15-16
		(*b*) Describe any methods used to examine subgroups and interactions	14-16
		(*c*) Explain how missing data were addressed	N.A.
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed	N.A.
		Case-control study—If applicable, explain how matching of cases and controls was addressed	13
		Cross-sectional study—If applicable, describe analytical methods taking account of sampling strategy	15
		(*e*) Describe any sensitivity analyses	N.A.

Results

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	N.A.
		(b) Give reasons for non-participation at each stage	N.A.
		(c) Consider use of a flow diagram	N.A.
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	N.A.
		(b) Indicate number of participants with missing data for each variable of interest	N.A.
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	N.A.
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	N.A.
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	N.A.
		Cross-sectional study—Report numbers of outcome events or summary measures	N.A.
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	N.A.
		(*b*) Report category boundaries when continuous variables were categorized	N.A.
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	N.A.
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	N.A.

Discussion

Key results	18	Summarise key results with reference to study objectives	17
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	17
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	N.A.
Generalisability	21	Discuss the generalisability (external validity) of the study results	17

Other information

Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	19

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.