



Malaria risk factors in pregnant women and infants in Benin

Violeta Moya-Alvarez

► To cite this version:

Violeta Moya-Alvarez. Malaria risk factors in pregnant women and infants in Benin. Santé publique et épidémiologie. Université Pierre et Marie Curie - Paris VI, 2015. English. NNT : 2015PA066539 . tel-01319033

HAL Id: tel-01319033

<https://theses.hal.science/tel-01319033>

Submitted on 20 May 2016

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THESE DE DOCTORAT DE L'UNIVERSITE PIERRE ET MARIE CURIE

Spécialité Epidémiologie

Ecole doctorale 393 Epidémiologie et Sciences de l'Information Biomédicale

Présentée par

Mme. Violeta MOYA-ALVAREZ

Pour obtenir le grade de

DOCTEUR de l'UNIVERSITÉ PIERRE ET MARIE CURIE

FACTEURS DE RISQUE DE PALUDISME CHEZ LA FEMME ENCEINTE ET LE JEUNE ENFANT AU BENIN

soutenue le 6/10/2015 devant le jury composé de :

M. le Dr. Michel Cot

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38 A mis abuelos y mi familia, por la brújula y el amor. Als meus pares, per la llibertat.

39 A la Isa, per l'alegria i amb les llàgrimes del record.

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“Do you see the story? Do you see anything? It seems to me I am trying to tell you a dream--making a vain attempt, because no relation of a dream can convey the dream-sensation, that commingling of absurdity, surprise, and bewilderment in a tremor of struggling revolt, that notion of being captured by the incredible which is the very essence of dreams...”

Joseph Conrad, *Heart of Darkness and the Congo Diary*

« —Mire vuestra merced —respondió Sancho— que aquellos que allí se parecen no son gigantes, sino molinos de viento, y lo que en ellos parecen brazos son las aspas, que, volteadas del viento, hacen andar la piedra del molino. —Bien parece —respondió don Quijote— que no estás cursado en esto de las aventuras: ellos son gigantes; y si tienes miedo quítate de ahí, y ponte en oración en el espacio que yo voy a entrar con ellos en fiera y desigual batalla ».

"Ninguna ciencia, en cuanto a ciencia, engaña; el engaño está en quien no la sabe."

Miguel de Cervantes

« Au milieu de l'hiver, j'apprenais enfin qu'il y avait en moi un été invincible"

Albert Camus

95Remerciements

96Aux femmes et nouveau-nés d'Allada. Je remercie également chacun des travailleurs des centres de santé
97d'Allada, Attogon, et Sékou pour leur travail sans lequel cette thèse n'aurait jamais été possible.

98Mes remerciements vont d'abord à Michel Cot. A piloto diestro, no hay mar siniestro, comme on dit...
99(quand le pilote est adroit, il n'y a pas de mer sinistre). En effet, merci Michel de m'avoir guidée au milieu
100des tempêtes et quand le vent soufflait contre nous ou bien quand je perdais le Nord... Merci des conseils,
101de ta compréhension, et de m'avoir posé les bonnes questions, énigmes dont je déchiffre encore la
102dimension. Merci de m'avoir remise sur la bonne route quand j'étais déboussolée, au long de ces années.
103Merci de t'être placé dans l'échange et le dialogue. J'ai tellement appris sur l'épidémiologie, la déontologie
104et l'épistémologie que tu en serais même content. Merci de ta sagesse.

105Je remercie les membres du jury de m'avoir fait l'honneur d'avoir consacré de leur temps à ce travail: les
106Docteurs Jean-François Etard et Bruno Pradines en tant que rapporteurs, et les Docteurs Clara Menéndez,
107Guillaume Leloup et Gérard Bréart en tant qu'examinateurs. Je remercie également Thierry Fusai et Xavier
108Duval d'avoir été mes tuteurs.

109Je remercie très fortement Florence Bodeau-Livinec de m'avoir offert l'accès au plomb et à bien d'autres
110éléments essentiels pour arriver à ma pierre philosophale... la fin de cette thèse.

111Merci à Smaila, Manfred et Gino, sans qui cette thèse n'existerait simplement pas. Merci de votre aide, aussi
112précieuse et fondamentale depuis le début jusqu'à la fin. Merci des échanges et d'avoir été là.

113Je veux remercier Philippe Deloron, Jean-Philippe Chippaux, et le Professeur Achille Massougbodji pour leur
114implication et pour leur appui dans le projet.

115Je remercie également Maud Subtil, Sarah Kitar, Karine Laboux, Evelyne Guilloux et Lydie Martorana de leur
116aide essentielle pendant la thèse, à tout moment.

117Thank you very much to Michael, the brightest PhD student, for the always interesting exchanges, but most
118of all for your kindness and support. Thanks for being there during the complicated, sad and joyful moments.

119Au Bénin et à Paris, je remercie Gilles, qui m'a aidée précieusement à déchiffrer les données et rendre
120statistiquement possible les incertitudes de ma thèse. Gilles, ça a été un plaisir de travailler avec toi. Merci
121de ta bonne humeur et de ta patience. Je fermerai la porte en sortant. Un très grand merci à Valérie, pour
122tes conseils, ton accueil, et ton aide très inspiratrice. Merci beaucoup aussi à Jean-Yves, dont la bonne
123humeur et le bon jugement ont illuminé mon chemin, toujours. Merci à Nadine pour son accueil chaleureux
124et protecteur à Cotonou et son aide toujours très effective. J'adresse un très vif remerciement à toute
125l'équipe de l'IRD au Bénin, tous, mais je salue spécialement Joseph et Pépin.

126Evidemment je remercie aussi Brigitte et Jean-Christophe, pour leurs sages conseils qu'il s'agisse du travail ou
127de grandir dans la vie. Merci à Jean-Gérard, pour les critiques (sur mon français, sur le cinéma...), les
128impressions, et tellement d'autres choses. Je remercie vivement André, dont les conseils m'ont aidée à
129décoder les secrets de Stata. Merci évidemment à Jacqueline et Audrey, qui m'ont offert des données clé
130et aussi des mots et des sourires qui m'ont toujours apaisée. Et bien sûr merci à Sylvain et à Aline, du bon
131sens et de la gentillesse de l'autre côté du bureau. Merci aussi particulièrement à Pascale et David, de leur
132bonne humeur et leur aide.

133Merci à toute l'équipe de l'unité à Paris, de votre accueil, vos encouragements et les innombrables conseils
134apportés, et surtout les conversations qui me sont toujours aussi chères: Florence, Murielle, Talleh, Anaïs,
135Sayeh, Carine, Magalie, Kossiwa, Romain, Nicaise, Azizath, Aicha, Komi, Alexandre, Rafiou, Alexandra,
136Rachida, Charles, Augustin, Ibrahim, Emmanuelle, Jocelyne, Carmen, Charles, Edwige, Sandrine, Jessica,
137Isabelle, Nicolas et les nouvelles arrivées Margaux, Sonia, et Claire. Un remerciement très particulier à la
138generation team, Guillaume et Laure, qui ne m'ont jamais laissé tomber du mauvais côté de la force.

139Un vif remerciement à Yves Martin-Prével de ses sages conseils et de son aide à tout moment. Je remercie
140également Yoann Madec, Loïc Chartier, et Arnaud Fontanet, de leur aide avant et durant la thèse.

141Bien évidemment, je remercie tout particulièrement Bich-Tram, Gwladys, Célia et Séb de leur aide
142constante, de leur accueil chaleureux, de leurs mots précis et gentils, et de m'avoir fait un espace où je me
143suis sentie bienvenue et protégée, et où j'ai vraiment envie de retourner.

144Merci Jérôme (Clain), des critiques, des blagues et de tous tes conseils depuis Madagascar. Merci d'être

145mon ami, mais, je ne t'ai toujours pas vu sur le mur...

146Plus que tout, je veux remercier encore mes co-thésards, avec qui j'ai partagé expériences et bureau.
147Merci Abdoulaye, Ghislain et Géraud de votre aide dans mon travail et des moments passés en dehors. Je
148ne peux que me réjouir de la grande chance que j'ai eue de partager ma thèse avec la douceur et la
149lumière de Julie et de Tania, personnes exceptionnelles cachées dans la salle des thésards. Merci de votre
150incroyable disposition pour donner toujours un coup de main, un sourire ou un éclat de rire. Les midis au
151Funzy ont été un point d'ancrage très important pour moi, et vous l'êtes et le serez toujours.

152Grand merci à la MES de la CIUP pour m'avoir permis d'y passer des grands moments et d'y habiter
153pendant ma thèse, merci notamment à Asa Ekwall, Manuela, Faty et Marc, sans qui cela n'aurait pas été
154possible. Grand merci à la MES team, Ingrid, Sophie, Laura, Niko. Sans vous cela n'aurait évidemment pas
155été aussi drôle ni joyeux. Vivre avec vous a été une des meilleures expériences de ma thèse. Ingrid, gracias
156por esos momentos puerta-puerta que duran toda la noche. Sophie, merci encore pour tout, à Genève,
157Valence... Grazie tanto a te, Camilla. Sei fata di una verità e tu una grandezza senza parangone. Grazie per
158tutti i momenti di luce e di ombre, ma sempre coperti di tenerezza e sapienza.

159Sophie et Grég, merci d'avoir accueilli la petite, merci des batailles. Sophie, la grande sœur, merci de ton
160accueil, toujours lors des incalculables circonstances, et de la joie. Grégoire, merci du Nord-Kivu et du
161partage des fonds laissés par les partants, et pas que. Merci de ton humour qui m'égaye et me pseudo-
162vexe depuis depuis. Tu as signé sur le livre d'invités de la Suède, enfin.

163Also would like to really thank Malena, Séphora, Franz, Josh, Katriina, Davina, Joni, Kaisa, Blair, Maren,
164Dimitri, for helping me during these years. Thanks to the beautiful Liz, for your help with my articles, and for
165the *underground* moments. Je tiens à remercier également Jocya et Christophe, et je salue nos
166retrouvailles! Grand merci aussi à François, qui me manque trop souvent, mais qui est toujours là, et
167comment. Arnaud, merci d'être toujours à l'écoute, des conseils et des rires. Clo, Laura, Chacha, Juliette,
168merci de votre soutien depuis le début. Jérôme B., merci. Des années, de ton soutien inconditionnel et de
169toujours avoir cru en moi. Gracias Natasha, por todo, siempre. En Navidad y en Seattle. Un placer, un gusto,
170un honor. Mil gracias. Merci Liem Binh, d'être aussi drôle, intelligent, et surtout d'être mon ami.

171Pasteurñoles, nunca una palabra implicó... ilusión, risa, complicidad, comprensión, ánimo, abrazos,
172compartir, amistad... Gracias Isa, Laura, Biel, Jota, Pedro, Raúl, Marc, Oriol, Ana, Marta, Toni, Charming,
173Gonzo, Casuso, Marga, Joaki, Rocío, Sonia, Alfonso, Pablo, Elena, Silvia, Max, Inés, Mer... Gracias, de
174corazón. Ya sabéis lo que hay y lo que hemos pasado, que nos quiten lo bailao. Os espero siempre pa
175enseñaros las fotos de mis vacaciones, pero la próxima vez pondré las fuentes!

176Mats, thank you very much for your help in so many important moments. Thank you for including me in the
177best unexpected trips and travels, waiting for many more to come.

178Camille, soleil du Sud, qui m'a hébergée et réchauffée avec soin et joie. Merci de m'héberger pour
179l'écriture des articles, de m'avoir aidée pour arriver au labo... je compte sur toi toujours, pour qu'on se
180retrouve ailleurs dans le monde (chez Marco par exemple ;)).

181Moltes gràcies també a l'Helena, perquè m'has ajudat molt (des fa més de 25 anys!) Moltes gràcies,
182Andrea i Sophie, pels petits moments entre Barcelona i París... Gracias Blanca, por tanto, por venir a
183Barcelona, a París, por ser tan maravillosa y gran amiga. Gracias Hele!! Gracias Isaac por hacerme y
184dejarme siempre un sitio. Gràcies Gemma, porque es lo que tiene el ruso. Carlitos, el chico que vuela alto y
185llega lejos. Gracias por tu amistad. Gràcies també a tu, Marta, per les illes retrobades. Gracias Sergio por
186venir al rescate, por ser tan maravilloso y tan guay. Oriol, el meu oràcul... kairós et non chronos. Gràcies,
187orfebre de melodies que acaricien i curen. Mari-Paz, Blanca. Porque siempre os he encontrado a la vuelta
188de la esquina. Carlos, des d'Estrasburg fins a la plaça d'Oscà, moltes gràcies pels consells, els riures i els
189ànims. David, tantes gràcies per tot, des de fa tant! La teva fè en mí és per mi una responsabilitat només a
190l'altura de la gran persona que ets. Dani, gràcies pels camins que has enlluernat. Jonathan, mi Emile
191Sinclair, gracias por los descubrimientos. En Benín, España o en París seguiremos buscando a nuestra Maga.

192Je remercie très fort les USMAiens, qui m'ont montré qu'à côté de la falaise il n'y a pas le vide, mais un
193espace à remplir d'amitié et de bonheur. Vous m'avez encouragée, assurée, soignée et donné la joie de
194vivre. Merci à Lucie, ma fleur du désert, belle et forte, pour tout. Merci à Rose, artiste de la vie. Merci aux
195deux de me laisser une place dans la scène et de me rendre juste heureuse. Richard, de l'ambrosie en
196essence. Merci de ta force pour l'ascension aux plus belles montagnes. Merci de ton honnêteté et de ta
197tendresse. Merci de m'aider à chercher le Nord... au canal de l'Ourq, dans un bus vers Sarcelles et surtout,

198ailleurs dans la vie et partout dans le monde. Merci des lunes audoniennes, des soirées sur le guidon, de la
199magie des loups des Pyrénées, d'être toujours au pied de la voie. Mylène, merci des étreintes qui
200réchauffent le cœur. Victor, merci de tes paroles et de tes gestes précis et précieux, à chaque instant,
201même dans une voiture en panne. Isa, merci de nos longues discussions. Lucie C, Julie, Vincent, Emilie,
202David, Christian, John, Thibaut, Olivier merci pour tout, de m'avoir accueillie, de m'avoir fait rire autant, de
203m'avoir donné de la force, USMA-force!

204Irene, la mujer de los sueños de los valientes. Mi remanso de paz allén de los mares. Nunca podré
205agradecerte suficientemente lo que me has cuidado estos años, y tu paciencia. Cctkta, guapa.

206Alessandro, Paola, grazie mille per accogliermi sempre a Bo. Bacioni grandi.

207Ale, ça va? grazie per la grande bellezza, grazie de ne pas être gêné, grazie per tutto il resto...da tanto!
208Bon courage!

209Celia, muchísimas gracias por tu ayuda y tu amistad, durante la tesis y siempre más allá.

210A special thanks to Jessica, for your amazing help with the English in my articles, but most important for your
211incredible friendship.

212Berta, espero tornar a viure algun cop a Barcelona només per poder estar més aprop teu. Ets un tresor
213reservat als que et coneixem, t'estimem molt. Moltes gràcies per aquests anys i pels que queden per venir.

214Benjamin, rayonnant Solal des Solal, la plus belle découverte... de la manne tombée du ciel thaï. Merci,
215merci d'avoir traversé les frontières, d'avoir triché un peu, d'avoir changé les normes, d'avoir pris des
216risques, merci d'y croire vraiment, merci de vouloir jouer avec moi, merci du bonheur... mais qu'est-ce
217qu'on a eu de la chance... You're a loving cup, gimme shelter!

218Estimat Xavi, germà (de fang), somos chenoas, i Déu a Sicília, i Itàlia, i el camino, i como te vea por Madrid,
219i no pot ser veritat, i l'Oscà, i Roger Poma, i « lleig », i fuig, i si fos un guió, i si « sho fó », i els plans subtils, i o és
220fàcil, o no és i amb tu sempre ho ha estat tant... Moltíssimes gràcies, per tot, des de sempre i per sempre.

221Enfin je tiens à remercier les trois personnes, autre que Michel, sans qui ma thèse serait encore, au mieux, un
222projet en cours. Ils m'ont aidé à surpasser les rapides et les crocodiles de la traversée... Merci aux trois
223mousquetaires : Blandine, Pierre et Aurélie.

224Pierre, Julien S., ma pierre angulaire, mon ange gardien... Merci de ta patience. Merci de ton aide pendant
225ces années. Merci d'avoir récupéré ma dégainé, des morceaux de bonheur et des étreintes rétentrices. Tu
226es la raison pure et la joie de vivre à la fois, l'ami dont l'on rêve. Illaliq'a kariban!

227Aurélie, drôle et douce comme notre soirée « crêpe au chocolat » à Cotonou, merci. Merci de tes conseils
228scientifiques et autres, d'être toujours là, pour les bo-buns, les concerts, les conversations chargées et celles
229de 15 minutes. Que de nouvelles aventures nous attendent, qui nous feront rire comme toujours. Merci!

230Blandine... tu m'as offert la phrase *leitmotiv* de ma thèse: aux projets que l'on fait, et à la vie qui nous
231change tous les chemins. Tu m'as donné du souffle, et tu m'as montré que la marée pousse les étoiles vers
232la plage. Notre voyage de Saint-Germain à Télégraphe était un voyage au bout de la nuit... Merci de
233chaque nouvelle journée, des brouillons, de ta sagesse, du "grand bonheur", du fou rire. Merci des
234discussions « Sciences et Vie », et d'éclairer mon esprit, mes articles et mon chemin pendant ces années.

235También quiero dar las gracias, muchas y fuertes, a mi familia, por haber estado siempre ahí. Todos habéis
236sido muy importantes, ya lo sabéis. Gracias a vosotros que me habéis enseñado el cuestionamiento, la
237reflexión, la auto-crítica, y que no hay que tener miedo a preguntar, y a cuestionar la ortodoxia para
238obtener respuestas, y que no siempre hay que fiarse de lo que emana de la superficie, esa punta de
239iceberg.. Gracias por haberme enseñado el valor de la creación, la valentía, la libertad y la alegría.

240

241Violeta, octobre 2015.

243 Liste de publications et communications de la thèse:

244 ³⁵₁₇ Publications:

245 "Does iron increase the risk of malaria in pregnancy?" Violeta Moya-Alvarez, Gilles Cottrell, Smaila
246 Ouédraogo, Manfred Accrombessi, Achille Massougbdgi, and Michel Cot, Open Forum Infect
247 Dis (Spring 2015) 2 (2):doi: 10.1093/ofid/ofv038

248 "Pregnancy-associated malaria and malaria in infants: an old problem with present consequences".
249 Violeta Moya-Alvarez, Rosa Abellana, Michel Cot. *Malaria Journal*. 2014; 13:271.

250

251 ³⁵₁₇ Articles sous révision

252

253 "Iron levels and malaria in infants: the dangerous liaisons" Violeta Moya-Alvarez, Florence Bodeau-
254 Livinec, Michel Cot. (Under review in *Nutrition reviews*).

255 "Elevated blood lead levels are associated with reduced risk of malaria in Beninese infants" Violeta
256 Moya-Alvarez, Michael Osei Mireku, Pierre Ayotte, Michel Cot, Florence Bodeau-Livinec. (Under review
257 in *Plos One*).

258 "The effect of iron levels and IPTp on malaria risk in infants: a prospective cohort study in Benin"
259 Violeta Moya-Alvarez, Gilles Cottrell, Smaila Ouédraogo, Manfred Accrombessi, Achille Massougbdgi,
260 and Michel Cot. (Under review in *Pediatrics*).

261

262 ³⁵₁₇ Communications:

263 "High folate levels are not associated to increased risk of malaria but to reduced anemia rates in the
264 context of high dosed folate supplements and SP-IPTp in Benin" Violeta Moya-Alvarez, Smaila
265 Ouédraogo, Manfred Accrombessi, and Michel Cot. Poster presentation, Meeting of the American Society
266 of Tropical Medicine and Hygiene 2015, Philadelphia, PA.

267 "Iron levels and IPTp extent are associated with higher malaria risk during infancy in Benin"
268 Violeta Moya-Alvarez, Gilles Cottrell, Smaila Ouédraogo, Manfred Accrombessi, Achille Massougbdgi,
269 and Michel Cot. Oral presentation, Meeting of the American Society of Tropical Medicine and Hygiene
270 2014, New Orleans, LA.

271 "Lead levels are associated with a certain protection for malaria risk during infancy in Benin"
272 Violeta Moya-Alvarez, Michael Osei Mireku, Pierre Ayotte, Michel Cot, Florence Bodeau-Livinec. Oral
273 presentation, Meeting of the American Society of Tropical Medicine and Hygiene 2014, New Orleans, LA.

274 "Total body iron and IPTp calendar are associated with *Plasmodium falciparum* parasitemia during
275 the first year of life in Benin" Violeta Moya Alvarez, Smaila Ouédraogo, Florence Bodeau-Livinec,
276 Gilles Cottrel, Michel Cot. Poster presentation. 8th European Congress on Tropical Medicine and
277 International Health, Copenhagen 2013

278

279 ³⁵₁₇ Récompenses:

280 Prix à la meilleure communication, Journées EHESP 2014.

281 Bourse de voyage pour l'ASTMH 2014, EHESP.

282 Bourse de voyage pour l'ASTMH 2015, EHESP.

283 Laboratoire d'accueil

284 UMR 216- Mère et enfant face aux infections tropicales - MERIT

285 Institut de recherche pour le développement

286 Université Paris Descartes

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290	Sigles et abréviations
291	ACTs : Artemisinin combination therapy
292	AGP : α -1-glycoprotein
293	AHR: Adjusted hazard ratio
294	AL: Artemether-lumefantrine
295	ANC : Ante-natal care
296	ANV : Ante-natal visit
297	aOR: Adjusted odds ratio
298	APEC: Anaemia in pregnancy: aetiology and consequences
299	AQ: Amodiaquine
300	aRR: Adjusted relative risk
301	AS : Artesunate
302	BMI : Body Mass Index
303	CDC: Centers for disease control and prevention
304	CRP: C- reactive protein
305	CQ: Chloroquine
306	DALY: Disability-adjusted life year
307	DHA: Di-hydro arthemisininine
308	EDCTP: European and Developing Countries Clinical Trials Partnerships
309	ELISA: Enzyme-linked immunosorbent assay
310	Hb: Hemoglobin
311	HIV : Human Immunodeficiency virus
312	HR: Hazard ratio
313	Ig: Immunoglobulin
314	IPTp: Intermittent preventive treatment in pregnancy
315	IRD : Institut de recherche pour le développement
316	IST : Intermittent screening and testing
317	ITN : Insecticide-treated net
318	IUGR : Intra-uterine growth retardation

- 319 LBW: Low birth weight
- 320 MCV : Mean corpuscular volume
- 321 MeSH: Medical Subjects Headings
- 322 MiPc: Malaria in pregnancy consortium
- 323 MiPPAD: Malaria in pregnancy preventive alternative drugs
- 324 MPAC: Malaria Policy Advisory Committee
- 325 MQ : Mefloquine
- 326 OMS : Organisation mondiale de la santé
- 327 OR: Odds ratio
- 328 PAM: Pregnancy associated malaria
- 329 PM: Placental malaria
- 330 PNLP : Programme national de lutte contre le paludisme
- 331 PQ : Priperaqueine
- 332 RDT : Rapid diagnostic test
- 333 RR: Relative risk
- 334 SGA: Small for gestational age
- 335 SP: Sulphadoxine-pyrimethamine
- 336 SPR: Slide positivity rate
- 337 sTfR : Seric transferrin receptor
- 338 TBS : Thick blood smear
- 339 TPI : Traitement préventif intermittent pendant la grossesse
- 340 UMR216 : Unité mixte de recherche 216
- 341 VIH : Virus de l'immunodéficience humaine
- 342 WHO : World Health Organisation
- 343 ZPP: H: Zinc protoporphyrin/hemoglobin ratio
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348 **Malaria risk factors for pregnant women and infants in Benin.**

349 **Abstract**

350 In Benin malaria and nutritional deficiencies are the main diseases contributing to the disease burden.

351 Therefore, preventive strategies targetting both diseases have been deployed for over 10 years.

352 Pregnancy-associated malaria (PAM) is responsible for maternal anaemia, placental malaria and low

353 birth weight (<2500g), contributing to enhance maternal and child morbidity and mortality. To

354 prevent PAM, the World Health Organization recommends the intermittent preventive

355 treatment during pregnancy (IPTp). In Benin it consists in the administration of two curative doses of

356 sulfadoxine/pyrimethamine (SP) at least one month apart and starting at the second trimester of

357 pregnancy. Considering that IPTp has an effect on PAM, and thereby influences the exposure of the

358 foetus to the parasite, we wanted to investigate the possible effect of IPTp on malaria in infants.

359 In parallel, iron supplements are recommended during pregnancy to prevent maternal anemia. Some

360 pediatricians give iron supplements to infants as well. As there is some epidemiological evidence that

361 iron might enhance malaria episodes and their severity we wanted to analyse the association of iron

362 levels with malaria in pregnancy and infancy. Therefore, we analysed data from a cohort study of

363 1005 pregnant women conducted from 2010 to 2012 in Allada (Benin), and data of the first 400

364 infants born to this cohort of mothers, who were followed for a year.

365 First, we showed that interval length between IPTp doses (the number of days between doses) was

366 inversely correlated with malaria risk and *P. falciparum* parasitemia, possibly due to the reduction of

367 the exposure of the foetus to the parasite in utero, which thereby hinders a possible immune tolerance

368 process.

369 We also found that iron levels during pregnancy and infancy were associated to increased malaria

370 risk and *P. falciparum* parasitemia, with a possible dose effect.

371 In a context of growing resistance to SP, a strategy based on more than 2 doses of SP should be

372 encouraged to confer an optimal protection to pregnant women. In addition, complementary

373 interventional data are needed to determine the benefits and risks of differently dosed iron

374 supplements, in order to ascertain their impact on infant health in malaria-endemic regions.

375 Key words: pregnancy-associated malaria, IPTp, malaria in infants, iron supplements, iron deficiency

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Résumé de la thèse

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398 I. Introduction

399 Les principales causes de morbidité et de mortalité en Afrique sub-Saharienne sont les maladies
400 infectieuses et les déficiences nutritionnelles. Les femmes et les enfants de moins de cinq ans y
401 sont particulièrement vulnérables. D'après l'OMS, entre 2000 et 2012, les carences nutritionnelles,
402 les maladies infectieuses, ainsi que la morbidité périnatale représentent la plupart des causes de
403 mortalité chez les enfants et les jeunes femmes .

404 L'anémie, dont la première cause est la carence en fer, est définie par l'OMS par des taux
405 d'hémoglobine $< 11 \text{ g / l}$. C'est une des maladies liées aux carences nutritionnelles les plus
406 prévalentes dans le monde : on estime qu'au début du XXI siècle, 25 % des enfants seraient
407 anémiés . La prévalence de l'anémie gestationnelle au Bénin est très élevée avec une estimation
408 dépassant 65 % . Pour pallier ce problème d'anémie chez la femme enceinte, une supplémentation
409 en fer a été activement recommandée par l'OMS depuis les années 1990. De fait, une méta-
410 analyse Cochrane effectuée en 2012 montre que la supplémentation en fer est associée à une
411 réduction de 70 % du risque d'anémie et de 57 % du risque de carence en fer . Au Bénin, des
412 suppléments de 200 mg de sulfate ferreux et 5 mg de folate jusqu'à 45 jours après l'accouchement
413 sont donnés aux femmes enceintes systématiquement.

414 Cependant différentes études épidémiologiques suggèrent que des niveaux de fer élevés auraient
415 un effet délétère sur le risque de paludisme . Néanmoins, l'absence d'étude de cohorte
416 longitudinale chez la femme enceinte et chez l'enfant reste un obstacle important pour établir un
417 lien entre les niveaux de fer et un risque accru de paludisme.

418 Etant donné qu'au Bénin une supplémentation en fer est donnée systématiquement aux femmes
419 enceintes, et que le paludisme est endémique dans la région, notre premier objectif était d'analyser
420 l'association entre les niveaux de fer et le paludisme gestationnel dans une cohorte prospective de
421 femmes enceintes.

Chez les enfants béninois des taux d'anémie supérieurs à 80 % ont été reportés . Néanmoins, il n'y pas à ce jour au Bénin de recommandation officielle concernant la supplémentation en fer chez l'enfant, même si l'OMS recommande un supplément quotidien de 12,5 mg de fer chez les enfants âgés entre 6 et 24 mois dans des contextes où la prévalence d'anémie dépasse 40 % .

Par ailleurs, au Bénin, la principale cause de mortalité des enfants de moins de cinq ans reste le paludisme. Environ 21 % des décès infantiles dans ce pays sont dus au paludisme, maladie responsable de 22,8 % des années de vie perdues en 2010 . En définitive, malgré une prévalence d'anémie infantile et une mortalité causée par le paludisme très importantes, aucune recommandation nationale concernant les suppléments en fer n'est proposée. Pour ces raisons, nous avons cherché à identifier l'accroissement du risque de paludisme chez le nourrisson en lien avec des niveaux élevés de fer plasmatique.

En parallèle, afin de réduire les effets du paludisme gestationnel, le Ministère de la Sante du Bénin a mis en place une stratégie de traitement préventif intermittent (TPI) du paludisme pendant la grossesse. Ce traitement, par son effet sur le parasite, permet de réduire l'anémie maternelle, mais aussi le paludisme placentaire, la prématurité, et le petit poids à la naissance . Ainsi, outre les suppléments de sulfate ferreux et de folate, 1500 / 75 mg de sulphadoxine-pyrimethamine (SP) sont prescrits aux femmes enceintes béninoises en tant que TPI. Ce traitement s'administre en deux doses à un mois d'écart au minimum, dont la première le plus tôt possible au cours du deuxième trimestre de grossesse.

Ainsi, le paludisme gestationnel étant associé au paludisme de l'enfant , il est possible que les interventions modifiant l'exposition au parasite, aient aussi un effet sur le paludisme de l'enfant. Cet aspect a été peu investigué jusqu'à présent : notre deuxième objectif a donc consisté en l'analyse de l'effet du TPI sur le risque de paludisme chez l'enfant pendant la première année de vie.

Finalement, des chercheurs travaillant sur la même cohorte avaient trouvé des niveaux élevés de plomb chez ces enfants. Les niveaux élevés de plomb, comme le paludisme, ont un effet sévère sur

448le développement de l'enfant et sont associés à des taux très importants d'anémie. Par ailleurs,
449Nriagu avait trouvé un effet significatif du paludisme sur la plombémie chez des enfants
450nigériens . Pour ces raisons notre troisième objectif était d'évaluer l'effet des niveaux élevés de
451plomb sur le risque palustre.

452Afin d'atteindre ces objectifs, nous avons étudié les indicateurs palustres ainsi que les niveaux de
453fer sériques chez 1005 femmes enceintes. Ces femmes étaient recrutées par les études APEC
454(Anemia in pregnancy : etiology and consequences) et MiPPAD (Malaria in pregnancy preventive
455alternative drugs, <http://clinicaltrials.gov/ct2/show/NCT00811421>). Cette dernière étant plus
456spécifiquement un essai clinique comparant l'efficacité de la sulphadoxine-pyriméthamine
457(1500/75 mg par dose) et la méfloquine (15 mg/kg). Les critères d'inclusion des femmes étaient :
458l'absence de prise de TPI, de traitement anti-helminthique ou de suppléments en fer ou acide
459folique. Un dépistage du VIH était également proposé aux femmes.

460Après l'accouchement, nous avons suivi 400 de leurs enfants (200 enfants de mères anémiées à
461l'accouchement, et 200 enfants de mères non-anémiées à l'accouchement) pendant toute leur
462première année de vie. Les niveaux de plomb des enfants ont également été analysés à 12 mois.

463Ces études ont été réalisées entre janvier 2010 et mai 2012 dans trois cliniques d'Allada, une
464région semi-rurale 50 km au Nord de Cotonou, où le paludisme est principalement dû à
465*Plasmodium falciparum*. La transmission du paludisme à Allada est pérenne avec des pics
466saisonniers : entre avril et juillet et entre octobre et novembre.

467Notre suivi dans le temps de la femme enceinte et de l'enfant comprenant des données répétées,
468nous avons utilisé des modèles multiniveaux avec un intercept aléatoire au niveau individuel. Plus
469précisément, nous avons utilisé comme variables dépendantes : i) la possibilité d'avoir ou pas une
470goutte épaisse positive pendant le suivi et ii) la parasitémie (évaluée par microscopie) au cours du
471suivi.

472Pour évaluer l'effet du plomb sur le risque palustre, nous avons utilisé une régression logistique
473sur la possibilité d'avoir ou pas une goutte épaisse positive à 12 mois au cours du suivi, ainsi

474qu'une régression linéaire en utilisant la parasitémie à 12 mois (évaluée par microscopie) comme
475variable dépendante.

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477 II. Etat de l'art

4781. Effet des niveaux de fer de la femme enceinte sur le paludisme gestationnel

479Une méta-analyse Cochrane a montré de manière convaincante les bénéfices associés à la
480supplémentation en fer. En effet, les suppléments en fer pendant la grossesse sont associés à une
481réduction de 70 % du risque d'anémie et à une réduction de 57 % de la carence en fer comparé à
482des contrôles. Cependant, le fer est un cofacteur de la croissance de *Plasmodium* , et ces
483suppléments pourraient entraîner une augmentation du risque palustre dans les zones d'endémie.

484Bien que les essais cliniques ne montrent pas d'augmentation de la morbidité liée à la
485supplémentation, la carence en fer est associée à un moindre risque d'épisodes palustres . Même si
486les différences ne sont pas statistiquement significatives, les taux de ferritine des femmes avec un
487placenta infecté par *Plasmodium falciparum* sont systématiquement plus élevés que chez les
488femmes sans infection placentaire dans toutes les études dans des pays avec des transmissions
489aussi diverses que la Tanzanie , le Gabon , le Malawi , la Gambie , ou le Kenya . Une méta-
490analyse récente, bien que concluant à l'absence de preuve épidémiologique pour conclure à une
491augmentation de risque palustre liée aux suppléments , montre que la carence en fer, mesurée par la
492ferritine sérique, est associée à un moindre risque de paludisme gestationnel. En outre, la plupart
493des études n'évaluant les niveaux de fer sériques qu'à l'inclusion des femmes ou lors de
494l'accouchement, il serait utile de mener des études de cohortes avec un suivi systématique des
495niveaux de fer pendant tout le déroulement de la grossesse.

496Un autre élément important concerne la manière d'évaluer les taux de fer. Une combinaison
497d'indicateurs est souhaitable d'après l'OMS en dépit de l'existence d'un marqueur «gold
498standard», l'hémoglobine. En conséquence le Comité Technique de l'OMS recommande le suivi

des niveaux de fer par l'hémoglobine, le volume corpusculaire moyen (VCM), le récepteur soluble de la transferrine (sTfR), la ferritine sérique, et la protoporphyrine des globules rouges mesurée par le ratio zinc protoporphyrine/hémoglobine (ZPP :H) .

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2. Effet des niveaux de fer de l'enfant sur le paludisme

Chez le jeune enfant, l'épisode palustre est défini par une température $> 37.5^{\circ}\text{C}$ et une goutte épaisse positive dans les 48 h. La carence en fer est définie par des niveaux de ferritine sérique $< 12\text{ }\mu\text{g / ml}$ ou $< 15\text{ }\mu\text{g / ml}$ dans la plupart d'études. Une première révision d'enquêtes menées entre 2001 et 2003 au Kenya d'enfants âgés de 8 mois à 8 ans décrit une protection significative contre le paludisme chez les enfants carencés en fer (ratio d'incidence ajusté (RI) = 0,7 ; IC 95 % (0,51; 0,99)). Une enquête plus récente menée en Tanzanie (2012), a également montré que la carence en fer était associée à un moindre risque de parasitémie (OR = 0,15 ; IC 95 % (0,12; 0,19)), d'hyperparasitémie (définie par un nombre de parasites $> 2500 / 200$ globules blancs) (OR = 0,04 ; IC 95 % (0,02; 0,07)) et de paludisme sévère (OR = 0,25 ; (IC 95 % (0,14; 0,46)).

Quant aux études sur la supplémentation, un essai clinique randomisé contre placebo en 1995 en Tanzanie n'avait pas montré de différences significatives relatives au risque palustre entre les enfants de 8 à 24 mois . Néanmoins, en 2003 l'essai clinique de Pemba (Tanzanie) avait montré une augmentation très importante du risque palustre parmi les 2413 enfants âgés de 0 à 35 mois, de la cohorte . Plus précisément, le risque d'hospitalisation par paludisme était significativement supérieur (RR = 1,18 ; IC 95 % (1,02; 1,36)), ainsi que le risque de paludisme cérébral (RR = 1,22 ; IC 95 % (1,02; 1,46)) chez les enfants supplémentés. Cette étude avait fait modifier les recommandations de l'OMS dans le sens d'une restriction des suppléments en fer uniquement aux enfants carencés .

Concernant l'importance des niveaux de fer de départ pour la supplémentation, lors d'une étude au Ghana en 2010, les enfants ayant une carence en fer et de l'anémie avait un risque significativement réduit de paludisme comparés aux enfants ayant reçu du placebo (RR = 0,67 ; IC

52595 % (0,5; 0,88). Cependant, en Tanzanie en 2008 un essai de supplémentation en zinc et autres
526nutriments (dont le fer), avait décrit que les enfants carencés étaient significativement plus à risque
527de paludisme lors de la supplémentation (Rapport de risque =1,41 ; IC 95 % (1,09; 1,82)). En
528effet, la question reste ouverte et les résultats des différents essais cliniques se révèlent à nouveau
529contradictaires. Une revue Cochrane a tenté de trancher ce débat en analysant les données de
53045.353 enfants de 71 essais cliniques différents. Après s'être concentré sur les 13 études les plus
531fiables, cette révision conclut qu'il n'y a pas de différences statistiquement significatives
532concernant les épisodes palustres entre les enfants supplémentés par rapport aux enfants ayant reçu
533un placebo (RR = 0,99 ; IC 95 % (0,9; 1,09). Nonobstant, cette revue décrit un risque de
534paludisme plus élevé chez les enfants supplémentés, en l'absence de surveillance épidémiologique
535ou de traitement. Une évaluation de l'augmentation du risque en prenant en compte les niveaux de
536fer de départ, mesurés par la ferritine, reste également à faire.

537En conclusion, l'interprétation dans le sens d'une augmentation du risque de paludisme associée
538aux niveaux de fer diffère entre les études observationnelles et les essais cliniques. Globalement,
539les études observationnelles décrivent une certaine protection contre le paludisme chez les enfants
540carencés en fer. En parallèle, des anciennes études sur l'administration de suppléments en fer
541rapportent un risque de paludisme accru lié à la supplémentation, comme le fait l'étude de Pemba,
542qui a une puissance statistique notable. Pourtant, les essais cliniques les plus récents, réalisés dans
543le contexte d'une prophylaxie anti-palustre effective, ne montrent pas d'augmentation de risque
544significative de paludisme liée à la supplémentation. Pour toutes ces raisons, il est nécessaire
545d'analyser une cohorte prospective où l'on évalue les niveaux de fer lors de chaque épisode
546palustre afin de pouvoir conclure sur l'association entre paludisme et niveaux de fer.

547La révision de la littérature sur l'association entre les niveaux de fer et le risque palustre a fait
548l'objet d'un article actuellement sous révision dans le journal « *Nutrition reviews* ».

551 Le paludisme gestationnel est défini comme l'infection du sang périphérique ou placentaire par
552 *Plasmodium falciparum* par l'OMS. Ayant un effet délétère sur la santé de la femme enceinte et de
553 l'enfant, le paludisme gestationnel constitue un problème majeur de santé publique dans le monde.
554 A lui seul, il est responsable de 125 millions de grossesses à risque par an . L'exposition à
555 *Plasmodium in utero* dépend de la transmission et des mesures de contrôle qui modifient cette
556 exposition. Le TPI, une des plus importantes stratégies de contrôle, diminue la parasitémie
557 périphérique de la mère ainsi que le paludisme placentaire, modifiant significativement la réponse
558 immunitaire du fœtus *in utero* .

559 Une analyse globale de quatre études fondatrices réalisée en 2007 a déterminé que l'administration
560 du TPI, constitué de deux doses de SP, est associée à une réduction du risque de paludisme
561 placentaire correspondant à un risque relatif (RR) = 0 , 48 comparé à l'administration d'un
562 placebo, ou comparé au seul traitement des accès cliniques .

563 Le paludisme gestationnel est lié pendant les premiers mois de vie à un risque accru de paludisme
564 chez le jeune enfant . En effet, il est associé à un risque augmenté de paludisme congénital, à un
565 plus grand nombre d'épisodes palustres pendant l'enfance, à un plus grand risque d'anémie, et
566 enfin à des épisodes de fièvre non palustres .

567 Comme l'a révélé une étude réalisée en 1997 au Cameroun, le paludisme gestationnel est corrélé
568 avec des épisodes palustres plus précoces chez le nourrisson . Plus précisément, cette étude a
569 trouvé que l'infection placentaire par *Plasmodium falciparum* était significativement liée au
570 paludisme de l'enfant âgé de quatre à six mois : à six mois, 36 % des enfants avec un placenta
571 infecté avaient déjà subi un épisode palustre, alors que seulement 14 % des enfants avec un
572 placenta non-infecté avait souffert un épisode palustre (valeur $p < 0.05$). En outre, la parasitémie
573 était plus élevée chez les enfants issus d'un placenta infecté entre 5 et 8 mois, que chez les enfants
574 dont le placenta n'était pas infecté. En 2002-2004, une étude effectuée en Tanzanie a confirmé ces
575 résultats et déterminé un hazard ratio (HR) de 1,41 (intervalle de confiance (IC) 95 % (1,01; 1,99))
576 jusqu'à la première parasitémie chez les enfants nés avec un placenta infecté par rapport aux

577autres. Plus récemment, au Mozambique, il a été observé que les enfants des mères ayant subi des
578épisodes palustres pendant la grossesse et / ou un placenta infecté, présentaient significativement
579plus d'épisodes palustres pendant l'enfance (Odds ratio (OR) = 1,96 ; IC 95 %, (1,13; 3,41), et OR
580= 4,63 ; IC 95 % (2,10; 10,24)), respectivement . Enfin, une étude réalisée en 2009 au Bénin a
581confirmé le lien entre le paludisme placentaire et le paludisme chez l'enfant, en s'appuyant sur un
582suivi entomologique et environnemental rigoureux . Cette étude a montré que les enfants issus
583d'un placenta infecté dormant sous une moustiquaire imprégnée ont significativement plus de
584risques de contracter le paludisme que les enfants dont le placenta n'était pas infecté (rapport de
585risque = 2,13 ; IC 95 % (1,24; 3,67)). Cette étude a considéré également d'autres facteurs de
586risque comme la transmission, la saisonnalité, le nombre d'*Anopheles*, et des facteurs obstétricaux.
587Cette même étude a montré que les enfants présentaient une sensibilité accrue à des parasites
588possédant les mêmes antigènes que ceux auxquels ils avaient été exposés *in utero*, ce qui suggère
589l'existence d'un processus de tolérance immunitaire . Enfin, plusieurs études ont mis en évidence
590une réduction du transfert d'anticorps au fœtus associée au paludisme gestationnel, ce qui
591augmenterait la susceptibilité de l'enfant au parasite . Cependant les mécanismes
592physiopathologiques de ce processus n'ont pas encore été élucidés.

593En définitive, le paludisme gestationnel détermine l'exposition foetale à *P. falciparum* et il est
594corrélé à un risque accru de paludisme pendant l'enfance, probablement suite à un processus de
595tolérance immunitaire *in utero*. Le TPI, en réduisant l'exposition au parasite, pourrait également
596diminuer la morbidité associée au paludisme gestationnel. Ceci implique une réduction des taux de
597petit poids à la naissance, de la prématurité, du retard de croissance intra-utérin et de la mortalité
598périnatale dans des contextes où la résistance à la SP n'est pas encore très présente.

599La révision de la littérature sur le lien entre le paludisme gestationnel et le paludisme chez l'enfant
600a fait l'objet d'un article publié dans le journal « *Malaria Journal* ».

601 4. Autres facteurs ayant un effet sur le paludisme de l'enfant : le cas du plomb.

602En parallèle à notre étude, des collègues ont retrouvé dans la même cohorte d'enfants des niveaux

603de plomb très élevés. Des niveaux élevés de plomb sont associés à un risque accru d'anémie et à
604des troubles neurologiques, symptomatologie également présente dans le paludisme. Ceci est
605d'autant plus préoccupant que la pathologie se concentre aussi dans la tranche d'âge de 12 à 36
606mois, période particulièrement délicate pour les enfants impaludés. Enfin, Nriagu avait trouvé en
6072008 un effet négatif significatif du paludisme sur les niveaux de plomb, alors que la prévalence
608de niveaux de plomb élevés en Afrique de l'Ouest est très importante. Pour ces raisons, nous
609voulions déterminer la nature de l'association entre les niveaux de plomb et le risque palustre tout
610en considérant d'autres facteurs de risque de paludisme.

611

612 III. Résultats

613 1. Effet des niveaux de fer sur le paludisme gestationnel

614A la première consultation anténatale 1005 femmes enceintes ont été incluses et 941 ont été suivis
615jusqu'à l'accouchement. Pendant le suivi, 29 % des femmes enceintes ont eu au moins un épisode
616palustre. La moyenne, de gouttes épaisses positives était de 0,52 (écart-type = 1,23), avec une
617médiane de 0 (1er quartile=0, 3eme quartile=1) et une étendue de 0 à 6.

618Après utilisation de modèles multi-niveaux à intercept aléatoire chez les mères, les valeurs élevées
619de la concentration de fer (approximées par le logarithme en base 10 de la ferritine corrigé par
620l'inflammation) étaient associées significativement au risque d'avoir une goutte épaisse positive
621(OR ajusté = 1,75 ; IC 95 % (1,46; 2,11) ; valeur $p < 0,001$) et à une parasitémie par *P. falciparum*
622plus importante (estimateur beta = 0,22 ; IC 95 % (0,18; 0,27) ; valeur $p < 0,001$). De plus, les
623femmes carencées en fer étaient significativement à moindre risque d'avoir une goutte épaisse
624positive et une parasitémie élevée (valeur $p < 0,001$ dans les deux cas). Plus précisément, ces
625modèles comprennent les résultats de 2227 gouttes épaisses et 2227 frottis sanguins de 826
626femmes. Des niveaux élevés de fer étaient également significativement associés au risque de
627paludisme placentaire (OR ajusté = 2,02 ; IC 95 % (1,43; 2,86) ; valeur $p < 0,001$) et de petit poids
628à la naissance (OR ajusté = 1,69 IC 95 % (1,28; 2,22) ; valeur $p < 0,001$).

629 En parallèle, des niveaux élevés de folate étaient significativement associés à un moindre risque
630 d'avoir une goutte épaisse positive (OR ajusté = 0,37 ; IC 95 % (0,19; 0,70) ; valeur p = 0,002), et
631 à une moindre parasitémie (estimateur beta = -0,20 ; IC 95 % (-0,37; -0,08) ; valeur p < 0,001). Un
632 statut socio-économique élevé était associé à un moindre risque de paludisme et à une moindre
633 parasitémie par *P. falciparum* (OR ajusté = 0,82 ; IC 95 % (0,69 ; 0,96) ; valeur p = 0,02, et
634 estimateur beta = -0,05 ; IC 95 % (-0,09; -0,01) ; valeur p = 0,01, respectivement). Egalement, un
635 jeune âge de la mère, un âge gestationnel précoce et un processus inflammatoire actif, étaient
636 statistiquement liés au risque palustre et à une parasitémie élevée.

637 Ces résultats ont fait l'objet d'un article publié dans le journal « *Open Forum Infectious*
638 *Diseases* ».

639 2. Effet du TPI et des niveaux de fer sur le paludisme de l'enfant

640 A l'accouchement, 10,9% des placentas étaient infectés par *Plasmodium falciparum*, même si
641 aucun cas de paludisme congénital n'a été trouvé. Parmi les 400 enfants inclus à la naissance, 324
642 ont été suivis au long des 12 mois de suivi. Pendant la première année de vie 40% des enfants ont
643 eu au moins un épisode palustre. En moyenne, les enfants ont eu 0,64 gouttes épaisses positives
644 (écart-type = 0,92), avec une étendue de 0 à 4. Plus concrètement, 60,25 % des enfants n'ont eu
645 aucune goutte épaisse positive pendant le suivi, 22 % en ont eu 1, 12,5 % en ont eu 2, 4,5 % en ont
646 eu 3, et 0,75 % des 400 enfants en ont eu 4.

647 Il n'y avait pas de différences significatives entre les femmes ayant reçu un TPI à base de SP et les
648 femmes ayant reçu de la MQ. Néanmoins, l'intervalle entre deux prises du TPI était
649 significativement associé à un moindre risque de paludisme (OR ajusté = 0,87 ; IC 95 % (0,76 ;
650 0,99) ; valeur p = 0,04) et à une parasitémie plus basse (estimateur beta = -0,06 ; IC 95 % (-0,1 ;
651 -0,01) ; valeur p < 0,001).

652 Dans des modèles multi-niveaux à intercept aléatoire réalisés chez les enfants, les niveaux de fer
653 de élevés (approximés par le logarithme en base 10 de la ferritine corrigé par l'inflammation)
654 étaient associés significativement au risque d'avoir une goutte épaisse positive (OR ajusté = 2,77 ;

655IC 95 % (1,95 ; 3,96) ; valeur $p < 0,001$) et à une parasitémie par *P. falciparum* plus élevée

656(estimateur $\beta = 0,38$; IC 95 % (0,29 ; 0,47) ; valeur $p < 0,001$).). Plus précisément, ces

657modèles comprennent les résultats de 746 gouttes épaisses et de 746 frottis sanguins de 329

658enfants. Egalement, les enfants carencés en fer étaient significativement à moindre risque d'avoir

659une goutte épaisse positive et une parasitémie élevée (valeur $p < 0,001$ dans les deux cas). En

660parallèle, la présence d'un processus inflammatoire actif était associée à un risque accru d'avoir

661une goutte épaisse positive (OR ajusté = 4,37 ; IC 95 % (2,20 ; 8,65) ; valeur $p < 0,001$) et une

662parasitémie élevée (estimateur $\beta = 0,77$; IC 95 % (0,53 ; 1,01) ; valeur $p < 0,001$).

663Par ailleurs, des niveaux de folate maternels élevés et la présence d'helminthes chez la mère à

664l'accouchement étaient significativement associés à un risque accru d'avoir une parasitémie élevée

665pendant la première année de vie (estimateur $\beta = 0,34$; IC 95 % (0,01 ; 0,66) ; valeur $p = 0,04$,

666et estimateur $\beta = 0,88$; IC 95 % (0,20 ; 1,57) ; valeur $p = 0,03$, respectivement). Un statut

667socio-économique bas était aussi lié à une parasitémie élevée (estimateur $\beta = 0,12$; IC 95 %

668(0,01 ; 0,23) ; valeur $p = 0,03$). Le volume des précipitations, indicateur du risque anophélien, était

669marginalelement associé à un risque élevé de paludisme (OR ajusté = 1,06 ; IC 95% (0,99 ; 1,11) ;

670valeur $p = 0,06$) et à une parasitémie plus importante (estimateur $\beta = 0,03$; IC 95% (-0,00 ;

6710,06) ; valeur $p=0,06$).

672Finalement, les enfants avec des niveaux de ferritine dans les deux derniers quartiles étaient

673significativement plus à risque de paludisme.

674Ces résultats ont fait l'objet d'un article actuellement sous révision dans le journal « *Pediatrics* ».

675

676 3. Effet des niveaux de plomb sur le paludisme de l'enfant

677A 12 mois, 25 des 203 enfants pour qui les niveaux de plomb avaient été évalués (12,5 %), avaient

678une goutte épaisse positive, avec une parasitémie moyenne de 13460 parasites / μ l. Les niveaux de

679plomb élevés sont définis par le CDC comme des niveaux de plomb sanguin $> 5 \mu\text{g} / \text{dL}$. Trente-

680neuf enfants (19 %) avaient des niveaux de plomb toxiques, définis par des niveaux de plomb
681sanguin $> 10 \mu\text{g} / \text{dL}$. Lors de l'analyse multivariée par régressions logistique et linéaire
682respectivement, des niveaux de plomb élevés étaient associés à un moindre risque de goutte
683épaisse positive, (OR ajusté = 0,98 ; IC 95 % (0,96 ; 0,99) ; valeur $p = 0,02$) et à une moindre
684parasitémie par *P. falciparum* (estimateur beta = -0,003 ; IC 95 % (-0,006 ; -0,001) ; valeur $p =$
6850,04). Les niveaux élevés de plomb, étaient aussi statistiquement corrélés à un moindre risque de
686goutte épaisse positive, (OR ajusté = 0,38 ; IC 95 % (0,15 ; 0,99) ; valeur $p = 0,048$) et à une
687moindre parasitémie par *P. falciparum* (estimateur beta = -0,44 ; IC 95 % (-0,84 ; -0,04) ; valeur p
688= 0,03). D'autres facteurs ont été trouvés associés à un risque accru de paludisme : les niveaux
689élevés de fer (estimés par le logarithme en base 10 de la ferritine) (OR ajusté = 2,46 ; IC 95 %
690(1,01 ; 6,05) ; valeur $p = 0,05$) et les niveaux élevés de folate, statistiquement liés à une plus
691grande parasitémie par *P. falciparum* (estimateur beta = 0,0003 ; IC 95 % (0,0001 ; 0,006) ; valeur
692 $p = 0,04$).

693Ces résultats ont fait l'objet d'un article actuellement sous révision dans le journal « *Plos One* ».

694

695 IV. Discussion

696 1. Effet des niveaux de fer sur le paludisme gestationnel

697Le fait de retrouver une association très significative entre les niveaux de fer et le risque palustre
698chez la femme enceinte est d'autant plus important qu'une récente méta-analyse avait conclu qu'il
699n'y avait pas d'éléments suffisants pour évaluer ce lien. En effet, les niveaux de fer et le risque
700palustre n'avaient jamais été analysés de manière conjointe tout au long d'un suivi de cohorte
701pendant la grossesse, en dépit de l'importance donnée aux suppléments en fer pour corriger
702l'anémie gestationnelle dans les zones d'endémie palustre. De plus, nous avons également trouvé
703que les niveaux de fer étaient statistiquement associés au paludisme placentaire et au petit poids de
704naissance, ce qui illustre l'effet délétère des niveaux élevés de fer de la mère sur la santé de
705l'enfant également.

706 Le fait que la carence en fer confère une protection contre le risque palustre pendant le suivi et que
707 les niveaux de fer n'aient pas été trouvés significativement associés avec le risque palustre chez
708 les femmes carencées suggère l'existence d'un seuil à partir duquel les niveaux de fer
709 deviendraient délétères. En effet, une étude a montré une augmentation du risque palustre à partir
710 de 30 jours de supplémentation en Afrique. Nos résultats sont en outre cohérents avec la
711 littérature, qui décrit une protection conférée la carence en fer, bien que les essais cliniques (menés
712 dans le contexte de mesures préventives importantes) ne montrent pas d'augmentation
713 significative du risque.

714 Des explications plausibles pour expliquer l'augmentation du risque liée à des niveaux de fer
715 élevés résultent, au niveau de l'hôte, de l'interférence de *Plasmodium* avec le système immunitaire
716 et de son intervention dans l'inhibition de l'absorption de fer. En outre, les niveaux élevés de fer
717 rendraient plus difficile l'activation des macrophages et, de fait, le fer non lié à la transferrine est
718 corrélé avec la sévérité du paludisme.

719 En conclusion, l'interaction entre les niveaux de fer et le risque palustre est complexe et
720 ambivalente en raison des besoins augmentés de fer pendant la grossesse et d'autre part de
721 l'augmentation de risque palustre que supposent des taux élevés. Pour ces raisons, une recherche
722 plus approfondie est nécessaire afin de lever cette ambiguïté dans un contexte d'anémie
723 gestationnelle fortement prévalente.

724 2. Effet du TPI et des niveaux de fer sur le paludisme de l'enfant

725 L'intervalle entre deux prises de TPI ainsi que les niveaux de fer sont associés au risque palustre
726 pendant la première année de vie, en considérant aussi bien la probabilité de survenue d'une
727 goutte épaisse positive que la parasitémie par *Plasmodium falciparum*.

728 Le paludisme gestationnel étant connu comme influençant l'état de santé de l'enfant¹⁰, il est
729 plausible qu'une intervention préventive chez la mère ait également un effet sur le paludisme de
730 l'enfant. Nos résultats sont de ce point de vue cohérents avec la littérature. Borgella et al. ont
731 trouvé que les infections pendant le dernier trimestre de grossesse étaient associées à un risque

732accru d'infection (OR = 4,2 ; CI 95 % (1,6; 10,5) ; valeur p = 0,003) ainsi que d'épisode palustre
733(OR = 4,6 ; CI 95 % (1,7; 12,5) ; valeur p = 0,003) . En outre, Huynh et al. avaient décrit que le
734calendrier du TPI et les infections au premier trimestre de grossesse étaient liés à un plus grand
735risque de petit poids à la naissance (-98,5 g; valeur p = 0,03) . Par contre, Harrington avait trouvé
736en Tanzanie que le TPI était associé à des épisodes palustres plus précoces parmi les enfants issus
737d'un placenta infecté . Néanmoins, on retrouve une résistance très importante à la SP dans le
738Nord-Est de la Tanzanie, et la même équipe a déjà montré que, dans cette région, le TPI se révèle
739inefficace . Dans cette population, le TPI est associé à une grande fréquence d'allèles de résistance
740à la SP, à une densité parasitaire plus importante. Ces arguments renforcent indirectement
741l'hypothèse du processus de tolérance immunitaire *in utero*. Dans tous les cas, le fait que
742l'augmentation de la durée totale du TPI (par espacement ou ajout de nouvelles prises) ait un effet
743protecteur sur le paludisme de l'enfant est plutôt rassurant à la lumière des nouvelles
744recommandations de l'OMS en faveur d'un renforcement du rythme d'administration de la SP.
745En parallèle, nous avons trouvé une association très significative entre les niveaux de fer et le
746risque palustre (prévalence et densité parasitaire) pendant toute la première année de vie en
747prenant en compte d'autres facteurs de risque environnementaux, socio-économiques, et
748obstétricaux. La carence en fer avait en particulier un effet protecteur pendant tout le suivi. Plus
749précisément, les enfants avec de faibles niveaux de fer, dans le premier quartile de l'échantillon,
750étaient significativement à moindre risque d'épisodes palustres et avaient une densité parasitaire
751significativement plus basse que les autres.

752Dans la littérature cet effet protecteur de la carence en fer est souvent évoqué. Dans une revue
753Cochrane étudiant l'effet de la supplémentation en fer chez les enfants en zone d'endémie palustre
754aucune augmentation de risque palustre n'avait été mise en évidence . Cependant, comme déjà dit,
755les niveaux de fer ne sont pas déterminés longitudinalement.

756Malgré ces résultats, les suppléments en fer ont des bénéfices indéniables pour la santé des
757enfants. Une méta-analyse leur attribue une protection très significative contre l'anémie (RR =

7580,61 ; IC 95 % (0,50; 0,74), n=4825) et contre la carence en fer (RR = 0,14 IC 95% (0,10; -0,22),
759n=2145) . Etant donné qu'il n'est pas possible de pondérer les risques et les avantages des
760suppléments car très difficilement quantifiables, les mesures antipaludiques doivent être sans
761doute encouragées.

762 3. Effet des niveaux de plomb sur le risque palustre

763Les proportions très importantes d'enfants avec des niveaux de plomb élevés (63 %) et avec des
764niveaux toxiques (19 %) plaident pour la prise en considération du rôle des niveaux de plomb dans
765la morbidité liée aux maladies infectieuses chez les enfants. L'effet protecteur du plomb associé au
766risque palustre est plutôt rassurant en raison de l'importante prévalence des niveaux élevés de
767plomb en Afrique de l'Ouest. Au Nigéria, on retrouve 55 % d'enfants avec des niveaux toxiques .
768Néanmoins, en dépit de cette étude qui met en évidence en analyse univariée un effet du
769paludisme sur les niveaux de plomb , notre travail est le premier à décrire un effet des niveaux
770élevés de plomb sur le paludisme. Le mécanisme explicatif de l'interférence entre plomb et
771paludisme serait un effet toxique du métal sur le parasite dans le globule rouge,
772l'immunorégulation, et l'inhibition de l'utilisation du fer par le parasite qui se produit dans un
773contexte d'élévation de la plombémie.

774En outre, le fer comme le plomb sont associés significativement au paludisme, mais aussi à
775l'anémie. En conséquence, il est nécessaire de considérer l'impact sur la morbidité lié au fer
776comme au plomb dans les stratégies dédiées à la lutte contre l'anémie.

777 V. Conclusion

778L'impact du paludisme gestationnel n'implique pas seulement le paludisme placentaire, la
779prématurité ou le petit poids à la naissance, mais aussi un risque accru de paludisme pendant
780l'enfance, probablement suite à un processus de tolérance immunitaire *in utero*. Par conséquent,
781les interventions s'attaquant au paludisme placentaire devraient également avoir un effet sur le
782paludisme chez l'enfant. En effet, l'augmentation de la durée d'administration du TPI (par
783exemple par augmentation du nombre de prises) permettrait d'allonger la période pendant laquelle

784l'enfant est protégé et serait ainsi associée à un moindre risque d'épisodes palustres et à une
785parasitémie par *Plasmodium falciparum* moins importante. Néanmoins dans notre étude, ni le
786moment d'administration du TPI, ni le type de régime (SP ou MQ) ne paraissent avoir d'effet sur
787le paludisme de l'enfant.

788L'association entre les niveaux de fer et le risque palustre pendant la grossesse et l'enfance est
789d'autant plus importante que le contexte d'endémie palustre est associé à une prévalence
790importante d'anémie, rendant les suppléments d'autant plus nécessaires. D'où l'importance de
791montrer ce risque augmenté dans une cohorte prospective chez la femme enceinte et aussi chez
792l'enfant.

793Chez la femme enceinte, même dans le cadre de l'utilisation de moustiquaires et du TPI, nous
794avons montré l'impact des niveaux élevés de ferritine sur le risque d'épisodes palustres et de
795densités parasitaires élevées, ainsi que sur le paludisme placentaire et le petit poids à la naissance.

796Chez l'enfant les mêmes résultats sont retrouvés, ce qui devrait être pris en compte pour élaborer
797les politiques de supplémentation et des nouveaux essais cliniques, qui devraient aussi élargir les
798marqueurs du monitoring du fer.

799

800 VI. Perspectives

801Compte tenu de l'effet probable du paludisme gestationnel sur le paludisme de l'enfant, de
802nombreux arguments plaident en faveur d'une initiation des stratégies préventives contre le
803paludisme gestationnel dès la période pré-conceptionnelle afin de mieux protéger la mère et
804l'enfant. La recherche opérationnelle sur les différentes stratégies de TPI en fonction du contexte
805de résistance à la SP avec des doses élargies devrait fournir des connaissances supplémentaires.
806Ainsi, des analyses coût-efficacité du dépistage et du traitement au niveau communautaire
807pourraient également se révéler très utiles pour les décideurs en santé publique. Le fait que les
808enfants aient une susceptibilité accrue aux parasites portant les mêmes allèles que ceux auxquels

809ils avaient été exposés *in utero*, est également encourageant pour la poursuite de recherches
810explorant le processus de tolérance immunitaire.

811D'autres aspects comme les conséquences neurocognitives du paludisme ou l'effet des
812polymorphismes d'HLA-G sur les symptômes du paludisme mériteraient des recherches plus
813approfondies.

814Sur un plan pratique, la possibilité d'un effet-dose des niveaux de fer sur le risque d'infection
815palustre devrait justifier la réalisation d'essais cliniques de supplémentation avec des doses
816différentes pour en évaluer l'efficacité sur les indicateurs hématologiques.

817Quant à la détermination de méthodes permettant d'obtenir des indicateurs fiables de la charge en
818fer de l'organisme, nous pensons que l'évaluation du fer dans les suivis de populations devrait
819intégrer un dosage de l'hépcidine ainsi que les marqueurs recommandés l'OMS, et inclure des
820marqueurs de l'inflammation comme la CRP ou l'AGP.

821Finalement, si les femmes avaient des niveaux de fer suffisants avant la grossesse, on pourrait
822envisager de diminuer le dosage des suppléments recommandés, ce qui diminuerait les
823inconvenients liés à l'administration de fortes doses de fer. D'autres pratiques comme le clampage
824retardé du cordon ombilical pourraient être appliquées dans le cas des enfants.

825Dans tous les cas, des niveaux de fer suffisants sont vitaux pour la mère et l'enfant, et ils doivent
826être atteints de toutes les manières possibles. En conséquence, les stratégies de contrôle et de
827prévention doivent être optimisées afin d'assurer un risque minimal pendant la grossesse et
828l'enfance.

831

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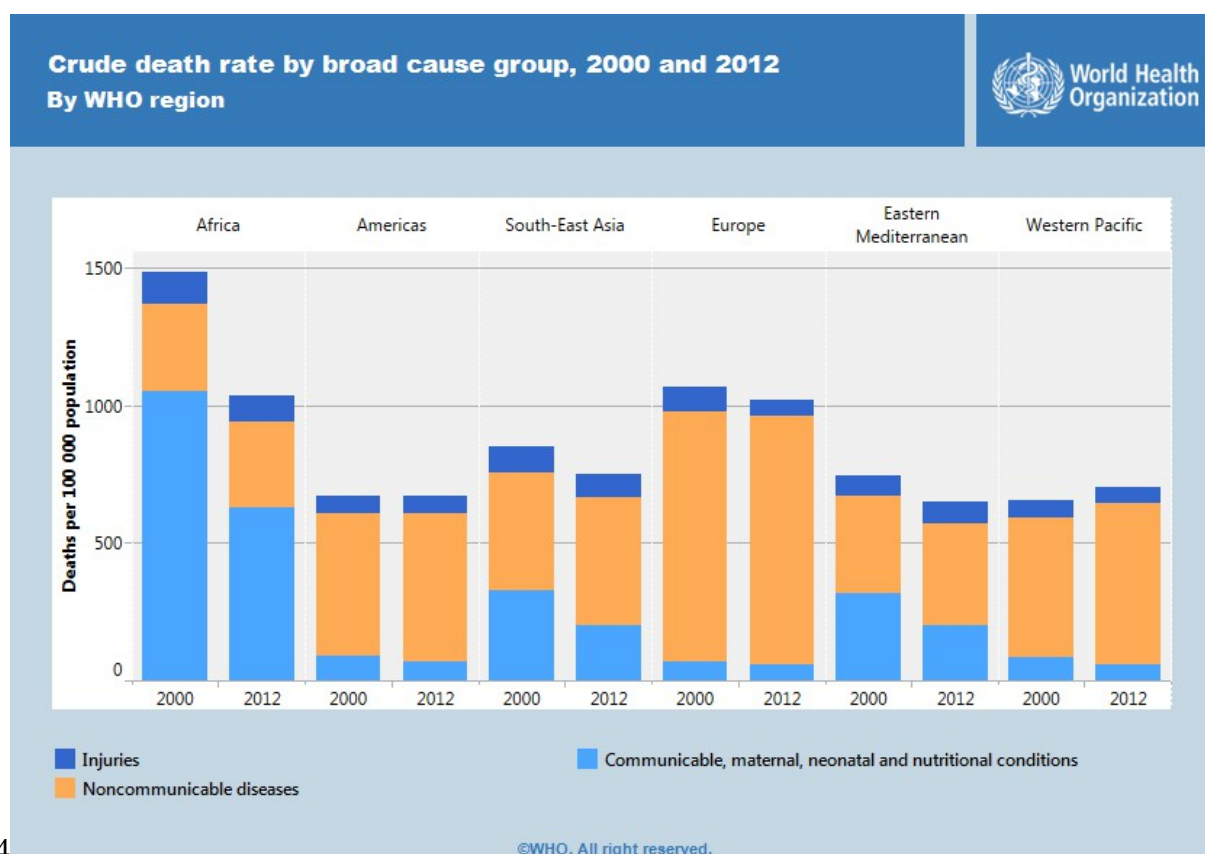
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I. Introduction

996I. 1. The global burden of disease in Africa

997The global burden of disease in the African continent is mainly driven by infectious diseases
 998and nutritional deficiencies, the pregnant women and the children under 5 years being the
 999most vulnerable groups in the population. In the African region communicable, maternal and
 1000nutritional conditions gather the largest proportion of the crude death rate by broad cause
 1001group between 2000 and 2012 (Figure 1). More precisely, in 2012, out of the 1000 deaths per
 1002100,000 people, approximately 60% were due to communicable, maternal and/or nutritional
 1003conditions, whereas communicable diseases gathered 30% and injuries 10%, respectively.



1004

1005Figure 1. Crude death rate by broad cause group between 2000 and 2012, WHO 2013.

1006Hence, maternal and infant health have been prioritized in public health policies. Indeed, they
 1007are at the heart of 4 out of the 8 Millenium Development Goals (i.e. to promote gender
 1008equality and empower women, to reduce child mortality, to improve maternal health, and to
 11947

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122

1009combat HIV/AIDS, malaria, and other diseases). To be most effective, public health strategies

1010need to target the main causes of disease underlying the impaired health status of pregnant

1011women and children.

1012I. 2. The burden of disease in Benin

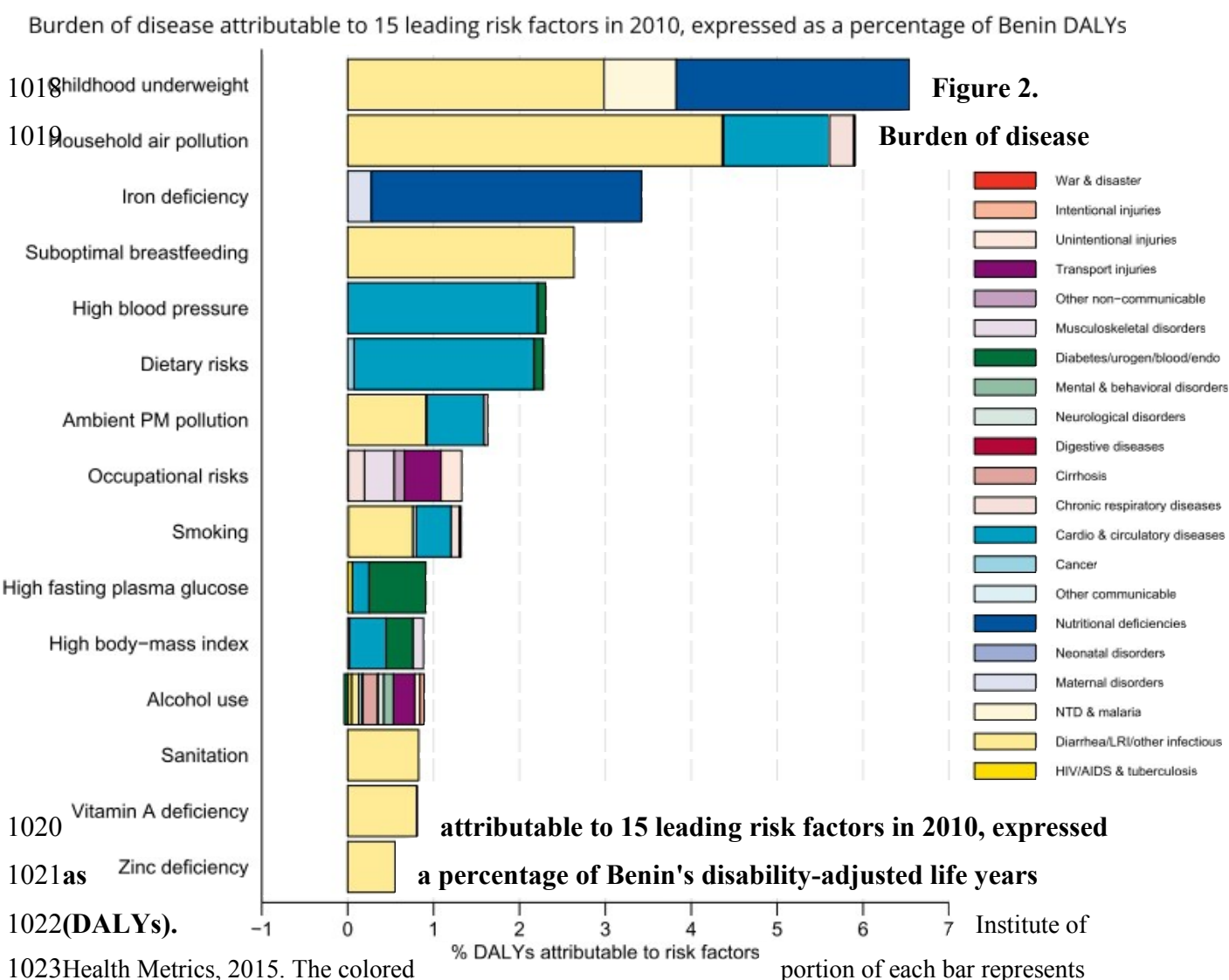
1013In Benin, the three risk factors that account for most of the disease burden (in disability-

1014adjusted life years (DALYs)) are childhood underweight, household air pollution from solid

1015fuels, and iron deficiency (defined by WHO as serum ferritin levels<15µg/l) (Figure 2). The

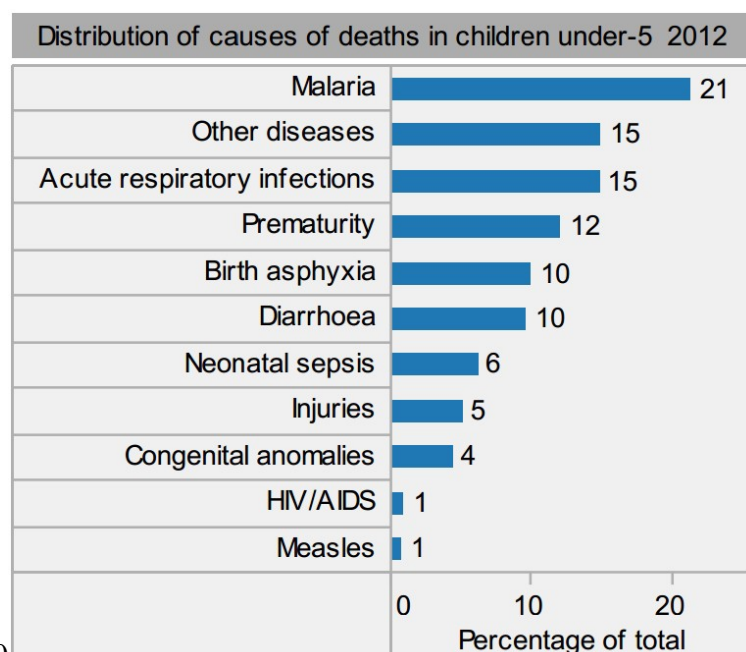
1016leading risk factors for the burden of diseases in children under 5 and adults aged 15-49 years

1017were childhood underweight and iron deficiency, respectively, in 2010.



1024the specific diseases attributable to that risk factor while bar size represents the percentage of DALYs
1025linked to specific risk factors.

1026Albeit the high disease burden gathered by nutritional deficiencies, mortality rates in children
1027under 5 years of age in Benin are driven mainly by malaria (Figure 3). Over 21% of child
1028deaths are caused by malaria, which, in addition, is also responsible for 22.8% of life years
1029lost (LYY) in 2010.



1030

1031**Figure 3. Distribution of causes of deaths in children under 5 years in Benin (2012).**
1032**WHO, 2014.**

1033

1034Therefore, not only globally but also in Benin do nutritional deficiencies and malaria lead

1035morbidity and mortality rates in children under 5 years. For these reasons, substantial efforts

1036have been made by to fight these diseases in Benin.

1037For further knowledge, information on malaria physiopathology is explained in Box 1.

1038Complementary information about the epidemiology of malaria in Benin is presented in Box

10392.

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Box 1. Malaria: Physiopathology and *Plasmodia* life cycle:

Malaria is a human disease caused by a eukaryotic unicellular parasite from the genus *Plasmodium*. There are 5 different *Plasmodia* species that can infect Humans: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*. In Benin the majority of the disease burden is caused by *P. falciparum*, and in this dissertation we will focus on *P. falciparum* malaria. This parasite is transmitted from one infected human host to another human by the bite of the mosquito vector, the female *Anopheles*. *P. falciparum* has a sexual reproduction in the *Anopheles* and an asexual reproductive phase in the human host. After the infectious *Anopheles* bite, the parasites (known as sporozoites at that stage of the life cycle) reach the hepatocytes within which they multiply. After one to two weeks, the infected hepatocytes explode and liberate hepatic merozoites parasites into the blood. The parasites infect then the red blood cells (RBC), where they develop as trophozoites and, after having multiplied, they become schizontes. Upon RBC rupture, erythrocytic merozoites are liberated into the blood and will infect other RBC. After several cycles of erythrocytic multiplication do gametocytes appear. Gametocytes are the sexual form of *Plasmodium*, and they are absorbed by the mosquito bite. After sexual reproduction and then maturation in the gut and salivary glands of the mosquito, respectively, they are injected by the female *Anopheles* to another human host.

Box 2. Malaria in Benin: Epidemiology

Benin is a West-African republic whose surface is 114 762 km². In 2013 the Beninese population was about 10.3 million people, half of them living in the countryside. It has a low Human Development Index (HDI, ranged 165th according to the HDI).

In 2013, according to the WHO World Malaria Report, it is still considered a high transmission country, i.e., there are >1 case per 1000 population per year. Even if there are some infections by *P. vivax*, WHO considers that in Benin almost 100% of malaria cases are due to *P. falciparum*. The main vectors are *A. gambiae*, *A. funestus*, and *A. melas*. In 2014 there were 1,078,834 confirmed cases and 2,288 reported deaths due to malaria. Intermittent preventive treatment in pregnancy (IPTp) against malaria was introduced in 2005, and Insecticide residual spraying (IRS) started to be implemented in 2006. The policy of free distribution of insecticide treated nets (ITNs) was adopted in 2007. The first-line treatment according to Beninese guidelines is arthemether-lumefantrine (AL), and in case of failure and/or severe malaria quinine (QN) is the molecule recommended. Artesunate (AS) is also recommended for severe malaria. Allada, the site of our research study, is a semi-rural area of 91,778 inhabitants located 50 km North of Cotonou (Benin). Malaria has a perennial transmission pattern with two transmission peaks corresponding to the rainy seasons in April-July and October-November. As in the rest of Benin, *Plasmodium falciparum* is the species responsible for the majority of infections. Source: WHO. *World Malaria Report 2014*. Beninese Ministry of Health.

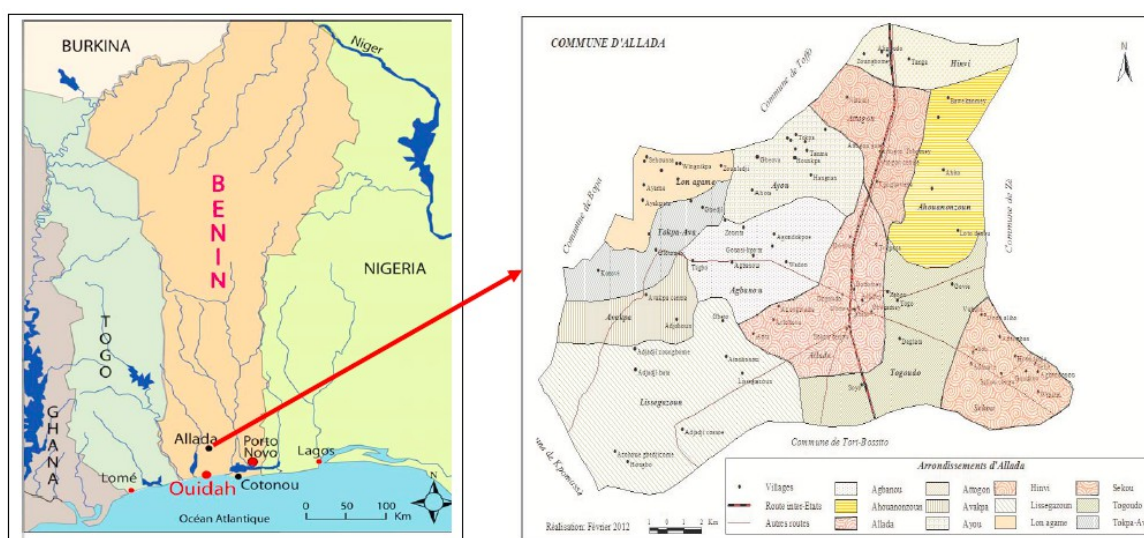


Figure 4. Map and plan of the district of Allada. Source: Institut Géographique National du Bénin.

1043**I. 3. Preventive strategies to tackle the disease burden during**

1044**pregnancy and infancy in Benin**

1045As stated previously, in Benin the main contributors to disease burden during infancy are

1046malaria and nutritional deficiencies.

1047However, there are no official preventive strategies regarding malaria in infants.

1048With regard to nutritional diseases, underweight and iron-deficiency anemia are the main

1049conditions contributing to enhance nutritional deficiencies.

1050Underweight is significantly linked to low birth-weight (LBW, defined as birth-

1051weight<2500g), whose rates in Benin reached 13% in 2012. Indeed, infants with lower birth

1052weights are likely to remain shorter and lighter throughout childhood compared to infants

1053without LBW, especially those having experienced intra-uterine growth retardation (IUGR,

1054defined as birth-weight below the 10th percentile of a reference weight distribution according

1055to gestational age). In addition, LBW is significantly associated to increased morbidity and

1056mortality. Furthermore, LBW and malnutrition have a synergistic relationship with infectious

1057diseases.

1058From the epidemiological perspective, LBW is correlated with pregnancy associated malaria

1059(PAM), low maternal body-mass index (BMI), and maternal micronutrient deficiencies.

1060Therefore, interventions during pregnancy to fight LBW include the prevention of PAM, low

1061BMI and maternal micronutrient deficiencies. To prevent the consequences of PAM, the

1062Ministry of Health implements an intermittent preventive treatment in pregnancy (IPTp)

1063against malaria. This intervention consists in 1500/75 mg of sulphadoxine-pyrimethamine

1064(SP). Usually, it is joint to the anti-helminth parasitic preventive treatment of 600 mg of

1065albendazole, although other treatments are available (appendix 3). Thereby Benin follows

1066WHO recommendations encouraging IPTp with SP for all pregnant women as early as
1067possible in the second trimester, and at each scheduled antenatal care (ANC) visit at least one
1068month apart in areas of moderate to high malaria transmission. Supplementary information on
1069PAM can be found in Box 2, but briefly, PAM by *Plasmodium falciparum* involves the
1070adherence of *Plasmodium* to the placenta, and it is thought this might entail reduced
1071nutritional exchanges between mother and foetus. Consequently, IUGR and prematurity
1072(defined as gestational term less than 37 weeks), the two main mechanisms underlying LBW,
1073are more likely to appear. IPTp should reduce plasmodial parasitemia in the mother's blood,
1074and thereby hinder the red blood cells (RBC) sequestration in the placental intervillous space.
1075Consequently, foeto-maternal exchanges should improve and, consequently, LBW rates
1076should diminish.

Box 3. Pregnancy associated malaria: basic concepts

In pregnancy associated malaria (PAM) erythrocytes infected with *P. falciparum* accumulate in the placenta through adhesion to molecules such as chondroitin sulphate A. Antibody recognition of placental infected erythrocytes is dependent on gravidity, and could protect from malaria complications. Moreover, the parasite gene *var2csa* has been associated with placental malaria, suggesting that its protein product might be an appropriate vaccine candidate. On the contrary, the understanding of placental immunopathology in the context of PAM and how this contributes to anaemia and low birth-weight has not been elucidated so far; although we know that inflammatory cytokines produced by T cells, macrophages, and other cells play a major role.

The symptoms and complications of PAM vary according to malaria transmission intensity in the given geographical area and according to the individual's level of acquired immunity. In high-transmission settings, where levels of acquired immunity tend to be high, *P. falciparum* infection is usually asymptomatic in pregnancy. Yet, parasites may be present in the placenta and contribute to maternal anaemia even in the absence of documented peripheral parasitaemia. In high-transmission settings, the adverse effects of *P. falciparum* infection in pregnancy are most pronounced for women in their first pregnancy. In low-transmission settings, where women of reproductive age have relatively little acquired immunity to malaria, malaria in pregnancy is associated with anemia, an increased risk of severe malaria, and it may lead to spontaneous abortion, stillbirth, prematurity and low birth weight. In such settings, malaria affects all pregnant women, regardless of the number of times they have been pregnant. Sources: WHO, Rogerson

To fight nutritional deficiencies during pregnancy, the Beninese Ministry of Health prioritized the prevention of anemia (defined by WHO as hemoglobin (Hb) <11g/l). Therefore, it

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1080recommends supplements of 200 mg of ferrous sulphate and 5 mg of folate given daily until
108145 days after delivery.

1082Indeed, anemia, including iron-deficiency anemia, constitute a public health concern not only
1083during pregnancy but also during infancy. Despite the lack of official recommendations, in
1084case of iron deficiency anemia, Beninese paediatricians give daily supplements of iron of 10
1085mg/kg/day and 0.5 mg/kg/day of folic acid during 2 months, every 6 months, starting at 6
1086months of age until 5 years. This is similar to WHO guidelines, which recommend 12.5 mg
1087iron and 50µg folic acid to prevent anaemia in children 6-24 months. In case of low birth-
1088weight (LBW), defined by birth weight<2500g, supplements start at 2 months. Concrete
1089details on accurate recommendations of the national Beninese program against malaria are
1090given in the appendix 3.

1091However, there is some epidemiological evidence that suggests that iron supplements could
1092have an effect on malaria appearance and severity. Considering that iron supplements are
1093given systematically during pregnancy in Benin, and that malaria is endemic in the region, we
1094wanted to investigate the possible effect of iron levels on PAM. Furthermore, we wanted to
1095analyse the effect of the infant iron levels on malaria in infants as malaria is the first cause of
1096infant mortality, and there are no national guidelines on the iron supplementation policy in
1097infants.

1098In parallel, as PAM seems to have a significant effect on malaria in infants, and IPTp has an
1099impact on secondary malaria outcomes (such as LBW and anaemia), we wanted to investigate
1100the possible impact of IPTp on malaria in infants during the first year of life.

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II. State of the art

1116**II.1. Effect of preventive public health interventions during pregnancy**
1117**on pregnancy associated malaria: evidence of protective measures and**
1118**iron levels.**

1119**II.1.1. Effect of IPTp on PAM outcomes: clinical malaria in pregnancy,**
1120**placental malaria, and low birth-weight.**

1121**II.1.1.a. Epidemiological evidence**

1122Pregnancy associated malaria (PAM) is defined as peripheral or placental infection by
1123*Plasmodium*, and it constitutes a stake of interest for infant health as its consequences may
1124attain 125 million pregnancies at risk of malaria infection every year. More precisely it is
1125estimated that 32 million women become pregnant every year in Sub-Saharan Africa endemic
1126countries. The prevalence of malaria in pregnancy is influenced by transmission, the
1127immunity of the mother and protective measures. The main protective interventions against
1128PAM are insecticide-treated nets (ITNs) and intermittent preventive treatment (IPT). IPT is a
1129widespread preventive strategy to fight malaria consisting in the administration of a curative
1130dose of an effective anti-malarial drug, regardless of the presence of *Plasmodium* in the blood,
1131to prevent the effects of the disease. A landmark review gathering evidence on PAM between
11321985 and 2000 in Sub-Saharan Africa stated a median prevalence of PAM of 27.8% among
1133all gravidae. In low transmission African settings the median prevalence peripheral infection
1134was 13.7% and placental malaria median prevalence was 6.7%. In general, recent studies
1135report a significant decline in prevalence following IPTp implementation since the beginning
1136of the XXI century. A systematic review and meta-analysis of trials determining whether
1137regimens containing 3 or more doses of SP for IPTp were associated with a higher birth
1138weight or lower risk of LBW than standard 2-dose regimens showed that the 2⁴-dose group

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1139 had less placental malaria (RR=0.51; 95% CI (0.38; 0.68) in 6 trials, 63 vs 32 per 1000;

1140 absolute risk reduction, 31 per 1000 (95%CI (20; 39)). However, the augmented efficacy

1141 related to higher doses is mostly observed in the case of clinical trials rather than in studies

1142 issued from public health program implementations. Finally, the additional protection of the

1143 joint use of ITNs with IPTp-SP is significant only in certain trials, but reported ITN use

1144 ranges from 5% to 25%, and it might not be sufficient to show an effect.

1145 In short the prevalence of PAM has evolved according to transmission and protective

1146 measures like IPTp or ITN use. Further elements like gestation and the moment of infection

1147 during pregnancy have shown to influence its pathologic consequences as well. Because

1148 immunity develops during the first pregnancy, primigravidae are especially at higher risk for

1149 PAM.

1150 Finally the timing of high parasitemia infections during pregnancy entails different effects on

1151 PAM outcomes like anemia or LBW. Therefore, the administration of IPTp at different

1152 moments determines different protection patterns for the infant.

1153 As a result of these concurrent realities, we have to consider other determinants of PAM, such

1154 as transmission, IPTp regime and gestity, to better understand the shades of the influence of

1155 IPTp on PAM.

1156 **II.1.1.b. Effect of IPTp on PAM**

1157 WHO recommends in areas of moderate to high malaria transmission, IPTp with SP for all

1158 pregnant women as early as possible in the second trimester, and at each scheduled antenatal

1159 care visit at least one month apart. The different IPTp regimes implemented in the African

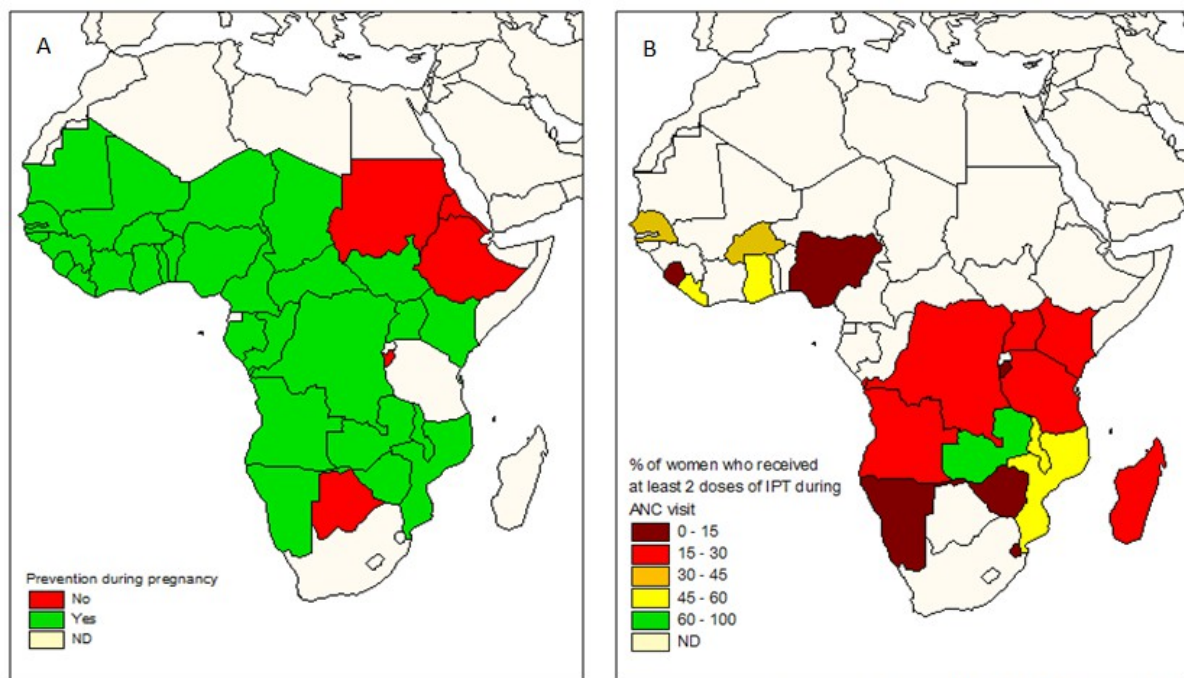
1160 region are described in Figure 4.

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1162 **Figure 5. Different IPTp regimes implemented in Sub-Saharan Africa**

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Source: WHO World Malaria Report 2013

1164 Effective IPTp clears placental parasitemia and consequently modifies the exposure to
 1165 malaria antigens. As a result, a significant reduction in placental malaria and maternal
 1166 parasitemia has been extensively described in founding literature. Compared to case
 1167 management or placebo in pregnant women, 2-dose IPTp with sulfadoxine-pyrimethamine
 1168 (SP) reduced significantly placental malaria according to a review on 4 studies (relative risk
 1169 (RR)=0.48). In a randomised, double blind, placebo-controlled trial with joint use of ITNs in
 1170 Mozambique, SP-IPTp (1-2 doses) was not associated with placental malaria ($p = 0.964$),
 1171 defined as the presence of parasites and/or pigment in the histological examination, and/or in
 1172 the impression smear. Nevertheless, the SP group showed a 40% reduction (95% CI (7.40;
 1173 61.20); p -value = 0.02) in the incidence of clinical malaria during pregnancy, and reductions
 1174 in the prevalence of peripheral parasitemia (7.10% vs 15.15%) (p -value=0.001), and of
 1175 actively infected placentas, defined as presence of parasites (7.04% vs 13.60%) (p -value=
 1176 0.002), (Table1).

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Table 1. Influence of pregnancy associated malaria on malaria in infants

Cohort	Study design and sample size	Time period	Transmission setting	Malaria prevention strategy during pregnancy	Treatment drug regime	Proportion of maternal peripheral parasitemia at delivery	Proportion of placental parasitemia	Proportion of neonatal parasitemia	Infant follow-up period	Median time to first parasitemia (days, min, max)	Association of infant malaria with PAM	Early infant parasitemia <3 months
Mangochi ⁶⁹ (Malawi)	Clinical trial on comparative efficacy of CQ or MQ; infant cohort follow-up (1766 women at delivery and 1289 infants)	1988-1990	Perennial with seasonal peaks	CQ and MQ	CQ	CQ: 20.3% MQ: 4.1%	CQ: 25.1% MQ: 6.2%	CQ: 8.6% MQ: 3.1%	12 months	199 (192-207)	at 3 months: 1.1 (0.7-1.9)	18.5%
Ebolowa ¹³ (Cameroon)	Infant cohort follow-up (197)	1993-1995	Perennial with seasonal peaks	CQ	CQ		22.84% (Primigravid: 69%; Multigravid: 31%)		24 months	PM+: 217; PM-:350	at 6 months: PM+: 36%; PM-: 14%, p<0.05 at 2 years: PM+: 46.5%; PM-: 38.5%, p=0.6	≈12%
Muheza ¹⁴ (Tanzania)	Infant cohort follow-up (453)	2002-2004	Perennial with seasonal peaks (400 infective mosquito bites each year)	SP (area with 68% resistance 14-day treatment failure rate)			15.2% (Primigravid≤2: 24%; Multigravid>2: 5.6%)		12 months	266 (238-294) PM-:273 (245-322) PM+: 244 (147-266);	Primigravidae: PM+:AOR=0.21, (0.09-0.47) PM-: Reference*** Multigravidae: PM+: AOR=1.59, (1.16-2.17) PM-:AOR=0.67, (0.50-0.91)	PM+ ≈20%; PM-≈10%
Lambarene ¹⁵ (Gabon)	Infant cohort follow-up (527)	2002-2004	Perennial	No		10.5%*	9.48%		30 months	Primigravidae: PM+:107 (83-139) PM-:102 (29-205) Multigravidae: PM+:111 (13-189) PM-:92 (27-208)	PM+:AOR= 2.1, (1.2-3) PM-: Reference**	PM+ ≈2%; PM-≈0%
Manhiça ²⁹ (Mozambique)	Clinical trial on the efficacy of SP compared to placebo; infant cohort follow-up (1030 women at delivery and 997 infants)	2003-2005	Perennial with seasonal peaks	ITNs vs ITNs+SP	SP-AQ	ITNs+ placebo:15.15% ITNs+SP: 7.1%	ITNs+ placebo:52.27% ITNs+SP: 52.11%	ITNs+ placebo:1.15% ITNs+SP: 0.92%	12 months		Clinical PAM: AOR=1.96 (1.13-3.41) Acute PM: AOR= 4.63 (2.1-10.24) Chronic PM: AOR=3.95 (2.07-7.55) PM-: Reference	
Tori Bossito ^{17,28} (Benin)	Infant cohort follow-up (550)	2007-2008	Perennial with seasonal peaks (400 infective mosquito bites each year)	SP	AL		11%	0.83%	12 months	PM+: 34 (4-83); PM-: 43 (4-85)	ITN:AOR=2.13 (1.24-3.67) No ITN: AOR=1.18 (0.60-2.33)	20.3%
Mono ³⁵ (Benin)	Mother and infant cohort follow-up (218)	2008-2010	Mesoendemic (1-35 bites/person/year)	SP	Quinine or SP		3.67%		12 months	PAM+: 362 (18-390) PAM-: 365 (64-449)	PAM during the 3rd trimester of pregnancy: AOR= 4.6 (1.7; 12.5) PAM during the 1st and 2nd trimesters non significant	

PM: Placental malaria, PAM: Pregnancy associated malaria and AOR: Adjusted Odds Ratio

* data from a reference article

**the association between placental malaria and malaria in the child was only statistically significant for children who were randomized to receive the sulphadoxine-pyrimethamine intervention (adjusted Hazard ratio (aHR)=3 (1.5-6))

***Analysis of the effect of IPTp on parasitemia of the offspring was performed for 882 women of this cohort. Among them, 21.6% received no IPTp, 42% one dose, and 36.4% two or more doses.

1178In Mali placental parasitemia was significantly reduced by SP-IPTp (aOR=0.69) when
1179compared to weekly chloroquine (CQ) and confirmed higher SP efficacy compared to CQ
1180already reported in Malawi. A recent meta-analysis has concluded to significant PM reduction
1181for 3 doses of SP compared to 2 doses which approaches the current WHO recommendations.

1182Problems related to reduced compliance with drug regimes and the increasing resistance to
1183anti-malaria drugs bring up the complexity of IPTp management at present. A 2007 meta-
1184analysis confirmed that SP IPTp continued to benefit pregnant women in areas of up to 39%
1185resistance to SP by day 14 in children, and similar results were found in Benin, where rates of
1186in vivo resistance to SP were estimated to be 50% by day 28 of treatment in infants, and yet
1187SP IPTp succeeded to prevent LBW. However, studies published more recently display
1188contradictory results. A study in Malawi, where there is a strong fixation of the resistant
1189quintuple mutant (mutations at *dhfr* codons 51, 59, and 108 and *dhps* codons 437 and 540),
1190showed that the number of IPTp doses has a protective effect on birth outcomes but not on
1191placental infection. More concretely, there were significantly less small for gestational age
1192(SGA) rates in offspring of primigravid women having received ≥ 2 doses of SP compared to
11930-1 doses even if peripheral parasitemia was significantly higher among women having
1194received ≥ 2 doses of SP. Indeed, the effects of resistance on malaria clinical outcomes
1195become more frequent in more recent studies from East Africa. In a Tanzanian site with high
1196SP resistance (14-day parasitologic SP treatment failure rate in children of 68%), IPTp was
1197not associated with a reduction in the odds of PM, LBW or maternal anemia. Furthermore, it
1198was associated with increased odds of fetal anemia and severe malaria among the offspring
1199(AOR=2.31). IPTp in this setting was associated with an increased risk of severe malaria
1200overall. Nevertheless, a recent longitudinal study showed no significant increase of malaria at
1201delivery after IPTp treatment albeit the increasing prevalence and fixation of SP-resistant *P.*
1202*falciparum* haplotypes in another area in Malawi. In conclusion, evidence on the present

1203 efficacy of SP-IPTp regimes is inaccurate but resistance to SP is spreading. And close

1204 monitoring of its efficacy is necessary to determine if or when the treatment failure of SP-

1205 IPTp detected by some recent studies is generalized at the population level and, in this case, a

1206 switch to other drug regimes would become necessary.

1207 Furthermore, effective IPTp diminishes PM and malaria associated morbidity like LBW, pre-

1208 term delivery, IUGR and perinatal mortality in areas where resistance to SP is not highly

1209 significant. Even the Malaria Policy Advisory Committee (MPAC) concluded that there is

1210 currently insufficient data to determine at what level of resistance IPTp-SP should be

1211 interrupted in the absence of an established and effective alternative. Yet, the influence of

1212 different IPTp regimes on malaria morbidity in infants remains a question for further research.

1213 The concrete effect of resistance and the ongoing immune tolerance process *in utero* are

1214 neither elucidated so far. Further evidence lacks as well on the importance of the timing of

1215 infection during pregnancy and infant malaria morbidity for instance. There is some evidence

1216 that earlier administration of IPTp has a positive effect on birth outcomes like LBW,

1217 nevertheless, it seems that later dosing provides a better protection at delivery. This is one

1218 reason because of which the administration of 3 doses instead of 2 shows better clinical

1219 outcomes. In addition, the implementation of different IPTp regimes, in the context of

1220 different resistance patterns, entails novel stakes to the question regarding the adequate IPTp

1221 policy according to the transmission and resistance setting. For instance, intermittent

1222 screening and testing (IST) has been applied successfully in an area of moderately high

1223 malaria transmission in Ghana. IST consists in screening for malaria infection using a malaria

1224 rapid diagnostic test (RDT) at scheduled antenatal clinic visits and subsequently treating

1225 positive women with an effective anti-malarial drug. Currently, the DHA-PQ IST is a

1226 proposed alternative to IPTp in areas with substantial resistance against IPTp regimes.

1227 However, at present conclusive evidence on IST efficacy is lacking in African regions and

1228further efficacy studies should be conducted.

1229The evaluation of its efficacy in other transmission settings is necessary to ascertain its utility

1230as an effective tool for the control of PAM.

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II.1.2. Effect of iron levels on PAM

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II.1.2.a. Iron markers

1233Before analyzing the effect of iron levels on PAM, it is useful to discern the specific

1234information provided by the different iron markers. A joint summary is presented in table 2.

Table 2: Iron indicators selected by the WHO-CDC Technical Consultation for iron assessment			
Indicator	Refers to	Threshold values (venous blood of persons residing at sea level)	Other valuable information
Hemoglobin	Anaemia	For anaemia: children aged 6 months to 6 years: 11g/100ml children aged 6–14 years: 12g/100ml adult males: 13g/100ml adult females, non-pregnant: 12g/100ml adult females, pregnant: 11g/100ml	The assessment of hemoglobin alone can provide only a rough estimate of the likely prevalence of iron deficiency anaemia (IDA). The absence of a consistent standard for identifying iron deficiency contributes to confound the analyses on the relationship between anaemia and IDA prevalence rates
Zinc protoporphyrin (ZPP)	Iron deficient erythropoiesis	>70-80 µmol/mol for infants	In the last step in hemoglobin synthesis, the enzyme ferrochetalase inserts iron. A lack of iron available to ferrochetalase during the early stages of iron deficient erythropoiesis results in a measurable increase in the concentration of zinc protoporphyrin, as trace amounts of zinc are incorporated into protoporphyrin instead. The normal ratio of iron to zinc in protoporphyrin is about 30 000:1. Thresholds for ZPP vary between 40 and 70 µmol/ mol haem depending on whether the cells have been washed before the assay or not
Mean cell volume (MCV)	Red blood cell size, anaemia characteristics. Microcytic anaemia is a sign of iron deficiency anaemia, whereas macrocytic anaemia indicates deficiency of vitamin B12 or folate	<67-81fl	Even if MCV is used widely for the evaluation of nutritional iron deficiency, low values are not specific to iron deficiency, but they are also found in thalassaemia and in about 50% of people with anaemia due to inflammation

Transferrin receptor in serum (STR)	Inadequate delivery of iron to bone marrow and tissue	It is not possible to assign a single threshold value that would be accurate for all commercial kits. Approximately: During severe beta thalassaemia the sTfR concentration is >100 mg/l During severe iron deficiency anaemia it is >20–30 mg/l	sTfR is sensitive to erythropoiesis due to any cause. Hence, it cannot be interpreted as an indicator of solely iron deficiency erythropoiesis. Its concentration increases in individuals with stimulated erythropoiesis, such as haemolytic anaemia and sickle cell anaemia. Indeed, acute or chronic inflammation and the anaemia of chronic disease, malaria, malnutrition, age and pregnancy may modify significantly sTfR. There is a lack of standardization between different commercial kits for measuring the concentration of transferrin receptor
Serum ferritin (SF)	Iron deficiency. SF is an iron storage protein that provides iron for haem synthesis when required.	Iron deficiency anaemia: SF concentration <12–15 µg/l.	Needs to be corrected upon inflammation. In clinical malaria a high SF values result from the destruction of red blood cells, an acute phase response, suppressed erythropoiesis, and ferritin released from damaged liver or spleen cells. However, in “holo-endemic” settings, the influence of parasite load on SF appears to be restrained and reliable after correction. The changes in SF concentration during development from birth to old age reflect changes in the amounts of iron stored in tissues

Source: Report of a technical consultation on the assessment of iron status at the population level. WHO-CDC, 2004

1235

1236The joint WHO-CDC Technical Consultation for iron assessment selected 5 different

1237indicators as good iron markers: hemoglobin, mean cell volume (MCV), (sTfR)

1238concentration, serum ferritin concentration, and red cell protoporphyrin (measured by the zinc

1239protoporphyrin/hemoglobin ratio (ZPP:H)). Hemoglobin is deeply useful in the monitoring of

1240health status and its determination is easy to realize on the field. Although it is a basic

1241fundamental haematological indicator, it is not specific as an iron marker because of the

1242multiple causes of anaemia and the physiological variations with regard to sex, age or

1243ethnicity. Therefore, it can be misleading for the extrapolation of conclusive results. Mean

1244cell volume accuracy is limited in the context of thalassemia and malaria as inflammation

1245serum transferrin receptor modifies significantly its values. Due to its physiopathological

1246pathway, serum transferrin receptor is also influenced by the haemolysis of malaria, and its

1247determination method is not always standardized nor cost-effective.

1248Serum ferritin is a precise indicator of iron storages in healthy individuals and it can be

1249corrected according to other inflammation proteins. It provides further information as it also

1250shows different patterns of behaviour depending on the aetiology of anaemia. In an iron

1251supplementation study in children, Doherty et al. compared the erythrocyte incorporation of

1252oral iron supplement in 37 Gambian children 8 to 36 months old with anaemia after malaria

1253treatment, to supplemented control children with IDA but no recent malaria. The non-malaria

1254control children showed progressively increased serum ferritin whereas the post-malarial

1255children showed decreased serum ferritin levels. Serum ferritin levels became similar in both

1256groups only by day 15 and 30. This is thought to be due to the normalization of the immune

1257response and to the normalization of the acute phase proteins following the malaria treatment.

1258Indeed serum ferritin is an acute phase protein. Hence, serum ferritin is either corrected upon

1259inflammation (with correction factors according to C-reactive protein (CRP) or α -1-

1260glycoprotein (AGP) levels), or samples with high acute inflammation proteins are

1261systematically excluded. Nevertheless the exclusion of samples with increased inflammation

1262might entail a subsequent bias in the context of malaria, as samples with high ferritin would

1263be systematically excluded as well. Despite its limited accuracy in case of inflammation,

1264ferritin is a consistent extended iron marker.

1265Along with ferritin, ZPP:H ratio is the most frequently used indicator for iron assessment. The

1266chelation of ferrous iron by protoporphyrin is the final step for the heme synthesis. In iron

1267deficiency zinc is chelated as iron is not available and ZPP formation is decreased. In the iron-

1268deficient parasitized RBC, the increased ZPP could bind to heme crystals, and inhibit the

1269formation of hemozoin. Longstanding inflammation processes, thalassaemia, and

1270asymptomatic *P. falciparum* parasitemia might also show elevated ZPP:H ratios, and

1271consequently be erroneously associated to iron deficiency. In addition there is no standardized

1272corrections applicable to ZPP:H ratios in the context of long-term inflammation processes.

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1273 Finally high lead levels interfere with ZPP:H, and polluted regions frequently overlap with

1274 malaria endemic settings. However, the impact of inflammation on ZPP:H is not as important

1275 as on serum ferritin.

1276 A novel marker has recently emerged as an alternative indicator: hepcidin. Hepcidin is a

1277 peptide hormone, which plays a crucial role in iron regulation and is determinant in the

1278 malaria infection process. Hepcidin binds ferroportin, it increases in response to inflammation

1279 and blocks iron entry into the plasma. It has been proposed as a good marker for iron levels,

1280 especially because it might be up-regulated after malaria episodes compared to other markers

1281 of iron-deficiency. Therefore, a priori, it might permit to distinguish between iron-deficiency

1282 and malaria related anaemia. However, hepcidin shows a non-linear association with anaemia

1283 in the context of malaria albeit its significant association with parasitemia in children.

1284 Furthermore, in Kenya it was increased on admission at hospital for *P. falciparum* malaria

1285 and was significantly associated with parasite density, but hepcidin levels were very low in

1286 severe malaria anaemia. In addition, its accuracy as an iron marker has been recently

1287 questioned as it has been shown that it is associated with the anti-inflammatory response but

1288 not with iron or anaemic status among malarial Nigerian children. Hence, further studies with

1289 more statistical power should be encouraged to ascertain its utility as an iron marker.

1290 In conclusion, complementary indicators are needed for the accurate assessment of iron status.

1291 In this respect, inflammation parameters are necessary to correct ferritin levels in the context

1292 of malaria, and further research is expected in order to determine precisely the utility of

1293 hepcidin in iron assessment in the context of malaria. It is also important to highlight the

1294 danger of categorising non-iron deficient individuals as "iron-replete", as limits for iron

1295 deficiency are not rigid and should be considered with caution and in relation to the clinical

1296 and environmental settings.

1297 II.2.2.b. Effect of iron levels on PAM: epidemiological evidence

1298 To certainly ascertain the effect of iron on PAM, it is essential to consider both the effect of
1299 iron levels at baseline and with no intervention, and also the effect of iron supplements on
1300 PAM as both measures embody different information.

1301 With regard to iron supplementation during pregnancy, its benefits for reducing iron related
1302 diseases are undeniable. A Cochrane review showed supplementation was associated to a 70%
1303 decreased risk of anaemia and to a 57% reduced risk of iron deficiency at delivery compared
1304 to controls. However, epidemiological studies have set into question the inviolability of the
1305 benefits of iron supplementation in the context of malaria-endemic countries. In a recent
1306 meta-analysis of the association between malaria and iron status or supplementation, data
1307 were reported to be insufficient for assessing the potential for an increased risk of *P.*
1308 *falciparum* infection. In addition, iron deficiency at baseline was associated with a decreased
1309 malarial risk in pregnancy when measured by ferritin, which is a robust indicator for iron
1310 levels.

1311 Although iron supplementation trials do not show augmented malaria morbidity associated
1312 with iron supplements, iron deficiency is correlated with lower odds of malarial episodes. Iron
1313 deficiency was statistically linked to reduced risk of placental malaria in Tanzania. Ferritin
1314 was also higher among placenta-infected mothers in Gabon and zinc protoporphyrin in
1315 Malawi, but these differences were not statistically significant. Similar results were found in
1316 clinical trials in The Gambia or Kenya. The recent meta-analysis on malarial risk and iron
1317 status suggested a possible but not significant difference in placental malaria associated with
1318 iron supplementation depending on sickle cell genotype. However, these studies report iron
1319 levels only at enrolment, at delivery, or both, and the limited sample sizes may be insufficient
1320 to show a statistically significant effect.

1321 **II.2.2.c. Effect of iron levels on PAM: physiopathology and further perspectives**

Possible explanations for the increased malarial risk associated with iron levels are related to malaria physiopathology in both the host and the parasite. At the host level, iron inhibits the synthesis of nitric oxide by inhibiting the expression of inducible nitric oxide synthase (iNOS), and thereby interferes with macrophage-mediated cytotoxicity against *Plasmodium*. Moreover, non-transferrine bound iron (NTBI) is involved in the severity of malaria. Indeed, *Plasmodium* has the capacity of acquiring iron in a transferrin-independent pathway.

In any case, the lack of complete follow-up of women through pregnancy is an important obstacle for the assessment of the influence of iron levels on *P.falciparum* malaria. In the majority of the studies included in the meta-analysis, iron was only determined either at enrolment, at delivery, or both, as already said. In the only prospective cohort malaria was analysed solely with regard to the first episode of the pregnancy. Furthermore, the authors themselves have underlined that the present evidence is inconclusive. Hence, the continuous monitoring of iron levels in the context of a PAM episode, might allow us to provide important supplementary evidence on the effect of iron levels on PAM.

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II.2. Malaria risk factors in infants: Effect of PAM and iron levels on malaria episodes and *Plasmodium falciparum* parasitemia.

II.2.1. Effect of PAM and IPTp on malaria in infants

II.2.1.a. Epidemiological evidence of PAM and IPTp

As already described in the pregnancy section, the impact of PAM on the infants includes low birth weight (LBW) (mainly induced by intra-uterine growth retardation (IUGR) and to a lesser extent pre-term delivery), stillbirth, reduced anthropometric parameters, increased mother-to-child HIV transmission, congenital malaria and fetal anemia. Taking all these

1345effects into account, PAM would be responsible for 75,000 to 200,000 deaths in infants in
1346Sub-Saharan Africa.

1347But beyond this indirect effect on infant mortality and morbidity, the impact of the exposure
1348to parasites *in utero* on the parasitemia of the infant arises in epidemiological studies as a risk
1349factor for increased susceptibility to malaria among the offspring. In this respect, research on
1350whether infants of primigravid women will be possibly at higher risk for subsequent malaria
1351as a result of reduced antibody transfer is still ongoing. In parallel, a significant reduction in
1352placental malaria and maternal parasitemia has been extensively described in founding
1353literature following the implementation of IPTp programs. Finally, the timing of high
1354parasitemia infections during pregnancy entails different effects on the infant. Therefore, the
1355administration of IPTp at different moments determines different protection patterns for the
1356infant.

1357Placental malaria (presence of parasites in the placenta) is shown to be an important
1358trademark for increased susceptibility to malaria during infancy, possibly due to its role as a
1359surrogate of the maternal infection. It has been associated with congenital malaria, increased
1360malaria episodes, anaemia, and non-malaria fever episodes in infants.

1361Congenital malaria is defined as the presence of asexual parasites in the cord blood or in the
1362peripheral blood during the first week of life. It is the result of transplacental transmission of
1363parasites just before or during delivery. Congenital malaria rates range between 0,83-5,96%
1364of total births in recent epidemiological studies. Nevertheless, the introduction of molecular
1365techniques has increased the detection of cord blood parasitemia raising prevalence rates up to
136633%. Although it might entail clinical important consequences in some cases and should be
1367considered in the differential diagnostic of neonatal fever in endemic countries, congenital
1368malaria does not seem to constitute an epidemic emergency at present. Nevertheless, we
1369should consider that symptomatic congenital malaria is more frequent in unstable malaria

1370transmission settings compared to high transmission settings.

1371Placental malaria is consistently associated with susceptibility to malaria with regard to both
1372first event and overall clinical episodes. In a landmark longitudinal cohort of infants in
1373Cameroon placental *P. falciparum* infection was associated with infant malaria between 4 and
13746 months, and parasitemia rates were higher between 5 to 8 months in offspring of placenta-
1375infected mothers independently of congenital infection. A study in Tanzania found an
1376interaction between gravidity and placental malaria. Albeit the lowest odds for offspring of
1377primigravid placenta infected pairs, multigravid gestation among placenta positive pairs was
1378the highest (Adjusted Odds Ratio (aOR=1.59)). Nevertheless epidemiological studies show
1379overall increased susceptibility to malaria among primigravidae (Table 1).

1380With regard to the early appearance of parasites in infants, the above mentioned study in
1381Tanzania reported a 1.41 estimated hazard ratio (HR) of first parasitemia for offspring of
1382mothers with *P. falciparum* placental infection, after adjustment for gravidity, transmission
1383season at time of birth, area of residence, and bed net usage. In Gabon a significant correlation
1384was also found (adjusted HR (aHR)=2.1) after adjustment for gravidity, season of birth, area
1385of residence, IPTp versus placebo, and ITNs. In Tori Bossito (Benin) the consistent
1386entomologic and environmental follow-up of infants confirmed the link between PM and
1387malaria in infants controlling for transmission intensity (aHR=2.13) for infants sleeping in a
1388house with an ITN, even after control for season, number of anopheles, antenatal care visits
1389and maternal severe anaemia, compared with infants whose mothers did not have placental
1390malaria at delivery. In addition, this cohort reports an increased susceptibility of infants to *P.*
1391*falciparum* parasites with antigens to which they were previously exposed *in utero*,
1392suggesting an immune tolerance process undergoing during pregnancy. PAM has also been
1393correlated to reduced transfer of maternal antibodies to the foetus, and this would increase the
1394infant susceptibility to parasites. Consistent with the idea that the type, the timing and the

1395duration of exposure to the parasite *in utero* determine susceptibility to malaria, infections

1396occurring during the 3rd trimester are associated with increased risk of infection and clinical

1397malaria during the first year of life in another study in Mono (Benin). In parallel, there is also

1398a first scientific evidence on the fact that HLA-G polymorphisms could be associated with

1399different malaria susceptibility.

1400But the effect of PAM may entail consequences for the morbidity and mortality of the infant

1401also in a broad manner. Indeed, both acute placental malaria and cord blood parasitemia have

1402been found associated with increased mortality. Moreover, placental malaria was a significant

1403risk factor for mortality in general during the first year of life in a study in Malawi. In

1404Mozambique infant mortality was also significantly associated with malaria infection of the

1405placenta (p-value<0.012) after adjustment for HIV status, LBW, maternal clinical malaria

1406during pregnancy, fetal anemia and IPTp regime. And mortality risk was significantly higher

1407(odds ratio (OR)=5.08) for infants issued of acute infection of the placenta at delivery.

1408Placental malaria was also correlated with non-malaria infections in the Tori Bossito cohort

1409infants during the first 18 months of life, suggesting that immune tolerance could also imply

1410immunity in a more general manner besides malaria specific immunity.

1411Even if complete explanation of the physiopathology of PAM has not been found so far to our

1412knowledge, *in utero* exposure to malaria might be correlated with placental sequestration of

1413erythrocytes, and the immune tolerance process might depend on the type of malaria antigen

1414in contact with the foetus, the amount and the duration of the exposure, and the moment of

1415exposure during pregnancy. However these parameters are modified by the introduction of

1416intermittent preventive treatment in pregnancy (IPTp). Indeed, intermittent preventive

1417treatment in pregnancy modifies parasite exposure to the fetus. Hence, IPTp may introduce

1418substantial changes in the epidemiologic pattern of malaria in infants, possibly as the result of

1419an ongoing process of immune tolerance to antigens *in utero*. However, little evidence exists

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1420on the subject at present.

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1422**II.2.2. Effect of iron on malaria in infants**

1423**II.2.2.a. Effect of iron levels on malaria in infants: epidemiological evidence**

1424Observational studies display information reflecting the association between iron and malaria
1425based on the real circumstances of the field, but accurate iron monitoring is not commonly
1426realized on a systematic basis in this context. Clinical trials focus rather on the effect of
1427supplements and investigate the possible consequences for malaria outcomes of the iron
1428supplementation policy, but their methodological protective constraints do not reflect the
1429epidemiological reality of malaria endemic settings. Indeed, both approaches assemble
1430different but important information and, therefore, both should be considered for the analysis
1431of the iron-malaria link. The results of the main studies on the malaria-iron relationship in
1432infants are presented in table 3.

Table 3. Effect of iron supplements on malaria incidence

Study site	Country	Year	Type of study	Malaria transmission	Number of individuals included	Follow-up period	Age at supplements	Iron deficiency or anaemia indicator	Relationship with malaria	Effects on anaemia and iron indicators
Aware	Somalia	1975	placebo controlled trial	perennial	137	30 days		Hemoglobin <11 g/dl Serum iron concentration <4.48 µmol/l	In univariate analysis: Placebo group 2/66; Iron supplemented group: 21/71	Mean hemoglobin (g/dl) Before treatment: Placebo 8.1±0.7 Iron 8.3±0.6 After treatment: Placebo 8.7±0.9 Iron 12.3±1.1 Mean serum Fe (µmol/l) Before treatment: Placebo 3.4±0.57 Iron 3.6±0.52 After treatment: Placebo 3.9±0.7 Iron 13.1±0.93
Madang	Papua New-Guinea	1980-1981	matched randomized prospective trial	perennial with seasonal peaks	486	12 months	2 months	Hemoglobin, transferrin saturation, serum ferritin (log)	At 6 months: OR=1.78 (CI 1.02; 3.1) At 12 months: OR=1.95 (CI 1.21; 3.13)	Mean hemoglobin at 6 months (g/dl): Placebo 9.82 (1.39) Iron 9.14 (1.09) (p<0.001) Mean hemoglobin at 12 months (g/dl): Placebo 9.78 (1.36) Iron 9.32 (1.34) (p<0.002)
Ifakara	Tanzania	1995-1996	randomised placebo-controlled trial	perennial and intense	832	minimum of 52 up to a maximum of 153 weeks	8 to 24 weeks	Hemoglobin	PE with regard to the 1st malaria episode compared to placebo Daily iron and weekly placebo: 11% (CI 21.8; 35) Daily placebo + weekly Deltaprim 59.4% (CI 41.1; 72%) Daily iron + weekly Deltaprim 65.9% (CI 49.6; 77)	PE with regard to the severe anaemia (PCV <25%) compared to placebo Daily iron and weekly placebo: 32.1% (CI 4.9; 51.6) Daily placebo + weekly Deltaprim 59.8% (CI 41.1; 72.6) Daily iron + weekly Deltaprim 68.5% (CI 52.3; 79.2)
Ngerenya	Kenya	2001-2003	observational study	perennial with seasonal peaks	240	2 cross-sectional surveys at 6 and 12 months after enrolment	no supplements	ID: plasma ferritin <12 µg/ml in association with TFS <10%	Adjusted IRR in iron-deficient children = 0.7 (CI 0.51; 0.99)	No supplements
Pemba	Tanzania	2002-2003	randomised placebo-controlled trial	holoendemic with year-round transmission and seasonal peaks	24076	until discharge or death	20 weeks	ID: zinc protoporphyrin >80 µmol/mol haeme Anaemia: hemoglobin 70-100 g/L	Overall adverse events, deaths, and admissions to hospital caused by malaria compared to placebo Iron and folic acid: RR= 1.16 (CI 1; 1.34) Iron, folic acid, and zinc: RR=1.16 (CI 1.01; 1.34)	Non significant trend for smaller proportion of children with anaemia among all admissions compared to placebo
Muheza	Tanzania	2002-2005	observational study	intense	785	at birth until 3 years	no supplements	ID: ferritin concentration <30 ng/mL when CRP <8.2 µg/mL or ferritin concentration <70 ng/mL when CRP >8.2 µg/mL	Children with ID OR=0.15 (CI 0.12; 0.19) and 3.9 fold lower parasite count (P<0.01) compared with iron replete children Children with ID, for Hyperparasitemia (= parasitemia >2500/200 WBC) OR=0.04 (CI 0.02; 0.07) and for severe malaria OR=0.25 (CI 0.14; 0.46) compared to iron-replete	No supplements

Study site	Country	Year	Type of study	Malaria transmission	Number of individuals included	Follow-up period	Age at supplements	Iron deficiency or anaemia indicator	Relationship with malaria	Effects on anaemia and iron indicators
Handeni	Tanzania	2008-2009	randomised placebo-controlled trial	intense	612	median follow-up 331 days	6-60 months	ID: plasma ferritin concentration <12 µg/L	<p>Compared to placebo: All malaria episodes: Zinc group: AHR=0.99 (CI 0.82; 1.18) Multi-nutrients without zinc: AHR=1.04 (CI 0.87; 1.23) Multinutrients with zinc: AHR=1.14 (CI 0.96; 1.35) First malaria episodes: Zinc group: AHR=1.12 (CI 0.86; 1.44) Multi-nutrients without zinc: AHR=1.35 (CI 1.05; 1.73) Multinutrients with zinc: AHR=1.38 (CI 1.07; 1.77) Number of episodes with versus without multinutrients Iron deficient: HR=1.41 (1.09; 1.82) Iron replete: HR=0.93 (0.77; 1.13)</p>	<p>*Difference relative to placebo (95%CI), Hemoglobin concentration (g/l) Micronutrients without zinc: 106.6 (10.7) *2.6 (0.8; 5.2) Micronutrients with zinc: 107.5 (11.4) *3.5 (0.8; 6.1) Geometric mean ferritin concentration (µg/l) All children Micronutrients without zinc: 57.1 (0.03) *24.5 (14.8; 36.2) Micronutrients with zinc: 57.2 (0.03) *24.6 (14.8; 36.3) without inflammation: Micronutrients without zinc: 43.9 (0.03) *19.5 (11.3; 28.6) Micronutrients with zinc: 51.1 (0.03) *26.7</p>
Brong-Ahafo	Ghana	2010	double blind, cluster-randomized trial	perennial with seasonal peaks	1958	6 months	6 to 35 months	ID: plasma ferritin concentration <12 µg/L	<p>Malaria risk for iron supplemented group compared to placebo: Malaria risk for all children RR=1 (CI 0.81; 1.23) RR for malaria with ID and without inflammation=0.81 (CI 0.63; 1.03) RR for iron replete children without inflammation=0.92 (CI 0.81; 1.06)</p>	
Cochrane Review		2011	systematic Cochrane review	variable upon studies	45,353 children under 18 years of 71 trials	until June 2011	different supplements: iron, iron and folic acid, iron and anti-malarials	depending on the trial hemoglobin, iron and ferritin	<p>For clinical malaria iron alone compared to placebo RR=0.99 (CI 0.9; 1.09) For clinical malaria iron alone compared to placebo among non-anaemic children at baseline RR=0.97 (CI 0.86; 1.09) For clinical malaria iron alone compared to placebo among infants <2 years RR=0.94 (CI 0.82; 1.09)</p>	<p>Iron versus placebo or no treatment, iron plus folic acid versus placebo or no treatment, iron plus antimalarial treatment or antimalarial treatment alone versus placebo or no treatment, iron versus placebo or no treatment in the treatment of proven malaria</p>

AHR: Adjusted hazard ratio; AOR: Adjusted odds ratio; HR: Hazard ratio; ID: Iron deficiency; IRR: Incidence rate ratio; OR: Odds ratio; PE: Protective efficacy; RR: Relative risk; sTfR: serum transferrin receptor

1435 Clinical malaria is the consequence of the asexual cycle of *Plasmodia* parasites in the RBC. It
1436 constitutes the main outcome of the majority of the observational studies and it is currently
1437 defined as temperature $>37.5^{\circ}$ or 38° C within the previous 48 hours and a blood film positive
1438 for blood-stage asexual parasites. In this respect, a study gathering evidence from two cross-
1439 sectional observational surveys from 2001 to 2003 in Kenya among children aged 8 months to
1440 8 years reported significant protection among iron deficient children (Adjusted incidence rate-
1441 ratio (IRR)= 0.7, 95%CI (0.51; 0.99) with ferritin $<12\mu\text{g/ml}$ and transferrin saturation $<10\%$).
1442 Furthermore, iron status was inversely correlated with malaria-specific immunoglobulins.
1443 Similar results were found in an observational cohort study in Tanzania among children
1444 between birth and 3 years. Iron deficiency (defined by ferritin concentration corrected on
1445 CRP) was also associated with a significant protection with regard to lower odds of malaria
1446 parasitemia (OR=0.15, 95%CI (0.12; 0.19)), lower odds of hyperparasitemia
1447 (parasites $>2500/200$ white blood cells (OR=0.04, 95%CI (0.02; 0.07)), and lower odds of
1448 severe malaria (OR=0.25, 95%CI (0.14; 0.46)) after adjustment for possible confounders.

1449 In a pioneer randomized placebo controlled trial in Tanzania in 1995 in infants between 8 and
1450 24 weeks of age, no increased susceptibility to malaria was observed among iron
1451 supplemented children with regard to first or only malaria episode compared to placebo
1452 (protective efficacy (PE)= 12.8%, 95%CI (-12.8; 32.5)). Albeit this first reassuring result,
1453 supplementation effects on children health status were re-evaluated after the Pemba trial. In
1454 2002-2003 a randomised, double blind, placebo-controlled trial, gathered medical evidence on
1455 all-cause morbidity and mortality among over 24,000 children up to 35 months daily
1456 supplemented with folic acid and iron, iron, folic acid, zinc or placebo in Pemba, Tanzania. In
1457 the same cohort, a sub-study among 2413 children addressed the impact of supplements on
1458 haematological status, zinc, malaria prevalence, and infectious disease morbidity. Combined
1459 groups of supplemented children had significant higher risk for serious clinical events

1460resulting from malaria compared to placebo (RR=1.16, 95%CI (1.02; 1.32)). Malaria related
1461hospital admissions were also significantly higher (RR=1.18, 95%CI (1.02; 1.36)) among
1462supplemented children. In the case of cerebral malaria, the RR of the iron and folic acid
1463group, was also significant compared to placebo (RR=1.22, 95%CI (1.02; 1.46)). In addition,
1464another deeply relevant aspect of the malaria-iron association was first raised up: the
1465importance of the iron levels at baseline. Iron-deficient children at baseline, defined by zinc
1466protoporphyrin>80 $\mu\text{mol/mol}$ haeme, had a reduced risk of malaria-related adverse events
1467when supplemented compared to placebo (RR=0.56, 95%CI (0.32; 0.97)). Due to the
1468increased morbidity found in this trial, the WHO recommendations restrained supplements to
1469iron deficient children in malaria endemic regions.

1470Nevertheless, more recent studies report different results. A study in Tanzania in 2008-2009
1471investigated the consequences of micronutrient supplementation in 612 children between 6
1472and 60 months. While there was no significant increase in overall malaria episodes among
1473supplemented children compared to placebo, multi-nutrient supplementation was associated to
1474a 41% increase in the overall number of malaria episodes in children with iron deficiency
1475(HR=1.41, 95%CI (1.09; 1.82)), whereas there was no significant impact among the iron-
1476replete children (p-value for difference in effect=0.01).

1477In 2010 in Ghana, in a double blind, cluster randomized trial providing a micronutrient
1478powder (MNP) with or without iron, 1958 infants of 6 to 35 months of age were followed for
14796 months and no significant increase in malaria risk was observed compared to placebo
1480(RR=1, 95%CI (0.81; 1.23)). No significant association with increased malaria was described
1481among iron-replete children, with or without concomitant anaemia (RR=0.83, 95%CI (0.64;
14821.08) and RR=1.04, 95%CI (0.82; 1.32), respectively). However, supplemented children with
1483both iron deficiency and anaemia showed significantly reduced risk of malaria (RR=0.67,
148495%CI (0.5; 0.88)) compared to placebo.

1485Because of these a priori contradictory results of the studies, a Cochrane review of 2011

1486analysed 71 trials collecting evidence on 45,353 children. For the 13 trials selected, the

1487Cochrane review concluded to an absence of significant differences in clinical malaria rates

1488between iron and placebo (RR=0.99, 95%CI (0.9; 1.09)). No statistical differences were

1489found neither among supplemented infants (children<2years) (RR=0.94, 95%CI (0.82; 1.09))

1490nor for severe malaria (RR=0.91, 95%CI (0.76; 1.08)) compared to placebo. Furthermore, no

1491statistical difference was found among non-anemic children at baseline (RR=0.97, 95%CI

1492(0.86; 1.09)). However, analyses on iron deficiency defined by ferritin were not realized.

1493Even if it is difficult to screen children for iron status at the population level, information on

1494the effect of iron deficiency is relevant to develop useful supplement strategies based on

1495scientific accurate evidence. Finally, this Cochrane meta-analysis describes increased risk for

1496clinical malaria among iron or iron plus folic acid supplemented children in the absence of

1497malaria surveillance and treatment.

1498Beyond clinical malaria, it is necessary to consider also malaria mortality to capture broader

1499aspects of the iron-malaria association. In the context of the clinical trial with iron

1500supplements in Pemba, mortality due to malaria was higher (although not significantly higher)

1501among supplemented children compared to placebo (RR=1.08, 95%CI (0.84; 1.40)). Among

1502children supplemented with iron and folic acid, there was a significant increased risk for

1503cerebral malaria as a cause of death compared to placebo (RR=1.70, 95%CI (1.08; 2.68)). The

1504iron and folic acid supplemented children were 12% more likely to suffer an adverse event

1505resulting in hospitalisation or death (95%CI (2; 23)) compared to placebo and all-cause

1506mortality was also significantly higher (OR= 1.61, 95%CI (1.03; 2.52)). Iron deficiency and

1507moderate anaemia at baseline were significantly associated to lower rate of adverse events

1508(death or severe morbidity leading to admission) among supplemented children compared to

1509placebo. Further extensive studies on the impact of iron supplements on malaria attributable

1510mortality are scarce due to the difficulty of attributing correctly the cause of death in endemic
1511settings and, hence, it is difficult to accurately assess the interaction between malaria and
1512infection with regard to mortality. In addition more statistical power is needed as iron
1513measures are rare and death is also a rare event.

1514In a good attempt to clarify finally the conundrum, the Cochrane meta-analysis on the impact
1515on iron supplements addressed certainly this question but did not provide a definite answer. In
1516this review, the relative risk for all-cause mortality was not estimable. However, it was
1517capable of displaying useful information with regard to transmission settings. Mortality was
1518not significantly different between hyper- and holo-endemic areas (Risk difference= 1.93 per
15191000 children, 95% CI (-1.78; 5.64)).

1520In summary, the risk for clinical malaria differs according to iron status between
1521observational studies and clinical trials on iron supplementation. Overall, observational
1522studies describe a certain protection for malaria risk among iron deficient children. In parallel,
1523meaningful ancient studies report increased susceptibility to clinical malaria among iron
1524supplemented children, and so does the Pemba trial, which has a considerable statistical
1525power. However, other recent clinical trials with important malaria monitoring and protective
1526measures, show no significant increase for malaria risk among iron supplemented children
1527and neither does the Cochrane review. Albeit the absence of overall significance, the cross-
1528sectional studies in Tanzania report also significant earlier malaria among supplemented
1529children.

1530**II.2.2.b. Effect of iron levels on malaria in infants: physiopathology and further** 1531**perspectives**

1532As in the case of PAM, the physiopathology of malaria infection involves a direct interaction
1533between *Plasmodia* and iron. This aspect has already been detailed for PAM, but briefly, only

1534within the infected RBC, *P. falciparum*, the parasite responsible for most malaria cases,
1535consumes up to 80% of the hemoglobin. In addition, the parasite sequestration in the intestinal
1536blood vessels impairs the optimal nutritional absorption. Furthermore, non-transferrine bound
1537iron (NTBI) is associated to increased severity of the malaria episode and to reduced
1538performance of the immune function. Beyond these direct interactions, further clinical
1539conditions, such as certain genetic variants, interfere to determine the association between
1540malaria and iron levels. Indeed, genetic variants are estimated to be responsible for over 25%
1541of the variation in susceptibility to malaria. In this respect sickle hemoglobin is a significant
1542example, but evidence on the possible interaction between sickle cell hemoglobin and iron
1543availability to *Plasmodium* is lacking. In any case, genetic protection against malaria is
1544thought to be rather multigenic. As in the case of the pregnant women, other co-morbidities,
1545such as HIV, bacterial and helminthic infections are also correlated with both iron and
1546malaria.

1547Evidence on the effect of iron levels on malaria risk is subject to certain limitations, such as
1548methodological study constraints, homogenous measurement of iron and haematological
1549indicators, the effect of different transmission patterns, and further possible confounders.

1550In effect, statistical limitations are inherent to ethical research studies. Clinical trials display
1551results based on intensively monitored parameters. In most of them prophylactic protection by
1552ITNs or preventive treatment for malaria is more frequent among enrolled patients than in
1553observational studies, and treatment is also given as soon as a case is confirmed. As a
1554consequence, it is difficult to disentangle the possible protective effect of IDA from the
1555protection given by protective measures, especially in the case of severe malaria or
1556hyperparasitemia in clinical trials. Preventive measures reduce the number and the severity of
1557malaria episodes and, hence, statistical power decreases, as does the force of the association.
1558The dimension of the association, or its absence, should be ideally assessed in the conditions

1559in which population undergo the malaria burden and the nutritional interventions.

1560Nevertheless, accurate iron monitoring is not realized systematically and malaria episodes are
1561not always captured by demographic or surveillance data. In addition, observational studies
1562that do not provide treatment are unethical in malaria endemic countries with limited access to
1563health care. However, surveillance data or data issue of demographic surveys may be useful to
1564get a basic idea on malaria risk and haematological indicators.

1565With regard to the epidemiological indicators, malaria infection outcomes (clinical malaria
1566and parasitemia) reflect more specifically the malaria-iron relationship, and mortality reflects
1567rather a broad association between iron and pathogens. In addition, its assessment is difficult
1568because of diagnostic reasons, and evidence lacks with regard to specific malaria deaths
1569related to iron supplements.

1570The transmission setting constitutes an additional important stake of the question. Disease
1571burden in children after iron supplementation does certainly differ in the absence of malaria
1572compared to malaria endemic settings. The existence of a possible malaria prevalence
1573threshold at which iron supplements start to have a deleterious effect on infant health requires
1574as well further research.

1575Other methodological obstacles contribute to the inconclusive results of the analyses of the
1576association between iron and malaria risk. Analyses in the clinical trials are seldom adjusted
1577on other significant co-variables and odds ratios (OR) and relative risks originate often from
1578univariate analyses. In addition, the exclusion of the children with inflammation in some
1579studies might have introduced a bias in the interpretation of results concerning the children
1580with the most severe disease, as inflammation is predominantly present in these more severe
1581cases.

1582Finally, the haematological indicators at baseline show contradictory results in literature at

1583present. Indeed, a clinical trial describes a significant protection against malaria among
1584supplemented children with both anaemia and iron deficiency. However, a study in Tanzania
1585observed an increase in malaria risk among iron-deficient infants. Similar results are found in
1586pregnant women. Indeed, there might be a possible protective role of anaemia or iron
1587deficiency in the context of iron supplementation. In case of anaemia the incorporated iron
1588might be used for hemoglobin synthesis whereas in the context of iron deficiency with no
1589anaemia at baseline the incorporated iron might entail an increase in NTBI, enhancing
1590parasite growth. More extensive research including different iron deficiency indicators is
1591needed to advance in the knowledge in this aspect. Yes it is essential to ascertain the meaning
1592of the information provided by the different iron markers used in the research studies to better
1593unravel the iron-malaria conundrum.

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1595**II.3. Complementary factors associated with malaria risk in infants:** 1596**the case of lead**

1597Simultaneously to our study in the same cohort another epidemiological project was
1598evaluating the effect of lead on the neurocognitive development in children. Our colleagues
1599found out lead levels were particularly high in the infants of our cohort. Nriagu had found in
1600Nigeria that malaria had a significant effect on lead levels in univariate analysis. In addition,
1601elevated blood lead levels (BLL) carry a significant burden of disease in Western Africa and
1602malaria is the first cause of infant mortality in Benin. Therefore, we aimed at assessing the
1603possible association of lead levels with malaria risk considering other major malarial risk
1604factors.

1605**II.3.1. Lead levels and malaria: clinical and epidemiological background**

1606Elevated lead levels have severe harmful effects on infant health. They are associated with
1607impaired neurocognitive development, anemia (due to either disruption of heme synthesis or
1608hemolysis), and renal and gastro-intestinal effects. Although high blood lead levels (BLL)
1609(BLL >100 µg/dl) can entail acute neurologic symptoms, such as ataxia, hyperirritability,
1610convulsions, coma, and death, BLL as low as 10 µg/dl have been also correlated with poor
1611neurocognitive outcomes and behavioral disorders. Indeed, the Center for Disease Control
1612(CDC) reduced the reference level of blood lead from 10 µg/dl to 5 µg/dl in 2012. This is of
1613special concern in young children as neuro-cognitive impairment has been found to be
1614associated with the degree of exposure to lead between the ages of 12 and 36 months. Albeit
1615the severe impact of elevated lead levels on infant health, epidemiological studies of lead
1616levels in Sub-Saharan Africa are limited. Data from the few existing studies, published in a
1617systematic review on BLL among Sub-Saharan children, suggest an alarming burden of
1618disease. This review reported a BLL weighted mean of 13.1 µg/dl which increases up to 16.2
1619µg/dl considering solely studies with robust quality BLL analyses. In addition, the prevalence
1620of BLL >10 µg/dl ranged from 7.0% to 70.9% in six of the studies reviewed. Recent mass
1621level intoxications reported in Senegal and Nigeria further raise the public health concern
1622about lead levels in West Africa. Notwithstanding these concerns, infectious diseases, mainly
1623malaria, lead the disease burden in West Africa. In Benin, malaria is the main cause of
1624mortality among children less than 5 years and there were over 1.5 million cases in 2012.
1625Both malaria and lead poisoning can have severe hematologic and neurologic symptoms on
1626children and development disruptions. Because of the recent evidence on the role of the
1627complement system in the regulation of neurodevelopment, it has been proposed that
1628excessive complement activation induced by placental malaria may disrupt normal
1629neurodevelopment resulting in neurocognitive impairment of infants exposed to *Plasmodia in*
1630*utero*.

1631Epidemiologically, malaria and lead poisoning may not only overlap geographically, but they
1632have major impact on the health of children, especially those under 5 years. Consequently,
1633their possible association may have an effect on one of the most vulnerable age groups in the
1634population, and it could have severe long-term implications for the development of the
1635children. Furthermore, Nriagu found a significant effect of malaria on the children lead levels
1636in different areas of Nigeria. Concern has been repeatedly raised up on the importance of
1637alarmingly high anemia rates in West Africa, and both malaria and EBLL are associated with
1638increased anemia rates. However, no evidence exists at present on the possible joint effect of
1639lead and *P.falciparum*. To our knowledge, no published study exists on lead levels in Benin,
1640and in particular, on the effects of lead levels on malaria risk in infants.

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III. Objectives

1646In Benin, the prevalence of anemia during pregnancy is over 60%. The main causes of anemia
1647in pregnancy are malaria and helminth infections. To fight nutritional deficiencies during
1648pregnancy, the Beninese Ministry of Health prioritized the prevention of anemia (defined by
1649WHO as hemoglobin (Hb) <11g/l). Therefore, it recommends supplements of 200 mg of
1650ferrous sulphate and 5 mg of folate given daily until 45 days after delivery.

1651Indeed, anemia, including iron-deficiency anemia, constitute a public health concern not only
1652during pregnancy but also during infancy. As said, albeit the lack of official
1653recommendations, in case of iron deficiency anemia, Beninese paediatricians give daily
1654supplements of iron of 10 mg/kg/day and 0.5 mg/kg/day of folic acid during 2 months, every
16556 months, starting at 6 months of age until 5 years. This is similar to WHO guidelines, which
1656recommend 12.5 mg iron and 50µg folic acid to prevent anaemia in children 6-24 months. In
1657case of low birth-weight (LBW), defined by birth weight<2500g, supplements start at 2
1658months.

1659Nevertheless, some epidemiological evidence suggests that iron supplements could influence
1660malaria episodes and severity. In addition, a recent meta-analysis declares that the present
1661epidemiological evidence is inconclusive to ascertain a possible increased risk of PAM
1662associated with iron supplements during pregnancy. Indeed, the lack of prospective follow-up
1663cohorts is a considerable obstacle to come to a conclusion on the issue. Considering that iron
1664supplements are given systematically during pregnancy in Benin, and that malaria is endemic
1665in the region, our first objective was **to analyse the possible effect of iron levels on PAM** in
1666the context of a prospective follow-up of pregnant women. Furthermore, we wanted **to**
1667**investigate the effect of the infant iron levels on malaria in infants** as malaria is the first
1668cause of infant mortality, and there are no national guidelines on the iron supplementation
1669policy in infants.

1670In parallel, PAM appearance and severity seems to be associated with increased malaria risk

1671in infants, and IPTp has an impact on secondary malaria outcomes (such as LBW and

1672anaemia). Hence, our second objective was **to investigate the possible impact of IPTp on**

1673**malaria in infants during the first year of life.**

1674Finally, a research group working on the same cohort found very high rates of elevated blood

1675lead levels in the infants. Both malaria and elevated lead levels have a severe impact on the

1676infant health. In addition Nriagu had found a significant effect of malaria on the children lead

1677levels in different areas of Nigeria. Therefore, our third objective was **to assess the possible**

1678**effect of elevated lead levels on malaria in infants**, as their possible association may have

1679severe long-term implications for the development of the children. Indeed, no published study

1680exists on lead levels in Benin, and in particular, on the effects of lead levels on malaria risk in

1681infants.

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IV. Methods

1688To investigate our objectives, we conducted our research in the context of the clinical trial

1689MiPPAD and a nested study APEC.

1690The clinical trial MiPPAD (Malaria in pregnancy preventive alternative drugs,

1691<http://clinicaltrials.gov/ct2/show/NCT00811421>) was conceived to compare the efficacy and

1692safety of IPTp with SP (1500/75 mg per dose) and mefloquine (15 mg/kg taken either in

1693simple or split intake).

1694The study APEC (Anemia in pregnancy: etiology and consequences) was a nested study to

1695MiPPAD that analysed parameters relevant to the anemia status of both the pregnant women

1696and infants.

1697More precisely, in the context of both studies in Benin, 1005 pregnant women and 400 of

1698their offspring (200 born to mothers with anemia at delivery, and 200 born to mothers without

1699anemia at delivery) were followed through pregnancy and the first year of life, respectively.

1700The APEC study was conducted in three maternity clinics in the district of Allada, between

1701January 2010 and May 2012. Allada is a semi-rural area of 91,778 inhabitants located 50 km

1702North of Cotonou (Benin). Malaria has a perennial transmission pattern with two transmission

1703peaks corresponding to the rainy seasons in April-July and October-November. *Plasmodium*

1704*falciparum* is the species responsible for the majority of infections.

1705The eligibility criteria included no intake of IPTp, iron, folic acid, vitamin B12, or anti-

1706helminthic treatment. All women were offered confidential pre-test HIV counselling and

1707thereafter informed consent was obtained.

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1709IV. 1. Cohort follow-up methods

1710Clinical and biological follow-up:

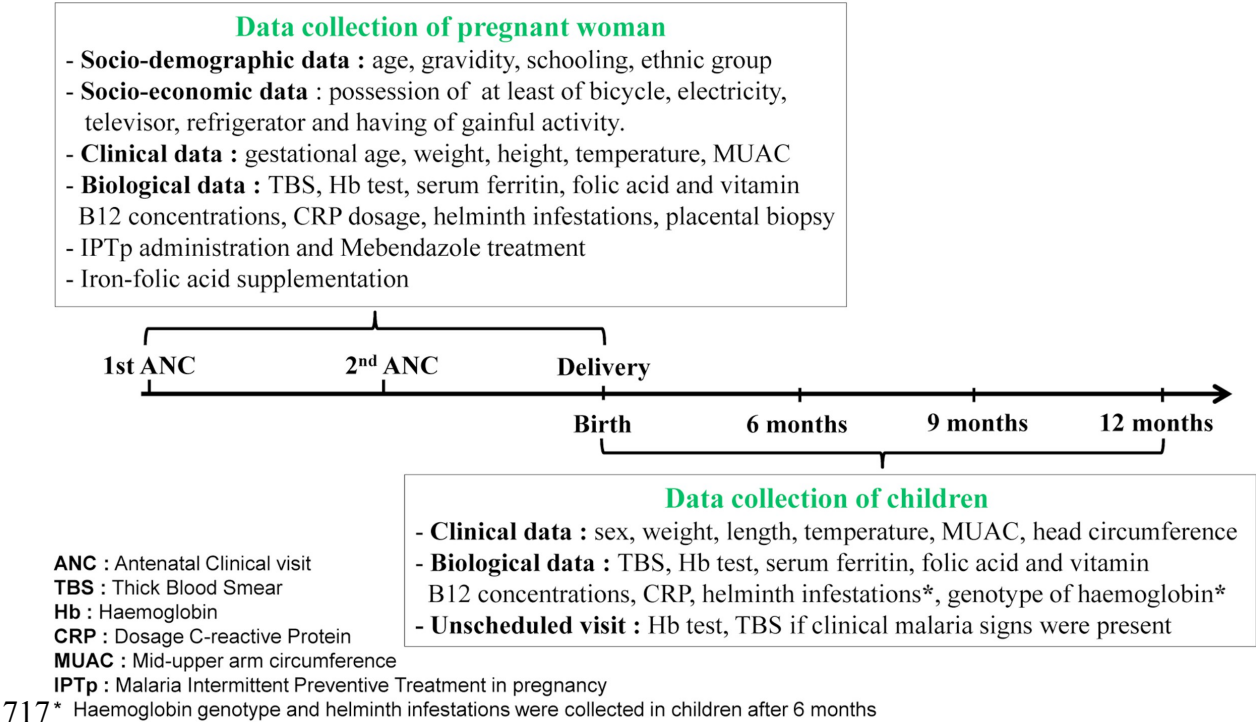
1711During follow-up, socio-demographic, economic, clinical and biological data were collected

1712in mothers at 1st antenatal clinical visit (ANC), 2nd ANC and delivery. The same data were

1713also recorded in infants at birth, 6, 9 and 12 months of life. In case of sickness, both pregnant

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1714women and infants came to the clinics for clinical examination. In these unscheduled visits,
1715haemoglobin concentration and blood smear were performed when malaria signs were
1716present. Concrete clinical and biological exams are summarized in Figure 6.



1718**Figure 6: Clinical and biological exams during the follow-up through pregnancy and**
1719**infancy.** (Figure realized by M. Accrombessi)

1720After obtaining informed consent, sociodemographic and socioeconomic characteristics of the
1721women were collected at enrolment. At the 1st ANC visit, women were examined and
1722gestational age, middle upper arm circumference (MUAC), weight and height were recorded.
1723This information, except for height, was also collected at 2nd ANC and delivery. Gestational
1724age was determined from fundal height measurement by bimanual palpation and following
1725McDonald's rules. Weight and height in pregnant women were respectively measured to the
1726nearest 0.1 kg using an electronic scale (Seca corp., Hanover, MD) and to the nearest 0.1 cm
1727by using a bodymeter device (Seca 206 Bodymeter; Seca corp.). These parameters were
1728measured twice by nurses, and the mean of both measurements was calculated.
1729At birth, newborn's sex, weight, length, head circumference and axillary temperature were

1730collected. Weight was measured using an electronic baby scale (SECA type 354) with a
 1731precision of 10 g and length was measured to the nearest 1 mm with a locally manufactured
 1732wooden measuring scale according to the criteria recommended by WHO. At the 6, 9 and 12
 1733months systematic visits, the possible history of fever within the previous 24 hours, malaria
 1734treatment or hospitalization since the last visit and use of insecticide-treated nets were
 1735investigated and recorded.

1736Concerning the blood and stool sample collection, 8 ml of mother's venous blood were
 1737collected at 1st ANC, 2nd ANC visit and at delivery. The same volume was also collected on
 1738cord blood at birth and on infant's venous blood at 6, 9 and 12 months of life. All the samples
 1739were used to look for malaria parasitaemia, to determine C-reactive protein (CRP),
 1740micronutrient (serum ferritin, folic acid and vitamin B12) and Hb concentration and to
 1741genotype Hb. At delivery, samples (biopsy and impression smear) were collected from the
 1742placenta for parasitological evaluation. A container was also given to the woman to collect
 1743infant's stools in search of intestinal helminths.

1744On unscheduled visits, Hb dosages and thick blood smears were performed in infants with
 1745clinical signs of malaria (history of fever in the last 24 hours or temperature $\geq 37.5^{\circ}\text{C}$ and
 1746pallor).

1747Laboratory methods. The Hb level was measured with a Hemo-Control photometer (EKF
 1748Diagnostics, Magdeburg, Germany) device. A daily calibration of the Hemo-Control device
 1749was performed by the laboratory technicians. In addition, an external quality control was
 1750made by sending one of 10 consecutive samples to the Allada Central Hospital laboratory,
 1751where dosages were assessed using a hematology analyser (Erma Laboratory, Tokyo, Japan).
 1752Hb genotypes were determined by alkaline electrophoresis on cellulose acetate (Helena
 1753laboratories, Beaumont, TX).

1754Serum ferritin, folic acid, and vitamin B12 concentrations were measured using a
 1755microparticle enzyme and fluorescence polarization immunoassay (AxSym Immuno-Assay

1756Analyser, Abbott Laboratories). CRP concentration was determined by rapid slide test (CRP
1757Latex; Cypress Diagnostics Inc.) to correct the effect of inflammatory syndromes on ferritin
1758concentrations.

1759The Determine (HIV1 and 2 kit; Abbott Laboratories) and Bioline (HIV1 and 2 3.0 kit;
1760Bioline, Taunton, MA) rapid tests were used to detect HIV infections using a serial testing
1761algorithm.

1762The Lambaréné technique was used to analyse peripheral malaria infection in blood smears.
1763It consists of spreading a calibrated 10 μ l amount of blood on a slide's rectangular area of 1.8
1764cm² (1.8 x 1 cm). The slide was stained with Giemsa and read at a magnification of 1,000 \times
1765with an oil immersion lens. A multiplication factor was applied to the average parasitemia/
1766field to determine the number of parasites/ μ l. The Lambaréné method detection threshold has
1767been estimated to be 5 parasites/ μ L.

1768Placental biopsies (2.5 x 2.5 cm³), collected at delivery for histology assessment, were
1769immediately put in 50 ml of 10% buffered formalin. It was then stored at 4°C in a refrigerator
1770until the placental tissue was processed at the pathology department. The maximum delay
1771before fixation was of 5 days. Placental malaria infection was defined as the presence of
1772parasites with /without pigment or pigment confined to fibrin in the histological examination.
1773Placental histology was examined without knowledge of the peripheral blood smears results.
1774In addition, an external quality control was made on 100% of positive slide and 10% of
1775negative slide in reference laboratory to Barcelona Centre for International Health Research
1776(CRESIB), Hospital Clínic-Universitat de Barcelona. Infestations by helminths were assessed
1777by using the Kato-Katz concentration method (Vestergaard Frandsen, Lausanne, Switzerland).
1778Environmental data: As no entomological data was available, we used rain quantity instead as
1779a surrogate for the anopheline presence. Because of the anopheline timeliness, rain was
1780calculated as the mean rainfall of the 7 days prior to the two weeks before the consultation.

1781Ethics statement

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1782These studies were approved by the Ethics Committee of the Health Sciences Faculty of
1783Cotonou in Benin. Before each inclusion, all participants involved in our study provided their
1784written informed consent to participate in this study. The study was also explained in the local
1785language to the participant, and her voluntary consent was obtained. In case the woman could
1786not read,an impartial witness was involved in the process. Mothers were free to interrupt their
1787participation at any time in the study.

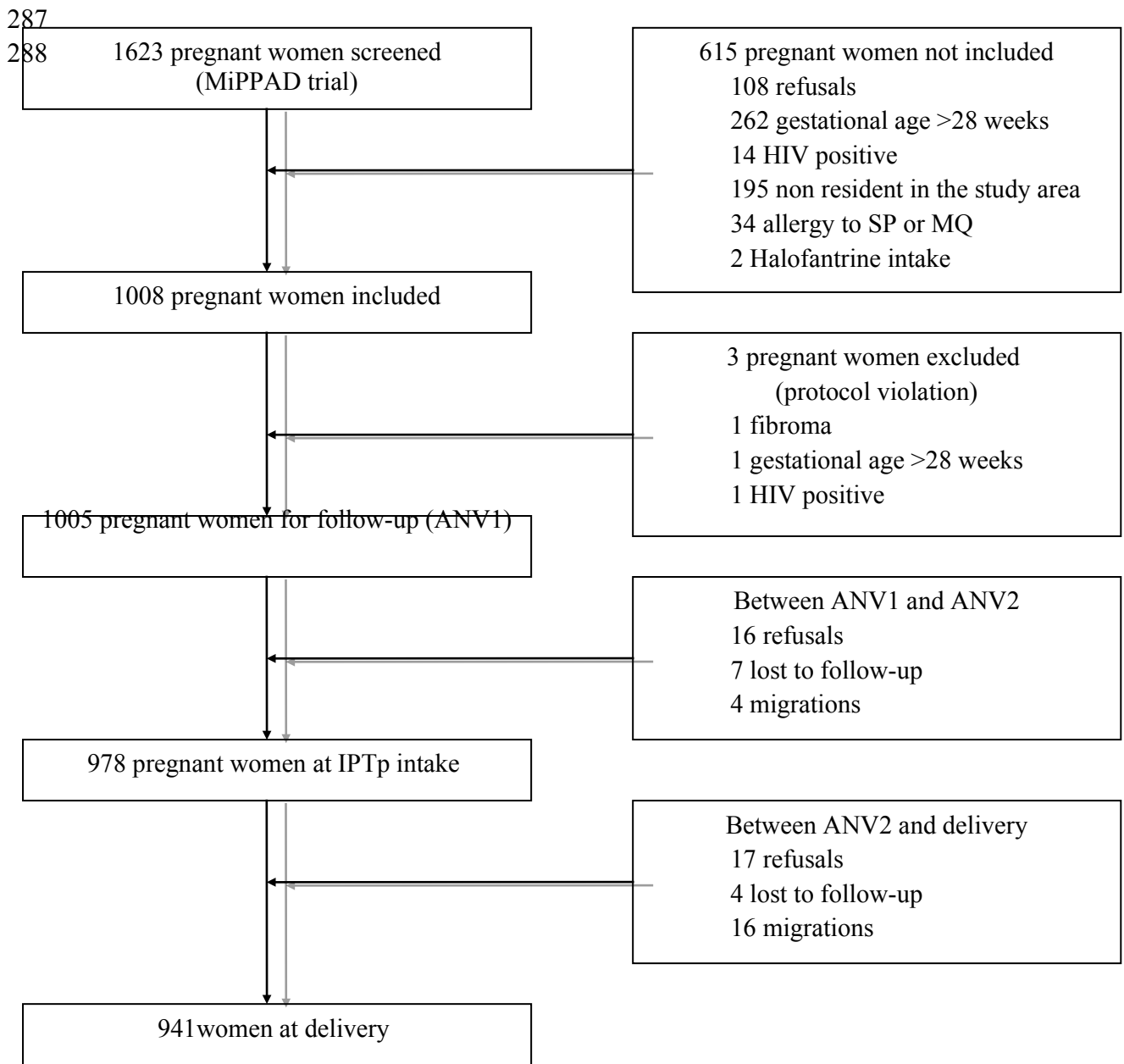
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1789**IV. 2. Cohort follow-up**

1790The follow-up of pregnant women and infants are described in Figure 7 and Figure 8,
1791respectively.

1792In the case of pregnant women, the lost to follow-up were below 10%. Therefore, no data
1793treatment was applied. In the case of infants, multiple imputation technique was used and
1794results did not differ significantly.

1795The sample size of the presented tables are below the sample size presented in these diagrams,
1796as often measures were not always available for each sample of every participant during the
1797follow-up.



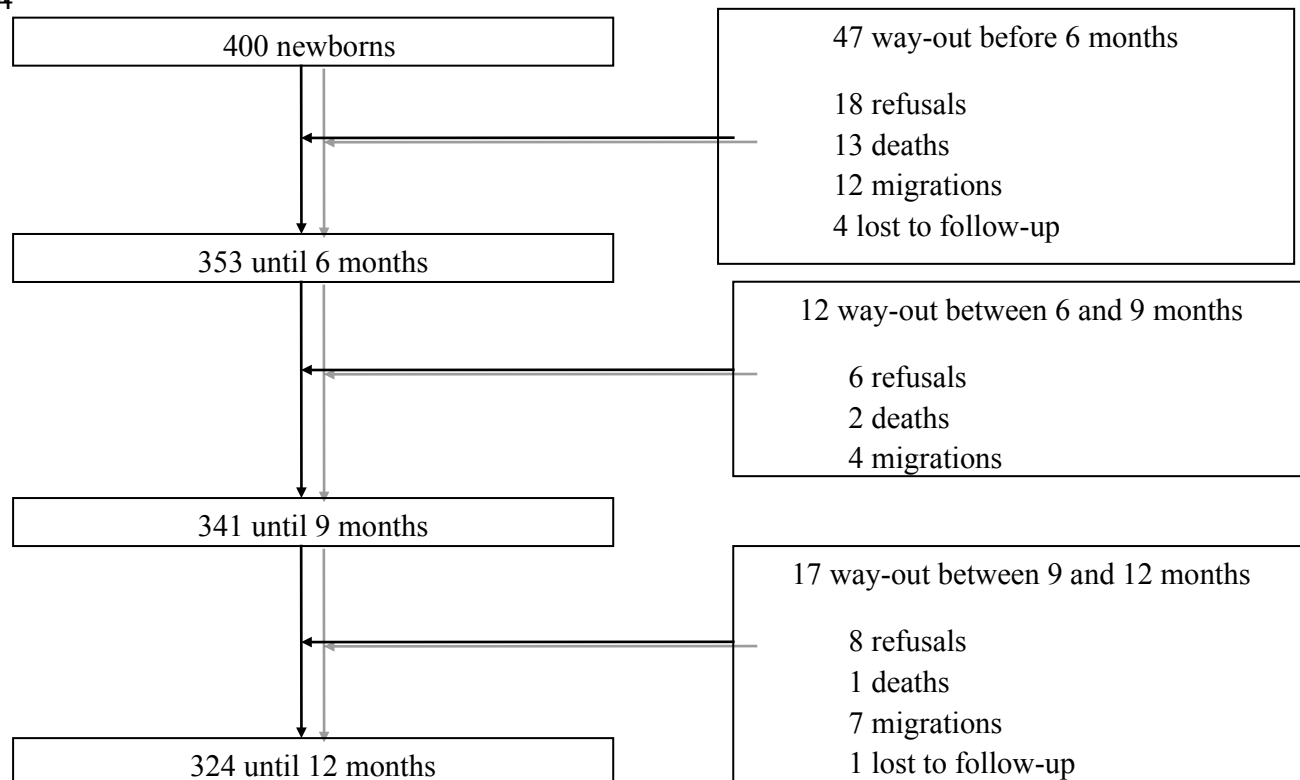
1799**Figure 7: Follow-up of pregnant women**

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1805**Figure 8: Follow-up of infants**

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1807**IV. 3. Definitions**

1808PAM was defined as peripheral or placental infection by *Plasmodium* while PM was defined
1809as presence of *Plasmodium* in the placenta.

1810LBW corresponds to newborn weights <2500g, and prematurity refers to offspring born prior
1811to 37 weeks of gestation.

1812Anemia was defined by Hb levels below 11g/l for both pregnant women and infants. Between
1813birth and 6 months anemia was defined by Hb below 140 g/l.

1814Severe, moderate and mild anemia were defined as Hb concentrations <80 g/l, 80-99 g/l, and
1815100-109 g/l, respectively, following WHO criteria.

1816Inflammation was determined by C-reactive protein (CRP) levels ≥ 5 mg / ml. We corrected
1817serum ferritin in the context of inflammation following the procedure inspired by the meta-
1818analysis by Thurnham before conducting the analyses, so we multiplied serum ferritin by 0.76
1819in the presence of *Plasmodia* without inflammation, and we multiplied serum ferritin by 0.53

1820in case of concurrent *Plasmodia* infection and inflammation.

1821ID was then defined as corrected serum ferritin <15 µg/l in pregnant women and corrected
1822serum ferritin concentration <12 µg/l in infants. Iron deficiency anemia (IDA) was defined as
1823Hb<110 g/l with ID.

1824Folic acid deficiency was defined as a serum concentration<6 ng/ml. Vitamin B₁₂ deficiency
1825was defined as a serum concentration<150 pg/ml. Intestinal helminth infestations were
1826diagnosed by the presence of intestinal helminth eggs in the stool sample.

1827To estimate pre-pregnancy body mass index (BMI), all pregnant women included in the study
1828had a gestational age less than 28 weeks. From the end of the first trimester of gestation, it
1829was estimated that pregnant women gained on average 1 kg per month until delivery.

1830We used the gestational age at inclusion to estimate approximately the weight that women
1831were supposed to have gained since the beginning of the pregnancy. This amount was then
1832subtracted from the weight on the day of inclusion to obtain a rough estimate of the weight
1833before pregnancy. BMI was calculated as the weight in kilograms divided by the square of the
1834height in meters (kg/m²).

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1836IV. 4. Statistical analyses

1837Data were double entered and analysed with *ACCESS2003* and *STATA12.0* (Stata Corp,
1838College Station, USA).

1839Continuous variables were analysed as follows: polynomes were considered and the number
1840of monomes was held depending on the adequacy of the polynome to the variable. More
1841concretely, only maternal age squared was retained as a squared variable.

1842Then, all continuos variables were also split into categories, and depending on the adequacy
1843of the case, their were either kept as a continuous variable or as categories in the final model.

1844Kruskal-Wallis test was used to analyse continuous variables. Chi-square test was used for
1845comparing categorical variables by gravidity status or infant age, respectively.

1846Socio-economic items (home possession of latrines, electricity, a refrigerator, a television, a
 1847vehicle with at least two wheels, being married, and working outside the home) were plotted
 1848into a multiple correspondence analysis. Then, a predictor was created to synthesize the
 1849information, and was kept as the final socio-economic index.

1850In the pregnant women follow-up, univariate analysis was conducted to assess the association
 1851of all variables with positive smear and maternal peripheral parasitaemia using multilevel
 1852models with a random intercept at the individual level. More precisely, we used the following
 1853co-variables: age (years), age squared, ethnic group, socio-economic index, gravidity,
 1854gestational age (weeks), number of antenatal visits, BMI, maternal hemoglobin, maternal
 1855anaemia, iron levels, folic acid, vitaminB12, folic acid and vitaminB12 deficiencies, socio-
 1856economic index, IPTp regime, IPTp interval length (number of days between IPTp doses),
 1857IPTp timing, and Kato-Katz positivity.

1858Thereafter, two different multilevel models regressions were built: the first on the risk of
 1859having a positive blood smear during the follow-up period and the second on *P.falciparum*
 1860parasite density. Both models included the smears and blood films of both systematic and
 1861unscheduled visits. The variables with p-values<0.2 in univariate analysis were included in
 1862the multilevel models. Maternal age squared was used due to the quadratic relationship of age
 1863with the malarial risk. Preliminary fixed effects analyses were realized using the maximum
 1864likelihood method, and variance components were estimated using the restricted maximum
 1865likelihood method. However, for both the analysis of the possibility of a positive blood smear
 1866and for the analysis of parasite density, random coefficient models were used as they were
 1867statistically better than fixed effects according to AIC and BIC criteria. The Akaike
 1868information criterion (AIC) and the Bayesian information criterion (BIC) compare maximum
 1869likelihood models. More precisely, random intercept was applied in both cases at the
 1870individual level as the effect of the variables is correlated within the women. Random slope
 1871was applied to gestational age as the effect of gestational age might vary differently according

1872to the timing of the measure. Multivariable linear regression was used in the analysis of birth
 1873weight, and logistic regression was used for PM and LBW assessment. Certain variables were
 1874forced into the model because of their meaning in the analyses according to the literature:
 1875socio-economic status and rainfall in the case of malarial indicators, and BMI in the case of
 1876LBW. Manual backward selection procedure was performed and statistical significance was
 1877set at $P < 0.05$. The presented p-values and the significance threshold were two-sided.

1878In the infant follow-up, univariate analysis was conducted to assess the association of all
 1879variables with positive smear and the infant peripheral parasitaemia using multilevel models
 1880with a random intercept at the individual level. More precisely, we used the following
 1881co-variables: sex, low birthweight ((LBW), weight < 2500 g), preterm birth (gestational age $<$
 188237 weeks), fever (temperature $> 37.5^{\circ}\text{C}$), inflammation syndrome, placental malaria status, age
 1883(months), ethnic group, socio-economic index, gestational age at birth (weeks), maternal
 1884hemoglobin at delivery, maternal anaemia at delivery, hemoglobin, iron levels, folic acid,
 1885vitaminB12, folic acid and vitaminB12 deficiencies, IPTp regime, IPTp interval length
 1886(number of days between IPTp doses), IPTp timing, and Kato-Katz positivity.

1887Thereafter, two different multilevel models regressions were built: the first on the risk of
 1888having a positive blood smear during the follow-up period and the second on *P.falciparum*
 1889parasite density. Both models included the smears and blood films of both systematic and
 1890unscheduled visits. The variables with p-values <0.2 in univariate analysis were included in
 1891the multilevel models. Preliminary fixed effects analyses were realized using the maximum
 1892likelihood method, and variance components were estimated using the restricted maximum
 1893likelihood method. However, for both the analysis of the possibility of a positive blood smear
 1894and for the analysis of parasite density, random coefficient models were used as they were
 1895statistically better than fixed effects according to AIC and BIC criteria. More precisely,
 1896random intercept was applied in both cases at the individual level as the effect of the variables
 1897is correlated within the infant. Random slope was applied to age as the effect of age might

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1898 vary differently according to the timing of the measure. Finally, iron levels were also analysed
1899 as a variable with categories corresponding to the 4 quartiles.

1900 In any case, to take into account the fact that parasites are absent at birth, we excluded the
1901 malaria measurements at birth from the hierarchical mixed model.

1902 Certain variables were forced into the model because of their meaning in the analyses
1903 according to the literature: socio-economic status and rainfall in the case of malarial
1904 indicators. Manual backward selection procedure was performed and statistical significance
1905 was set at $P < 0.05$. The presented p-values and the significance threshold were two-sided.

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V. Results

1914 **V.I. LITERATURE REVIEW**

1915 To better analyse the data of the study in Benin, and to better understand the relationships
1916 between gestational malaria, iron levels, and malaria in infants, we conducted a consistent
1917 literature review of the epidemiologic evidence regarding these issues. In this first part of the
1918 section, I will present the result of the work on reviewing 1. The influence of gestational
1919 malaria on malaria in infants; and 2. The association of iron levels with malaria.

1920 The number of articles presented in the “references” section of the articles is limited by the
1921 journal requirements. Moreover, we have not kept in our reviews all articles read related to
1922 the subject. The articles kept for review are presented in the “references” section of the
1923 articles below. The complete list of all articles considered for the reviews can be accessed
1924 online in my Mendeley webpage. More precisely, the articles considered for the article on the
1925 influence of gestational malaria on malaria in infants can be found in the files “mother”,
1926 “placenta”, “child”, “PAM”, and “parasitemia”. The complete list of the articles that were first
1927 selected for the article reviewing the evidence on the association of iron levels with malaria
1928 can be found in the same Mendeley webpage in the file “iron”. References, figures and tables
1929 in this section are independent of those in the whole dissertation as they are presented at the
1930 end of each article.

1931 Both articles have no date restriction, meaning that articles were considered irrespective of the
1932 date of appearance. However, the time period during which we conducted our research is
1933 limited to certain months, which is described in each article.

1934 Even if both review articles are not meta-analyses, we wanted to mention that publication
1935 bias was not addressed to give complementary information to the reader.

1936 Finally, to give a more accurate idea of what our articles add to the previous state of art, we
1937 have added a little paragraph at the end of the article summary.

1938**V.I.1. Pregnancy associated malaria and malaria in infants: an old**
1939**problem with present consequences.**

1940

1941**Summary of the article:** We wanted to analyse the impact of PAM and IPTp on malaria
1942outcomes during pregnancy, and during the first year of life in infants. Consequently, it was
1943necessary to imbalance the present knowledge on pregnancy-related factors that influence
1944malaria in infants, including the effect of control interventions and novel research
1945perspectives. We realized a review on the subject that was published in June 2014 in the
1946Malaria Journal.

1947Therefore, we analysed between the 10th January 2012 and the 9th June 2014 1,136 articles
1948published in PubMed, the Cochrane Library, Global Health and WHO databases. The search
1949terms used were the Medical Subjects Headings (MeSH) “Parasitemia” OR “Malaria” OR
1950“Anaemia”. Complementary articles, reports, and studies were identified through review and
1951citations. Finally, 355 articles were selected for final review.

1952PAM, defined as peripheral or placental infection by *Plasmodium*, constitutes a major public
1953health concern due to its significant adverse health effects on both the mother and the foetus.
1954Epidemiological studies estimate 32 million women become pregnant every year in malaria
1955endemic sub-Saharan Africa countries. Pregnant women are increasingly susceptible to
1956malaria infection since *Plasmodium falciparum*, the most common parasite responsible for
1957malaria in Africa, avoids spleen clearance through expression of proteins that bind to the
1958chondroitin sulphate A (CSA) in the placental intervillous space. Thus, PAM determines
1959foetal exposure to *P. falciparum in utero* and it is consistently associated with an increased
1960malaria risk during infancy. PAM has been associated with congenital malaria, increased
1961malaria episodes, anaemia, and non-malaria fever episodes. Although a complete explanation
1962of the physiopathology of PAM has not yet been elucidated, *in utero* exposure to malaria is

1963probably nonetheless correlated with placental sequestration of erythrocytes. The immune
1964tolerance process would plausibly depend on the type of malaria antigen in contact with the
1965foetus, the amount and the duration of the exposure, and the timing of exposure during
1966pregnancy. Indeed, the interaction between gestation and infection timing during pregnancy
1967has been previously shown to influence the pathologic consequences for the offspring. A
1968specific immunity develops during the first pregnancy and, hence, primigravidae and their
1969infants are at higher risk of PAM compared to multigravidae, the infants mainly as a result of
1970reduced antibody transfer. Finally, the timing of PAM results in different effects on both the
1971mother and the foetus with regard to LBW and anaemia rates.

1972With regard to control strategies, effective IPTp diminishes PM and malaria associated
1973morbidity such as LBW, pre-term delivery, IUGR, and perinatal mortality in areas where
1974resistance to SP is not highly significant. Still, the influence of different IPTp regimes on
1975malaria morbidity in infants remains a question for further research.

1976Further evidence is also needed on the importance of the timing of infection during pregnancy
1977and infant malaria morbidity. In addition, the implementation of different IPTp regimes
1978should be adapted according to transmission and the SP-resistance pattern. Furthermore,
1979preventive strategies should start during the pre-conceptual period or as soon as possible, as
1980there is evidence of increased infant susceptibility to parasites carrying antigens to which they
1981were exposed while *in utero*. Moreover, the role of protective maternal antibodies has to be
1982clarified yet. Operational research on different preventive IPT regimes and cost effectiveness
1983analysis for community-level IST interventions should be also encouraged.

1984Ultimately, the long-term neuro-cognitive consequences of placental malaria, as well as the
1985influence of HLA-G polymorphisms on subsequent malaria symptoms would significantly
1986contribute to better identify malaria risk factors in infants.

1987**What the article adds to the previous state of art:** Albeit the important prevalence of PAM,
1988no review gathering the epidemiologic evidence on the effect of PAM on malaria in infants
1989had been conducted. In addition, we include consistent information on the possible
1990physiopathological hypothesis undergoing in this interaction. Furthermore, we describe in
1991which manner malaria control strategies might also have an effect and the increasing
1992importance of resistance against SP in Africa. Finally, we present research gaps, such as the
1993influence of HLA-G on symptoms, the neuro-cognitive effect of malaria, and the lack of
1994consistent evidence regarding IST.

1995

1996

1997NB: The following article summarizes the state of the art of the topic. Consequently,
1998substantial information has already been explained in the “State of the art” section.

1999

REVIEW

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Pregnancy-associated malaria and malaria in infants: an old problem with present consequences

Violeta Moya-Alvarez^{1,2,3*}, Rosa Abellana⁴ and Michel Cot^{1,2}

Abstract

Albeit pregnancy-associated malaria (PAM) poses a potential risk for over 125 million women each year, an accurate review assessing the impact on malaria in infants has yet to be conducted. In addition to an effect on low birth weight (LBW) and prematurity, PAM determines foetal exposure to *Plasmodium falciparum in utero* and is correlated to congenital malaria and early development of clinical episodes during infancy. This interaction plausibly results from an ongoing immune tolerance process to antigens *in utero*, however, a complete explanation of this immune process remains a question for further research, as does the precise role of protective maternal antibodies. Preventive interventions against PAM modify foetal exposure to *P. falciparum in utero*, and have thus an effect on perinatal malaria outcomes. Effective intermittent preventive treatment in pregnancy (IPTp) diminishes placental malaria (PM) and its subsequent malaria-associated morbidity. However, emerging resistance to sulphadoxine-pyrimethamine (SP) is currently hindering the efficacy of IPTp regimes and the efficacy of alternative strategies, such as intermittent screening and treatment (IST), has not been accurately evaluated in different transmission settings. Due to the increased risk of clinical malaria for offspring of malaria infected mothers, PAM preventive interventions should ideally start during the preconceptional period. Innovative research examining the effect of PAM on the neurocognitive development of the infant, as well as examining the potential influence of HLA-G polymorphisms on malaria symptoms, is urged to contribute to a better understanding of PAM and infant health.

Keywords: Pregnancy-associated malaria, Immune tolerance, Intermittent preventive treatment in pregnancy, Parasitaemia, Infancy, Sulphadoxine-pyrimethamine

Background

Pregnancy-associated malaria (PAM), defined as peripheral or placental infection by *Plasmodium*, presents as a major public health concern due to significant adverse health effects on both the mother and the foetus. Women are increasingly susceptible to malaria infection during pregnancy since *Plasmodium falciparum*, the most common parasite responsible for malaria, avoids spleen clearance through expression of proteins that bind to the chondroitin sulphate A (CSA) in the placental intervillous space [1-3]. Consequently, the foetus is initially exposed to

malaria *in utero*. Epidemiological studies estimate 125 million pregnancies are at risk of malaria infection every year [4] within a purposed estimate of 32 million women who become pregnant every year in malaria endemic sub-Saharan Africa countries [5].

The effects of pregnancy-associated malaria on infants include stillbirth, congenital malaria, foetal anaemia, and low birth weight (LBW), caused by intra-uterine growth retardation (IUGR) and pre-term delivery [6-11]; considering the subsequent adverse health outcomes PAM related deaths would account for 75,000 to 200,000 infant deaths in sub-Saharan Africa [12].

Ultimately, pregnancy-associated malaria determines foetal exposure to *P. falciparum in utero*. Indeed, placental malaria is identified as a significant indicator for increased susceptibility to malaria during infancy [13-18]. In turn,

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PAM control strategies modify foetal exposure to *P. falciparum* in utero.

The prevalence of PAM is influenced by transmission, the immunity of the mother, and protective measures, such as insecticide-treated nets (ITNs) or intermittent preventive treatment in pregnancy (IPTp) [10]. Despite the considerable literature on PAM epidemiological and clinical outcomes, no clear conclusions regarding PAM's effect on malaria in infants have been accurately reported. Further well-known risk factors for malaria in infants include high transmission or HIV co-morbidity [19], but exploring PAM influence on malaria risk during infancy could significantly contribute to better understand *Plasmodium* infection among infants. This review aims to revisit the present evidence on pregnancy-related factors that influence malaria in infants, including the effect of control interventions and novel research perspectives. Results are presented by the following topic areas: the epidemiological evidence on the effect of PAM on malaria in the offspring, the risk factors determining exposure to *Plasmodium* in utero, with special regard to control interventions, such as IPTp and ITNs, and the influence of the increasing resistance to SP-IPTp on malaria perinatal outcomes. Finally, new research perspectives to examine the effect of PAM on infant health are discussed.

Methods: search strategy and selection criteria

A systematic literature specifying the epidemiology of malaria in infants with a focus on malaria risk factors in infants, was realized between the 10th January 2012 and the 9th June 2014 utilizing PubMed, the Cochrane Library, Global Health and World Health Organization regional databases. In total, 1,136 articles in English, French, Spanish and Portuguese were classified for review. A combination of standardized terms were used as search criteria; concerning PubMed, the search terms utilized were the Medical Subjects Headings (MeSH) "Parasitaemia" OR "Malaria" OR "Anaemia". In addition, complementary articles, reports, and studies were identified through review and citations. Search criteria for relevant PAM and IPTp studies accepted all designs with the sole caveat that they originated from a malaria endemic country. Three-hundred and fifty-five articles were selected for final review. Due to the limited number of studies and reviews, no sensitivity analysis was realized. No date restrictions were applied and publication bias was not addressed.

Pregnancy-associated malaria and malaria in infants: epidemiological evidence

Pregnancy-associated malaria is consistently associated with an increased malaria risk during infancy [13-16,18] and has been associated with congenital malaria, increased malaria episodes, anaemia, and non-malaria fever episodes

in infants [10,20]. The principal findings of several reviewed studies are presented in Table 1.

Congenital malaria, defined as the presence of asexual *P. falciparum* parasites in the cord blood or in the peripheral blood during the first week of life [25], is the result of transplacental transmission of parasites just before or during delivery. Congenital malaria rates range between 0.83 and 5.96% [17,25-29] in recent epidemiological studies. The introduction of molecular techniques has increased the detection of cord blood parasitaemia raising prevalence rates to 33% [30]. Congenital malaria might entail clinically relevant symptoms in some cases, such as high fever and convulsions, anaemia, hepatosplenomegaly, jaundice, anorexia, vomiting, diarrhoea, drowsiness, pallor, respiratory distress, and cyanosis [30,31]. Although congenital malaria is an important factor in the differential diagnostic of neonatal fever in endemic countries, severe symptoms are rare and, hence, it does not appear to constitute an epidemic at present.

PAM is also associated with earlier episodes, as well as overall clinical malaria episodes, in infants [13-15,17,23]. In a landmark longitudinal cohort study of infants in Cameroon, placental *P. falciparum* infection was associated with infant malaria between four and six months, and parasitaemia rates were higher between five to eight months in offspring of placenta-infected mothers compared to offspring of mothers without placental infection independently of congenital infection [13] (at 6 months: PM+: 36%; PM-: 14%, $p < 0.05$). A study in Tanzania found an interaction between gravidity and placental malaria. The findings demonstrated that the offspring of multigravid women with placental malaria had the highest odds of subsequent malaria episodes (Adjusted Odds Ratio (AOR) = 1.59) 95% confidence interval (CI) 1.16–2.17 [14], and the lowest odds were attributed to offspring of primigravid placenta infected mothers.

Regarding the early appearance of parasites in infants, the above mentioned study in Tanzania reported a 1.41 estimated hazard ratio (HR) (95% CI 1.01–1.99) of first parasitaemia for offspring of mothers with *P. falciparum* placental infection after adjusting for gravidity, transmission season at time of birth, area of residence, and bed net usage [14]. In Gabon, a significant correlation between placental malaria and the first malaria episode was also found (adjusted HR (AHR) = 2.1; 95% CI 1.2–3.7) after adjustment for gravidity, season of birth, area of residence, IPTp versus placebo, and ITNs [15]. A more recent study in Mozambique found that infants born to women who had clinical malaria during pregnancy, or acute placental infection, had an increased risk of clinical malaria during infancy (OR = 1.96; 95% CI, 1.13–3.41, and OR = 4.63; 95% CI 2.10–10.24, respectively) [22]. Furthermore, a cohort study conducted in Tori Bossito (Benin) confirmed the link between PM

Table 1 Influence of maternal parasitemia on malaria in infants

Cohort	Study design and sample size	Time period	Transmission setting	Malaria prevention strategy during pregnancy	Treatment drug regime	Proportion of maternal peripheral parasitemia at delivery	Proportion of placental parasitemia	Proportion of neonatal parasitemia	Infant follow-up period	Median time to first parasitemia (days, min, max)	Association of infant malaria with PAM	Early infant parasitemia <3 months
Mangochi [21] (Malawi)	Clinical trial on comparative efficacy of CQ or MQ; infant cohort follow-up (1766 women at delivery and 1289 infants)	1988-1990	Perennial with seasonal peaks	CQ and MQ	CQ	CQ: 20.3% MQ: 4.1%	CQ: 25.1% MQ: 6.2%	CQ: 8.6% MQ: 3.1%	12 months	199 (192-207)	at 3 months: 1.1 (0.7-1.9)	18.5%
Ebolowa [13] (Cameroon)	Infant cohort follow-up (197)	1993-1995	Perennial with seasonal peaks	CQ	CQ		22.84% (Primigravid: 69%; Multigravid: 31%)		24 months	PM+: 217; PM-: 350	at 6 months: PM+: 36%; PM-: 14%, $p < 0.05$ at 2 years: PM+: 46.5%; PM-: 38.5%, $p = 0.6$	≈12%
Muheza [14] (Tanzania)	Infant cohort follow-up (453)	2002-2004	Perennial with seasonal peaks (400 infective mosquito bites each year)	SP (area with 68% resistance 14-day treatment failure rate)			15.2% (Primigravid ≤ 2: 24%; Multigravid > 2: 5.6%)		12 months	266 (238-294) PM+: 273 (245-322) PM-: 244 (147-266);	Primigravidae: PM+: AOR = 0.21, (0.09-0.47) PM-: Reference*** Multi-gravidae: PM+: AOR = 1.59, (1.16-2.17) PM-: AOR = 0.67, (0.50-0.91)	PM+: ≈20%; PM-: ≈10%
Lambaréné [15] (Gabon)	Infant cohort follow-up (527)	2002-2004	Perennial	No		10.5%*	9.48%		30 months	Primigravidae: PM+: 107 (83-139) PM-: 102 (29-205) Multi-gravidae: PM+: 111 (13-189) PM-: 92 (27-208)	PM+: AOR = 2.1, (1.2-3) PM-: Reference**	PM+: ≈2%; PM-: ≈0%
Manhiça [22] (Mozambique)	Clinical trial on the efficacy of SP compared to placebo; infant cohort follow-up (1030 women at delivery and 997 infants)	2003-2005	Perennial with seasonal peaks	ITNs vs ITNs+SP	SP-AQ	ITNs+ placebo: 15.15% ITNs+SP: 7.1%	ITNs+ placebo: 52.27% ITNs+SP: 52.11%	ITNs+ placebo: 1.15% ITNs+SP: 0.92%	12 months		Clinical PAM: AOR = 1.96 (1.13-3.41) Acute PM: AOR = 4.63 (2.1-10.24) Chronic PM: AOR = 3.95 (2.07-7.55) PM-: Reference	

Title: *Prevalence of maternal parasitemia on malaria in infants (Continued)*

Year	Study	Design	Sample Size	Intervention	Control	Outcome	Significance
2004	Wang et al.	Randomized controlled trial	1000	1000	1000	1000	1000
2005	Wang et al.	Randomized controlled trial	1000	1000	1000	1000	1000
2006	Wang et al.	Randomized controlled trial	1000	1000	1000	1000	1000
2007	Wang et al.	Randomized controlled trial	1000	1000	1000	1000	1000
2008	Wang et al.	Randomized controlled trial	1000	1000	1000	1000	1000
2009	Wang et al.	Randomized controlled trial	1000	1000	1000	1000	1000
2010	Wang et al.	Randomized controlled trial	1000	1000	1000	1000	1000
2011	Wang et al.	Randomized controlled trial	1000	1000	1000	1000	1000
2012	Wang et al.	Randomized controlled trial	1000	1000	1000	1000	1000
2013	Wang et al.	Randomized controlled trial	1000	1000	1000	1000	1000
2014	Wang et al.	Randomized controlled trial	1000	1000	1000	1000	1000
2015	Wang et al.	Randomized controlled trial	1000	1000	1000	1000	1000
2016	Wang et al.	Randomized controlled trial	1000	1000	1000	1000	1000
2017	Wang et al.	Randomized controlled trial	1000	1000	1000	1000	1000
2018	Wang et al.	Randomized controlled trial	1000	1000	1000	1000	1000
2019	Wang et al.	Randomized controlled trial	1000	1000	1000	1000	1000
2020	Wang et al.	Randomized controlled trial	1000	1000	1000	1000	1000

PM: Placental malaria; PAM: Pregnancy associated malaria and AOR: Adjusted Odds Ratio.

*Data from a reference article.

*The association between placental malaria and malaria in the child was only statistically significant for children who were randomized to receive the subcutaneous pyrimethamine intervention (RR=3.15).

^{***} Analysis of the effect of Pp on parasitaemia of the offspring was performed for 382 women of this cohort. Among them, 216/9 received no Pp, 42/9 one dose, and 36/4 two or more doses.

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341
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2006

V. Results

and malaria in infants through consistent entomologic and environmental follow-up [17,23]. The study findings on infants sleeping in a house with an ITN confirmed the link between PM and malaria controlled for transmission intensity, seasonality, number of anopheles, antenatal care (ANC) visits, and maternal severe anaemia (AHR = 2.13; 95% CI 1.24–3.67) compared with infants whose mothers did not have placental malaria at delivery. This cohort study additionally reports an increased susceptibility of infants to *P. falciparum* parasites with antigens to which they were previously exposed *in utero* suggesting an immune tolerance process undergoing during pregnancy [32]. PAM has also been associated with a reduction in maternal antibody transfer to the foetus [33,34], hence increasing infant susceptibility to parasites [35,36]. Consistent with the notion that the type, timing, and the duration of exposure to the parasite *in utero* determine susceptibility to malaria, infections occurring during the third trimester are associated with increased risk of infection and clinical malaria during the first year of life according to another study in the province of Mono (South Benin) [24].

Nevertheless, the effect of PAM on infant health may involve an overall increased morbidity and mortality. Placental malaria was additionally correlated with non-malaria infections in the Tori Bossito cohort infants during the first 18 months of life suggesting that immune tolerance could also imply immunity in a more general manner besides malaria specific immunity [20]. Moreover, placental malaria posed a significant risk factor for overall mortality during the first year of life [37] in a study in Malawi, and another study from Mozambique [22] identified both acute placental malaria and cord blood parasitaemia with increased infant mortality. More precisely, in this study from Mozambique infant mortality was also significantly associated with malaria infection of the placenta (p -value < 0.012) after adjustment on HIV status, LBW, maternal clinical malaria during pregnancy, foetal anaemia and IPTp regime. The risk of dying during infancy was increased among infants born to women with acute placental infection (OR = 5.08; 95% CI 1.77–14.53), as well as among infants with parasitaemia in the cord blood (OR = 19.31; 95% CI, 4.44–84.02).

A possible explanation for different immune tolerance effects of PAM relates to HLA-G polymorphisms and their association with different malaria susceptibility [38]. HIV infection influences as well a woman's susceptibility to malaria, and this is of major concern as both diseases overlap considerably in sub-Saharan Africa. Consistent evidence suggests both infections interact synergistically and result in poorer health outcomes [39]. PAM is more frequent among HIV infected women in comparison to non-infected women, and can increase

maternal HIV load [40–42]. PAM in HIV-positive pregnant women is further associated with higher risk of both anaemia and LBW [40,43–45]. This results in overall increased maternal and infant mortality [46,47].

A potential long-term consequence of PAM concerns neuro-cognitive impairment of infants exposed to malaria *in utero*. Due to recent evidence concerning the role of the complement system in the regulation of neurodevelopment, it has been proposed that excessive complement activation induced by placental malaria may disrupt normal neurodevelopment resulting in neuro-cognitive impairment of infants exposed to *Plasmodium in utero* [48].

Although a complete explanation of the physiopathology of PAM has not yet been understood, *in utero* exposure to malaria is probably nonetheless correlated with placental sequestration of erythrocytes. The immune tolerance process would plausibly then depend on the type of malaria antigen in contact with the foetus, the amount and the duration of the exposure, and the timing of exposure during pregnancy [16,49]. The interaction between gestation and infection timing during pregnancy has been previously shown to influence the pathologic consequences for the offspring. Due to the particular physiopathology of PAM, a specific immunity develops during the first pregnancy [10] and, hence, primigravidae are at higher risk of PAM compared to multigravidae [10]. In this respect, infants of primigravid women are also at higher risk of subsequent malaria in comparison to infants of multigravid women, mainly as a result of reduced antibody transfer [11]. Finally the timing of malaria episodes during pregnancy results in different effects on both the mother and the foetus; parasitaemia appears to be higher during the first and second trimesters, even if follow-up on *P. falciparum* parasitaemia during the first trimester has seldom been complete [10,50–53]. Essentially, the administration of IPTp at different moments determines different protection patterns for the infant [50] and, in parallel, a significant reduction in placental malaria and maternal parasitaemia has been extensively described [54] following the implementation of PAM control interventions. As a result of the different infant malaria outcomes depending on PAM and IPTp, and considering the body of the available research, the following questions are posed: How does exposure *in utero* to *P. falciparum* influence malaria in infants? How do control interventions modify in turn the impact of PAM on clinical malaria in infants?

Pregnancy associated malaria and control interventions: effect on perinatal malaria outcomes

Foetal exposure to *Plasmodium in utero* primarily depends on transmission and control interventions.

Preventive measures substantially alter the interaction between exposure and immunity. IPT is a widespread preventive strategy to fight malaria and involves the administration of a curative dose of an effective anti-malarial drug, regardless of the presence of *Plasmodium* in the blood, to prevent the disease [19]. IPT measures decrease parasitaemia, and consequently influence the immunity response of the infant to *Plasmodium in utero* through maternal intermittent preventive treatment in pregnancy (IPTp). Therefore, WHO recommends IPTp with SP for all pregnant women as early as possible in the second trimester, and at each scheduled antenatal care visit at least one month apart in areas of moderate to high malaria transmission [55], IPTp strategies are however not yet completely deployed in malaria endemic regions and the implementation of IPTp interventions interfere with PAM outcomes. Figure 1 presents the main characteristics concerning implementation of IPTp programmes in Africa.

Effective administration of IPTp clears placental parasitaemia and consequently modifies the exposure to malaria antigens resulting in a significant reduction in placental malaria and maternal parasitaemia [54]. Compared to case management or placebo in pregnant women, a two-dose IPTp regime with sulphadoxine-pyrimethamine (SP) significantly reduced placental malaria according to a review on four studies (relative risk (RR) = 0.48) [56]. In a randomized, double blind, placebo-controlled trial with joint use of ITNs in Mozambique, SP-IPTp (1-2 doses) was correlated to a significant decrease only in active placental malaria [57] (Table 1). In Mali, placental parasitaemia was significantly reduced by SP-IPTp (AOR = 0.69) when compared to weekly administered chloroquine (CQ) [58] and confirmed higher SP efficacy compared to CQ already reported in Malawi [59]. A recent meta-analysis has concluded significant reduction in PM after three doses of SP compared to two doses [54], which corresponds to current WHO recommendations.

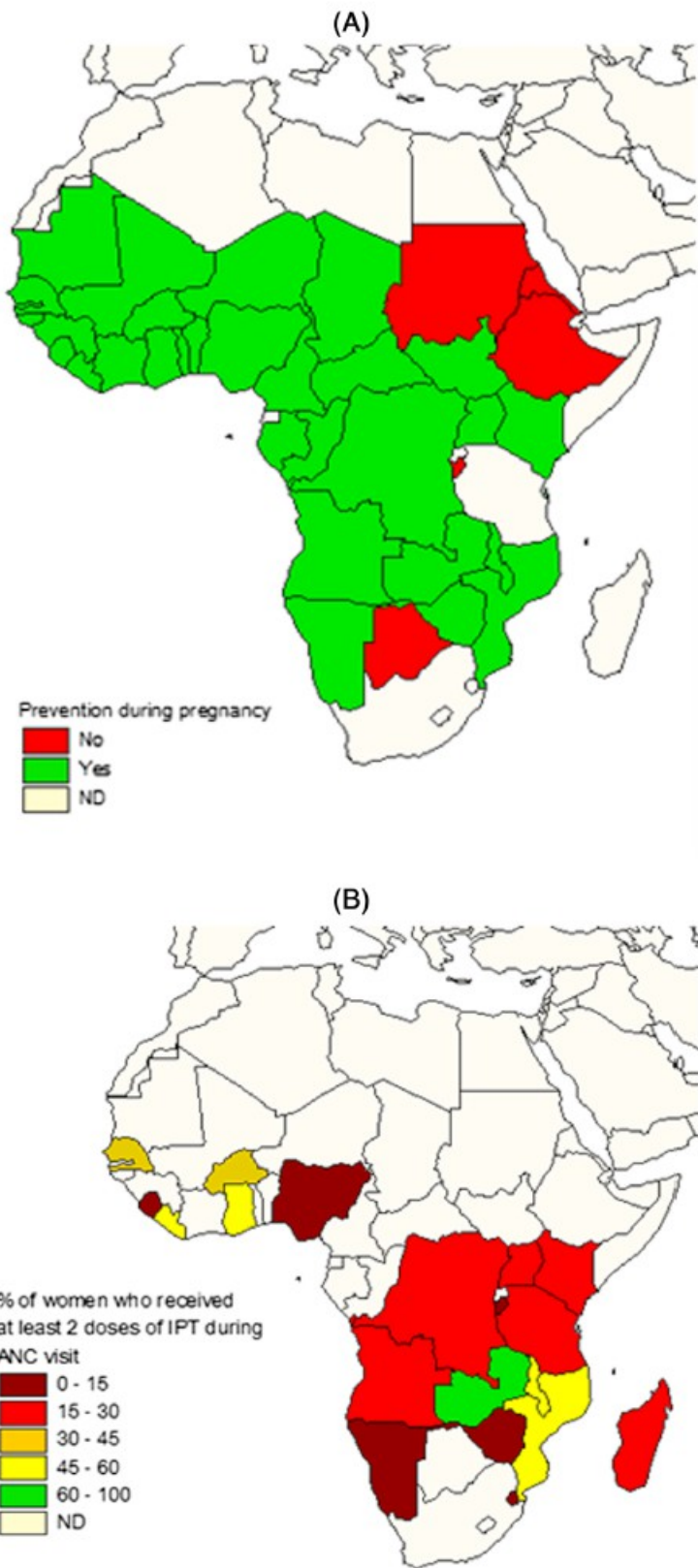
A comprehensive review encompassing published studies conducted between 1985 and 2000 found a PAM prevalence range of 10% to 65% among all gravidae [12], with a median prevalence of 27.8% [10]. In low-transmission African settings, the median prevalence peripheral infection was 13.7% and the placental malaria median prevalence was 6.7% [10]. Recent studies however reported a significant decline in prevalence following PAM control interventions. The protection of joint ITNs with IPTp-SP use is significant in only certain trials, yet reported ITN use ranges from 5 to 25%, and this might not be sufficient enough to show an effect [60]. An article reviewed the influence of preventive measures on PAM during a decade, effectively 2002 to 2012, and reported placental malaria rates ranging from 2 to 29% among women treated with less than three doses (mainly two) of sulphadoxine-

pyrimethamine (SP) compared to 2 to 8% among women receiving more than or equal three doses (mainly three) [60]. A novel study included within the afore mentioned review describes a two-fold lower prevalence of placental malaria in the three-dose SP group compared to the two-dose SP group (adjusted prevalence ratio = 0.48) [61]. Even if the augmented efficacy associated with higher doses is predominately observed in clinical trials rather than in studies of public health programme implementations [60], the emergence of SP resistance is certainly shaping the efficacy of IPTp, and consequently its influence on the malaria burden in infants.

IPTp and malaria in infants: when protection encounters resistance

Reduced compliance with drug regimes and the increasing resistance to anti-malaria drugs highlight the complexity of IPTp management at present. A 2007 meta-analysis confirmed that SP IPTp continued to benefit pregnant women in areas of up to 39% resistance to SP, measured by *in vivo* resistance at day 14 of treatment in children [56]. Similar results were found in Benin, where rates of *in vivo* resistance to SP were estimated to be 50% by day 28 of treatment in infants, and yet SP IPTp succeeded to prevent LBW [62]. However, studies published more recently display contradictory results. A study in Malawi, where there is a strong fixation of the resistant quintuple mutant, shows significantly reduced small for gestational age (SGA) rates in offspring of primigravid women having received ≥ 2 doses of SP compared to 0-1 doses [63]. On the other hand, peripheral parasitaemia was significantly higher among women having received ≥ 2 doses of SP. Indeed, the effects of resistance on malaria clinical outcomes become more frequent in more recent studies from East Africa. In a Tanzanian site with high SP resistance (14-day parasitologic SP treatment failure rate in children of 68%), IPTp was not associated with a reduction in odds of PM, LBW or maternal anaemia. Furthermore, it was associated with increased odds of foetal anaemia and severe malaria among the offspring (AOR = 2.31) [64]. IPTp in this setting was associated with an overall increased risk of severe malaria [64,65].

However a recent longitudinal study revealed no significant increase of malaria at delivery after IPTp treatment, albeit the increasing prevalence and fixation of SP-resistant *P. falciparum* haplotypes in another area in Malawi [66]. Evidence for the present efficacy of SP-IPTp regimes is inconclusive but resistance to SP is spreading. Close monitoring of its efficacy is therefore necessary to determine if or when the treatment failure of SP-IPTp detected by some recent studies has become generalized at the population level, thus necessitating a switch to alternative drug regimes. Nevertheless, the



Source: WHO World Malaria Report 2013

Figure 1 Intermittent Preventive Treatment in pregnancy in Africa. Source: World Malaria report 2013. WHO publications 2013. **A.** Implementation of intermittent preventive treatment in pregnancy in Africa. **B.** The percentages of women having received at least 2 doses of IPTp are approximated data issue of the latest demographic surveys of the countries represented.

Malaria Policy Advisory Committee (MPAC) cited a current paucity of data in order to determine the precise level of resistance obliging interruption of IPTp-SP treatment, especially in the absence of an established and effective alternative [55].

Currently, the Intermittent Screening and Treatment (IST) is a proposed alternative to IPTp in areas with substantial resistance against IPTp regimes. IST consists in screening for malaria infection using a malaria rapid diagnostic test (RDT) at scheduled antenatal clinic visits and subsequently treating positive women with an effective anti-malarial drug [67]. However, extensive evidence on IST efficacy is lacking in African regions and further efficacy studies should be conducted in broader geographical regions [68].

In summary, PAM determines foetal exposure to *P. falciparum* *in utero* and is hence correlated to congenital malaria and earlier development of clinical episodes in infancy, possibly as the consequence of an immune tolerance process *in utero*. Effective IPTp diminishes PM and malaria associated morbidity such as LBW, pre-term delivery, IUGR, and perinatal mortality in areas where resistance to SP is not highly significant. Yet the influence of different IPTp regimes on malaria morbidity in infants remains a question for further research. The concrete effect of resistance and the ongoing immune tolerance process *in utero* have not been presently explored. Further evidence is also lacking on the importance of the timing of infection during pregnancy and infant malaria morbidity. There exists some evidence that earlier administration of IPTp has a positive effect on birth outcomes like LBW, nevertheless, later dosing provides a more continuous protection [50], thus necessitating the administration of three doses instead of two for improved clinical outcomes. In addition, the implementation of different IPTp regimes should be adapted according to transmission and the SP-resistance pattern. For example, IST has been applied successfully in an area of moderately high malaria transmission in Ghana [67]. IST should be further explored and its efficacy should be evaluated in other transmission settings to ascertain its utility as an effective tool for the control of PAM.

Conclusions

This review on the impact of PAM on malaria in infants substantiates the complexity of the subject and the necessity of a holistic approach for fighting malaria. In addition, research gaps should be fulfilled to enhance malaria outcomes. Strategies should start during the pre-conceptual period or at least during pregnancy, as there is evidence of increased infant susceptibility to parasites carrying antigens to which they were previously exposed while *in utero*. A complete explanation of the immune process remains a

question for further research as well as the precise effect of the timing of *in utero* exposure to the parasite. Furthermore, the role of protective maternal antibodies has not yet been clarified. Operational research on different preventive IPT strategies should also be continuously conducted, and cost effectiveness analysis for community-level IST interventions should be investigated.

Finally, novel aspects of research on PAM should be further explored. Due to the long-term impact of placental malaria's possible neuro-cognitive consequences, the scientific community should prioritize studies investigating this interaction. An exploration of the influence of HLA-G polymorphisms on subsequent malaria symptoms would serve as well as an important contribution for infant malaria risk factors.

Abbreviations

ACT: Artemisinin-based combination therapy; AHR: Adjusted hazard ratio; AL: Artemether-lumefantrine; ANC: Antenatal care; AOR: Adjusted odds ratio; AQ: Amodiaquine; CQ: Chloroquine; HR: Hazard ratio; IPTp: Intermittent preventive treatment in pregnancy; IST: Intermittent Screening and Treatment; ITNs: Insecticide-treated nets; IUGR: Intra-uterine growth retardation; LBW: Low birth weight; MeSH: Medical Subjects Headings; MPAC: Malaria Policy Advisory Committee; MQ: Mefloquine; OR: Odds ratio; PAM: Pregnancy associated malaria; PM: Placental malaria; RDT: Rapid diagnostic test; RR: Relative risk; SGA: Small for gestational age; SP: Sulphadoxine-pyrimethamine; SPR: Slide positivity rate.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

VMA gathered and selected the articles, realized the article database and drafted the manuscript. RA realized the figure and helped to draft the manuscript. MC participated in the design and coordination of the article and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Jessica Barry read and edited the manuscript making valuable linguistic corrections. Philippe Deloron and Adrian Luty made valuable contributions to the intellectual content of the article. José Juan Lopez-Rubio, Isabel Puigdomènech and Laura Gomez-Valero contributed materials essential for the study. Violeta Moya-Alvarez is funded by the Doctoral Network of the Ecole des Hautes Etudes en Santé Publique and the Direction Générale de l'Armement. Rosa Abellana is funded by the Universitat de Barcelona. Michel Cot is funded by the Institut de Recherche pour le Développement and the Université Pierre et Marie Curie.

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Received: 22 March 2014 Accepted: 26 June 2014
Published: 11 July 2014

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doi:10.1186/1475-2875-13-271

Cite this article as: Moya-Alvarez et al: Pregnancy-associated malaria and malaria in infants: an old problem with present consequences. *Malaria Journal* 2014 **13**:271.

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2013 **V.I.2. Malaria and iron levels: where do we stand?**

2014

2015 **Summary of the article:** We wanted to analyse the state of art concerning the impact of iron
2016 levels on malaria risk during infancy. Consequently, we realized a systematic literature search
2017 on iron deficiency, anaemia, and malaria risk factors in infants between the January 2012 and
2018 April 2014. We used PubMed, the Cochrane Library, Global Health and the World Health
2019 Organization regional databases. In total, 398 articles in English, French, and Spanish were
2020 considered for review according to the specificity of the subject. No date restrictions were
2021 applied. We used Standardised terms and subsequent related citations and links as search
2022 criteria. In the case of PubMed, the search terms were the Medical Subjects Headings
2023 (MeSH) "Parasitemia" OR "Malaria" OR "Anemia, Iron deficiency". Two hundred and
2024 ninety-four articles were selected for final review. With regard to clinical trials, all study
2025 designs were accepted with the sole restriction of precedence from a malaria endemic country.
2026 No restriction with regard to the type of iron supplement intervention was applied (food
2027 based, ferrous sulphate, NaFeEDTA etc.).

2028 Observational studies describe a certain protection for malaria risk among iron deficient
2029 children, and ancient clinical trials report increased susceptibility to clinical malaria among
2030 iron-supplemented children. Nevertheless neither recent clinical trials with important malaria
2031 monitoring and protective measures, nor the Cochrane review show significant increase for
2032 malaria risk among iron-supplemented children. Evidence on the effect of iron levels on
2033 malaria risk is subject to limitations, such as the interference of protective measures, and the
2034 lack of homogenous iron markers and haematological indicators. The effect of the previous
2035 haematological and infectious health status, including the chronicity of iron deficiency and the
2036 possible threshold effect of iron levels, needs to be investigated in the context of a gold
2037 standard combination of iron markers taking into account both parasitological and clinical

2038malaria outcomes. Further epidemiological elements, such as age of the children, immunity
2039status, hemoglobinopathies, or the transmission setting should be considered as well. Finally,
2040it is essential to ponder the possible benefits of iron supplementation for anaemia and child
2041neurocognitive development beyond its possible deleterious effect.

2042**What the article adds to the previous state of art:** Albeit the important meta-analyses on
2043the association of malaria risk with iron, a qualitative review summarizing the complexity of
2044this relationship was yet to be conducted. Indeed, we do not analyse the power of each study.
2045However, we identify the fact that studies, which do not report increased malaria risk
2046associated to iron supplements, have strong protective measures. Furthermore, we bring up
2047the lack of prospective cohorts analyzing the association. Finally, we describe the important
2048obstacle of not having a gold standard indicator of iron levels and we suggest some proposals
2049for further research.

2050NB: The following article summarizes the state of the art of the topic. Consequently,
2051substantial information has already been explained in the “State of the art” section.

2053 Article under review in *Nutrition reviews*:

2054

2055 **Malaria and iron levels: the dangerous liaisons?**

2056 Violeta Moya-Alvarez, Florence Bodeau-Livinec, Michel Cot.

2057

2058 **Abstract:** Malaria is the disease with the highest infant morbidity and mortality (WHO

2059 estimates 207 million cases and 627,000 deaths in 2012), and it raises the burden of anaemia

2060 in low-income countries, where 40% of children are anaemic according to WHO estimates.

2061 Anaemia compromises immunity, and iron deficiency anaemia (IDA) has long-term

2062 permanent neuro-cognitive consequences. However iron has been pointed out as an important

2063 co-factor for *Plasmodium falciparum*, the main parasite responsible for malaria, raising fears

2064 that current iron supplementation policies might be harmful. Albeit the complexity of the

2065 effect of iron levels on malaria risk, an accurate review clarifying their epidemiological

2066 association and assessing the different novelties on iron markers has yet to be conducted.

2067 Observational studies describe a certain protection for malaria risk among iron deficient

2068 children, and ancient clinical trials report increased susceptibility to clinical malaria among

2069 iron supplemented children. Nevertheless neither recent clinical trials with important malaria

2070 monitoring and protective measures, nor the Cochrane review show significant increase for

2071 malaria risk among iron supplemented children. Evidence on the effect of iron levels on

2072 malaria risk is subject to limitations, such as the interference of protective measures, and the

2073 lack of homogenous iron markers and haematological indicators. The effect of the previous

2074 haematological and infectious health status, including the chronicity of iron deficiency and the

2075 possible threshold effect of iron levels, needs to be investigated in the context of a gold

2076 standard combination of iron markers taking into account both parasitological and clinical

2077 malaria outcomes. Further epidemiological elements, such as age of the children, immunity

2078 status, hemoglobinopathies, or the transmission setting should be considered as well. Finally,

2079it is essential to ponder the possible benefits of iron supplementation for anaemia and child
2080neurocognitive development beyond its possible deleterious effect.

2081 ³⁵₁₇ *Title. Iron and malaria: the dangerous liaisons?*

2082

2083 ³⁵₁₇ *Abstract.*

2084Malaria raises the burden of anaemia in low-income countries, where 40% of children are
2085anaemic (WHO,2012). Moreover, iron is an important co-factor for *Plasmodium falciparum*,
2086raising fears that iron supplementation might be harmful. We realized a systematic literature
2087search to review the present knowledge on the malaria-iron association considering recent
2088novelties and substantial qualitative information. Observational studies describe a certain
2089protection among iron deficient children, and ancient clinical trials report increased
2090susceptibility among iron supplemented children. Nevertheless, neither recent clinical trials,
2091nor the 2011 Cochrane review show significant increased malaria risk associated with iron
2092supplements. Evidence on the effect of iron on malaria is subject to limitations, such as the
2093interference of protective measures, the limited follow-up of the children, and the lack of
2094homogenous iron indicators. The effect of the previous haematological and infectious health
2095status and the possible threshold effect of iron levels need to be investigated in the context of
2096a gold standard combination of iron markers. Finally, it is necessary to ponder the benefits of
2097iron supplementation.

2098 ³⁵₁₇ *Key words.* Iron, malaria, iron supplements, iron indicators, anaemia.

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2100

2101 **Introduction**

2102 Malaria is the disease with the highest infant morbidity and mortality worldwide. In 2012 there
2103 were over 207 million cases and 627,000 deaths according to WHO estimates [1]. The burden of
2104 disease involves major constraints for public health and also for development in low-income
2105 countries, where infants constitute the most numerous age-group in society. Malaria entails the
2106 haemolysis of red blood cells (RBC) and the suppression of erythropoiesis resulting in important
2107 anaemia. Iron deficiency, defined by WHO by serum ferritin levels $<15\mu\text{g/l}$ [2], remains the main
2108 cause of anaemia, affecting over 2 billion people globally and it is indeed the most common
2109 nutritional deficiency. Iron deficiency anaemia (IDA) hinders the correct psychomotor
2110 development and has important long-term permanent consequences for the neuro-cognitive
2111 performance of the children [3]. According to WHO, 40% of children in low-income countries are
2112 estimated to be anaemic. Both malaria and iron deficiency affect mainly infants, pre-school
2113 children and pregnant women. Furthermore both diseases overlap geographically. Anaemia is not
2114 only a consequence of malaria, but anaemia compromises immunity and predisposes to infections.
2115 As a consequence, iron supplements have been recommended by WHO guidelines to fight the
2116 impaired health status of anaemic children [4]. More precisely, daily supplements of 12.5 mg iron
2117 and $50\mu\text{g}$ folic acid are encouraged to prevent anaemia in children 6-24 months where anaemia
2118 prevalence $>40\%$ or from 6 to 12 months in settings with low prevalence of anaemia. In case of
2119 low birth-weight supplements should start at 2 months. However iron has also been pointed out as
2120 an important co-factor for infective agents. Since Kochan first described the term “nutritional
2121 immunity” to describe the importance of iron deficiency as a defensive mechanism against
2122 bacteria in 1973 [5], controversy on the role of iron in infections in general, and specially in
2123 malaria, has been always present. Iron repletion was described as a risk factor for malaria in 1975
2124 [6] and for infection in general in 1978 [7]. While other articles had reported a deleterious effect

2125of high iron levels regarding the risk of malaria [8,9], the study realized in Pemba [10], Tanzania,
2126where malaria was highly prevalent, found a 12% increased mortality among iron supplemented
2127children in 2002-2003. Hence, substantial changes in iron supplementation guidelines ensued and
2128iron supplementation was restricted to iron deficient children [11]. Nevertheless universal and
2129systematic iron levels screening is highly difficult on the field. And the epidemiology of malaria
2130infections is substantially variable among transmission settings. Indeed, evidence should be
2131applied according to epidemiological infective settings and the effective control interventions
2132available. Therefore, we aim at reviewing the present knowledge on malaria-iron associations
2133taking into account the endemic setting, the recent novelties on markers of iron repletion, and the
2134forthcoming epidemiological challenges to elaborate a balanced analysis of the malaria risk
2135associated to iron. The main objectives are first, to clarify the association between iron and
2136malaria and to analyse the nature and the extent of their possible interactions; and second, to
2137identify the most adequate iron marker for both research and clinical purposes in order to optimize
2138the interventions tackling these complex but extended diseases.

2139

2140**Methods: Search strategy and selection criteria**

2141A systematic literature search on iron deficiency, anaemia, and malaria risk factors in infants was
2142realized between the January 2012 and April 2014 using PubMed, the Cochrane Library, Global
2143Health and the World Health Organization regional databases. In total, 398 articles in English,
2144French, and Spanish were considered for review according to the specificity of the subject. No
2145date restrictions were applied. Standardised terms and subsequent related citations and links were
2146used as search criteria. In the case of PubMed, the search terms were the Medical Subjects
2147Headings (MeSH) "Parasitemia" OR "Malaria" OR "Anemia, Iron deficiency". Two-hundred and
2148ninety-four articles were selected for final review. With regard to clinical trials, all study designs
2149were accepted with the sole restriction of precedence from a malaria endemic country. No
2150restriction with regard to the type of iron supplement intervention was applied (food based, ferrous

2151sulphate, NaFeEDTA etc.). Even if all studies on the iron-malaria association were considered,
2152only the studies concerning infants are included in the article. In addition, special attention was
2153given to epidemiological studies focused specifically on the iron-malaria association reporting
2154concrete haematologic and parasitological indicators (haemoglobin, ferritin, blood smear,
2155parasitaemia). Publication bias is not addressed.

2156

2157**Malaria and iron levels: evidence from epidemiological studies**

2158Because of its importance for public health, the attempt to unravel the complexity of malaria in
2159infants has brought up new aspects that influence parasitemia beyond entomological or
2160immunological factors and preventive interventions. Malaria endemic countries carry a significant
2161burden of nutritional deficiencies that a priori predispose to diseases. Numerous studies have been
2162carried out in these malaria endemic regions in order to observe the consequences of iron repletion
2163and supplementation policies on the appearance and severity of malaria. Effects are however
2164difficult to quantify and results should be interpreted according to their outcomes and their
2165measure indicators. To address the effect of iron levels on malaria risk, it is necessary to identify
2166the most significant outcomes illustrating the interaction between iron and malaria, and to analyse
2167the adequacy of the biological parameters used for measurement.

2168Therefore, certain methodological elements need to be taken into account. Iron deficiency is
2169defined according to different biological parameters across the studies. The demographic
2170characteristics of the population, the methodology of the study (clinical trial or observational
2171study), and the duration of the follow-up period need to be considered as well. Only the answers to
2172these questions can give further light on the question to prioritize public health policies.

2173

2174**Malaria indicators and iron: epidemiological evidence**

2175The physiopathology of malaria infection involves a direct interaction between Plasmodia and
2176iron. Only within the infected RBC, *P. falciparum*, the parasite responsible for most malaria cases,

2177consumes up to 80% of the haemoglobin [12]. In addition, the parasite sequestration in the
2178intestinal blood vessels impairs the optimal nutritional absorption [13]. Furthermore, non-
2179transferrine bound iron (NTBI) is associated to increased severity of the malaria episode and to
2180reduced performance of the immune function [14–16]. Beyond these direct interactions, further
2181clinical conditions, such as certain genetic variants, interfere to determine the association between
2182malaria and iron levels. Indeed, genetic variants are estimated to be responsible for over 25% of
2183the variation in susceptibility to malaria [17]. In this respect sickle haemoglobin is a significant
2184example, but protection is thought to be rather multigenic [18]. Other co-morbidities, such as HIV,
2185bacterial and helminthic infections are also correlated with both iron and malaria [19–21].
2186There are certainly numerous pathways in which iron and malaria interact. Consequently, it is
2187necessary to analyse their association with a holistic approach that arises from the epidemiological
2188pattern of infections on the field. Table 1 summarizes the landmark studies on iron levels and
2189malaria in different malaria endemic regions.

2190Observational studies display information reflecting the association between iron and malaria
2191based on the real circumstances of the field, but accurate iron monitoring is not commonly
2192realized on a systematic basis in this context. Clinical trials focus rather on the effect of
2193supplements and investigate the possible consequences for malaria outcomes of the iron
2194supplementation policy, but their methodological protective constraints do not reflect the
2195epidemiological reality of malaria endemic settings. Indeed, both approaches assemble different
2196but important information and, therefore, both should be considered for the analysis of the iron-
2197malaria link.

2198Clinical malaria is the consequence of the asexual cycle of Plasmodia parasites in the RBC. It
2199constitutes the main outcome of the majority of the observational studies and it is currently
2200defined as temperature $>37.5^{\circ}$ or 38° C within the previous 48 hours and a blood film positive for
2201blood-stage asexual parasites. In this respect, two cross-sectional observational surveys from 2001
2202to 2003 in Kenya among children aged 8 months to 8 years reported significant protection among

2203iron deficient children (Adjusted incidence rate-ratio (IRR)= 0.7 (95%CI 0.51;0.99) with
2204ferritin<12µg/ml and transferrin saturation<10%) [22]. Furthermore, iron status was inversely
2205correlated with malaria-specific immunoglobulins. Similar results were found in an observational
2206cohort study in Tanzania [23] among children between birth and 3 years. Iron deficiency (defined
2207by ferritin concentration corrected on CRP) was also associated with a significant protection with
2208regard to lower odds of malaria parasitemia (OR=0.15 (95%CI 0.12;0.19)), lower odds of
2209hyperparasitemia (parasites>2500/200 white blood cells (OR=0.04 (95%CI 0.02;0.07)), and lower
2210odds of severe malaria (OR=0.25 (95%CI 0.14;0.46)) after adjustment for possible confounders.
2211In a pioneer randomized placebo controlled trial in Tanzania in 1995 in infants between 8 and 24
2212weeks of age, no increased susceptibility to malaria was observed among iron supplemented
2213children with regard to first or only malaria episode compared to placebo (protective efficacy
2214(PE)= 12.8% (CI -12.8;32.5) [24]. Albeit this first reassuring result, supplementation effects on
2215children health status had to be re-evaluated after the Pemba trial. In 2002-2003 a randomised,
2216double blind, placebo-controlled trial, gathered medical evidence on all-cause morbidity and
2217mortality among over 24,000 children up to 35 months daily supplemented with folic acid and
2218iron, iron, folic acid, zinc or placebo¹⁰ in Pemba, Tanzania. In the same cohort, a sub-study
2219among 2413 children addressed the impact of supplements on haematological status, zinc, malaria
2220prevalence, and infectious disease morbidity. Combined groups of supplemented children had
2221significant higher risk for serious clinical events resulting from malaria compared to placebo
2222(RR=1.16, CI 1.02; 1.32). Malaria related hospital admissions were also significantly higher
2223(RR=1.18, (95%CI 1.02; 1.36)) among supplemented children. In the case of cerebral malaria, the
2224RR of the iron and folic acid group, was also significant compared to placebo (RR=1.22 (CI 1.02;
22251.46). In addition another deeply relevant aspect of the malaria-iron association was first raised
2226up: the importance of the iron levels at baseline. Iron-deficient children at baseline, defined by
2227zinc protoporphyrin>80 µmol/molhaeme, had a reduced risk of malaria-related adverse events
2228when supplemented compared to placebo (RR=0.56, 95%CI 0.32; 0.97). Due to the increased

2229morbidity found in this trial, the WHO recommendations restrained supplements to iron deficient
2230children in malaria endemic regions [25].

2231Nevertheless, as previously said, more recent studies report different results. A study in Tanzania
2232in 2008-2009 investigated the consequences of micronutrient supplementation in 612 children
2233between 6 and 60 months [26]. While there was no significant increase in overall malaria episodes
2234among supplemented children compared to placebo, multi-nutrient supplementation was
2235associated to a 41% increase in the overall number of malaria episodes in children with iron
2236deficiency (HR=1.41 (95%CI 1.09; 1.82)), whereas there was no significant impact among the
2237iron-replete children (p-value for difference in effect=0.01).

2238In 2010 in Ghana, in a double blind, cluster randomized trial providing a micronutrient powder
2239(MNP) with or without iron, 1958 infants of 6 to 35 months of age were followed for 6 months
2240and no significant increase in malaria risk was observed compared to placebo (Risk ratio (RR)=1
2241(95%CI 0.81;1.23)) [27]. No significant association with increased malaria was described among
2242iron replete children, with or without concomitant anaemia (RR=0.83 (95%CI 0.64;1.08) and
2243RR=1.04 (95%CI 0.82;1.32), respectively). However, supplemented children with both iron
2244deficiency and anaemia showed significantly reduced risk of malaria RR=0.67 (95%CI 0.5;0.88)
2245compared to placebo.

2246Because of these a priori contradictory results of the studies, a Cochrane review of 2011 analysed
224771 trials collecting evidence on 45,353 children [28]. For the 13 trials selected, the Cochrane
2248review concluded to an absence of significant differences in clinical malaria rates between iron
2249and placebo (RR=0.99, 95%CI 0.9; 1.09). No statistical differences were found neither among
2250supplemented infants (children<2years) (RR=0.94 (95%CI 0.82; 1.09) nor for severe malaria
2251(RR=0.91 (95%CI 0.76; 1.08)) compared to placebo. Furthermore, no statistical difference was
2252found among non-anaemic children at baseline (RR=0.97 (95%CI 0.86; 1.09). However, analyses
2253on iron deficiency defined by ferritin were not realized. Even if it is difficult to screen children for
2254iron status at the population level, information on the effect of iron deficiency is relevant to

2255develop useful supplement strategies based on scientific accurate evidence. Finally, this Cochrane
2256meta-analysis describes increased risk for clinical malaria among iron or iron plus folic acid
2257supplemented children in the absence of malaria surveillance and treatment.

2258Beyond clinical malaria, it is necessary to consider also malaria mortality to capture broader
2259aspects of the iron-malaria association. In the context of the clinical trial with iron supplements in
2260Pemba, mortality due to malaria was higher (although not significantly) among supplemented
2261children compared to placebo (RR=1.08, (95%CI 0.84; 1.40)). Among children supplemented with
2262iron and folic acid, there was also a significant increased risk for cerebral malaria as a cause of
2263death compared to placebo (RR=1.70, 95%CI 1.08; 2.68). The iron and folic acid supplemented
2264children were 12% more likely to suffer an adverse event resulting in hospitalisation or death
2265(95%CI 2;23) compared to placebo and all-cause mortality was also significantly higher: OR=
22661.61 (95%CI 1.03; 2.52). Iron deficiency and moderate anaemia at baseline were significantly
2267associated to lower rate of adverse events (death or severe morbidity leading to admission) among
2268supplemented children compared to placebo. Further extensive studies on the impact of iron
2269supplements on mortality to malaria are scarce due to the difficulty of attributing correctly the
2270cause of death in endemic settings and, hence, it is difficult to accurately assess the interaction
2271between malaria and infection with regard to mortality. In addition more statistical power is
2272needed as iron measures are rare and death is also a rare event.

2273In a good attempt to clarify finally the conundrum, the Cochrane meta-analysis²⁸ on the impact on
2274iron supplements addressed certainly this question but did not provide a definite answer. In this
2275review, the relative risk for all-cause mortality was not estimable. However, it was capable of
2276displaying useful information with regard to transmission settings. Mortality was not significantly
2277different between hyper- and holo-endemic areas (Risk difference= 1.93 per 1000 children (95%
2278CI -1.78; 5.64).

2279In summary, the risk for clinical malaria differs according to iron status between observational
2280studies and clinical trials on iron supplementation. Overall, observational studies describe a certain

2281protection for malaria risk among iron deficient children. In parallel, meaningful ancient studies
2282report increased susceptibility to clinical malaria among iron supplemented children^{7,8}, and so
2283does the Pemba trial, which has a considerable statistical power. However, other recent clinical
2284trials with important malaria monitoring and protective measures, show no significant increase for
2285malaria risk among iron supplemented children^{26,27} and neither does the Cochrane review²⁸.
2286Albeit the absence of overall significance, the cross-sectional studies in Tanzania report also
2287significant earlier malaria among supplemented children²⁶.
2288Evidence on the effect of iron levels on malaria risk is subject to certain limitations, such as
2289methodological study constraints, homogenous measurement of iron and haematological
2290indicators, the effect of different transmission patterns, and further possible confounders.
2291In effect, statistical limitations are inherent to ethical research studies. Clinical trials display
2292results based on intensively monitored parameters. In most of them prophylactic protection by
2293ITNs or preventive treatment for malaria is more frequent among enrolled patients than in
2294observational studies, and treatment is also given as soon as a case is confirmed. As a
2295consequence, it is difficult to disentangle the possible protective effect of IDA from the protection
2296given by protective measures, especially in the case of severe malaria or hyperparasitemia in
2297clinical trials. Preventive measures reduce the number and the severity of malaria episodes and,
2298hence, statistical power decreases as does the force of the association. The dimension of the
2299association, or its absence, should be ideally assessed in the conditions in which population
2300undergo the malaria burden and the nutritional interventions. Nevertheless, accurate iron
2301monitoring is not realized systematically and malaria episodes are not always captured by
2302demographic or surveillance data. In addition, observational studies that do not provide treatment
2303are unethical in malaria endemic countries with limited access to health care. However,
2304surveillance data or data issue of demographic surveys may be useful to get a basic idea on
2305malaria risk and haematological indicators.
2306With regard to the epidemiological indicators, malaria infection outcomes (clinical malaria and

2307parasitaemia) reflect more specifically the malaria-iron relationship, and mortality reflects rather a
2308broad association between iron and pathogens. In addition its assessment is difficult because of
2309diagnostic reasons, and evidence lacks with regard to specific malaria deaths related to iron
2310supplements.

2311The transmission setting constitutes an additional important stake of the question. Disease burden
2312in children after iron supplementation does certainly differ in the absence of malaria compared to
2313malaria endemic settings [29]. The existence of a possible malaria prevalence threshold at which
2314iron supplements start to have a deleterious effect on infant health requires as well further
2315research.

2316Other methodological obstacles contribute to the inconclusive results of the analyses of the
2317association between iron and malaria risk. Analyses in the clinical trials are seldom adjusted on
2318other significant co-variables and odds ratios (OR) and relative risks (RR) originate often from
2319univariate analyses. In addition, the exclusion of the children with inflammation in some studies
2320might have introduced a bias in the interpretation of results concerning the children with the most
2321severe disease, as inflammation is predominantly present in these more severe cases.

2322Finally, the haematological indicators at baseline show contradictory results in literature at
2323present. Indeed, a clinical trial describes a significant protection against malaria among
2324supplemented children with both anaemia and iron deficiency [27]. However a study in Tanzania
2325observed an increase in malaria risk among iron-deficient infants²⁶. Similar results are found in
2326pregnant women [30]. Indeed, there might be a possible protective role of anaemia or iron
2327deficiency in the context of iron supplementation. In case of anaemia the incorporated iron might
2328be used for haemoglobin synthesis whereas in the context of iron deficiency with no anaemia at
2329baseline the incorporated iron might entail an increase in NTBI, enhancing parasite growth. More
2330extensive research including different iron deficiency indicators is needed to advance in the
2331knowledge in this aspect. Yes it is essential to ascertain the meaning of the information provided
2332by the different iron markers used in the research studies to better unravel the iron-malaria

2335Iron status assessment and iron markers: the Rosetta stone to understanding

2336In order to better discern the importance of iron levels for malaria morbidity and mortality, the

2337determination of iron levels requires precision and consensus among researchers. The

2338understanding of the nature and the meaning of the different iron and haematological markers is

2339necessary as the definition of common indicators might enable the extrapolation of results and

2340improve their interpretation. Therefore, it becomes a prerequisite to remind briefly the

2341physiopathology of iron involved in the *P. falciparum* infection process.

2342Iron has multiple effects on malaria physiopathology: it interacts with the host's immunity but also

2343with the parasite. With regard to the host immunity, iron interferes with zinc and with the

2344inducible nitric oxide synthase (iNOS). In parallel, the host inflammation process increases

2345hepcidin, a hormone regulating iron disposal in plasma, in order to block iron absorption. Thus it

2346was first reported that by inhibiting the absorption of zinc, iron would alter the immune response

2347to infection [31], but recent studies describe no improvement in infection outcomes in zinc

2348supplemented children [26]. In addition, iron inhibits the synthesis of nitric oxide, an anti-

2349infectious agent [32], even if the subsequent consequences for malaria are not fully understood.

2350At the host level the interaction of iron and *P. falciparum* is also significantly determined by the

2351NTBI, involved in parasite metabolism. Hepatocytes take up faster NTBI than transferrin-bound

2352iron [33] and, in animal models, the supply of iron contributes to the penetration of hepatocytes by

2353*Plasmodium* and stimulates their growth to merozoites [34]. Furthermore NTBI is involved in

2354parasite sequestration of malaria-infected erythrocytes in the capillaries of the brain and intestine

2355through up-regulation of ICAM-1 and is thus linked to severe malaria [14–16].

2356The biological indicators reflect the different pathways in which iron interferes with malaria

2357infection, and their choice as iron markers in research studies are crucial to determine the meaning

2358of the results. The joint WHO-CDC Technical Consultation for iron assessment selected 5

2359different indicators as good iron markers: haemoglobin, mean cell volume (MCV), (sTfR)

2360concentration, serum ferritin concentration, and red cell protoporphyrin (measured by the zinc

2361protoporphyrin/haemoglobin ratio (ZPP:H) [35,36]). Table 2 summarizes the main characteristics

2362of these markers. Haemoglobin is deeply useful in the monitoring of health status and its

2363determination is easy to realize on the field. Although it is a basic fundamental haematological

2364indicator, it is not specific as an iron marker because of the multiple causes of anaemia and the

2365physiological variations with regard to sex, age or ethnicity. Therefore, it can be misleading for

2366the extrapolation of conclusive results. Mean cell volume accuracy is limited in the context of

2367thalassemia and malaria as inflammation serum transferrin receptor modifies significantly its

2368values. Due to its physiopathological pathway, serum transferrin receptor is also influenced by the

2369haemolysis of malaria, and its determination method is not always standardized nor cost-effective

2370[37].

2371Serum ferritin is a precise indicator of iron storages in healthy individuals and it can be corrected

2372according to other inflammation proteins. It provides further information as it also shows different

2373patterns of behaviour depending on the aetiology of anaemia [16]. In an iron supplementation

2374study, Doherty et al. compared the erythrocyte incorporation of oral iron supplement in 37

2375Gambian children 8 to 36 months old with anaemia after malaria treatment, to supplemented

2376control children with IDA but no recent malaria [38]. The non-malaria control children showed

2377progressively increased serum ferritin whereas the post-malarial children showed decreased serum

2378ferritin levels. Serum ferritin levels became similar in both groups only by day 15 and 30. This is

2379thought to be due to the normalization of the immune response following the malaria treatment

2380[16]. Indeed, serum ferritin is an acute phase protein. Hence, serum ferritin is either corrected

2381upon inflammation (with correction factors according to C-reactive protein (CRP) or α -1-

2382glycoprotein (AGP) levels), or samples with high acute inflammation proteins are systematically

2383excluded. Nevertheless the exclusion of samples with increased inflammation might entail a

2384subsequent bias in the context of malaria, as samples with high ferritin would be systematically

2385excluded as well. Despite its limited accuracy in case of inflammation, ferritin is a consistent
2386extended iron marker.

2387Along with ferritin, ZPP:H ratio is the most frequently used indicator for iron assessment. The
2388chelation of ferrous iron by protoporphyrin is the final step for the heme synthesis. In iron
2389deficiency zinc is chelated as iron is not available and ZPP formation is decreased. In the iron-
2390deficient parasitized RBC, the increased ZPP could bind to heme crystals, and inhibit the
2391formation of hemozoin [12]. Longstanding inflammation processes, thalassaemia, and
2392asymptomatic *P. falciparum* parasitemia might also show elevated ZPP:H ratios, and consequently
2393be erroneously associated to iron deficiency [26]. For this reason Oppenheimer suggested that the
2394benefit of iron supplementation in the Pemba sub-study might be due to the selection of
2395individuals who were thalassemic or sickle cell carriers (WHO/UNICEF/IVACG Innocenti
2396Conference on Micronutrients and Health: Emerging Issues Related to Supplementation, 2005). In
2397addition there is no standardized corrections applicable to ZPP:H ratios in the context of long-term
2398inflammation processes. Finally high lead levels interfere with ZPP:H, and polluted regions
2399frequently overlap with malaria endemic settings. However the impact of inflammation on ZPP:H
2400is not as important as on serum ferritin.

2401A novel marker has recently emerged as an alternative indicator: hepcidin. Hepcidin is a peptide
2402hormone which plays a crucial role in iron regulation and is determinant in the malaria infection
2403process. Hepcidin binds ferroportin [39], it increases in response to inflammation and blocks iron
2404entry into the plasma. It has been proposed as a good marker for iron levels, especially because it
2405might be up-regulated after malaria episodes compared to other markers of iron-deficiency [16].
2406Therefore, a priori, it might permit to distinguish between iron-deficiency and malaria related
2407anaemia. However, hepcidin shows a non-linear association with anaemia in the context of malaria
2408albeit its significant association with parasitemia [40,41]. Furthermore, in Kenya it was increased
2409on admission at hospital for *P. falciparum* malaria and was significantly associated with parasite
2410density, but hepcidin levels were very low in severe malaria anaemia [41]. In addition, its

2411 accuracy as an iron marker has been recently questioned as it has been shown that it is associated
2412 with the anti-inflammatory response but not with iron or anaemic status among malarial Nigerian
2413 children [42]. Hence, further studies with more statistical power should be encouraged to ascertain
2414 its utility as an iron marker.

2415 In conclusion, complementary indicators are needed for the accurate assessment of iron status. In
2416 this respect, inflammation parameters are necessary to correct ferritin levels in the context of
2417 malaria, and further research is expected in order to determine precisely the utility of hepcidin in
2418 iron assessment in the context of malaria. It is also important to highlight the danger of
2419 categorising non-iron deficient infants as "iron-replete", as limits for iron deficiency are not rigid
2420 and should be considered with caution and in relation to the clinical and environmental settings.

2421 Conclusion

2422 Iron physiopathology interacts with *P. falciparum* at different levels. Therefore, the iron balance
2423 influences the appearance and the evolution of the infection with regard to both the immune
2424 system and the parasite. As a consequence, it is important to analyse in which manner providing
2425 supplementary iron has an effect on immunity and on invading pathogens taking into account the
2426 previous haematological and infectious health status of the infants.

2427 With regard to epidemiological studies, malaria risk should be assessed with regard to both
2428 clinical episodes and *P. falciparum* density to monitor accurate measures of the impact of iron.
2429 Further epidemiological elements should be taken into account to analyse the effect of iron on *P.*
2430 *falciparum* parasitemia: age of the children, immunity status, or hemoglobinopathies should be
2431 considered as well to give further light on the subject. Indeed, Sazawal et al. have already
2432 underlined that reviews do not always assess separately studies from malaria-endemic areas with
2433 different transmission or studies in different age groups [10]. In addition, meta-analyses should
2434 differentiate studies in which iron was given as treatment for anaemia and studies for prevention
2435 of iron deficiency. Adjustment on other causes of iron deficiency and anaemia, such as nutritional
2436 deficiencies, helminthic infections or haemoglobinopathies should be compulsory as well.

2437 In general, observational studies display a certain protection against malaria among iron-deficient
2438 children. However, iron assessment including multiple markers must be introduced yet on a
2439 systematic basis among all study designs to guarantee a solid accuracy of the iron-malaria
2440 association, especially in relation to haematological indicators at baseline. Corollary to this
2441 question is the necessity to find a gold standard or a best iron marker combination. Ferritin and
2442 haemoglobin are still at the core of the haematological assessment, but the role of hepcidine must
2443 be further investigated in the context of large epidemiological studies in parallel to other best
2444 known iron indicators like ferritin, haemoglobin or ZPP:H.

2445 In any case, the budget and technology constraints will determine the implementation of this
2446 screening strategy, and blood test to determine iron levels should become more affordable. For
2447 these reasons, targeting low-birth weight infants for iron supplements has been proposed since
2448 they are at higher risk for iron deficiency and anaemia [4]. Still, low birth weight is associated to
2449 increased mortality, and the effect of iron on infection can further contribute to the deterioration of
2450 the infant health status when malaria treatment is not available.

2451 Another aspect, which should be further investigated, is the link between the chronicity of iron
2452 deficiency and the response to iron supplementation and infection, especially as chronic effects of
2453 inflammation might modify the malaria-iron association. Furthermore, the existence of
2454 *P.falciparum* strains requiring more or less iron should be investigated, as well as the possible
2455 selection of *P.falciparum* drug-resistant strains in the context of increased iron availability.

2456 With regard to transmission, the existence of a possible malaria prevalence threshold at which iron
2457 supplements start to have a deleterious effect on infant health requires as well further
2458 investigation.

2459 Finally, when analyzing the effect of iron on infant health, it is essential to take into account the
2460 possible benefits of iron supplementation for anaemia and child neurocognitive development
2461 beyond its deleterious effect. According to the Cochrane review [28], iron supplements given as
2462 part of the treatment for anaemia resulted in higher increases in haemoglobin than iron given as

2463prophylaxis for anaemia in both malaria hyper- and holo-endemic areas. Indeed, joint malaria
2464treatment and iron supplements reduce malaria rates significantly compared to no prophylaxis.
2465Nevertheless, it is important for both preventive and treatment iron supplementation policies to
2466consider the poor utilization of the iron intake by the body until one week after the malaria
2467episode. In conclusion, the joint treatment for malaria along with oral iron supplements seems to
2468improve anaemia without increased risk for malaria.

2469Acknowledgements: We want to thank Jessica Barry for the valuable linguistic contributions to the manuscript. We
2470want to thank Yves Martin-Prevel for his technical support.

2471Funding and sponsorship: Violeta Moya-Alvarez has been sponsored by the “Ecole des Hautes Etudes en Santé
2472Publique-DGA” grant.

2473The authors declare no conflict of interests.

2474**Tables:**

Table 1. Effect of iron supplements on malaria incidence

V. Results

Study site	Country	Year	Type of study	Malaria transmission	Number of individuals included	Follow-up period	Age at supplements	Iron deficiency or anaemia indicator	Relationship with malaria	Effects on anaemia and iron indicators
Awara	Somalia	1975	trial	perennial	137	30 days		Hemoglobin<11g/dl Serum iron concentration<4.48µmol/l Transferrin saturation<15% Peripheral blood smear with microcytic hypochromasia	In univariate analysis: Placebo group 2/66; Iron supplemented group: 21/71	Mean hemoglobin (g/dl) Before treatment: Placebo 8.1±0.7 Iron 8.3±0.6 After treatment: Placebo 8.7±0.9 Iron 12.3±1.1 Mean serum Fe (µmol/l) Before treatment: Placebo 3.4±0.57 Iron 3.6±0.52 After treatment: Placebo 3.9±0.7 Iron 13.1±0.93 Mean % saturation transferrin Before treatment: Placebo 7±1.4 Iron 7±1.8 After treatment: Placebo 8±0.7 Iron 31±1.4
Madang	Papua New-Guinea	1980-1981	prospective trial	perennial with seasonal peaks	486	12 months	2 months	Hemoglobin, transferrin saturation, serum ferritin (log)	At 6 months: OR=1.78 (CI 1.02; 3.1) At 12 months: OR=1.95 (CI 1.21; 3.13)	Mean hemoglobin at 6 months (g/dl): Placebo 9.82 (1.39) Iron 9.14 (1.09) (p<0.001) Mean hemoglobin at 12 months (g/dl): Placebo 9.78 (1.36) Iron 9.32 (1.34) (p<0.002)
Ifakara	Tanzania	1995-1996	randomised placebo-controlled trial	perennial and intense	832	minim of 52 up to a maximum of 153 weeks	8 to 24 weeks	Hemoglobin	PE with regard to the 1st malaria episode compared to placebo Daily iron and weekly placebo: 11% (CI 21.8; 35) Daily placebo +weekly Deltaprim 59.4% (CI 41.1; 72) Daily iron + weekly Deltaprim 65.9% (CI 49.6; 77)	PE with regard to the severe anaemia (PCV<25%) compared to placebo Daily iron and weekly placebo: 32.1% (CI 4.9; 51.6) Daily placebo +weekly Deltaprim 59.8% (CI 41.1; 72.6) Daily iron + weekly Deltaprim 68.5% (CI 52.3; 79.2)
Ngerenya	Kenya	2001-2003	observational study	perennial with seasonal peaks	240	2 cross-sectional surveys at 6 and 12 months after enrolment	no supplements	ID: plasma ferritin<12µg/ml in association with TFS<10%	Adjusted IRR in iron-deficient children=0.7 (CI 0.51; 0.99)	No supplements
Pemba	Tanzania	2002-2003	randomised placebo-controlled trial	holoendemic with year-round transmission and seasonal peaks	24076	until discharge or death	20 weeks	ID: zinc protoporphyrin >80µmol/mol haeme Anaemia: hemoglobin 70-100 g/L	Overall adverse events, deaths, and admissions to hospital caused by malaria compared to placebo Iron and folic acid: RR= 1.16 (CI 1; 1.34) Iron, folic acid, and zinc: RR=1.16 (CI 1.01; 1.34) Children with ID OR=0.15 (CI 0.12; 0.19) and 3.9 fold lower parasite count (P<0.001) compared with iron replete children Children with ID, for Hyperparasitemia (= parasitemia>2500/200 WBC) OR=0.04 (CI 0.02; .07) and for severe malaria OR=0.25 (CI 0.14; 0.46) compared to iron-replete	Non-significant trend for smaller proportion of children with anaemia among all admissions compared to placebo
Muheza	Tanzania	2002-2005	observational study	intense	785	at birth until 3 years	no supplements	ID: ferritin concentration <30 ng/mL when CRP <8.2 µg/mL or ferritin concentration <70 ng/mL when CRP >8.2 µg/mL		No supplements

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V. Results

Study site	Country	Year	Type of study	Malaria transmission	Number of individuals included	Follow-up period	Age at supplements	Iron deficiency or anaemia indicator	Relationship with malaria	Effects on anaemia and iron indicators
Handeni	Tanzania	2008-2009	randomised placebo-controlled trial	intense	612	median follow-up 331 days	6-60 months	ID: plasma ferritin concentration <12 µg/L	<p>Compared to placebo:</p> <p>All malaria episodes:</p> <p>Zinc group: AHR=0.99 (CI 0.82; 1.18)</p> <p>Multi-nutrients without zinc: AHR=1.04 (CI 0.87; 1.23)</p> <p>Multinutrients with zinc: AHR=1.14 (CI 0.96; 1.35)</p> <p>First malaria episodes:</p> <p>Zinc group: AHR=1.12 (CI 0.86; 1.44)</p> <p>Multi-nutrients without zinc: AHR=1.35 (CI 1.05; 1.73)</p> <p>Multinutrients with zinc: AHR=1.38 (CI 1.07; 1.77)</p> <p>Number of episodes with versus without multinutrients</p> <p>Iron deficient: HR=1.41 (1.09; 1.82)</p> <p>Iron replete: HR=0.93 (0.77; 1.13)</p>	<p>*Difference relative to placebo (95%CI), Hemoglobin concentration (g/l)</p> <p>Micro nutrients without zinc: 106.6 (10.7) *2.6 (0.0; 5.2)</p> <p>Micro nutrients with zinc: 107.5 (11.4) *3.5 (0.8; 6.1)</p> <p>Geometric mean ferritin concentration (µg/l)</p> <p>All children</p> <p>Micro nutrients without zinc: 57.1 (0.03) *24.5 (14.8; 36.2)</p> <p>Micro nutrients with zinc: 57.2 (0.03) *24.6 (14.8; 36.3)</p> <p>without inflammation:</p> <p>Micro nutrients without zinc: 43.9 (0.03) *19.5 (11.3; 28.6)</p> <p>Micro nutrients with zinc: 51.1 (0.03) *26.7</p>
Brong-Ahafo	Ghana	2010	double blind, cluster-randomized trial	perennial with seasonal peaks	1958	6 months	6 to 35 months	ID: plasma ferritin concentration <12 µg/L	<p>Malaria risk for iron supplemented group compared to placebo:</p> <p>Malaria risk for all children RR=1 (CI 0.81; 1.23)</p> <p>RR for malaria with ID and without inflammation=0.81 (CI 0.63; 1.03)</p> <p>RR for iron replete children without inflammation=0.92 (CI 0.81; 1.06)</p>	
Cochrane Review		2011	systematic Cochrane review	variable upon studies	45,353 children under 18 years of 71 trials	until June 2011		different supplements: iron, iron and folic acid, iron and anti-malarials depending on the trial hemoglobin, iron and ferritin	<p>For clinical malaria iron alone compared to placebo RR=0.99 (CI 0.9; 1.09)</p> <p>For clinical malaria iron alone compared to placebo among non-anaemic children at baseline RR=0.97 (CI 0.86; 1.09)</p> <p>For clinical malaria iron alone compared to placebo among infants <2 years RR=0.94 (CI 0.82; 1.09)</p>	<p>Iron versus placebo or no treatment, iron plus folic acid versus placebo or no treatment, iron plus antimalarial treatment or antimalarial treatment alone versus placebo or no treatment, iron versus placebo or no treatment in the treatment of proven malaria</p>

AHR: Adjusted hazard ratio; AOR: Adjusted odds ratio; HR: Hazard ratio; ID: Iron deficiency; IRR: Incidence rate ratio; OR: Odds ratio; PE: Protective efficacy; RR: Relative-risk; STR: serum transferrin receptor

453 Table 2: Iron indicators selected by the WHO-CDC Technical Consultation for iron assessment

Indicator	Refers to	Threshold values (venous blood of persons residing at sea level)	Other valuable information
Hemoglobin	Anaemia	For anaemia: children aged 6 months to 6 years: 11g/100ml children aged 6–14 years: 12g/100ml adult males: 13g/100ml adult females, non-pregnant: 12g/100ml adult females, pregnant: 11g/100ml	The assessment of hemoglobin alone can provide only a rough estimate of the likely prevalence of iron deficiency anaemia (IDA). The absence of a consistent standard for identifying iron deficiency contributes to confound the analyses on the relationship between anaemia and IDA prevalence rates
Zinc protoporphyrin (ZPP)	Iron deficient erythropoiesis	>70-80 µmol/mol for infants	In the last step in hemoglobin synthesis, the enzyme ferrochetalase inserts iron. A lack of iron available to ferrochetalase during the early stages of iron deficient erythropoiesis results in a measurable increase in the concentration of zinc protoporphyrin, as trace amounts of zinc are incorporated into protoporphyrin instead. The normal ratio of iron to zinc in protoporphyrin is about 30 000:1. Thresholds for ZPP vary between 40 and 70 µmol/ mol haem depending on whether the cells have been washed before the assay or not
Mean cell volume (MCV)	Red blood cell size, anaemia characteristics. Microcytic anaemia is a sign of iron deficiency anaemia, whereas macrocytic anaemia indicates deficiency of vitamin B12 or folate	<67-81fl	Even if MCV is used widely for the evaluation of nutritional iron deficiency, low values are not specific to iron deficiency, but they are also found in thalassaemia and in about 50% of people with anaemia due to inflammation
Transferrin receptor in serum (STR)	Inadequate delivery of iron to bone marrow and tissue	It is not possible to assign a single threshold value that would be accurate for all commercial kits. Approximately: During severe beta thalassaemia the sTfR concentration is >100 mg/l During severe iron deficiency anaemia it is >20–30 mg/l	sTfR is sensitive to erythropoiesis due to any cause. Hence, it cannot be interpreted as an indicator of solely iron deficiency erythropoiesis. Its concentration increases in individuals with stimulated erythropoiesis, such as haemolytic anaemia and sickle cell anaemia. Indeed, acute or chronic inflammation and the anaemia of chronic disease, malaria, malnutrition, age and pregnancy may modify significantly sTfR. There is a lack of standardization between different commercial kits for measuring the concentration of transferrin receptor

Serum ferritin (SF)	Iron deficiency.	Iron deficiency anameia:	Needs to be corrected upon inflammation. In clinical malaria a high SF values result from the destruction of red blood cells, an acute phase response, suppressed erythropoiesis, and ferritin released from damaged liver or spleen cells. However, in “holo-endemic” settings, the influence of parasite load on SF appears to be restrained and reliable after correction.
	SF is an iron storage protein that provides iron for haem synthesis when required.	SF concentration<12–15 µg/l.	The changes in SF concentration during development from birth to old age reflect changes in the amounts of iron stored in tissues

Source: Report of a technical consultation on the assessment of iron status at the population level. WHO-CDC, 2004

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2575 **V.II. ARTICLES REPORTING ORIGINAL RESULTS**

2576 In this second part of the section, I will present the results of the study that we conducted in
2577 Benin. They respond to the objectives and they are structured as follows: 1. The influence
2578 of iron levels on malaria risk during pregnancy; 2. The association of iron levels and IPTp
2579 with malaria in infants; and 3. The association of elevated blood lead level with malaria in
2580 infants.

2581 As in the previous sub-section, references, figures and tables in this section are independent of
2582 those in the whole dissertation as they are presented at the end of each article.

2583 Finally, to give a more accurate idea of what our articles add to the previous state of art, we
2584 have added a little paragraphe at the end of the article summary.

2585

V.II.1. Iron levels and pregnancy associated malaria

Summary of the article: As explained in the introduction, cross-sectional studies report that iron might be associated with increased malaria morbidity, raising fears that current iron supplementation policies will cause harm in the present context of increasing resistance against intermittent preventive treatment in pregnancy (IPTp). Therefore, we wanted to assess the relation of iron levels with malaria risk during the entire pregnancy.

To investigate the association of maternal iron levels on malaria risk in the context of an IPTp clinical trial, 1005 human immunodeficiency virus-negative, pregnant Beninese women were monitored throughout their pregnancy between January 2010 and May 2011 in three maternities of the district of Allada. Allada is a semi-rural area of 91,778 inhabitants located 50 km North of Cotonou (Benin). Malaria has a perennial transmission pattern with two transmission peaks corresponding to the rainy seasons in April-July and October-November. *Plasmodium falciparum* is the species responsible for the majority of infections.

This study is a sub-study of the MiPPAD clinical trial, where 4,749 pregnant women were enrolled in an open-label randomized clinical trial conducted in Benin, Gabon, Mozambique, and Tanzania comparing 2-dose MQ or SP for IPTp and MQ tolerability of two different regimens. The study arms were: (1) SP, (2) single dose MQ (15 mg/kg), and (3) split-dose MQ in the context of long lasting insecticide treated nets. In the MiPPAD trial there was no difference on low birth weight prevalence (primary study outcome) between groups (360/2,778 (13.0%)) for MQ group and 177/1,398 (12.7%) for SP group (RR= 1.02, 95% CI (0.86; 1.22), p-value = 0.80 in the ITT analysis). Women receiving MQ had reduced risks of parasitemia (63/1,372 (4.6%) in the SP group and 88/2,737 (3.2%) in the MQ group (RR= 0.70, 95% CI (0.51; 0.96), p-value = 0.03) and anemia at delivery (609/1,380 (44.1%) in the SP group and 1,110/2743 (40.5%) in the MQ group (RR= 0.92, 95% CI (0.85; 0.99), p-value

2611= 0.03), and reduced incidence of clinical malaria (96/ 551.8 malaria episodes person/year
2612(PYAR) in the SP group and 130/1,103.2 episodes PYAR in the MQ group (RR= 0.67, 95%
2613CI (0.52; 0.88), p-value = 0.004) and all-cause outpatient attendances during pregnancy
2614(850/557.8 outpatients visits PYAR in the SP group and 1,480/1,110.1 visits PYAR in the
2615MQ group (RR= 0.86, 95%CI (0.78; 0.95), p-value =0.003). In the MiPPAD study there were
2616no differences in the prevalence of placental infection and adverse pregnancy outcomes
2617between groups. In conclusion women taking MQ IPTp (15 mg/kg) in the context of long
2618lasting insecticide treated nets had similar prevalence rates of low birth weight as those taking
2619SP IPTp. MQ recipients had less clinical malaria than SP recipients, and the pregnancy
2620outcomes and safety profile were similar. The conclusions of the MiPPAD trial do not support
2621a change in the current IPTp policy.

2622On the contrary to the MiPPAD trial, in our sub-study in Benin, named “Anaemia in
2623pregnancy: etiology and consequences (APEC)”, women were followed prospectively until
2624delivery through a close monitoring of their haematologic parameters as well, including
2625hemoglobin, serum ferritin and CRP in addition to the blood smear, blood film and Kato-Katz
2626exam. During the follow-up of the Beninese cohort, 29% of the women had at least 1 episode
2627of malaria. On average, women had 0.52 positive smears (95% CI (0.44; 0.60)).

2628Multilevel models with random intercept at the individual levels and random slope for
2629gestational age were used to analyse the factors associated with increased risk of a positive
2630blood smear and increased *Plasmodium falciparum* density. Indeed, high iron levels
2631(measured by the log10 of ferritin corrected on inflammation) were significantly associated
2632with increased risk of a positive blood smear (aOR = 1.75, 95% CI (1.46; 2.11), p-value
2633<0.001) and high *P. falciparum* density (beta estimate = 0.22, 95% CI (0.18; 0.27); p-value
2634<0.001) during the follow-up period adjusted on pregnancy parameters, comorbidities,
2635environmental and socioeconomic indicators, and IPTp regime. Furthermore, iron-deficient

2636 women were significantly less likely to have a positive blood smear and high *P. falciparum*
2637 density (p-value < 0.001 in both cases). Supplementary interventional studies are needed to
2638 determine the benefits and risks of differently dosed iron and folate supplements in malaria-
2639 endemic regions.

2640

2641 **What's known on this subject:** The prevalence of anemia in Sub-Saharan Africa is high.
2642 Malaria, helminth infection and iron deficiency are the main causes of gestational anemia.
2643 WHO recommends iron supplements and IPTp during pregnancy. However, the benefits of
2644 iron supplements are set into question in settings with high malaria incidence. Indeed,
2645 evidence is inconclusive, and prospective longitudinal data is lacking.

2646

2647 **What this study adds:** We show that elevated iron levels are associated with increased risk
2648 of malaria and *P. falciparum* density in a longitudinal prospective cohort during pregnancy in
2649 the context of ITN use, considering environmental, clinical and obstetric risk factors. Women
2650 with iron deficiency are significantly protected against malaria.

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V. Results

Does Iron Increase the Risk of Malaria in Pregnancy?

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Background. Pregnancy-associated malaria (PAM) remains a significant health concern in sub-Saharan Africa. Cross-sectional studies report that iron might be associated with increased malaria morbidity, raising fears that current iron supplementation policies will cause harm in the present context of increasing resistance against intermittent preventive treatment in pregnancy (IPTp). Therefore, it is necessary to assess the relation of iron levels with malaria risk during the entire pregnancy.

Methods. To investigate the association of maternal iron levels on malaria risk in the context of an IPTp clinical trial, 1005 human immunodeficiency virus-negative, pregnant Beninese women were monitored throughout their pregnancy between January 2010 and May 2011. Multilevel models with random intercept at the individual levels and random slope for gestational age were used to analyze the factors associated with increased risk of a positive blood smear and increased *Plasmodium falciparum* density.

Results. During the follow-up, 29% of the women had at least 1 episode of malaria. On average, women had 0.52 positive smears (95% confidence interval [CI], 0.44–0.60). High iron levels (measured by the log₁₀ of ferritin corrected on inflammation) were significantly associated with increased risk of a positive blood smear (adjusted odds ratio = 1.75; 95% CI, 1.46–2.11; *P* < .001) and high *P. falciparum* density (beta estimate = 0.22; 95% CI, 0.18–0.27; *P* < .001) during the follow-up period adjusted on pregnancy parameters, comorbidities, environmental and socio-economic indicators, and IPTp regime. Furthermore, iron-deficient women were significantly less likely to have a positive blood smear and high *P. falciparum* density (*P* < .001 in both cases).

Conclusions. Iron levels were positively associated with increased PAM during pregnancy in the context of IPTp. Supplementary interventional studies are needed to determine the benefits and risks of differently dosed iron and folate supplements in malaria-endemic regions.

Keywords. iron levels; pregnancy-associated malaria.

Pregnancy-associated malaria (PAM) remains a public health concern in sub-Saharan Africa with over 35 million

pregnant women at risk [1]. Pregnancy-associated malaria is defined as a peripheral or placental infection by *Plasmodium*, and it is correlated with increased maternal morbidity and mortality [2, 3] and severe anemia (defined as hemoglobin [Hb] <70 g/L or <80 g/L) [3]. Furthermore, PAM is associated with an increased risk for placental malaria (PM), prematurity and low birth weight (LBW) [3, 4]. Preventive strategies such as intermittent preventive treatment in pregnancy (IPTp) or insecticide-treated mosquito nets (ITNs) have shown their efficacy in reducing PAM and its subsequent morbidity [5, 6]. Indeed the World Health Organization (WHO) recommends IPTp with sulphadoxine-pyrimethamine (SP) for all pregnant women as early as possible in the

Received 4 December 2014; accepted 15 March 2015.

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DOI: 10.1093/ofid/ofv038

second trimester and at each scheduled antenatal visit (ANV) at least 1 month apart [7].

However, IPTp does not always completely clear *Plasmodium falciparum* parasitemia, and residual parasitemia increases as a consequence of the growing resistance [8]. In addition, the effect of residual parasitemia is not harmless [9, 10]. For these reasons, it is necessary to further investigate additional factors influencing *P. falciparum* parasitemia during pregnancy among women receiving IPTp.

Environmental, obstetric, and hematologic genetic risk factors for PAM have been extensively assessed. The association of gravidity with parasitemia increases with transmission [11], and a young maternal age (≤ 20 years) is also associated with increased malarial risk especially in high-transmission settings [12–14]. Pregnancy-associated malaria seems to vary depending on gestational age with the period before the first IPTp intake seemingly at particular risk [15]. Nevertheless, important knowledge gaps need to be filled with regard to the influence of nutritional indicators on PAM. This aspect is of special concern, because iron has been repeatedly linked to increased infectious morbidity, and it is simultaneously involved in the hematological outcomes of *P. falciparum* infection. A recent Cochrane review on iron supplementation during pregnancy found only 2 studies (of the 23 studies of malaria-endemic countries) that reported results concerning malarial infection. It concluded that there was no evidence that iron supplements were associated to PM [16]. However, an important cohort in Tanzania indicated that iron deficiency (ID) was significantly protective for PM in terms of both prevalence and severity [17]. Therefore, it is necessary to further investigate the association of iron and folate with malarial risk in a prospective longitudinal cohort during pregnancy. More precisely, the study of the influence of maternal iron and folate levels on *P. falciparum* parasitemia in the context of IPTp will help to better understand PAM and provide important knowledge on supplementary factors influencing malarial risk during pregnancy among women receiving IPTp.

The aim of our study was to investigate the relationship of maternal iron and folate levels with malarial risk and *P. falciparum* parasite density during pregnancy in the context of IPTp in Benin, taking into account environmental and obstetric risk factors and simultaneous comorbidities. In addition, we aimed to explore the association of iron and folate with PAM outcomes such as LBW and PM.

MATERIALS AND METHODS

Study Design

One thousand five human immunodeficiency virus (HIV)-negative pregnant women under 28 weeks of gestational age were observed until delivery in the context of the Anemia in Pregnancy: Etiology and Consequences (APEC) study, an observational study nested in the Malaria in Pregnancy Preventive

Alternative Drugs (MiPPAD) clinical trial (<http://clinicaltrials.gov/ct2/show/NCT00811421>). Further details are given in González et al [18].

Study Site and Population

The APEC study was conducted in 3 maternity clinics in the district of Allada, between January 2010 and May 2012. Allada is a semirural area of 91 778 inhabitants located 50 km North of Cotonou (Benin). Malaria has a perennial transmission pattern with 2 transmission peaks corresponding to the rainy seasons in April–July and October–November. *Plasmodium falciparum* is the species responsible for the majority of infections.

Further details of the study are described elsewhere [19], but, briefly, the eligibility criteria included no intake of IPTp, iron, folic acid, vitamin B12, or antihelminthic treatment. All women were offered confidential pretest HIV counseling and thereafter informed consent was obtained. The study was approved by the Ethics Committee of the Faculty of Medicine of Cotonou. Precise details of the follow-up are presented in Figure 1.

Study Procedures

Clinical Data Collection

The pregnant women were observed through 2 systematic ANV, the first taking place at inclusion, and through unscheduled visits in case of disease. The observations were completed after the women gave birth. At the first ANV, each woman was given an ITN, she was examined, and her clinical and gynecological histories were recorded. At each systematic ANV, 2-dose IPTp (1500/75 mg of SP per dose or 15 mg/kg mefloquine [MQ], either single or split intake) was administered 1 month apart, the first given to pregnant women after 15 weeks of gestation. Women were also systematically given 600 mg of albendazole as well as supplements of oral ferrous sulfate (200 mg per day) and folic acid (5 mg/day) for home treatment. In case of Hb concentration < 110 g/L, women were treated as follows: ie, they received 200 mg of oral ferrous sulfate twice a day for mild or moderate anemia (Hb between 70 and 110 g/L, according to the national recommendations in Benin); and they were referred to the tertiary hospital in case of severe anemia (Hb < 70 g/L, according to the national recommendations in Benin). In case of sickness, women were examined and, if necessary, treated in unscheduled visits. Clinical data were collected at each ANV, unscheduled visit, and at delivery.

Blood and Stools Samples Collection

At ANV1, ANV2, and at delivery, 8 mL venous blood were collected. A container was also given to the woman to collect stools to examine the presence of intestinal helminths. At delivery, a placental blood smear was performed to investigate the existence of PM. The study sample examination techniques have been described elsewhere [20]. Microbiological exams were realized as follows: the Lambaréné technique [21] was

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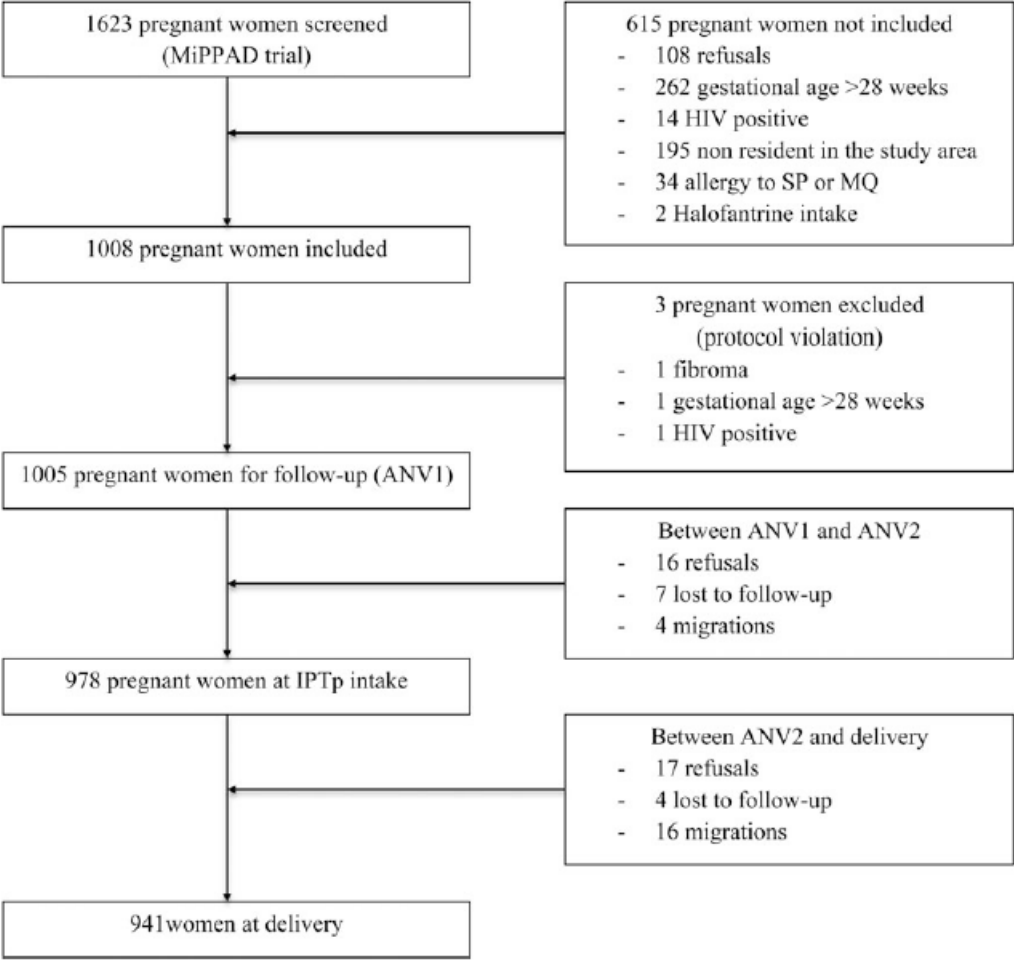


Figure 1. Study profile. Abbreviations: ANV, antenatal visit; HIV, human immunodeficiency virus; IPTp, intermittent preventive treatment; MiPPAD, Malaria in Pregnancy Preventive Alternative Drugs; MQ, mefloquine; SP, sulphadoxine-pyrimethamine.

used to assess malaria infection on thick smears; and helminthic infestations were assessed using the Kato-Katz concentration method.

Environmental Data

Because no entomological data were available, we used rain quantity instead as a surrogate for the anopheline presence. Because of the anopheline timeliness, rain was calculated as the mean rainfall of the 7 days before the 2 weeks before the consultation.

Definitions

Pregnancy-associated malaria was defined as peripheral or placental infection by *Plasmodium*, whereas PM was defined as presence of *Plasmodium* in the placenta. Low birth weight corresponds to newborn weights <2500 g, and prematurity refers to offspring born before 37 weeks of gestation. Severe, moderate, and mild anemia were defined as Hb concentrations <80 g/L, 80–99 g/L, and 100–109 g/L, respectively, following WHO criteria [22]. Inflammation was determined by C-reactive protein (CRP) levels ≥5 mg/mL. We corrected serum ferritin in the context of inflammation following the procedure inspired by

the meta-analysis by Thurnham et al [23] before conducting the analyses, so we multiplied serum ferritin by 0.76 in the presence of *Plasmodia* without inflammation, and we multiplied serum ferritin by 0.53 in case of concurrent *Plasmodia* infection and inflammation. Iron deficiency was then defined as corrected serum ferritin <15 µg/L. Iron deficiency anemia (IDA) was defined as Hb <110 g/L with ID. Folic acid deficiency was defined as a serum concentration <6 ng/mL. Vitamin B₁₂ deficiency was defined as a serum concentration <150 pg/mL. Intestinal helminth infestations were diagnosed by the presence of intestinal helminth eggs in the stool sample.

Socioeconomic items (home possession of latrines, electricity, a refrigerator, a television, a vehicle with at least 2 wheels, being married, and working outside the home) were plotted into a multiple correspondence analysis. Then, a predictor was created to synthesize the information, and it was kept as the final socioeconomic index.

Statistical Analysis

Data were double entered and analyzed with ACCESS2003 and STATA12.0 (StataCorp, College Station, TX). The

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508 Kruskal-Wallis test was also used to analyze continuous variables. The χ^2 test was used for comparing categorical variables by gravidity status. Univariate analysis was conducted to assess the association of all variables with positive smear and maternal peripheral parasitemia using multilevel models with a random intercept at the individual level. Thereafter, 2 different multilevel models regressions were built: the first on the risk of having a positive blood smear during the follow-up period and the second on *P. falciparum* parasite density. Both models included the smears and blood films of both systematic and unscheduled visits. The variables with $P < .2$ in univariate analysis were included in the multilevel models. Maternal age squared was used due to the quadratic relationship of age with the malarial risk. For both the analysis of the possibility of a positive blood smear and for the analysis of parasite density, random coefficient models were used because they were statistically better than fixed effects according to Akaike information criterion (AIC) and Bayesian information criterion (BIC). The AIC and BIC compare maximal likelihood models. More precisely, random intercept was applied in both cases at the individual level and random slope was applied to gestational age, because the effect of the variables might differ among women and the effect of gestational age might also vary differently according to the timing of the measure. Multivariable linear regression was used in the analysis of birth weight, and logistic regression was used for PM and LBW assessment. Certain variables were forced into the model because of their meaning in the analyses according to the literature: socioeconomic status and rainfall in the case of malarial indicators, and body mass index (BMI) in the case of LBW. The statistical significance in the final multivariable models was set to $P < .05$. The presented P values and the significance threshold were 2-sided.

RESULTS

Study Population

Between January 2010 and May 2011, 1005 pregnant women were included in the cohort, 978 continued until the second ANV (second IPTp dose), and 941 (93.63%) completed the follow-up until delivery. During the follow-up period, 29% of the women had at least 1 malarial episode. On average, women had 0.52 positive smears (standard deviation [SD] = 1.23, with a median of 0 [25th percentile = 0, 75th percentile = 1], and range of 0–6 positive smears). Demographic and clinical characteristics were statistically different in univariate analyses between primigravida, secundigravida, and multigravida women with regard to age, BMI, socioeconomic status, number of positive blood smears, PM, and LBW (Table 1). Sixty-nine of the 751 placentas analyzed had placental malaria (9.2%). The mean of positive blood smears during pregnancy was significantly higher for primi- and secundigravidae than for multigravidae (0.84, 0.86, and 0.32, respectively; $P < .01$). The percentage of women with placental malaria decreased as gravidity increased: placental malaria was found in 15.3% of primigravida, 13.4% of secundigravida, and 6% of multigravida women ($P < .01$). The proportion of LBW was also inversely correlated with gravidity: 18%, 10.7%, and 7.5% for primi-, secundi-, and multigravida women, respectively ($P < .01$). However, gravidity was not significant in the multivariable analysis of positive blood smears and parasite density after the inclusion of maternal age in the model (P value for gravidity in the multivariable model = .16 and .08, respectively; data not shown).

Follow-Up

Indicators of nutritional status such as folate, vitamin B12, and ferritin changed significantly during pregnancy (Table 2). The mean ferritin levels decreased after the first iron supplements

Table 1. Characteristics of the Study Population, by Gravidity Status^a

Characteristic	Primigravidae (n = 172, 18.45%)	Secundigravidae (n = 187, 20.06%)	Multigravidae (n = 573, 61.48%)	P Value
Age, years	20.10 (19.74; 20.46)	22.29 (21.80; 22.79)	28.77 (28.38; 29.16)	<.001
BMI before pregnancy (kg/m ²)	20.41 (19.98; 20.84)	20.66 (20.18; 21.13)	21.35 (21.02; 21.68)	.01
IPTp regime				
SP	56 (32.56%)	64 (34.22%)	198 (34.55%)	.89
MQ	116 (67.44%)	123 (65.78%)	375 (65.45%)	.89
Gestational age at ANV1 (weeks)	22.06 (21.52; 22.61)	22.11 (21.50; 22.71)	22.20 (21.87; 22.52)	.77
Gestational age at ANV2	28.41 (27.82; 29.01)	28.88 (28.33; 29.42)	28.97 (28.66; 29.28)	.21
Gestational age at delivery	38.37 (37.85; 38.89)	37.86 (37.38; 38.34)	38.20 (37.92; 38.48)	.42
Number of positive smears during pregnancy	0.84 (0.63; 1.05)	0.86 (0.63; 1.09)	0.32 (0.24; 0.40)	.42
Placental malaria	20 (15.27%)	20 (13.42%)	28 (5.97%)	.001
Low birth weight	31 (18.02%)	20 (10.70%)	43 (7.50%)	<.001

Abbreviations: ANV, antenatal visit; BMI, body mass index; IPTp, intermittent preventive treatment; MQ, mefloquine; SP, sulphadoxine-pyrimethamine.

^a For continuous variables, the mean is provided followed by the 95% confidence interval in brackets. For categorical variables, n is presented followed by the % in brackets.

Parameters	ANV 1 (n = 932)	ANV 2 (n = 906)	Delivery (n = 858)
Gestational age (weeks)	22.15 (21.90; 22.41)	28.85 (28.60; 29.09)	39.51 (39.34; 39.68)
Folate (ng/mL)	9.52 (9.12; 9.91)	10.47 (9.91; 11.02)	11.25 (10.09; 12.40)
Folate deficiency (serum folate <6 ng/mL)	294 (31.55%)	155 (17.09%)	330 (39.01%)
Vitamin B12 (pg/mL)	397.55 (385.34; 409.77)	370.36 (356.65; 384.06)	337.09 (322.20; 351.98)
Vitamin B12 deficiency (vitamin B12 <150 pg/mL)	32 (3.43%)	33 (3.64%)	62 (7.32%)
Ferritin (mg/L)	36.99 (34.24; 39.73)	25.10 (23.05; 27.14)	60.19 (54.58; 65.80)
Inflammation (CRP >5 mg/mL)	195 (20.92%)	110 (12.13%)	292 (34.11%)
Iron deficiency (corrected SF <15 µg/L)	277 (33.09%)	359 (44.16%)	183 (23.11%)
Hemoglobin (g/L)	10.30 (10.22; 10.38)	10.50 (10.43; 10.57)	11.16 (11.07; 11.26)
Anemia (Hb <110 g/L)	636 (68.24%)	589 (65.01%)	346 (40.37%)
Severe anemia (Hb <80 g/L)	32 (3.43%)	15 (1.66%)	20 (2.33%)
Positive blood smear	143 (15.34%)	35 (3.86%)	82 (9.56%)
<i>Plasmodium falciparum</i> parasitemia (parasites/µL)	382.40 (143.96; 620.84)	214.09 (36.19; 392.00)	3098.82 (1013.53; 5184.12)
Kato-Katz test positivity	104 (11.33%)	65 (7.30%)	28 (3.75%)

Abbreviations: ANV, antenatal visit; CRP, C-reactive protein; Hb, hemoglobin; SF, serum ferritin.

^aFor continuous variables, the mean is provided followed by the 95% confidence interval in brackets. For categorical variables, n is presented followed by the % in brackets.

were given at ANV1 from 37 mg/L (SD = 42.7) to 25.1 mg/L (SD = 31.3) at the second ANV, and then it increased up to 60.2 mg/L (SD = 83.1) at delivery. In parallel, the proportion of women with a positive smear decreased after IPTp (from 15.3% at ANV1 to 3.9% at ANV2), and then it increased again up to 9.6% at delivery. Nevertheless, the trend was slightly different concerning parasite density. *Plasmodium falciparum* parasite density was higher at ANV1 than at ANV2 (382.4, SD = 3709.2 and 214.1, SD = 2728.5 parasites/µL, respectively) but then rose up to 3098.8, SD = 31120.7 parasites/µL at delivery. There were no significant differences between SP and MQ IPTp with regard to the women malarial risk or parasite density within the whole follow-up period. There were no significant differences in ferritin levels or ID rates depending on the IPTp regime.

Malarial Outcomes

High iron levels (log₁₀ of ferritin corrected on inflammation) were significantly associated with increased risk of a positive blood smear (adjusted odds ratio [aOR] = 1.75; 95% CI, 1.46–2.11; *P* < .001) and *P. falciparum* parasite density (coefficient = 0.22; 95% CI, 0.18–0.27; *P* < .001) during the follow-up in logistic and linear-mixed multivariable models, respectively (Tables 3 and 4). More precisely, high corrected ferritin levels were associated with malaria risk at each visit unless the one following iron supplements (*P* value in univariate analysis = .07; data not shown). However, corrected ferritin was statistically associated with parasite density at each visit. Women with ID were significantly less likely to have a positive blood smear and a high *P. falciparum* density (*P* < .001; data not shown). In parallel, high folate levels were statistically associated with

decreased odds of a positive blood smear (aOR = 0.36; 95% CI, 0.19–0.70; *P* < .001) and to a lower *P. falciparum* parasite density (beta coefficient = −0.2; 95% CI, −0.37 to −0.08; *P* < .001). When adjusted on maternal age, gravidity was not significantly correlated with malaria risk or parasite density. Young maternal age, early gestational age, and inflammatory status were significantly positively correlated to increased malarial risk with regard to both having a positive smear and to higher parasite density. High socioeconomic status was associated with reduced malaria risk and *P. falciparum* parasite

Table 3. Multilevel Model on Factors Associated With Having Positive Blood Smears During Pregnancy

Factor	AOR (95% CI)	<i>P</i> Value
Ferritin corrected on inflammation (logarithm of µg/L)	1.75 (1.46; 2.11)	<.001
Folate (logarithm of ng/mL)	0.37 (0.19; 0.70)	.002
IPTp with MQ (SP = reference)	1.06 (0.76; 1.48)	.74
Gestational Age (weeks)	0.95 (0.93; 0.98)	.001
Maternal age (years)	0.64 (0.51; 0.82)	<.001
Maternal age squared (years)	1.01 (1.00; 1.01)	.004
Inflammatory process	5.41 (3.90; 7.70)	<.001
High socioeconomic status	0.82 (0.69; 0.96)	.02
Rain (mm)	0.99 (0.96; 1.03)	.75
Kato-Katz test positivity	0.98 (0.56; 1.70)	.93

Abbreviations: AOR, adjusted odds ratio; CI, confidence interval; IPTp, intermittent preventive treatment; MQ, mefloquine; SP, sulphadoxine-pyrimethamine.

^a Random intercept at the individual level and random slope for gestational age. Analysis on 2227 blood smears from 826 women.

Table 4. Multilevel Model on Factors Associated With *Plasmodium falciparum* Parasitemia (in Logarithm) During Pregnancy: Iron Levels Analysis^a

Factor	Coefficient (95% CI)	P Value
Ferritin corrected on inflammation (logarithm of µg/L)	0.22 (0.18; 0.27)	<.001
Folate (logarithm of ng/mL)	−0.23 (−0.37; −0.08)	.002
IPTp with MQ (SP = reference)	−0.01 (−0.09; 0.07)	.81
Gestational age (weeks)	−0.01 (−0.01; −0.002)	.01
Maternal age (years)	−0.15 (−0.21; −0.09)	<.001
Maternal age squared (years)	0.002 (0.001; 0.003)	<.001
Inflammatory process	0.62 (0.53; 0.71)	<.001
High socioeconomic index	−0.05 (−0.09; −0.01)	.01
Rain (mm)	−0.00 (−0.01; 0.01)	.98
Kato-Katz test positivity	−0.01 (−0.15; 0.13)	.90

Abbreviations: CI, confidence interval; IPTp, intermittent preventive treatment; MQ, mefloquine; SP, sulphadoxine-pyrimethamine.

^aRandom intercept at the individual level and random slope for gestational age. Analysis on 2227 blood smears of 826 women.

density (aOR = 0.82; 95% CI, 0.69–0.96; $P = .02$ and beta coefficient = −0.05; 95% CI, −0.09 to −0.01; $P = .01$, respectively).

High iron levels were also significantly associated with PM and LBW. More precisely, high levels of ferritin corrected on inflammation at delivery was strongly associated with placental malaria (aOR = 2.02; 95% CI, 1.43–2.86; $P < .01$) (Table 5, placental malaria). Similarly, corrected high ferritin at the ANV2 and at delivery were significantly correlated with increased odds of LBW (aOR = 1.59; 95% CI, 1.12–2.26 and aOR = 1.69; 95% CI, 1.28–2.22, respectively) (Table 5, low birth weight at delivery [birth weight <2500 g]).

We investigated further the differences in malarial risk factors stratifying between anemic- and nonanemic, and iron-deficient and noniron-deficient women (Table 6). In this analysis, we included women with ID defined by serum ferritin <15 µg/L

Table 5A. Logistic Regression on the Possibility of Having Placental Malaria^a

Factor	AOR (95% CI)	P Value
Socioeconomic index	1.26 (0.88; 1.79)	.20
Maternal age	0.94 (0.87; 1.00)	.06
Ferritin corrected on inflammation at delivery (logarithm)	2.02 (1.43; 2.86)	<.001
Inflammatory process at delivery	4.65 (2.32; 9.3)	<.001
Folate (logarithm) at ANV2	0.16 (0.03; 0.86)	.03
Number of maternal positive blood smears during pregnancy	2.51 (2.00; 3.15)	<.001

Abbreviations: ANV, antenatal visit; AOR, adjusted odds ratio; CI, confidence interval.

^aAnalysis on 689 placentas by blood smear. Pseudo R² = 0.43

Table 5B. Logistic Regression on the Possibility of Having Low Birth Weight at Delivery (Birth Weight <2500 g).^a

Factor	AOR (95% CI)	P Value
Socioeconomic index	0.91 (0.72; 1.19)	.55
Maternal BMI before pregnancy	0.92 (0.84; 1.00)	.06
Gestational age at the first ANV (and IPTp dose)	0.90 (0.85; 0.96)	<.001
Ferritin corrected on inflammation at ANV2 (logarithm)	1.59 (1.12; 2.26)	.01
Ferritin corrected on inflammation at delivery (logarithm)	1.69 (1.28; 2.22)	<.001
Positive blood smear at ANV2	2.88 (1.15; 7.22)	.02

Abbreviations: ANV, antenatal visit; AOR, adjusted odds ratio; BMI, body mass index; CI, confidence interval; IPTp, intermittent preventive treatment.

^aAnalysis on the birth weight of 763 infants. Pseudo R² = 0.11

at the moment of the malaria measure. Multilevel models showed corrected ferritin and CRP were equally significant for parasite density among anemic and non anemic women. However, folate was not correlated to parasite density in anemic women. In addition, iron levels were no longer associated with *P. falciparum* parasite density among iron-deficient women.

DISCUSSION

Benefits of iron supplementation during pregnancy for reducing iron related-diseases are undeniable. A Cochrane review showed supplementation was associated to a 70% decreased risk of anemia and to a 57% reduced risk of ID at delivery compared with controls [16]. However, epidemiological studies have questioned the benefits of iron supplementation in the context of malaria-endemic countries [24]. In a recent meta-analysis of the association between malaria and iron status or supplementation, data

Table 6. Multilevel Model on Factors Associated With Having Positive Blood Smears During Pregnancy Among Iron-Deficient Women^a

Factor	AOR (95% CI)	P Value
Ferritin corrected on inflammation (logarithm of µg/L)	0.96 (0.63; 1.47)	.86
Folate (logarithm of ng/mL)	0.69 (0.28; 1.73)	.43
Gestational age (weeks)	0.96 (0.90; 1.03)	.27
Maternal age (years)	0.70 (0.51; 0.97)	.03
Maternal age squared (years)	1.01 (0.99; 1.01)	.06
Inflammatory process	5.86 (3.54; 10.00)	<.001
Socioeconomic index	0.85 (0.67; 1.07)	.16

Abbreviations: AOR, adjusted odds ratio; CI, confidence interval; IPTp, intermittent preventive treatment; MQ, mefloquine; SP, sulphadoxine-pyrimethamine.

^aRandom intercept at the individual level and random slope for gestational age. Analysis on 1605 blood smears from 747 women.

521 were reported to be insufficient for assessing the potential for an
 522 increased risk of *P. falciparum* [25] infection. In addition, ID was
 associated with a decreased malarial risk in pregnancy when
 measured by ferritin, which is a robust indicator for iron levels
 [26, 27]. Indeed, the lack of complete follow-up of women
 through pregnancy is an important obstacle for the assessment
 of the influence of iron levels on *P. falciparum* malaria. In the
 majority of the studies included in the meta-analysis, iron was
 only determined either at enrollment, at delivery, or both. In the
 only prospective cohort [28], malaria was analyzed solely with
 regard to the first episode of the pregnancy.

In our study, we have assessed for the first time the influence
 of iron levels on malarial risk in a prospective longitudinal co-
 hort through pregnancy, considering the possibility of having a
 positive blood smear and *P. falciparum* parasite density. Indeed,
 iron levels, measured by ferritin corrected for inflammation,
 were significantly associated with malarial episodes and *P. fal-
 ciparum* density through the pregnancy period in the context
 of IPTp and ITN use. Furthermore, this association is strongly
 significant even after adjustment on inflammatory status. More-
 over, iron levels are significantly associated with placental ma-
 laria even after adjustment on maternal infection. Literature
 shows PM is associated with increased infant's susceptibility
 to the infection, translating into an increased number of clinical
 episodes [29–31]. Consequently, the association of high iron
 with placental malaria might contribute to enhance its conse-
 quences throughout the perinatal period. Finally, the associa-
 tion of maternal iron levels with LBW, possibly due to their
 relationship with PAM, suggests a broader impact of iron on in-
 fant health. Further details on the evolution of iron levels and
 anemia during pregnancy in this cohort are presented by Oué-
 draogo et al [19, 20, 32], but ID conferred protection against
 malaria through the entire follow-up. However, iron levels
 were no longer associated with *P. falciparum* parasite density
 among iron-deficient women, which suggests the possible exis-
 tence of a threshold level above which iron levels become dele-
 terious. Indeed, there was significant increased malarial risk
 above 30 days of supplementation in the stratified analysis of
 2 African surveys with high antimalarial preventive measures
 (relative risk = 1.42; 95% CI, 1.09–1.84) [25].

Our results are consistent with those in other studies. Al-
 though iron supplementation trials do not show augmented
 malaria morbidity associated with iron supplements, ID is cor-
 related with lower odds of malarial episodes [25]. Iron defi-
 ciency was statistically linked to reduced risk of placental
 malaria in Tanzania [17]. Ferritin was also higher among pla-
 centa-infected mothers in Gabon [33] and zinc protoporphyrin
 in Malawi [34], but these differences were not statistically signif-
 icant. Similar results were found in clinical trials in The Gambia
 [35] or Kenya [36]. The recent meta-analysis on malarial risk
 and iron status suggested a possible but not significant differ-
 ence in placental malaria associated with iron supplementation

depending on sickle cell genotype [25]. However, as stated pre-
 viously, these studies report iron levels only at enrollment, at de-
 livery, or both, and the limited sample might be insufficient to
 show a statistically significant effect.

Possible explanations for the increased malarial risk associated
 with iron levels found in our study are related to malaria patho-
 physiology in both the host and the parasite. At the host level,
Plasmodium interferes with the physiological iron distribution
 and use through hemolysis, release of heme, dyserythropoiesis,
 anemia, deposition of iron in macrophages, and inhibition of di-
 etary iron absorption [37]. Furthermore, the changes in iron me-
 tabolism during a malaria infection may modulate susceptibility
 to coinfections [37]. In addition, iron inhibits the synthesis of ni-
 tric oxide by inhibiting the expression of inducible nitric oxide
 synthase and thereby interferes with macrophage-mediated cyto-
 toxicity against *Plasmodium* [38]. Moreover, nontransferrin-
 bound iron is involved in the severity of malaria [39–41]. Indeed,
Plasmodium has the capacity of acquiring iron in a transferrin-
 independent pathway [42]. With regard to placenta, Penha-Gon-
 çalves et al [43] described in their preliminary results that iron
 overload in trophoblasts of *Plasmodium berghei*-infected placenta
 is associated with fetal death.

Accurate assessment of iron levels is challenging and no gold
 standard exists at present. We used serum ferritin to measure
 iron levels because it is a robust iron indicator and its frequent
 use in clinical studies facilitates the comparison of our results
 with other cohorts. To attenuate the interference of inflamma-
 tion on ferritin values (ferritin is an acute phase protein), we
 corrected ferritin upon inflammation (with correction factors
 according to CRP). Then, we included systematically inflamma-
 tion in the statistical models to capture its independent associ-
 ation with malarial risk.

Another important finding of our study is the association be-
 tween folate levels and PAM outcomes. High folate was corre-
 lated with reduced risk of malarial episodes, parasite density,
 and PM. Folate is an important cofactor used by (1) *P. falci-
 parum* in DNA synthesis and methylation and (2) mRNA trans-
 lation. Therefore, antifolates have been extensively used against
 malaria for nearly 70 years [44]. Hence, it is expected that folate
 levels are inversely correlated with malarial outcomes.

CONCLUSIONS

The interaction between iron and PAM is daunting because of
 the iron requirements during pregnancy and the fact that iron
 contributes to *P. falciparum* growth. In turn, this interaction is
 modified by malaria control interventions. Intermittent preven-
 tive treatment in pregnancy clears *Plasmodium* parasites and
 has a prophylactic effect on malarial episodes. Intermittent pre-
 ventive treatment in pregnancy and iron and folate supplements
 are given only at precise moments of pregnancy, whereas the
 impact of malaria on pregnancy outcomes are different

525 according to gestational age. For these reasons, it is important to
526 show that iron and folate levels are associated with increased
malarial risk in a prospective longitudinal cohort in the context
of both supplements and IPTp.

We show for the first time that high ferritin and low folate levels are associated with increased malarial risk during pregnancy period with regard to malarial episodes and *P. falciparum* parasite density in the context of IPTp and ITN use, even if positive smears diminish effectively after IPTp implementation. In addition, iron levels also have a significant association with important perinatal outcomes such as PM malaria and LBW. Our data also suggest there might be a threshold level above which iron has a deleterious impact on malarial risk. These results warrant additional epidemiological studies to evaluate the effect of different doses of iron and folate supplementation on maternal and infant health outcomes in malaria-endemic regions.

Acknowledgments

We thank Elizabeth Lim for reading and editing the manuscript and for making valuable linguistic corrections. We also thank the MiPPAD executive committee and Malaria in Pregnancy Consortium reviewers for valuable input in this work. Furthermore, we thank the women who participated in the study. Finally, we thank the midwives of the district of Allada and their assistants for help in conducting this study.

Financial support. This work was supported by the European and Developing Countries Clinical Trials Partnership (grant EDCTP-IP.07.31080.002; MiPPAD study "Malaria in Pregnancy Preventive Alternative Drugs," <http://clinicaltrials.gov/ct2/show/NCT00811421>), and the Malaria in Pregnancy Consortium, which is funded through a grant from the Bill and Melinda Gates Foundation to the Liverpool School of Tropical Medicine). V. M.-A. was funded by the Réseau doctoral de l'Ecole des Hautes Etudes en Santé Publique and the Direction Générale de l'Armement grant.

Potential conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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2662 V.II.2 Association of iron levels and interval length between IPTp

2663doses on malaria in infants during the first year of life

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2665**Summary of the article:** As already explained in the « State of art » section, epidemiological
2666studies have reported an increased malarial risk in infancy associated with high iron levels.
2667This aspect is of special concern in Benin, because malaria is the first cause of mortality of
2668infants under 5 years, and no national guidelines exist at present regarding iron supplements
2669in infancy.

2670To investigate the effect of iron on malaria risk during the first year of life, we used the data
2671of 400 infants (200 born of the anemic and 200 born of non anemic mothers) included in the
2672APEC (Anaemia in Pregnancy: Etiology and Consequences) study. In addition to the
2673mother's follow-up, clinical data of the infants were collected during systematic visits at 6, 9,
2674and 12 months in three clinics in the district of Allada (Allada, Attogon, Sékou). In case of
2675sickness infants were accurately examined in unscheduled visits and, if necessary, treated
2676according to the Beninese Ministry Health guidelines. In the unscheduled visits clinical and
2677biological exams were realized following the same protocol as systematic visits, i.e.,
2678anthropometric measures, and an extensive clinical examination were realized. In addition, 8
2679ml of venous blood (4ml in a dry tube and 4ml in an edta tube) were collected at each visit.
2680Haemoglobin, serum ferritin, CRP, vitamin B12, and folate levels were assessed. A container
2681was also given to the women to collect stools to examine the presence of intestinal helminths
2682in the infants. These containers were collected the following day by the study nurses within
2683the first 6 hours after emission.

2684During the first year of life, 40% of the infants had at least one malarial episode, with a range
2685of 0-4 positive smears. Offspring of mothers with longer IPTp protection (number of days
2686between IPTp doses) were significantly less likely to both have a positive smear (adjusted

2687odds ratio (aOR)=0.87, p-value=0.04) and high *P. falciparum* parasite density (beta

2688estimate=-0.06, p-value<0.001) during the entire follow-up period. Iron levels (measured by

2689the log of ferritin corrected on inflammation) were significantly associated with the risk of a

2690positive blood smear (aOR=2.77, p-value<0.001) and *P. falciparum* parasitaemia (beta

2691estimate=0.38, p-value<0.001). In multilevel model analysis, infants with iron levels in the

2692lowest quartile were significantly less likely to have a positive blood smear during the first

2693year of life (p-value<0.001), and the risk increases with higher iron levels.

2694We were surprised that the interval length between IPTp doses (i. e. the number of days

2695between doses) was associated with malarial risk and not with PAM. However, PAM might

2696not be symptomatic enough in the women of our cohort to make them go consult to the

2697clinics. Therefore, we might have lost valuable information during the mother's follow-up

2698and, hence, our data might not have enough power to show an effect. Nevertheless, it is

2699coherent that, knowing that the interval length between IPTp doses modifies the time of

2700exposure of the foetus to *Plasmodium*, it might have an effect of malaria in infants due to a

2701possible immune tolerance process.

2702Similarly to the mother's case, iron levels in infants were significantly associated with

2703increased malaria risk during the first year of life. Furthermore, our results suggest the

2704existence of dose effect of iron levels on malaria risk. Because of these results and the

2705previous literature on the topic, we think that additional epidemiological studies are required

2706to evaluate the effect of different doses of iron supplements on the infant health outcomes. In

2707addition, the comparison of cohorts in which iron is given with preventive purpose versus iron

2708given for the treatment of anaemia or iron deficiency (ID) is also interesting. Finally, public

2709policies should be encouraged to increase the observance of IPTp as it has a protective effect

2710not only in mothers but also in their offspring.

2711**What's known on this subject:** Malaria and iron deficiency are the main causes of anemia in

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2712infants. The benefits of iron supplements are questioned in malaria settings, but no

2713longitudinal data exist. Moreover, the influence of IPTp on malaria in infants has seldom been

2714analysed.

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2716**What this study adds:** We show that elevated iron levels and short interval between IPTp

2717doses are associated with increased risk of malaria and *P.falciparum* density in a longitudinal

2718prospective cohort during infancy in the context of ITN use, considering environmental,

2719clinical and obstetric risk factors.

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2723 Article under review in "*Pediatrics*"

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2725 **The effect of iron levels and IPTp on malaria risk in infants: a** 2726 **prospective cohort study in Benin**

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2728

2729 Violeta Moya-Alvarez, Gilles Cottrell, Smaila Ouédraogo, Manfred Accrombessi, Achille

2730 Massougbodgi, Michel Cot

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2733 **Abstract:**

2734 **Background:** In areas with high malaria and anaemia rates, intermittent preventive treatment

2735 in pregnancy (IPTp) and iron supplements are recommended by WHO. However, studies have

2736 set into question the inviolability of the benefits of iron supplementation in the context of

2737 malaria. In addition, pregnancy-associated malaria (PAM) has been found to be associated to

2738 malaria in infants, but epidemiological studies do seldom analyse the influence of IPTp. We

2739 investigated the effect of IPTp and iron levels during the first year of life on malarial risk.

2740 **Methods:** We followed 400 women and their offspring between January 2010 and May 2012

2741 in Allada (Benin). Environmental, obstetric and numerous clinical maternal and infant risk

2742 factors were considered.

2743 **Results:** In multilevel models, offspring of mothers with longer IPTp protection were

2744 significantly less likely to both have a positive smear (adjusted odds ratio (aOR)=0.87, p-

2745 value=0.04) and high *P.falciparum* parasitaemia (beta-estimate=-0.06, p-value<0.001). Iron

2746 levels were significantly associated with the risk of a positive blood smear (aOR=2.77, p-

2747 value<0.001) and *P.falciparum* parasitaemia (beta-estimate=0.38, p-value<0.001). Infants with

2748 iron levels in the lowest quartile were less likely to have a positive blood smear (p-

2749 value<0.001), and the risk increased with higher iron levels.

2750 **Conclusion:** Our results appeal for additional evaluation of different doses of iron

2751 supplements on the infant health outcomes. Thus, the comparison of cohorts in which iron is

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2752given with preventive purpose versus iron given for treatment is also required. Finally, the

2753observance of IPTp should be encouraged as it has a protective effect not only in mothers but

2754also in their offspring.

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2758Body of the article:

2759

2760Introduction

2761Infant health morbidity in Sub-Saharan Africa is mainly driven by infectious diseases and
2762nutritional deficiencies [1]. Indeed, malaria and anaemia (mainly due to iron deficiency) are
2763two leading pathologies contributing to enhance the disease burden among African infants
2764[2]. In 2013, malaria was responsible for an estimated 198 million cases and an estimated 584
2765000 deaths [3]. In addition, malaria causes anaemia, which is the second leading cause of
2766disability [4] and entails severe consequences for the development of the children [5].
2767Moreover, both diseases harm mainly children under 5 years of age. For these reasons, public
2768health strategies have been developed to tackle both diseases simultaneously.
2769To tackle anaemia WHO recommends the administration of 12.5 mg iron and 50µg folic acid
2770daily between 6 and 12 months [6]. However, in Benin this policy has not been implemented
2771so far, and, in general, Beninese paediatricians give a preventive treatment consisting in 10
2772mg/kg and day during 2 months every 6 months starting at 6 months of age until 5 years of
2773age. With regard to malaria, the present WHO recommendations for the control of malaria are
2774the use of insecticide treated nets (ITNs) and/or indoor residual spraying (IRS) for vector
2775control, and prompt access to diagnostic testing of suspected malaria and treatment of
2776confirmed cases.

2777Albeit the large implementation of these interventions, epidemiological studies have set into
2778question the inviolability of the benefits of iron supplementation in the context of malaria-
2779endemic countries [7], and iron deficiency has been associated to reduced malaria odds
2780among pregnant women and infants [8,9]. However, in a recent meta-analysis of the
2781association between malaria and iron status or supplementation in children, data were reported
2782to be insufficient for assessing the potential of an increased risk of *P.falciparum* infection [9].

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2783Indeed, evidence on the iron-malaria association lacks from prospective cohorts during
2784infancy.

2785In parallel, PAM is significantly associated to malaria in infants [10], but epidemiological
2786studies do seldom analyse its influence. Therefore, we investigated the effect of iron levels
2787during the first year of life on malarial risk in infants taking into account complementary
2788information on PAM, IPTp, environmental, socio-economic, and clinical indicators and co-
2789morbidity to better understand malaria risk factors in the context of the present malaria
2790control strategies.

2791Materials and methods

2792A prospective cohort of 400 infants was followed from birth to 12 months of age in the
2793context of the APEC study (Anaemia in Pregnancy: Etiology and Consequences). APEC
2794study is an ancillary survey nested within the MiPPAD trial in Benin (Malaria in Pregnancy
2795Preventive Alternative Drugs “<http://clinicaltrials.gov/ct2/show/NCT00811421>”). This study
2796was conducted in three clinics in the district of Allada, between January 2010 and May 2012.
2797Allada is a semi-rural area of 91,778 inhabitants located 50 km North of Cotonou (Benin).
2798Malaria has a perennial transmission pattern with two transmission peaks corresponding to the
2799rainy seasons in April-July and October-November. *Plasmodium falciparum* is the species
2800responsible for the majority of infections.

2801Complete details of MiPPAD are presented elsewhere [11], but, briefly, MiPPAD was a
2802randomized trial comparing the efficacy and safety of IPTp with SP (1,500/75 mg per dose)
2803and mefloquine (15 mg/kg per dose). At delivery placenta was examined in order to analyse
2804*P. falciparum* parasite infestation. Clinical data of the infants were collected during
2805systematic visits at 6, 9, and 12 months. In case of sickness infants were accurately examined
2806in unscheduled visits and, if necessary, treated according to Beninese guidelines. In the
2807unscheduled visits clinical and biological exams were realized following the same protocol as
2808systematic visits. All drugs prescribed to the infants during the follow-up were free of charge.

2809 For the purpose of the APEC sub-study, anthropometric measures, and an extensive clinical
2810 examination were realized during the visits. In addition, 8 mL of venous blood were collected
2811 at each visit. Haemoglobin, serum ferritin, CRP, vitamin B12, and folate levels were assessed.
2812 A container was also given to the women to collect stools that were collected the following
2813 day by the study nurses within the first 6 hours after emission. Microbiological exams were
2814 realized as follows: Lambaréné technique was used to assess malaria infection [12].
2815 Helminthic infestations were assessed using the Kato-Katz concentration method
2816 (VestergaardFrandsen kit®). In case of inflammation (CRP>5mg/l) serum ferritin was
2817 adjusted following the corrections recommended by Thurnham et al. in their meta-analysis
2818 [13] to avoid the extrinsic effect of inflammation on serum ferritin levels.
2819 We used rain quantity as a surrogate for the risk of exposure to anopheline bites. Because of
2820 the anopheline timeliness, rainfall quantity was calculated as the mean rain volume of the 7
2821 days prior to the two weeks before the consultation. It was independently assessed for each
2822 visit and each health centre of the study.
2823 Socio-economic status was assessed using a socio-economic index. The socio-economic index
2824 was created in a two-step process. First a multiple correspondence analysis of socio-economic
2825 items was performed. Then the first principal axis was used as an overall socio-economic
2826 index in the further regression analysis.
2827 Data were double entered and analysed with ACCESS 2003, and STATA 12.0 Software
2828 (Stata Corp, College Station, TX, USA). Then exploratory and univariate analyses were
2829 realized to assess the association of all variables with both infant positive smear and
2830 peripheral *P.falciparum* density at each visit (systematic or unscheduled visit). Chi-square and
2831 Kruskal-Wallis tests were used in the univariate analyses. For time-dependent variables,
2832 univariate analyses were realized using a random intercept model at the infant level.
2833 Thereafter, all variables with P values<0.2 were included in either a logistic or a linear
2834 multivariate multilevel model with a random intercept and slope at the infant level including

all visits for each infant, to explore the determinant of the probability of having a positive smear or peripheral *P.falciparum* parasitaemia, respectively. More precisely, a random slope was applied to the infant age, as the effect of the variables might differ between the infants. The statistical significance in the final multivariate models was set to $P < 0.05$ (two-sided tests). To evaluate the possible diverse effect of different iron levels on malaria risk, we run the same multilevel model considering the different quartiles of corrected ferritin. This study was approved by the Ethics Committee of the Faculty of Medicine of Cotonou. It was explained in the local language to the mothers and their voluntary consent was obtained before enrolment.

Results

Between January 2010 and 2012, 400 mother-infant pairs were included in the cohort. Three-hundred and twenty-seven infants continued to be followed-up until the first systematic visit at 6 months, 325 until the second visit at 9 months, and 324 completed the 12 month follow-up. At birth 10.9% of the infants were born from a malaria infected placenta, but no cord blood infection by *Plasmodium* was detected at the microscopy exam. The main characteristics of the infants at birth are presented in Table 1.

During the first year of life 40% of the infants had at least one malarial episode, with a range of 0-4 positive smears taking into account both systematic and unscheduled visits. More precisely, 60.25% of infants had no positive blood smear during the entire follow-up, 22% of infants had 1, 12.50% had 2, 4.5% had 3, and 0.75% had 4 positive blood smears during follow-up. The clinical and biological characteristics of the infants at the systematic visits are summarized in Table 2. The proportion of infants with a positive smear at the systematic visits remained constant along the follow-up (around 12% of the infants were infected at each visit). However, *P.falciparum* parasitaemia did change significantly during the first year of life. Among infants with a positive smear, the median *P.falciparum* density was 7597.5 parasites/mm³ (95% confidence interval (CI)= 17584.92; 97814.82) at 6 months, 14839

2861parasites/mm³ (95% CI= 45882.41; 263310.7) at 9 months, and 7919.5 parasites/mm³ (95%
2862CI= 26019.96; 136360.9) at 12 months.

2863In parallel, the mean haemoglobin values increased slightly, though not significantly, through
2864the follow-up (102.1 g/l, 102.9 g/l, and 103.6 g/l at the 6, 9, and 12 month systematic visits,
2865respectively).

2866Iron indicators decreased through the follow-up. The mean ferritin levels decreased after the 6
2867month visit from 605 µg/l (95% CI= 508; 702) to 455 µg/l (95% CI= 384; 526) at 9 months,
2868and then decreased again until 436 µg/l (95% CI= 350; 522) at 12 months. Iron deficiency
2869increased in parallel from 16% at 6 months, to 29% at 9 months, and up to 34% at 12 months.
2870During the first year of life malaria rates and *P. falciparum* parasitaemia were determined by
2871clinical, environmental and socio-economic factors, but pregnancy related aspects did also
2872influence significantly the malarial outcomes of the infant during the entire follow-up. The
2873risk factors for malaria and *P. falciparum* parasite density are presented in Table 3 and Table
28744, respectively.

2875There were no statistical differences in the number of positive smears or *P. falciparum*
2876density during the first year of life depending neither on the placental malarial status nor on
2877the intermittent preventive treatment in pregnancy (IPTp) regime of the mothers (either
2878sulphadoxine-pyrimethamine (SP) IPTp or mefloquine (MQ)). Nevertheless, the time interval
2879between IPTp doses of the mothers (number of days between IPTp doses), i.e. the period
2880during which the mothers were protected against malaria, was significantly associated with
2881the risk of malarial infection of the infant during the first year of life. Infants born to mothers
2882who had longer IPTp protection were significantly less likely to both have a positive smear
2883(adjusted odds ratio (aOR)=0.87, 95% CI= 0.76; 0.99, p-value=0.04) and high *P. falciparum*
2884parasite density (beta-estimate=-0.06, 95% CI= -0.10; -0.01, p-value<0.001) during the entire
2885follow-up. Higher maternal folate levels and helminth infection at delivery were also
2886significantly linked to increased parasite density during the first year of life (beta-

estimate=0.34, 95% CI=0.01; 0.66, p-value=0.04, and beta-estimate=0.88, 95% CI= 0.20; 1.57, p-value=0.03, respectively).

The clinical and nutritional status of the infant was also correlated with malarial risk. Iron levels (log of ferritin corrected on inflammation) were significantly associated with the risk of a positive blood smear (aOR=2.77, 95% CI= 1.95; 3.96, p-value<0.001) and *P. falciparum* parasite density (beta-estimate=0.38, 95% CI= 0.29; 0.47, p-value<0.001) during the first year of life. Infants with iron deficiency were significantly less likely to have a positive blood smear and a high *P. falciparum* density (p-value=0.01 in both cases). In parallel, ongoing inflammatory status of the infant (CRP>5mg/l) was significantly associated to an increased risk of positive blood smear (aOR=4.37, 95% CI= 2.20; 8.65, p-value<0.001) and to a higher *P. falciparum* parasite density (beta-estimate=0.77, 95% CI= 0.53; 1.01, p-value<0.001). The presence of other parasites such as intestinal helminths was not significantly associated with increased malaria risk. There were no statistical differences in malaria risk between the different age periods of the follow-up.

The rain volume (representing the anopheline risk) was marginally associated to increased malaria risk with regard to both increased risk of a positive smear (aOR=1.06, p-value=0.06), and to increased *P. falciparum* parasitaemia (beta-estimate=0.03, p-value=0.06).

Finally, we investigated further the differences in malarial risk factors considering the different quartiles of iron levels in infants to evaluate the possible different effects of iron on malaria risk depending on the different levels of iron. Indeed, infants with iron levels in the three upper quartiles had significantly higher risk of having malaria during the first year of life (table 5). Infants with iron levels in the upper quartiles had significantly higher *P. falciparum* parasite density.

Discussion

In this study, we evidenced the influence of two important factors related with malaria infection during the first year of life, time duration between two IPTp doses and iron levels.

2913More precisely, we found that the time period between IPTp doses (number of days) is

2914inversely correlated to malaria risk. When the period of time between IPTp doses is longer,

2915infants have significantly reduced risk of malaria during the first year of life. High iron levels

2916also have a significant effect on malaria severity in infants during the first year of life

2917considering both the possibility of having a positive blood smear and *P.falciparum* parasite

2918density.

2919PAM has been frequently correlated to an impaired health status of the offspring [10]. In a

2920recent follow-up of a mother-child cohort in Benin, Borgella et al. showed that infants born to

2921a mother with PAM during the third trimester of pregnancy had a significantly increased risk

2922of infection (OR=4.2 95% CI (1.6; 10.5), p-value=0.003) or of malaria episode (OR=4.6 95%

2923CI (1.7; 12.5), p-value=0.003), assuming the period covered by IPTp (2nd trimester of

2924pregnancy) was at low risk for malaria infection [14]. In addition, Huynh found IPTp calendar

2925was associated with consequences of malaria such as LBW and anaemia [15]. Considering

2926that PAM has a significant effect on malaria in infants and that IPTp, by preventing new

2927infections, has an impact on secondary malaria outcomes, such as LBW and anaemia, we

2928think that albeit their novelty our results are coherent with the existing literature. A single

2929discordant study in Tanzania found that IPTp could be associated with an overall increase of

2930severe malaria and earlier first malaria episodes in the offspring [16]. Such paradoxical results

2931could be explained by the high level of resistance to SP in this area of Tanzania [17,18].

2932Indeed, Dechavanne found increased susceptibility of infants to *P. falciparum* parasites with

2933antigens to which they were previously exposed in utero [19], suggesting the existence of an

2934in utero ongoing immune tolerance process. However, no evidence exists at present on its

2935concrete physiopathological pathways.

2936At present an adjustment of IPTp calendar to enhance protection is already ongoing in Benin.

2937In effect, Benin is setting a 3rd dose in the IPTp regime to implement the WHO new

2938recommendations.

2939 Another important result of our study is the significant association of iron levels with malaria
2940 risk. We have assessed the influence of iron levels on malarial risk with regard to the
2941 possibility of having a positive blood smear and *P.falciparum* parasite density throughout the
2942 first year of life in a prospective longitudinal cohort, considering environmental, socio-
2943 economic, and PAM factors. Iron levels, measured by ferritin corrected for inflammation, a
2944 consistent indicator of iron [20,21], were significantly associated with malarial episodes and
2945 *P.falciparum* parasitaemia. Furthermore, this association was significant even after
2946 adjustment on inflammatory status. Iron deficiency was associated to a significant protection
2947 through the entire follow-up. More precisely, infants with iron levels in the first quartile
2948 seemed to be significantly protected against malaria. Indeed, iron deficiency has frequently
2949 been linked to a certain protection against malaria [9]. Nevertheless, results on the effect of
2950 iron levels on malaria differ in the context of clinical trials with iron supplements. In a
2951 specific Cochrane review [9] no significant difference in clinical malaria episodes was
2952 detected between children supplemented with iron alone and those receiving a placebo (risk
2953 ratio (RR)=0.99, 95% CI (0.90; 1.09). However, the effect of iron deficiency was not
2954 assessed, and solid preventive measures against malaria were implemented in the clinical
2955 trials. Indeed, an increased risk of malaria with high iron levels was observed in trials that did
2956 not provide malaria surveillance and treatment, and the risk of malaria and parasitaemia was
2957 higher with high iron levels (RR=1.13, 95% CI (1.01; 1.26) [9]. Furthermore, in numerous
2958 studies included in the meta-analysis, iron was seldom determined longitudinally.
2959 Malaria physiopathology could explain the increased malarial risk associated with elevated
2960 iron levels. In effect, iron inhibits the synthesis of nitric oxide by inhibiting the expression of
2961 inducible nitric oxide synthase (iNOS) at the host level, and thereby interferes with
2962 macrophage-mediated cytotoxicity against *Plasmodium* [22]. Furthermore, non-transferrine
2963 bound iron (NTBI) is associated with the severity of malaria [23–25], and *Plasmodium* has
2964 the capacity of acquiring iron in a transferrin-independent pathway [26].

2965Albeit the hereby reported results, iron supplements have undeniable benefits for infants. A
29662013 meta-analysis showed supplementation was associated to a reduced risk of anaemia, of
2967iron deficiency, and of iron deficiency anaemia [27]. As pondering the advantages and risk of
2968iron supplements is daunting because they are not epidemiologically quantifiable, the
2969implementation of malaria protective strategies should be seriously encouraged.
2970Complementary findings of our study are the significant impact of maternal folate levels and
2971helminths at delivery on *P. falciparum* parasite density of the infant. Indeed, folate is an
2972important cofactor used by *P.falciparum* in DNA synthesis and methylation, and mRNA
2973translation. Therefore, high folate levels could enhance the immune tolerance process
2974undergoing PAM. With regard to the significant association between maternal helminths and
2975increased *P. falciparum* parasite density, this was already described In Uganda, where
2976Ndibazza et al. suggested this could be due to hyporesponsiveness of T-cells of the of the
2977infants previously exposed in utero to parasites [28]. Therefore, further epidemiological
2978evidence could be useful to analyse the extent of the immune tolerance process undergoing in
2979utero.

2980Conclusion

2981The impact of PAM on malaria in infants does not only involve placental malaria, prematurity
2982or LBW. PAM entails increased risk of malaria in infants and PAM preventive interventions
2983have a significant influence on malaria in infants as well. In our study, long IPTp interval is
2984associated to reduced malaria risk in infants during the first year of life. Public policies should
2985be encouraged to increase the observance of IPTp as it has a protective effect not only in
2986mothers but also in their offspring, as it has been recently recommended by the WHO [29].
2987Malaria risk during the first year of life is also associated with high ferritin levels in a
2988prospective longitudinal cohort considering complementary risk factors. Our data also suggest
2989that malaria risk increases with higher ferritin levels. Indeed, the interaction between iron and
2990malaria is complex because of the iron requirements during infancy and the fact that iron

2991 contributes to the parasite growth. These results appeal for additional epidemiological studies
 2992 to evaluate the effect of different doses of iron supplements on the infant infectious and
 2993 haematological outcomes. Complementary interventional data are needed to determine the
 2994 benefits and risks of differently dosed iron supplements, in order to ascertain their impact on
 2995 infant health in malaria-endemic regions. Finally, the epidemiological comparison of cohorts
 2996 in which iron is given as preventive intervention and cohorts in which iron is given solely on
 2997 the purpose of treatment for anaemia or ID should also be analysed.

2998

2999 Acknowledgments:

3000 Jessica Barry read and edited the manuscript making valuable linguistic corrections. We also thank the MiPPAD
 3001 executive committee and MiPc reviewers for valuable input in this work. We thank the women who participated
 3002 in the study. We also thank the midwives of the district of Allada and their assistants for their help in conducting
 3003 this study.

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3008 Tables:

Table 1. Clinical and biologic indicators of the infants at birth

	Mean or Proportion (95% CI)
Sex of the infants	Male: 183 (46.9%) Female: 207 (53.1%)
Gestational age at birth (weeks) (Ballard score)	38.1 (37.8; 38.4)
Weight (g)	3033.5 (2992; 3075)
Low birthweight (%) (birthweight<2500g)	9 (6.2; 11.9)
<i>P. falciparum</i> infected placenta (%)	10.9 (7.8; 13.9)
Haemoglobine (g/l)	139 (136.9; 141)

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V. Results

Serum ferritin (µg/l)	182.6 (165.5; 199.7)
Folate (ng/l)	16.5 (12.6; 20.4)

95% CI= 95% Confindence interval

Table 2. Clinical and biologic indicators of the infants during the follow up period (6, 9, 12 months)

Characteristics	6 months (n=327) Mean or Proportion (95% CI)	9 months (n=325) Mean or Proportion (95% CI)	12 months (n=324) Mean or Proportion (95% CI)
<i>P. falciparum</i> infection (%)	12.06 (8.45; 15.68)	12.00 (8.28; 15.52)	12.34 (8.70; 15.99)
Parasite density (nb/mm ³)	6960.862 (1869.05; 12052.19)	18392.52 (4791.55; 31993.49)	9794.40 (2764.46; 16824.35)
Haemoglobine (g/l)	102.22 (100.55; 103.88)	102.91 (101.32; 104.50)	103.80 (102.14; 105.47)
Anaemia (%) (Hb<110g/l)	66.99 (61.74; 72.23)	69.81 (64.65; 74.96)	64.86 (59.54; 70.17)
Mild anaemia (%) (Hb 100-109 g/l)	31.41 (26.23; 36.59)	34.09 (28.77; 39.41)	36.42 (31.06; 41.78)
Moderate anaemia (%) (Hb 80-109 g/l)	28.53 (23.48; 33.56)	30.52 (25.34; 35.69)	21.73 (17.13; 26.32)
Severe anaemia (%) (Hb<80 g/l)	7.05 (4.19; 9.90)	5.19 (2.70; 7.69)	6.70 (3.92; 9.50)
Corrected serum ferritin (µg/l)	604.58 (507.64; 701.52)	455.37 (384.27; 526.48)	436.16 (350.42; 521.90)
Iron deficiency (%) (corrected SF<15µg/l)	16.09 (8.21; 23.97)	29.63 (20.88; 38.38)	34.28 (25.06; 43.52)

95% CI= 95% Confidence interval; Hb: Haemoglobin; SF: Serum ferritin

Table 3. Multilevel model on factors associated with having positive blood smears during the first year of life

Factor	aOR (95% CI)	p-value
Infant factors		
Ferritin corrected on inflammation (logarithm of µg/l)	2.77 (1.95; 3.96)	<0.01
Inflammatory process (CRP>5mg/l)	4.37 (2.20; 8.65)	<0.01
Kato-katz test positivity	0.89 (0.33; 2.40)	0.82
Age 1-4 months (reference)		
Age 4-8 months	2.95 (0.41; 21.23)	0.28
Age 8-12 months	2.07 (0.25; 16.99)	0.50
Pregnancy associated factors		
IPTp extent (days between IPTp doses)	0.87 (0.76; 0.99)	0.04
Demographic and environmental factors		
Low socio-economic index	1.22 (0.89; 1.66)	0.20
Rain volume (mm)	1.06 (0.99; 1.11)	0.06

Random intercept at the infant level. Random slope for the age of the infants. Analysis on 746 blood smears from 329 infants.

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Table 4. Multilevel model on factors associated with *P.falciparum* parasitemia (in logarithm) during the first year of life

Factor	Beta estimate (95% CI)	p-value
Infant factors		
Ferritin corrected on inflammation (logarithm of µg/l)	0.38 (0.29; 0.47)	<0.01
Inflammatory process	0.77 (0.53; 1.01)	<0.01
Kato-katz test positivity	-0.20 (-0.58; 0.18)	0.30
Age of the infant (1-4 months (reference))		
Age of the infant 4-8 months	0.20 (-0.14; 0.54)	0.24
Age of the infant 8-12 months	-0.06 (-0.39; 0.26)	0.71
Pregnancy associated factors		
IPTp extent (days between IPTp doses)	-0.06 (-0.10; -0.01)	<0.01
Kato-katz test positivity of the mother at delivery	0.88 (0.20; 1.57)	0.03
Folate of the mother at delivery (Logarithm of ng/ml)	0.34 (0.01; 0.66)	0.04
Demographic and environmental factors		
Low socio-economic index	0.12 (0.01; 0.23)	0.03
Rain volume (mm)	0.03 (-0.00; 0.06)	0.06

Random intercept at the infant level. Random slope for age of the infant. Analysis on 542 blood smears of 236 infants.

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Table 5. Multilevel model on factors associated with malaria risk during the first year of life depending on the different iron levels

Factor	Multilevel model on the positive blood smear		Multilevel model on <i>P.falciparum</i> parasitaemia	
	aOR (95% CI)	p-value	Beta estimate (95% CI)	p-value
Infant factors				
Ferritin corrected on inflammation (logarithm of µg/L) 1st quartile	reference		reference	
Ferritin corrected on inflammation 2nd quartile	3.28 (1.20; 8.96)	0.02	0.06 (-0.26; 0.38)	0.73
Ferritin corrected on inflammation 3rd quartile	4.53 (1.75; 11.77)	<0.01	0.35 (0.03; 0.66)	0.03
Ferritin corrected on inflammation 4th quartile	6.16 (2.40; 15.81)	<0.01	0.55 (0.24; 0.87)	<0.01
Inflammatory process	4.37 (2.44; 7.80)	<0.01	0.76 (0.51; 1.01)	<0.01
Kato-katz test positivity	0.92 (0.36; 2.36)	0.86	-0.16 (-0.55; 0.24)	0.44
Age of the infant (1-4 months (reference))				
Age of the infant 4-8 months	3.39 (0.58; 19.71)	0.18	0.26 (-0.08; 0.61)	0.14
Age of the infant 8-12 months	2.95 (0.30; 29.24)	0.36	0.03 (-0.31; 0.36)	0.88
Pregnancy associated factors				
IPTp extent (days between IPTp doses)	0.88 (0.78; 0.99)	0.04	-0.06 (-0.10; -0.01)	0.01
Kato-katz test positivity of the mother at delivery			0.96 (0.24; 1.68)	<0.01
Folate of the mother at delivery (Logarithm of ng/mL)			0.32 (-0.02; 0.65)	0.06
Demographic and environmental factors				
Low socio-economic index	1.16 (0.87; 1.55)	0.32	0.12 (0.004; 0.23)	0.04
Rain volume (mm)	1.06 (0.99; 1.12)	0.06	0.03 (-0.004; 0.07)	0.08

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3110 **V.II.3 Other factors associated with malaria risk during infancy:** 3111 **the case of lead**

3112

3113 **Summary of the article:** As said in the “State of art” section, simultaneously to our study in
3114 the same cohort another epidemiological project was evaluating the effect of lead on the
3115 neurocognitive development in children. Our colleagues found out lead levels were
3116 particularly high in the infants of our cohort. Nriagu had found in Nigeria that malaria had a
3117 significant effect on lead levels in univariate analysis. In addition, elevated blood lead levels
3118 (BLL) carry a significant burden of disease in Western Africa and malaria is the first cause of
3119 infant mortality in Benin. Therefore, we aimed at assessing the possible association of lead
3120 levels with malaria risk considering other major malarial risk factors.

3121 Elevated lead levels have severe harmful effects on infant health. They are associated with
3122 impaired neurocognitive development, anemia (due to either disruption of heme synthesis or
3123 hemolysis), and renal and gastro-intestinal effects. Although high blood lead levels (BLL)
3124 (BLL >100 µg/dl) can entail acute neurologic symptoms, such as ataxia, hyperirritability,
3125 convulsions, coma, and death, BLL as low as 10 µg/dl have been also correlated with poor
3126 neurocognitive outcomes and behavioral disorders. Indeed, the Center for Disease Control
3127 (CDC) reduced the reference level of blood lead from 10 µg/dl to 5 µg/dl in 2012. This is of
3128 special concern in young children as neuro-cognitive impairment has been found to be
3129 associated with the degree of exposure to lead between the ages of 12 and 36 months. Albeit
3130 the severe impact of elevated lead levels on infant health, epidemiological studies of lead
3131 levels in Sub-Saharan Africa are limited. Data from the few existing studies, published in a
3132 systematic review on BLL among Sub-Saharan children, suggest an alarming burden of
3133 disease. This review reported a BLL weighted mean of 13.1 µg/dl which increases up to 16.2

3134 $\mu\text{g}/\text{dl}$ considering solely studies with robust quality BLL analyses. In addition, the prevalence
3135 of BLL $>10 \mu\text{g}/\text{dl}$ ranged from 7.0% to 70.9% in six of the studies reviewed. Recent mass
3136 level intoxications reported in Senegal and Nigeria further raise the public health concern
3137 about lead levels in West Africa. Notwithstanding these concerns, infectious diseases, mainly
3138 malaria, lead the disease burden in West Africa. In Benin, malaria is the main cause of
3139 mortality among children less than 5 years and there were over 1.5 million cases in 2012.
3140 Both malaria and lead poisoning can have severe hematologic and neurologic symptoms on
3141 children and development disruptions. Because of the recent evidence on the role of the
3142 complement system in the regulation of neurodevelopment, it has been proposed that
3143 excessive complement activation induced by placental malaria may disrupt normal
3144 neurodevelopment resulting in neurocognitive impairment of infants exposed to *Plasmodia in*
3145 *utero*.

3146 Epidemiologically, malaria and lead poisoning may not only overlap geographically, but they
3147 have major impact on the health of children, especially those under 5 years. Consequently,
3148 their possible association may have an effect on one of the most vulnerable age groups in the
3149 population, and it could have severe long-term implications for the development of the
3150 children. Furthermore, Nriagu found a significant effect of malaria on the children lead levels
3151 in different areas of Nigeria. Concern has been repeatedly raised up on the importance of
3152 alarmingly high anemia rates in West Africa, and both malaria and EBLL are associated with
3153 increased anemia rates. However, no evidence exists at present on the possible joint effect of
3154 lead and *P.falciparum*. To our knowledge, no published study exists on lead levels in Benin,
3155 and in particular, on the effects of lead levels on malaria risk in infants.

3156

3157 **What's known on this subject:** Malaria and elevated lead levels overlap geographically in
3158 West Africa. Both entail anemia and impaired neuro-cognitive development and their effect is

3159particularly severe in infants. Albeit the effect of malaria on lead levels found by Nriagu in
3160Nigeria, no evidence exists at present on the effect of lead levels on malaria risk.

3161

3162**What this study adds:** We show that the rate of elevated blood lead levels is very high in
3163Benin. In addition, we show that elevated lead levels give a certain protection to infants with
3164regard to the malaria risk, possibly due to a toxic effect of lead on *Plasmodium*. Furthermore,
3165we show that even in the context of high lead levels, iron levels are still significantly
3166associated to increased malaria risk.

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3169Article under review in *Plos One*:

3170Elevated blood lead levels are associated with reduced risk of malaria in Beninese infants

3171Violeta Moya-Alvarez, Michael Osei Mireku, Pierre Ayotte, Michel Cot, and Florence Bodeau-
3172Livinec.

3173Abstract

3174**Introduction:** Elevated blood lead levels (BLL) and malaria carry an important burden of
3175disease in West Africa. Both diseases might cause anemia and they might entail long-term
3176consequences for the development and the health status of the child. Albeit the significant
3177impact of malaria on lead levels described in Nigeria, no evaluation of the effect of elevated
3178BLL on malaria risk has been investigated so far.

3179**Materials and methods:** Between 2010 and 2012, 203 Beninese infants were followed
3180during the first year of life through three systematic visits at 6, 9, and 12 months, and
3181emergency unscheduled visits to evaluate their health status and gather clinical,
3182microbiological and hematological data. Blood lead levels were assessed at 12 months.

3183**Results:** At 12 months, the mean BLL of infants was 7.41 $\mu\text{g/dl}$ (CI: 65.2; 83), and 128
3184infants (63%) had elevated blood lead levels, defined by the CDC as $\text{BLL} > 5 \mu\text{g/dl}$. Lead
3185poisoning, defined as $\text{BLL} > 10 \mu\text{g/dl}$, was found in 39 infants (19%). Twenty-five infants
3186(12.5%) had a positive blood smear at 12 months and 144 infants were anemic (71%, $\text{Hb} < 110$
3187 g/l). Elevated blood lead levels were significantly associated with reduced risk of a positive
3188blood smear (aOR=0.98, p-value=0.02) and *P. falciparum* parasite density (beta-estimate=-
31890.003, Pvalue=0.048) in logistic and linear regression multivariate models, respectively
3190adjusted on clinical and environmental indicators.

3191**Conclusion:** Our study shows for the first time that BLL are negatively associated with

3192malarial risk considering other risk factors. Malaria is the main cause of mortality for infants
3193under 5 years worldwide, and lead poisoning is the 6th most important contributor to the
3194global burden of diseases measured in disability adjusted life years (DALYs) according to the
3195Institute of Health Metrics. In conclusion, environmental factors, such as lead levels, need to
3196be considered in the debate about iron supplements in malaria endemic countries.

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3198 **Body of the article**

3199 **Introduction**

3200 Elevated lead levels have severe harmful effects on infant health. Symptoms related to
3201 toxicity occur from mid to high levels of exposure and they depend on the amount of lead in
3202 the blood and tissues. High lead levels are associated with impaired neurocognitive
3203 development, anemia (due to either disruption of heme synthesis or hemolysis [1]), and renal
3204 and gastro-intestinal effects [2]. Although high blood lead levels (BLL) ($\text{BLL} > 100 \mu\text{g/dl}$) can
3205 entail acute neurologic symptoms, such as ataxia, hyperirritability, convulsions, coma, and
3206 death, BLL as low as $10 \mu\text{g/dl}$ have been also correlated with poor neurocognitive outcomes
3207 and behavioral disorders [3,4]. This is of special concern in young children as neuro-cognitive
3208 impairment has been found to be associated with the degree of exposure to lead between the
3209 ages of 12 and 36 months [5]. Indeed, the Center for Disease Control (CDC) reduced the
3210 reference level of blood lead from $10 \mu\text{g/dl}$ to $5 \mu\text{g/dl}$ [6] in 2012.

3211 Albeit the severe impact of elevated lead levels on infant health, epidemiological studies of
3212 lead levels in Sub-Saharan Africa are limited. Data from the few existing studies, published in
3213 a systematic review on BLL among Sub-Saharan children, suggest an alarming burden of
3214 elevated BLL. This review reported a BLL weighted mean of $13.1 \mu\text{g/dl}$, which increased up
3215 to $16.2 \mu\text{g/dl}$ considering solely studies with robust quality BLL analyses [7]. In addition, the
3216 prevalence of $\text{BLL} > 10 \mu\text{g/dl}$ exceeded 44% in all cases reviewed, with a maximum of 70.9%
3217 in Nigeria. Only one study in Kenya reported a relatively low prevalence (7%). Recent mass
3218 level intoxications reported in Senegal and Nigeria [8] further raise the public health concern
3219 about lead exposure in West Africa.

3220 In addition, malaria and lead poisoning overlap geographically. Indeed, infectious diseases,

3221mainly malaria, dominate the disease burden in West Africa [9]. In Benin, malaria is the main
3222cause of mortality among children less than 5 years and there were over 1.5 million cases in
32232012 [10]. As already explained, both malaria and lead poisoning can have severe
3224hematologic and neurologic symptoms on children and their development. Malaria and lead
3225poisoning may not only overlap, but they have major impact on the health of children,
3226especially those under 5 years. Consequently, their possible association may have an effect on
3227one of the most vulnerable age groups in the population, and it could have severe long-term
3228implications for the development of the children. Furthermore, Nriagu found a significant
3229effect in univariate analysis of malaria on the children lead levels in different areas of Nigeria
3230[11]. However, no evidence exists at present on the possible joint effect of lead and
3231*P.falciparum*. To our knowledge, no published study exists on lead levels in Benin, and in
3232particular, on the effects of lead levels on malaria risk in infants. Therefore we aim at
3233analyzing the effect of lead levels on malaria risk with regard to both the possibility of having
3234a positive smear and their effect on *P.falciparum* parasite density taking into account
3235hematological and parasitological factors.

3236Materials and methods

3237Data used in this study were obtained from two-hundred and three infants who were followed
3238from birth until 12 months of age in two embedded studies: the APEC study (Anemia in
3239Pregnancy: Etiology and Consequences) and the TOVI study. More precisely, a prospective
3240cohort of 400 infants was followed from birth to 12 months of age in the context of the APEC
3241study (Anaemia in Pregnancy: Etiology and Consequences). APEC study is an ancillary
3242survey nested within the MiPPAD study in Benin (Malaria in Pregnancy Preventive
3243Alternative Drugs “<http://clinicaltrials.gov/ct2/show/NCT00811421>”). This study was
3244conducted in three clinics in the district of Allada (Allada, Attogon, Sékou), between January
32452010 and May 2012. Allada is a semi-rural area of 91,778 inhabitants located 50 km North of

3246Cotonou (Benin). Malaria has a perennial transmission pattern with two transmission peaks
3247corresponding to the rainy seasons in April-July and October-November. *Plasmodium*
3248*falciparum* is the species responsible for the majority of infections. Complete details of
3249MiPPAD are presented elsewhere [12], but, briefly, MiPPAD was a randomized trial
3250comparing the efficacy and safety of intermittent preventive treatment in pregnancy (IPTp)
3251with sulphadoxine pyrimethamine (SP) (1,500/75 mg per dose) and mefloquine (15 mg/kg per
3252dose). At delivery placenta was examined in order to analyse *P. falciparum* parasite
3253infestation. All live born children of recruited pregnant women who survived to 12 months
3254were invited for neurocognitive assessment in the TOVI study, which evaluated the children
3255for cognitive and motor functions using the Mullen Scales of Early Learning as well as their
3256lead levels at 12 months [13]. The 203 infants of the sample correspond to the infants for
3257whom data at 12 months include a complete follow-up of lead and malaria indicators.

3258Clinical data of the infants were collected at systematic visits at 6, 9, and 12 months. After
3259delivery, in any case of sickness, infants were accurately examined and when necessary,
3260treated in unscheduled visits. All drugs prescribed to the infants during the follow-up were
3261free of charge. During the visits, anthropometric measures and clinical examination were
3262realized. Eight milliliters of venous blood were collected at each visit. Hemoglobin, serum
3263ferritin, CRP, vitamin B12, and folate levels were thereby assessed. At 12 months, lead levels
3264were also determined. A container was also given to the women to collect stools to examine
3265the presence of intestinal helminths in the infants. Microbiological exams were realized as
3266follows: Lambaréné technique was used to assess malaria infection on thick blood smears
3267[14]. To assess parasite density (in parasites/ μ L), a multiplication factor was applied to the
3268average parasitaemia/field. Helminthic infestations were assessed using the Kato-Katz
3269concentration method (VestergaardFrandsen kit®). Iron deficiency is defined according to
3270WHO standards by serum ferritin levels $<15\mu\text{g/l}$ [15].

3271 With regard to BLL, eight milliliters (ml) of venous blood were obtained from each
3272 participant, of which 4 ml were collected into a tube containing dipotassium EDTA and 4 ml
3273 into an iron-free dry tube. Blood samples were analysed at the Centre de Toxicologie, Institut
3274 National de Santé Publique du Québec (Québec, Canada), by inductively coupled plasma
3275 mass spectrometry (ICP-MS; Perkin Elmer Sciex Elan DRC II ICP-MS instrument) prior to
3276 20 fold dilution in ammonia 0.5% v/v and 0.1 % v/v surfactant Triton-X. The limit of detection
3277 for blood sample analysis was 0.2 µg/l.

3278 Because of the anopheline breeding cycle, the mean rainfall of the 7 days prior to the two
3279 weeks before the consultation was calculated. It was independently assessed for each health
3280 centre of the district of Allada.

3281 Socio-economic status was assessed using a socio-economic index created in a two-step
3282 process. First all socio-economic items (home possession of latrines, electricity, a refrigerator,
3283 a television, a vehicle with at least two wheels, being married, and working outside the home)
3284 were plotted into a multiple correspondence analysis. Then, two predictors were created to
3285 synthesize the information, and as the first captured the large majority of the information, it
3286 was withheld as the socio-economic index.

3287 Statistical analysis

3288 Data were double entered and analysed with ACCESS2003 and STATA12.0 softwares for
3289 Windows (Stata Corp, College Station, TX, USA). Univariate analysis was realized to assess
3290 the association of all variables with either the infant positive smear or peripheral *P.falciparum*
3291 density at the moment of lead assessment (at 12 months of age). Thereafter, all variables with
3292 P values < 0.2 were included in a multivariate model regression. Logistic regression was used
3293 to evaluate the determinants associated with a positive blood smear. Linear regression was
3294 used for the multivariate analysis of *P.falciparum* parasite density. Socio-economic status was

3295 forced into the model because of its known association with lead levels according to the

3296 literature [11]. The statistical significance in the final multivariate models was set to $P < 0.05$.

3297 Ethical considerations

3298 This study and the consent procedure regarding the women and their offspring were approved

3299 by the Ethics Committee of the Faculty of Medicine of Cotonou, Benin. It was explained in

3300 local language to the participant and her voluntary written consent was obtained and recorded

3301 in the clinic files before enrolment. In case the woman could not read, an impartial witness

3302 was included in the process. In the case of the inclusion of minor women, both their consent

3303 and the consent from the parents or legal guardians were obtained. Women were free to

3304 interrupt their participation at any time of the study.

3305 **Results**

3306 The BLL of 203 infants included in the APEC-cohort were obtained at the 12-month visit

3307 between April 2011 and May 2012. During the 12-month follow-up 84 infants (42%) had at

3308 least one malarial episode. More precisely, 60.25% of infants had no positive blood smear

3309 during the entire follow-up period, 22% of infants had 1, 12.50% had 2, 4.5% had 3, and

3310 0.75% had 4 positive blood smears during follow-up. The main malarial and hematological

3311 indicators as well as lead levels are presented in table 1. At the moment of lead assessment, 25

3312 out of 200 (12.5%) of the infants had a positive blood smear, with a mean parasite density of

3313 13460 (CI: 2775; 24145). Lead levels were high overall. The mean BLL of infants was 7.41

3314 $\mu\text{g/dl}$ (CI: 65.2; 83), and 128 infants (63%) had elevated blood lead levels, defined by the

3315 CDC as $\text{BLL} > 5 \mu\text{g/dl}$. Lead poisoning, defined as $\text{BLL} > 10 \mu\text{g/dl}$, was found in 39 infants

3316 (19%). With regard to the hematological indicators, 144 infants were anaemic (71%, $\text{Hb} < 110$

3317 g/l), and 85 were iron deficient (42%, CRP-corrected serum ferritin (SF) $< 15 \mu\text{g/l}$). The mean

3318 and median hemoglobin and ferritin values were 101.69 g/l and 104 g/l (CI: 99.51; 103.86),

3319 and 571 mg/l and 201.5 mg/l (CI: 429.67; 712.34), respectively.

3320 At 12 months, ferritine, folate, and CRP levels as well as rain quantity and low socio-

3321 economic status were associated in univariate analysis with increased malaria risk with regard

3322 to both the risk of having a positive smear and *P. falciparum* parasite density. In parallel,

3323 hemoglobin and lead levels were inversely correlated with malaria risk in univariate analysis.

3324 In univariate and multivariate analyses, girls were significantly less likely to have high *P.*

3325 *falciparum* parasitemia but there were no statistical differences in malaria risk between boys

3326 and girls with regard to the probability of having a positive smear. There were no statistical

3327 significant differences in malarial, lead, or hematologic indicators depending on the health

3328 care centre. Table 2 and table 3 describe risk factors associated with the possibility of having

3329 a positive blood smear and high *P. falciparum* parasitaemia with lead levels as a continuous

3330 variable, whereas table 4 and table 5 include the possibility of having elevated BLL.

3331 In multivariate analysis, high lead levels were significantly associated with reduced risk of a

3332 positive blood smear (p-value=0.02) and *P. falciparum* parasite density (p-value=0.048) in

3333 logistic and linear regression models, respectively. More precisely, no positive blood smear

3334 was found among infants with lead poisoning, and infants with elevated BLL (table 4) were

3335 significantly less likely to have a positive blood smear and a high *P. falciparum* density

3336 (aOR=0.38 95% CI (0.15; 0.99), and Coefficient=-0.44, p-value=0.03, respectively). Factors

3337 associated with increased malaria risk include high iron and folate levels and ongoing

3338 inflammatory process. In effect, elevated ferritin levels (log of ferritin corrected on

3339 inflammation) were associated with increased risk of a positive blood smear (aOR=2.46 (1.01;

3340 6.05), p-value=0.05). In addition, high folate levels were statistically associated to an

3341 increased *P. falciparum* parasite density (coefficient=0.0003, p-value=0.04).

3342 Discussion

3343The high proportion of infants with elevated BLL (63%) and lead poisoning (19%) plead for
3344the necessity of considering the possible influence of lead levels on the infant infectious
3345morbidity, especially with regard to malaria, the main cause of mortality in children<5 years.
3346In effect, high BLL were significantly associated with reduced malaria risk with regard to
3347both the possibility of having a positive blood smear and *P. falciparum* density. Concern has
3348been repeatedly raised up on the importance of alarmingly high anemia rates in West Africa
3349[16], and both malaria and elevated BLL are associated with increased anemia rates.

3350Similar prevalence of elevated BLL has been found in other West-African regions. The mean
3351BLL value for this study (7.4 µg/dl) is slightly lower than the mean BLL found by Nriagu et
3352al in Nigeria in 2008[11] (8.9 µg/dl). In Jos, Nigeria, another study reported an average BLL
3353of 11.2 µg/dl (range: 9.1–13.3 µg/dl), and that 55% had BLLs above 10 µg/dl [17]. Indeed,
3354the existing epidemiological evidence reveals the high prevalence of elevated BLL among
3355African infants. However, there is very limited evidence on their effect on malaria. Nriagu et
3356al described the inverse association of BLL and malaria in univariate analysis
3357(pvalue=<0.001). Our results not only confirm this association in univariate analysis, but they
3358are the first to evidence the significant effect of BLL on malarial risk. Furthermore, these
3359results show that elevated BLL are also associated with reduced probability of a positive
3360blood smear as well as reduced *P.falciparum* parasite density. As a consequence,
3361epidemiological evidence in our study rejects the possible synergistic effect of lead on
3362*P.falciparum* infection, but rather suggests a protective effect. Moreover, the high BLL
3363present in our sample raise concern on their possible harmful consequences for the infant
3364health.

3365The mechanism by which lead might influence malaria infection has not been elucidated so
3366far. However, Nriagu postulated that there are multiple levels at which lead can modulate the
3367specific host response to *Plasmodium* infection including alterations in heme synthesis,

3368immunoregulation, and iron metabolism.

3369Lead concentrates in red blood cells (RBC) in the context of lead poisoning [18]. The
3370accumulation of lead in the RBC, the main nutrition source of *Plasmodium*, may inhibit the
3371development of the parasite. Elevated intra-erythrocytic concentration of lead may interfere
3372with the development from the ring form to the schizont stage and, consequently, lead
3373exposure may be associated to reduced parasitemia in malaria-infected infants.

3374In addition, elevated BLL can exert a general effect on the immune regulatory
3375function[19,20]. In this respect, both lead poisoning and malaria favor the cytokine response
3376which, in turn, has an influence on the Th1/Th2 balance [21,22]. Indeed, a certain protection
3377against severe malaria has been described as a consequence of the Th2 response following the
3378alteration of the immune system induced by lead poisoning [23].

3379Alternatively, iron deficiency and hemoglobinopathies can foster the anti-parasite effect of
3380lead in the context of the blood stages of *P. falciparum*. Indeed, iron deficiency may interfere
3381with the proper use of iron by the parasite [24]. However, iron deficiency was not
3382significantly correlated with malaria risk in our analyses. Finally, high intra-erythrocyte lead
3383concentration can inhibit protein synthesis [25], and thereby interfere with the correct iron
3384utilization by *Plasmodia* [24].

3385With regard to iron levels, high iron levels have already been associated with increased
3386malaria morbidity [26]. This raises the concern on the iron supplements recommended by the
3387WHO when anemia prevalence >40%, which is the case of Benin. Furthermore, published
3388literature reports both iron and lead have a significant effect not only on malaria, but also on
3389anemia. Indeed, strategies to tackle anemia should consider not only iron supplementation but
3390public health policies should also imply the sources of elevated BLL.

3391Low folate levels are also associated to anemia. Nevertheless, high folate levels are associated

3392with high *P. falciparum* parasitemia as well. The significant role of high folate levels for
3393increased risk of *P. falciparum* parasite density is coherent with the literature. Indeed, anti-
3394folates are one of the most anti-malarial drugs used worldwide. Finally, boys displaying
3395higher *P. falciparum* parasite density than girls has already been described[27], may be due to
3396their higher exposure because of socio-behavioural habits.

3397**Conclusion**

3398Malaria is the main cause of mortality for infants under 5 years worldwide[10], and high iron
3399levels have been associated to increased malaria risk[26]. Therefore, in the context of limited
3400protection against malaria, iron supplements to fight anemia might entail some deleterious
3401effect for *P. falciparum* infections. As a consequence, giving supplements to infants that do
3402not need them might be harmful for their health status.

3403However, iron supplements are crucial to fight anemia especially in the context of elevated
3404BLL[28]. This is of special relevance, as iron deficiency is associated to increased lead
3405absorption[29]. Lead poisoning is the 6th most important contributor to the global burden of
3406diseases measured in disability adjusted life years (DALYs) according to the Institute of
3407Health Metrics, with Sub-Saharan African countries being predominantly responsible for the
3408global DALYs[30]. Lead poisoning entails severe consequences for the development of the
3409children and is associated with major health problems highly prevalent in West Africa, such
3410as anemia, having an important impact on the infants and their communities. In addition, iron
3411is essential for the neurocognitive development of the child brain.

3412In conclusion, environmental factors, such as lead levels, need to be considered in the debate
3413about iron supplements in malaria endemic countries.

3414

3415Acknowledgements:

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3416Elizabeth Lim read and edited the manuscript making valuable linguistic corrections. We also thank the
3417MiPPAD executive committee and MiPc reviewers for valuable input in this work. We thank the women who
3418participated in the study. We also thank the health workers of the district of Allada and their assistants for their
3419help in conducting this study.

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3423Tables:

Table 1. Clinical characteristics of the infants: malaria indicators and blood lead levels

Parameters	Mean or number of people affected
Malaria infection (%)	25 (12.5%)
<i>P.falciparum</i> density (parasites/ μ L)	13460 (CI:2775; 24145)
Blood lead levels (μ g/l)	74.1 (CI: 65.2;83)
Elevated blood lead levels (BLL>5 μ g/dl)	128 (63.05%)
Lead poisoning levels (BLL>10 μ g/dl)	39 (19.21%)
Haemoglobin (g/l)	101.69 (CI: 99.51; 103.86)
Anaemia (Hb <110 g/l)	144 (70.94%)
Ferritin (mg/l)	571 (CI: 429.67; 712.34)
Iron deficiency (corrected SF <15 μ g/l)	85 (42.93%)

Table 2. Logistic regression on the possibility of having a positive blood smear at 12 months

Factor	aOR (95% CI)	p-value
Blood lead levels (μ g/l)	0.98 (0.96; 0.99)	0.02
Ferritin levels (logarithm of the ferritin (mg/l) corrected on inflammation)	2.46 (1.01; 6.05)	0.05
Low socio-economic index	1.52 (0.95; 2.45)	0.08

Prob>chi2=0.0002 Number of observations=197

3424

**Table 3. Linear regression on factors associated with *P.falciparum* parasitemia
(logarithm of parasite density at lead assessment)**

Factor	Coefficient (95% CI)	p-value
Blood lead levels	-0.003 (-0.006; -0.0001)	0.04
Ongoing inflammatory process (CRP levels \geq 5 mg / ml)	0.72 (0.31; 1.14)	0.01
Ferritin levels (logarithm of the ferritin corrected on inflammation)	0.25 (-0.12; 0.61)	0.19
Folate levels (ng/ml)	0.0003 (0.0001; 0.006)	0.04
Sex of the infant (female)	-0.44 (-0.81; -0.06)	0.02
Low socio-economic index	0.04 (-0.14; 0.22)	0.64

Prob>F=0.00 Number of observations=196

Table 4. Logistic regression on the possibility of having a positive blood smear at 12 months with elevated BLL

Factor	aOR (95% CI)	p-value
Elevated blood lead levels ($\mu\text{g/l}$)	0.38 (0.15; 0.99)	0.048
Ferritin levels (logarithm of the ferritin (mg/l) corrected on inflammation)	2.86 (1.13; 7.27)	0.03
Low socio-economic index	1.42 (0.87; 2.32)	0.16
Inflammatory process (CRP levels ≥ 5 mg / ml)	3.09 (1.2; 7.93)	0.02

Prob>chi2=0.0005 Number of observations=197

**Linear regression on factors associated with *P.falciparum* parasitemia at 12 months with elevated BLL
(logarithm of parasite density at lead assessment)**

Factor	Coefficient (95% CI)	p-value
Elevated blood lead levels	-0.44 (-0.84; -0.04)	0.03
Inflammatory process (CRP levels ≥ 5 mg / ml)	0.65 (0.23; 1.06)	<0.01
Ferritin levels (logarithm of the ferritin corrected on inflammation)	0.22 (-0.15; 0.59)	0.24
Folate levels (ng/ml)	0.0004 (0.0001; 0.006)	0.02
Low socio-economic index	0.06 (-0.13; 0.24)	0.56

Prob>F=0.00 Number of observations=196

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VI. Discussion

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3521 **VI.1. Effect of preventive public health interventions during** 3522 **pregnancy on pregnancy associated malaria**

3523 **IV.1.1. Effect of IPTp on PAM outcomes**

3524 PAM has not a single dimension. It entails different clinical manifestations that depend, as
 3525 said in the second section, on transmission, immunity, and preventive strategies. Clinical
 3526 malaria, high parasite density, LBW, and placental malaria are the main symptoms of the
 3527 infection by *Plasmodium*. Therefore, a holistic analysis that includes every outcome issue of
 3528 PAM, should lead us to contemplate the multiple different dimensions of the protective effect
 3529 of IPTp.

3530 **IV.1.1.a. Effect of transmission and previous immunity**

3531 An initial approach of the results requires acknowledging the differences among women with
 3532 regard to transmission and immunity. Differences in transmission have been estimated by
 3533 rainfall, which is a useful tool to account for *anopheline* risk. Rainfall varies significantly
 3534 throughout the follow-up period. Nevertheless, it is not associated with malaria risk during
 3535 pregnancy nor with placental malaria or LBW. The possible effect of rain might be mitigated
 3536 by the effect of previous immunity. Indeed, there is some evidence suggesting a certain
 3537 protection for multiparous women because of the immunity due to previous gestations. In
 3538 effect, in univariate analysis, gravidity was associated with the age of the mother, BMI, socio-
 3539 economic status, number of positive blood smears, PM and LBW. In this respect, infants of
 3540 primigravid women will be possibly at higher risk for subsequent malaria as a result of
 3541 reduced antibody transfer, even if one study has shown the opposite results in women with no
 3542 infected placenta. As shown in the results section, the mean of positive blood smears during
 3543 pregnancy was significantly higher for primi- and secundigravidae than for multigravidae. In
 3544 addition, the percentage of women with placental malaria decreased significantly as gravidity

increased and the proportion of LBW babies was also inversely correlated with gravidity. However, gravidity was not significant in the multivariable analysis of positive blood smears and parasite density when considering maternal age. Furthermore, some studies suggest that age has an independent effect on immunity regardless of gestation. Therefore, the analyses include maternal age, as we estimated it was a better estimator for malaria risk in our study than gravidity.

IV.1.1.a. Effect of IPTp: absolute reduced risk, IPTp regime, and IPTp calendar

As there was no placebo group in our study, it is not possible to evaluate the absolute efficacy of IPTp. However, we can comment on the evolution of malarial risk after IPTp implementation. The proportion of women with a positive smear decreased after IPTp (from 15.3% at ANV1 to 3.9% at ANV2), and then increased again up to 9.6% at delivery. Nevertheless, the trend was slightly different concerning parasite density. *P.falciparum* parasite density was higher at ANV1 than at ANV2 (382.4, SD=3709.2 and 214.1, SD=2728.5 parasites/ μ L, respectively) but then rose up to 3098.8, SD=31120.7 parasites/ μ L at delivery. Indeed, 2-dose IPTp seems to be effective after the first dose but its protection is not long enough to control parasitemia at delivery.

Concerning parasitemia at delivery, in our Beninese sample there were no statistical differences between women who had received SP and women who had received MQ IPTp (RR=0.73, 95% CI (0.51; 1.06), p-value=0.14). Furthermore, neither there were significant differences among women with SP and MQ IPTp regimes with regard to LBW, nor with regard to placental malaria. More precisely, the adjusted RR for LBW was =1.06, 95% CI (0.7; 1.54), p-value=0.77) and RR for placental malaria was= 0.74, (95% CI (0.52; 1.06), p-value =0.10). Our study was a sub-study of the MiPPAD clinical trial, in which 4,749 pregnant women were enrolled in an open-label randomized clinical trial conducted in Benin, Gabon, Mozambique, and Tanzania comparing 2-dose MQ or SP for IPTp and MQ

tolerability of two different regimens. The multi-centre analyses show women receiving MQ had reduced risk of parasitemia (63/1,372 (4.6%) in the SP group and 88/2,737 (3.2%) in the MQ group (RR=0.70, 95% CI (0.51; 0.96), p-value =0.03), and reduced incidence of clinical malaria (96/551.8 malaria episodes person/year (PYAR) in the SP group and 130/1,103.2 episodes PYAR in the MQ group (RR=0.67, 95% CI (0.52; 0.88), p-value =0.004). In our sub-study in Benin women receiving the SP-IPTp regime had on average 0.5 positive blood smears whereas women receiving MQ had on average 0.54. Nevertheless, this difference is not statistically significant and MQ has been already found to be more effective against PAM than SP in other studies in Benin.

IPTp timeframe also seems to influence PAM outcomes. According to Huynh et al. an early intake of the first SP dose (up to 4 months of gestation) was associated with a lower risk of LBW compared to a late intake (6-7 months of gestation) (aOR= 0.5, p-value = 0.01) in an observational cohort of pregnant women. Even if our results show similar trends in the association of early IPTp and LBW in univariate analysis, we do not obtain the same significant results in multivariate analysis, when considering other risk factors, such as other important clinical outcomes, environmental indicators and obstetric parameters. Furthermore, we do not see a significant association of IPTp timing with positive blood smear during pregnancy, *P. falciparum* parasitemia or placental malaria.

In conclusion, we do not see differences in malarial risk, parasite density, LBW or placental malaria between IPTp regimes and IPTp timing. Additional epidemiological evidence concerning the effect of IPTp timing in PAM is required to conclude to consistent recommendations. Consequently, IPTp clinical trials should analyse the effect of different timing of IPTp on PAM outcomes beyond LBW and anemia at delivery.

VI.1.2. Effect of iron levels on PAM outcomes

As presented in the state of art section, iron levels are of crucial interest for public health strategies during pregnancy. Indeed, iron supplements alleviate anemia, but they might also trigger infectious agents to develop. Hence, the analysis of iron levels and the simultaneous co-infections need to be analysed in a prospective, longitudinal manner in order to capture the dynamics of the process and to be able to evaluate iron levels at the precise moment when infection takes place.

VI.1.2.a. Complementary aspects of the analysis of iron I: a foreword on ferritin and inflammation

As stated in the second section, ferritin is a consistent marker of iron levels. However, ferritin is also an acute phase protein and it is associated with the inflammatory response, which, in turn, increases in the context of malaria. Indeed, infections entail the activation of the inflammatory response and CRP levels increase as a consequence. To attenuate the interference of inflammation on ferritin values, we corrected ferritin upon inflammation (with correction factors according to CRP) following the correction suggested by Thurham meta-analysis. Conversely, there is substantial scientific evidence that significant differences in the inflammatory response of the individual will determine the development and severity of malaria. Kabyemela et al. showed that inflammatory status at birth (before any malaria infection) predicts malaria severity during infancy. Wilson et al. have also shown in pregnant women that elevated levels of IL-10 and G-CSF are associated with asymptomatic malaria. Furthermore, the results of Perera et al. suggest that the high circulating TNF-alpha levels and the inadequate IL-10 response in severe malaria patients carrying TNF2 allele could contribute to the development of severe falciparum malarial disease. Previously, May et al. had shown that plasma Interleukin-10 Tumor Necrosis Factor (TNF)— α ratio was associated with TNF promoter variants and predicted malarial complications. This was confirmed by Zhang et al., who described that interleukin-10 (IL-10) polymorphisms are associated with IL-10 production and clinical malaria in young children. CXCL9 expression is induced by IFN- γ ,

and the strong association between birth weight and placental CXCL9 is consistent with previous observations relating IFN- γ to poor pregnancy outcomes. For these reasons, although being aware of the risk of over-adjusting, we decided to include inflammatory status in the model to take into account the different degree of inflammatory response that might be associated with different malaria clinical severity. This might account for the different degree of inflammatory response the individual would develop, which is specific at the individual level. This is one of the reasons for setting an individual intercept at the individual level in the model.

VI.1.2.b. Epidemiological evidence

We analysed the association of iron levels with malarial risk in a prospective longitudinal cohort through pregnancy considering both the possibility of having a positive blood smear and *P.falciparum* parasite density. Indeed, iron levels, measured by ferritin corrected for inflammation, were significantly associated with malarial episodes and *P.falciparum* density through the pregnancy period in the context of IPTp and ITN use. Furthermore, this association is strongly significant even after adjustment on inflammatory status. Moreover, iron levels are significantly associated with placental malaria even after adjustment on maternal infection. Literature shows PM is associated with increased infant's susceptibility to the infection translating into increased number of clinical episodes. Consequently, the association of high iron with placental malaria might contribute to enhance its effect on malaria risk throughout the perinatal period. Finally, the association of maternal iron levels with LBW, possibly due to their relationship with PAM, suggests a broader impact of iron on infant health. Further details on the evolution of iron levels and anemia during pregnancy in this cohort are presented by Ouédraogo et al., but briefly, iron deficiency conferred protection against malaria through the entire follow-up. However iron levels were no longer associated with *P.falciparum* parasite density among iron deficient women, which suggests the possible existence of a threshold level above which iron levels become deleterious. Indeed there was

significant increased malarial risk above 30 days of supplementation in the stratified analysis of two African surveys with high antimalarial preventive measures (RR=1.42, 95% CI (1.09; 1.84)).

Our results are consistent with other studies. Although iron supplementation trials do not show augmented malaria morbidity associated with iron supplements, iron deficiency is correlated with lower odds of malarial episodes. Iron deficiency was statistically linked to reduced risk of placental malaria in Tanzania. Ferritin was also higher among placenta-infected mothers in Gabon and zinc protoporphyrin in Malawi, but these differences were not statistically significant. Similar results were found in clinical trials in The Gambia or Kenya. The recent meta-analysis on malarial risk and iron status suggested a possible but not significant difference in placental malaria associated with iron supplementation depending on sickle cell genotype. However, as already said, these studies report iron levels only at enrolment, at delivery, or both, and the limited sample might be insufficient to show a statistically significant effect.

Possible explanations for the increased malarial risk associated with iron levels found in our study are related to malaria pathophysiology in both the host and the parasite. At the host level, *Plasmodium* interferes with the physiological iron distribution and use through hemolysis, release of heme, dyserythropoiesis, anemia, deposition of iron in macrophages, and inhibition of dietary iron absorption. Furthermore, the changes in iron metabolism during a malaria infection may modulate susceptibility to co-infections. In addition, iron inhibits the synthesis of nitric oxide by inhibiting the expression of inducible nitric oxide synthase (iNOS), and thereby interferes with macrophage-mediated cytotoxicity against *Plasmodium*. Moreover, non-transferrin bound iron (NTBI) is involved in the severity of malaria. Indeed, *Plasmodium* has the capacity of acquiring iron in a transferrin-independent pathway(42).

VI.1.2.c. A comment on the specific characteristics of the individuals and their evolution

3672during pregnancy

3673The individual particularities of each pregnant woman and the physiopathological evolution
 3674of iron levels within the different periods of pregnancy need to be considered in the analysis
 3675of the association of iron levels with malarial risk. Therefore, a well-defined and concrete
 3676statistical approach is necessary: the multilevel model analysis. Multi-level models are
 3677particularly suited to the statistical analyses of prospective cohorts with repeated measures at
 3678the individual level, as they can take into account the specificities of each individual at
 3679different time measures.

3680In order to treat the evolution of iron levels, we planned a multi-step statistical analysis. First,
 3681we assessed the association of iron with malaria risk by trimester, and high ferritin was
 3682significant in the observations of the different trimesters with regard to both the possibility of
 3683having a positive smear and also *P.falciparum* parasite density. Thereafter, in the context of
 3684the multilevel analysis we included a categorical variable to account for the specific
 3685pregnancy trimester of each observation and this variable was not significant. Ferritin levels
 3686may also differ from one trimester to the other also because women take iron supplements.
 3687Indeed, when we conducted the univariable analyses on the association between ferritin and
 3688the possibility of a positive blood smear or parasite density for each visit, the only case in
 3689which ferritin levels and a positive blood smear were not significantly associated (p-
 3690value=0.07), still borderline, was in the visit following the iron supplements. However, the
 3691association between ferritin levels and parasite density for this same visit, i.e. before iron
 3692intake, was statistically significant (p-value=0.003). Then we tested the link between ferritin
 3693levels and gestational age and their association was significant, possibly due to the timing of
 3694the supplements.

3695With regard to the specific characteristic inherent to the physiopathology of each individual,
 3696including its own immunity, we decided to use multilevel models with a random intercept at

the individual level. More precisely, for both the analysis of the possibility of a positive blood smear and for the analysis of parasite density, random beta estimate models were used as they were statistically better than fixed effects according to AIC and BIC criteria. The Akaike information criterion (AIC) and the Bayesian information criterion (BIC) compare maximum likelihood models. More precisely, AIC and BIC are defined as: $AIC = -2 \cdot \ln(\text{likelihood}) + 2 \cdot k$, and $BIC = -2 \cdot \ln(\text{likelihood}) + \ln(N) \cdot k$, where k = number of parameters estimated, N = number of observations. AIC and BIC can be viewed as measures that combine fit and complexity. Fit is measured negatively by $-2 \cdot \ln(\text{likelihood})$; the larger the value, the worse the fit. Complexity is measured positively, either by $2 \cdot k$ (AIC) or $\ln(N) \cdot k$ (BIC).

In conclusion, random intercept was applied in both cases at the individual level and random slope was applied to gestational age, as the effect of the variables might differ between women and the effect of gestational age might also vary differently according to the timing of the measure. Certain variables were forced into the model because of their meaning in the analyses according to the literature: socio-economic status and rainfall in the case of malarial indicators, and BMI in the case of LBW.

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VI.2. Effect of preventive public health interventions on malaria in infants: the determinant print?

Public health interventions aim at improving the health status of the individuals and to avoiding the disease consequences. Pregnancy is a particular period in which the women are at special risk and the effect of diseases can harm both the mother and the foetus. Therefore, special caution needs to be paid when implementing any public health intervention during this critical period because of the possible long-term consequences.

VI.2.1. Effect of IPTp on malaria in infants

The case of pregnancy associated malaria is particularly sensible, as women are increasingly susceptible to malaria infection during pregnancy since *Plasmodium falciparum*, the most common parasite responsible for malaria, avoids spleen clearance through expression of proteins that bind to the chondroitin sulphate A (CSA) in the placental intervillous space. Consequently, the foetus is initially exposed to the effects of PAM *in utero*. Indeed, there is substantial epidemiological evidence that placental malaria is associated to increased susceptibility to malaria during infancy, possibly due to an ongoing immune tolerance process. Hence, it is reasonable that IPTp interventions, which have an impact on malaria parasitemia, would modify the immune tolerance process and thereby have an effect on malaria in infants.

VI.2.1.a. Epidemiological evidence

Indeed, our results show that IPTp has a significant effect on malaria severity in infants during the first year of life considering both the possibility of having a positive blood smear and *P.falciparum* parasite density.

PAM has been frequently correlated to an impaired health status of the offspring¹⁰. We found that the period of time between IPTp doses, i.e. the number of days between IPTp doses, is inversely correlated to malaria risk. When the period of time between IPTp doses is longer, infants have significantly reduced risk of malaria during the first year of life.

Albeit their novelty, our results are coherent with the existing literature, that suggests that IPTp in general might be associated with malaria risk in the infant. Indeed, Borgella found infants born to a mother with PAM during the third trimester of pregnancy had a significantly increased risk of infection (OR=4.2, 95% CI (1.6; 10.5), p-value = 0.003) or of malaria attack (OR=4.6, 95% CI (1.7; 12.5), p-value = 0.003). In addition, Huynh found IPTp calendar is associated with secondary malaria indicators like LBW and anaemia. More precisely, at the beginning of pregnancy, peripheral infections were associated with a decrease in mean birth

weight (-98.5 g; p-value = 0.03) and an increase in the risk of anemia at delivery (aOR = 1.6; p-value = 0.03). Infections in late pregnancy were related to a higher risk of maternal anemia at delivery (aOR = 1.7; p-value = 0.001). Considering that PAM has a significant effect of malaria in infants and that IPTp has an impact on secondary malaria outcomes, such as LBW and anaemia, our results are consistent with those of other studies. The only discordant study is the cohort followed by Harrington in NE Tanzania. Surprisingly, IPTp was also associated with earlier first malaria episode among mothers with placental malaria and increased overall odds of severe malaria among all offspring in the cohort. However, there is a strong resistance against SP IPTp in NE- Tanzania, and the same team has shown that IPTp in this area is ineffective. In addition, women with placental malaria in this population, IPTp was associated with increased drug resistance alleles, placental parasite density, and inflammation. These findings are consistent with parasite competitive facilitation, a phenomenon where drug pressure eliminates drug susceptible parasites, allowing drug resistant parasites to overgrow. Indeed, the association between IPTp and time to first parasitemia was restricted to offspring of women with placental malaria, which suggests that the discordant results might be due to the ineffective IPTp, and would speak in favor of the immune tolerance hypothesis suggested by our results.

Indeed, Dechavanne found in Benin increased susceptibility of infants to *P. falciparum* parasites with antigens to which they were previously exposed in utero, suggesting the existence of an in utero ongoing immune tolerance process. However, no evidence exists at present on its concrete physiopathological pathways. Following these results, an adjustment of IPTp calendar to enhance protection would be welcome in Benin. In effect, this intervention has already been recommended by WHO, which has recently outlined the convenience of a more frequent IPTp regime.

However, it is interesting to note that IPTp extent has an impact on malaria in infants whereas it has no effect on malaria clinical signs during pregnancy. Despite surprising, this might be

explained by the following reasons: first, the majority of women are multiparous, and even if they were not, clinical symptoms associated with PAM are rare or mild in this population. Hence, women might not come to the health centre during malaria episodes and we might have lost enough observations that could corroborate the association. Second, a recent article has shown that submicroscopic parasitemia has an impact on LBW, prematurity and maternal anemia but not with maternal malaria episodes. IPTp might clear numerous parasites and be effective enough to reduce PAM clinical episodes, but its protective effect on the infants might not last during the entire pregnancy period. During the time during which IPTp is no longer effective, even submicroscopic parasitemia might have an effect on the *in utero* exposition to the parasite and thereby an impact on immune tolerance and, consequently, on malaria in infants. Therefore, the extent of IPTp, i.e. the number of days between doses, which prolongs the time during which the foetus is protected, might entail a certain protection for the infant even if no significant protection is detectable on maternal clinical malaria.

VI.2.2. Effect of the infant iron levels on malaria in infants

As explained, the analysis of iron levels is really complex. Therefore, a consistent and multi-technical statistical approach was necessary. More precisely, we followed the same analytical approach that we used to analyse the maternal malaria risk.

VI.2.2.a. Statistical approach

First of all, exploratory and univariate analyses were realized to assess the association of all variables with both infant positive smear and peripheral *P.falciparum* density at each visit (systematic or unscheduled visit). Chi-squared and Kruskal-Wallis tests were used in the univariate analyses. When variables had several measures evolving during follow-up, univariate analyses were realized using a multilevel model with a random intercept at the infant level, as each infant has its own immunological, clinical and obstetric background. Thereafter, all variables with *P* values < 0.2 were included in a multivariate multilevel model

with a random intercept at the infant level, and considering all visits for each infant. Multilevel models with a random intercept at the infant level were applied to explore the determinant of both the possibility of having a positive smear and peripheral *P.falciparum* density, respectively. More precisely, random intercept was applied in both cases at the individual level and random slope was applied to the infant age, as the effect of the variables might differ among infants and the effect of the infant age might also vary differently according to the timing of the measure. The statistical significance in the final multivariate models was set to $P < 0.05$.

VI.2.2.b. Epidemiological evidence

We have assessed the influence of iron levels on malarial risk throughout the first year of life with regard to the possibility of having a positive blood smear and *P.falciparum* parasite density, considering environmental, socio-economic, and PAM factors, such as placental malaria or gestational age. Indeed, iron levels, measured by ferritin corrected for inflammation, a consistent indicator of iron levels, were significantly associated with malarial episodes and *P.falciparum* density. Furthermore, like in the case of the mothers, this association was strongly significant even after adjustment on inflammatory status. Iron deficiency conferred protection through the entire follow-up period. More precisely, infants with iron levels in the first quartile were significantly protected against malaria. Indeed, iron deficiency has frequently been linked to a certain protection against malaria. Nevertheless, results on the effect of iron levels on malaria differ in the context of clinical trials with iron supplements. In a specific Cochrane review no significant difference in clinical malaria episodes was detected between iron alone and placebo (RR=0.99, 95% CI (0.90; 1.09)). However, the effect of iron deficiency was not assessed, and solid preventive measures against malaria were implemented in the clinical trials. Indeed, an increased risk of malaria with iron was observed in trials that did not provide malaria surveillance and treatment, and the risk of malaria parasitemia was higher with iron (RR=1.13, 95% CI (1.01; 1.26)).

Furthermore, in numerous studies included in the meta-analysis, iron was seldom determined longitudinally.

Albeit the hereby reported results, iron supplements have undeniable benefits for infants. A 2013 meta-analysis showed supplementation was associated to a reduced risk of anaemia (RR=0.61, 95% CI (0.50; 0.74), n=4825), of iron deficiency (RR=0.30, 95% CI (0.15; 0.60), n=2464), and of iron deficiency anaemia (RR=0.14, 95% CI (0.10; -0.22), n=2145). As pondering the advantages and risk of iron supplements is daunting because they are not epidemiologically quantifiable, the implementation of malaria protective strategies should be seriously encouraged. Indeed, the Cochrane review shows no increased risk of malaria in infants implementing protective interventions.

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VI.2.3. Supplementary factors associated with malaria in infants: the case of lead

VI.2.3.a. Epidemiological evidence

The high proportion of infants with elevated blood lead levels (BLL) (63%) and lead poisoning (19%) plead for the necessity of considering the possible influence of lead levels on the infant infectious morbidity, especially with regard to malaria, the main cause of mortality in children <5 years. In effect, high BLL were significantly associated with reduced malaria risk with regard to both the possibility of having a positive blood smear and *P. falciparum* density. Concern has been repeatedly raised up on the importance of alarmingly high anemia rates in West Africa, and both malaria and elevated BLL are associated with increased anemia rates.

Similar prevalence of elevated BLL has been found in other West-African regions. The mean BLL value for this study (7.4 µg/dl) is slightly lower than the mean BLL found by Nriagu et al in Nigeria in 2008 (8.9 µg/dl). In Jos, Nigeria, another study reported an average BLL of

3848 11.2 µg/dl (range: 9.1–13.3 µg/dl), and that 55% had BLLs above 10 µg/dl. Indeed, the
 3849 existing epidemiological evidence reveals the high prevalence of elevated BLL among
 3850 African infants. However, there is very limited evidence on their effect on malaria. Nriagu et
 3851 al described the inverse association of BLL and malaria in univariate analysis (p-
 3852 value=<0.001). Our results not only confirm this association in univariate analysis, but they
 3853 are the first to evidence the significant effect of BLL on malarial risk. More precisely, these
 3854 results show that elevated BLL are also associated with reduced probability of a positive
 3855 blood smear as well as reduced *P.falciparum* parasite density. As a consequence,
 3856 epidemiological evidence in our study rejects the possible synergistic effect of lead on
 3857 *P.falciparum* infection, but confirms its significant protective effect. Moreover, the high BLL
 3858 present in our sample raise concern on their possible harmful consequences for the infant
 3859 health.

3860 The mechanism by which lead might influence malaria infection has not been elucidated so
 3861 far. However, Nriagu postulated that there are multiple levels at which lead can modulate the
 3862 specific host response to *Plasmodium* infection including alterations in heme synthesis,
 3863 immunoregulation, and iron metabolism.

3864 Lead concentrates in red blood cells (RBC) in the context of lead poisoning. The toxification
 3865 of the RBC, the main nutrition source of *Plasmodium*, may inhibit the development of the
 3866 parasite. More precisely, the elevated intra-erythrocytic concentration of lead may interfere
 3867 with the development from the ring form to the schizont stage and, consequently, lead
 3868 exposure may be associated to reduced parasitemia in malaria-infected infants.

3869 In addition, EBLL can exert a general effect on the immune regulatory function. In this
 3870 respect, both lead poisoning and malaria favor the cytokine response which, in turn, has an
 3871 influence on the Th1/Th2 balance. Indeed, a certain protection against severe malaria has been
 3872 described as a consequence of the Th2 response following the alteration of the immune

3873system operated by lead poisoning.

3874Alternatively, iron deficiency and hemoglobinopathies can foster the anti-parasite effect of
3875lead in the context of the blood stages of *P. falciparum*. Indeed, iron deficiency may interfere
3876with the proper use of iron by the parasite. However, iron deficiency was not significantly
3877correlated with malaria risk in our analyses. Finally, high intra-erythrocyte lead concentration
3878can inhibit protein synthesis, and thereby interfere with the correct iron utilization by
3879*Plasmodia*.

3880However, iron supplements are crucial to fight anemia especially in the context of elevated
3881BLL. This is of special relevance, as iron deficiency is associated to increased lead
3882absorption. Lead poisoning is the 6th most important contributor to the global burden of
3883diseases measured in disability adjusted life years (DALYs) according to the Institute of
3884Health Metrics, with Sub-Saharan African countries being predominantly responsible for the
3885global DALYs. Lead poisoning entails severe consequences for the development of the
3886children and is associated with major health problems highly prevalent in West Africa, such
3887as anemia, having an important impact on the infants and their communities. In addition, iron
3888is essential for the neurocognitive development of the child brain.

3889In conclusion, environmental factors, such as lead levels, need to be considered in the debate
3890about iron supplements in malaria endemic countries.

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VII. Conclusion

VII.1. Effect of pregnancy associated malaria and intermittent

preventive treatment on malaria in infants

The impact of PAM on malaria in infants does not only involve placental malaria, prematurity or LBW. PAM entails increased risk of malaria in infants, possibly due to an ongoing immune tolerance process *in utero*. As a consequence, interventions tackling at PAM have also an effect on malaria in infants. Effective administration of IPTp clears placental parasitemia and consequently modifies the exposure to malaria antigens *in utero* resulting in a significant protection for malarial episodes during infancy. Indeed, the interval between IPTp doses, which might reflect the time during which the foetus might be protected, is associated to a reduced risk of malaria during infancy with regard to both the possibility of having a positive smear and *P. falciparum* parasitemia. However, IPTp timing (the moment of pregnancy when IPTp is given) does not seem to have a significant effect on malaria outcomes of the infant in our study. Moreover, there are no significant differences in malarial risk during pregnancy or infancy depending on the IPTp regime (either SP or MQ).

The new WHO recommendations encourage IPTp with SP for all pregnant women as early as possible in the second trimester, and at each scheduled antenatal care visit at least one month apart in areas of moderate to high malaria transmission seems to improve the previous IPTp schedule (2 doses). IPTp strategies are however not yet completely deployed in malaria endemic regions and due to the insufficient implementation of IPTp the effect of this new policy on malaria in infants might be difficult to evaluate. In any case, the new recommendations are supposed to improve the disease burden associated to PAM.

VII.2. Effect of iron levels on malaria: evidence from pregnant women and infants.

The interaction between iron levels and malaria is daunting because of the iron requirements

during pregnancy and infancy, and because of the fact that iron contributes to *P.falciparum* growth. In addition, this interaction is modified by malaria control interventions. For these reasons it is important to find out whether iron levels are associated with increased malarial risk in a prospective longitudinal cohort in the context of both supplements and IPTp in pregnant women but also in infants.

High ferritin levels are associated with increased malarial risk during pregnancy with regard to malarial episodes and *P.falciparum* parasite density in the context of IPTp and ITN use, even if positive smears diminish effectively after IPTp implementation. In addition, iron levels have also a significant association with important perinatal outcomes like placental malaria and LBW. Our data also suggest there might be a dose effect of iron levels on malarial risk.

Even if infants are not supplemented with iron, malaria risk during the first year of life is also significantly associated with iron levels. High ferritin levels are associated with increased malarial risk throughout the first year of life with regard to malarial episodes and *P.falciparum* parasitemia considering other socioeconomic, environmental and clinical factors. We also find a dose effect of iron levels on malarial risk.

In conclusion, we observe increased malaria risk associated with high iron levels in both pregnant women and infants. Furthermore, we find a certain dose effect of iron levels on malaria risk. This might be considered in the implementation of public health supplement strategies during pregnancy and infancy.

Additionally, we find high folate and lead levels are associated to reduced malarial risk.

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VIII. Perspectives

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3957**VIII.1. The new WHO recommendations on IPTp in the context of** 3958**increasing resistance**

3959The effect on maternal and infant health of the extension by WHO of IPTp regime to a SP-
 3960dose at each ANV needs to be monitored. In theory LBW, prematurity and placental malaria
 3961but also malaria in infants should be carefully analysed to obtain an optimal timing and IPTp
 3962regime to optimize the protective effect of IPTp. However, operational research on the topic
 3963might be difficult to implement on the field, and the resulting data might be difficult to
 3964interpret. Protective strategies regarding iron levels should maybe start during the pre-
 3965conceptional period to better protect both mother and infant. Operational research on different
 3966preventive IPT strategies should also be continuously conducted, and cost effectiveness
 3967analysis for community-level IST interventions should be further investigated, considering as
 3968well that IST has no effect on sub-microscopic parasitemia, which might be troublesome
 3969when targeting the elimination.

3970In addition, as there is evidence of increased infant susceptibility to parasites carrying
 3971antigens to which they were previously exposed while *in utero*, further research should also
 3972tempt to explain the ongoing immune process. Furthermore, the role of protective maternal
 3973antibodies has not yet been clarified. An exploration of the influence of HLA-G
 3974polymorphisms on subsequent malaria symptoms would serve as well as an important
 3975contribution for infant malaria risk factors.

3976Finally, novel aspects of research on PAM should be further explored. Due to the long-term
 3977impact of placental malaria's possible neuro-cognitive consequences, the scientific
 3978community should prioritize studies investigating this interaction.

3979**VIII.2. Iron supplements in malaria endemic settings**

3980The significant association between iron levels and malarial risk in both pregnant women and
 3981infants appeals for additional epidemiological studies. Furthermore, the possible dose effect
 3982of iron levels for malaria risk, advocates for the evaluation of the effect of different doses of
 3983iron supplements on the infant infectious and haematological outcomes. Complementary
 3984interventional data are needed to determine the benefits and risks of differently dosed iron
 3985supplements, in order to ascertain their impact on infant health in malaria-endemic regions.
 3986Finally, the epidemiological comparison of cohorts in which iron is given as preventive
 3987intervention and cohorts in which iron is given solely on the purpose of treatment for anaemia
 3988or ID should be also analysed.

3989With regard to the difficulty of finding a gold standard for iron levels evaluation, a complete
 3990combination of iron markers is desirable. This evaluation should at least include the markers
 3991recommended by the joint WHO-CDC Technical Consultation for anaemia assessment
 3992(hemoglobin, mean cell volume (MCV), serum transferrin receptor (sTfR) concentration,
 3993serum ferritin concentration, and red cell protoporphyrin (measured by the zinc
 3994protoporphyrin/hemoglobin ratio (ZPP:H)) in addition to hepcidin, haptoglobin and
 3995inflammation indicators (C-reactive protein (CRP) and alpha-1-acid glycoprotein (AGP). The
 3996index sTfR/log ferritin adjusted on CRP has also been recommended.

3997In parallel, the association of iron with malaria risk might be different depending on malaria
 3998transmission patterns. Indeed, the dose-dependent effect, might be modified by the differences
 3999in the prevalence of *Plasmodium falciparum*, and this notable aspect has not been evaluated
 4000so far in clinical trials.

4001Finally, if women had sufficient pre-conceptual iron storages, iron supplements might not be
 4002necessary during pregnancy and the supplementary risk of adding iron, which could be used
 4003as a growing factor by the parasite, would not be necessary during that critical period.

4004In infants, iron storages depend on the mothers', but other strategies like delayed cord

4005clamping can also contribute to increase them.

4006In any case, sufficient iron levels are crucial for both the mother and the infant, and they need
4007to be reached in every possible manner. Therefore, malaria control interventions should be
4008optimized to better ensure a minimal infective risk during pregnancy and infancy.

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4491

X. Appendix

4497

Appendix1: Score of Ballard to determine gestational age

NEW BALLARD SCORE							
Neuromuscular Maturity							
Score	-1	0	1	2	3	4	5
Posture							
Square window (wrist)	>90°	90°	60°	45°	30°	0°	
Arm recoil		180°	140°-180°	110°-140°	90°-110°	<90°	
Popliteal angle	180°	160°	140°	120°	100°	90°	<90°
Scarf sign							
Heel to ear							

4498

Physical Maturity							
Skin	Sticky, friable, transparent	Gelatinous, red, translucent	Smooth, pink; visible veins	Superficial peeling and/or rash; few veins	Cracking, pale areas; rare veins	Parchment, deep cracking; no vessels	Leathery, cracked wrinkled
Lanugo	None	Sparse	Abundant	Thinning	Bald areas	Mostly bald	Maturity Rating
Plantar surface	Heel-toe 40-50 mm: -1 <40 mm: -2	>50 mm, no crease	Faint red marks	Anterior transverse crease only	Creases anterior 2/3	Creases over entire sole	
Breast	Imperceptible	Barely perceptible	Flat areola, no bud	Stippled areola, 1-2 mm bud	Raised areola, 3-4 mm bud	Full areola, 5-10 mm bud	Score
Eye/Ear	Lids fused loosely: -1 tightly: -2	Lids open; pinna flat; stays folded	Slightly curved pinna; soft; slow recoil	Well curved pinna; soft but ready recoil	Formed and firm, instant recoil	Thick cartilage, ear stiff	Weeks
Genitals (male)	Scrotum flat, smooth	Scrotum empty, faint rugae	Testes in upper canal, rare rugae	Testes descending, few rugae	Testes down, good rugae	Testes pendulous, deep rugae	-10 20
Genitals (female)	Clitoris prominent, labia flat	Clitoris prominent, small labia minora	Clitoris prominent, enlarging minora	Majora and minora equally prominent	Majora large, minora small	Majora cover clitoris and minora	-5 22
							0 24
							5 26
							10 28
							15 30
							20 32
							25 34
							30 36
							35 38
							40 40
							45 42
							50 44

Source: [Reprinted from *The Journal of Pediatrics*, 119(3), J.L. Ballard, J.C. Khoury, K. Wedif, C. Jarg, B.L. Walsman, and R. Lipp, "New Ballard Score Expanded to Include Extremely Premature Infants." Copyright 1991 by Mosby, Inc., with permission from Elsevier.]

Figure 1

4499

4500Appendix 2: Further details of the study APEC

4501The preparation of the APEC project started in 2008. Nineteen people (2 medical doctors, 8
4502nurses, 5 lab technicians and 4 supporting agents) formed the APEC-MiPPAD research team.
4503The study implied people from 33 different villages of the Allada region. Even if the follow-
4504up took place in three maternities (Sékou, Allada, and Attogon), the laboratory was in the
4505centre of Sékou. In APEC the first woman was recruited on the 15th January 2009 and the last
4506one delivered on the 10th January 2012.

4507In case of illness each case has been managed according to Beninese guidelines.

4508Uncomplicated malaria has been treated in the different maternities and complicated malaria
4509cases have been referred either to the Hospital of Calavi, to the Hôpital de la mère et de
4510l'enfant Lagune, or to the Centre hospitalier universitaire-Hubert Koutoukou Maga in
4511Cotonou, where they have received quinine.

4512In case of severe anemia the patients have been transfused and detected and treated the cause
4513of anemia.

4514With regard to the lost-of follow-up, every trimester the entire cohort was controlled. A case
4515was determined to be lost of follow-up when there were no news of the mother or the infant
4516for longer than 3 visits. Each case was documented and the cause was also determined. In
4517case of death, the date, the cause, and the previous treatments were also investigated.

4518HIV rapid tests were proposed to the pregnant women after HIV counselling at the 1st ANC
4519visit. The tests realized were Determine® HIV-1/2 (Abbott Determine Kit HIV 1 and 2
4520package insert) et Bioline (SD Bioline Kit HIV 1 and 2 3.0 package insert). When the result
4521was positive, women were sent to the Hospital of Allada for an ELISA confirmation. In case
4522of confirmation of the diagnostic, women were treated and followed according to the
4523Beninese guidelines.

With regard to the diagnostic of malaria, the technique of Lambarené was employed: it consist in the analysis of 10 µL of blood on a surface of 1.8 cm² at the microscope. Afterwards the sample is colored with Giemsa. Then the mean number of parasites for each field is counted and then multiplied by a factor to obtain the mean number of parasites for each µL of blood. Parastiemia is determined by an estimation of the mean number of parasites per field. The number of fields to be counted depends on the parasite density:

more than 1000 parasites/ field: count 0.5 field

100 to 999 parasites/ field: count 1 field

10 to 99 parasites/ field: count 10 fields

1 to 9 parasites/ field: count 100 fields

The factor corresponds to the following microscopic factor:

Parasites / µl = parasites / field * µL / field where **µL / field**

Hemoglobin was determined by and hemoglobinometer needing 10 µL blood:

Hemo_Control® EKF Diagnostic, Germany). An internal control was realized every morning and an external control was realized by sending 10% of the samples to the health centre in Allada, where hemoglobin was dosed by an automat (Erma laboratory, Japan).

The hemoglobin type was determined by an electrophoresis on a cellulose acetate electric field using 50 µL blood (Helena Laboratories, USA).

To evaluate seric ferritin, folate and vitamine B12 the automate AxSYM (AxSYM, Abbott Diagnostic, USA) was used. An immunoenzymatic technique based on microparticules was used to determine the vitamine B12 concentration and a technique based on ionic capture was used to quatify the concentration of folate. Five-hundred µL serum were necessary to analyse these parameters.

4547CRP was determined by a qualitative-semiquantitative kit (Cypress Diagnostic). It is a
4548suspension of latex polystyrene particules covered with a specific anti-serum of IgG fraction
4549anti-human CRP. The test is positive is the concentration is equal or higher than 6 mg/l. The
4550sensibility of the test is 95,6% and the specificity 96,2%.

4551Helminth infections were analysed using the Kato-Katz technique. It consists in the
4552examination of a calibrated film of fecale substance previously impregnated in a chemical
4553solution. It can detect also eggs, especially ankylostome eggs, as well as the intensity of
4554helminth infection.

Appendix 3: PNLP recommendations:

Since 2006, the programme national de lutte contre le paludisme (PNLP), recommends:

- daily supplement of 200 mg ferrous sulfate (containing 120 mg iron and 5 mg of folic acid) for all pregnant women starting at the 1st ANC visit until 3 months after delivery.
- In case maternal hemoglobin <110 g/l the quantity of supplements are doubled and if maternal hemoglobin <70 g/l maternal transfusion is then encouraged.
- Anti-parasitic treatment starting at the 2nd trimester of pregnancy consists in either one-dosed 500 mg mebendazole or 600 mg albendazole (100mg twice a day during 3 days).
- IPTp with 1500mg sulfadoxine-75mg pyrimethamine twice during pregnancy, starting after the first trimester on month apart. The drug regime is augmented to 3 doses in case of HIV positive women.
- A rapid diagnostic test is realized to every pregnant woman in case of fever
- For uncomplicated malaria, since 2011, the PNLP recommends the ACT with 20mg artemether-120mg lumefantrine. In case of complicated malaria, the treatment consists in 8 mg/kg 3 times a day during one week.
- Insecticide treated nets. Since 2003 campaigns are organized to treat and repair ITN and/or change them.
- At each ANC visit, HIV screening is proposed for free.

4576 Cette thèse n'aurait pas eu lieu sans l'aide des bailleurs suivants :

4577 L'Ecole des Hautes Etudes en Santé Publique (EHESP) a financé la majorité de mon contrat

4578 doctoral, ainsi que les déplacements pour les Congrès de l'ASTMH en 2014 et 2015.

4579 Egalement, l'EHESP a financé les deux stages que j'ai réalisés au cours de ma thèse à la

4580 SMRU.

4581 La Direction Générale de l'Armement a financé également mon contrat doctoral.

4582 Le programme EDCTP (European and Developing Countries Clinical Trials Partnerships) de

4583 l'Union Européenne ainsi que la Fondation Bill et Melinda Gates ont financé les études

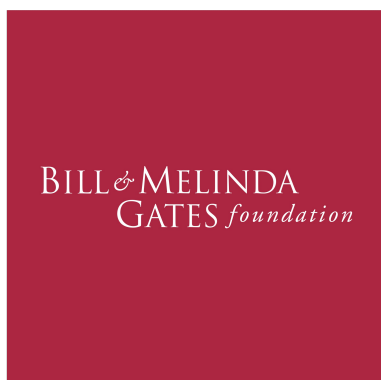
4584 MiPPAD et APEC.

4585 Le NIH a financé le projet TOVI.

4586 Je remercie vivement toutes ces institutions ainsi que les personnes qui y travaillent pour leur

4587 aide pendant ces années.

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4594

4595A ces mots, il partit, et le laissa là, avec, dans le coeur,
4596des pensées qui ne devaient pas se réaliser : Agamemnon se
4597disait qu'il prendrait la ville de Priam ce jour-là,
4598l'insensé, et il ignorait les desseins de Zeus, qui devait
4599encore infliger bien des douleurs et des gémissements aux
4600Troyens et aux Danaens, en de rudes mêlées. Il s'éveilla de
4601son sommeil, et la voix divine s'écoula autour de lui.