Malaria risk factors in pregnant women and infants in Benin

Violeta Moya Alvarez

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Mme. Violeta MOYA-ALVAREZ

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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	Directeur de thèse
M. le Dr. Jean-François Etard	Rapporteur
M. le Dr. Bruno Pradines	Rapporteur
Mme. le Dr. Clara Menéndez	Examinatrice
M. le Dr. Guillaume Leloup	Examinateur
M. le Dr. Gérard Bréart	Examinateur

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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

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Liste de publications et communications de la thèse:

Publications:

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

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

Articles sous révision

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

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

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

Communications:

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

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

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

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

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Prix à la meilleure communication, Journées EHESP 2014.

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Laboratoire d'accueil

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Faculté de pharmacie-laboratoire de parasitologie

4, avenue de l'observatoire

75270 Paris cedex 6

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290 Sigles et abréviations

291 ACTs : Arthemisin 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 arthemisinine

308 EDCTP: European and Developing Countries Clinical Trails Parternhsips

309 ELISA: Enzyme-linked immunosorbent assay

310 Hb: Hemoglobin

311 HIV : Human Inmunodeficiency 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

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LBW: Low birth weight

MCV : Mean corpuscular volume

MeSH: Medical Subjects Headings

MiPc: Malaria in pregnancy consortium

MiPPAD: Malaria in pregnancy preventive alternative drugs

MPAC: Malaria Policy Advisory Committee

MQ : Mefloquine

OMS : Organisation mondiale de la santé

OR: Odds ratio

PAM: Pregnancy associated malaria

PM: Placental malaria

PNLP : Programme national de lutte contre le paludisme

PQ : Priperaquine

RDT : Rapid diagnostic test

RR: Relative risk

SGA: Small for gestational age

SP: Sulphadoxine-pyrimethamine

SPR: Slide positivity rate

sTfR : Seric transferrin receptor

TBS : Thick blood smear

TPI : Traitement préventif intermittent pendant la grossesse

UMR216 : Unité mixte de recherche 216

VIH : Virus de l'immunodéficience humaine

WHO : World Health Organisation

ZPP: H: Zinc protoporphyrin/hemoglobin ratio

Malaria risk factors for pregnant women and infants in Benin.

Abstract

In Benin malaria and nutritional deficiencies are the main diseases contributing to the disease burden. Therefore, preventive strategies targetting both diseases have been deployed for over 10 years. Pregnancy-associated malaria (PAM) is responsible for maternal anaemia, placental malaria and low birth weight (<2500g), contributing to enhance maternal and child morbidity and mortality. To prevent PAM, the World Health Organization recommends the intermittent preventive treatmentduring pregnancy (IPTp). In Benin it consists in the administration of two curative doses of sulfadoxine/pyrimethamine (SP) at least one month apart and starting at the second trimester of pregnancy. Considering that IPTp has an effect on PAM, and thereby influences the exposure of the fœtus to the parasite, we wanted to investigate the possible effect of IPTp on malaria in infants. In parallel, iron supplements are recommended during pregnancy to prevent maternal anemia. Some pediatricians give iron supplements to infants as well. As there is some epidemiological evidence that iron might enhance malaria episodes and their severity we wanted to analyse the association of iron levels with malaria in pregnancy and infancy. Therefore, we analysed data from a cohort study of 1005 pregnant women conducted from 2010 to 2012 in Allada (Benin), and data of the first 400 infants born to this cohort of mothers, who were followed for a year.

First, we showed that interval length between IPTp doses (the number of days between doses) was inversely correlated with malaria risk and *P. falciparum* parasitemia, possibly due to the reduction of the exposure of the fœtus to the parasite in utero, which thereby hinders a possible immune tolerance process.

We also found that iron levels during pregnancy and infancy were associated to increased malaria risk and *P. falciparum* parasitemia, with a possible dose effect.

In a context of growing resistance to SP, a strategy based on more than 2 doses of SP should be encouraged to confer an optimal protection to pregnant women. In addition, complementary interventional data are needed to determine the benefits and risks of differently dosed iron supplements, in order to ascertain their impact on infant health in malaria-endemic regions.

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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I. Introduction

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

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

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

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

Chez les enfants béninois des taux d'anémie supérieurs à 80 % ont été reportés . Néanmoins, il n'y a 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 parallèlle, afin de réduir 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 a 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

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

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

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

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

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

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

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

II. Etat de l'art

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

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

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

Un autre élément important concerne la manière d'évaluer les taux de fer. Une combinaison d'indicateurs est souhaitable d'après l'OMS en dépit de l'existence d'un marqueur «gold standard», 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) .

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 ºC et une goutte épaisse positive dans les 48 h. La carence en fer est définie par des niveaux de ferritine sérique < 12 µg / ml ou < 15 µg / 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

595 % (0,5; 0,88). Cependant, en Tanzanie en 2008 un essai de supplémentation en zinc et autres nutriments (dont le fer), avait décrit que les enfants carencés étaient significativement plus à risque de paludisme lors de la supplémentation (Rapport de risque =1,41 ; IC 95 % (1,09; 1,82)). En effet, la question reste ouverte et les résultats des différents essais cliniques se révèlent a nouveau contradictoires. Une revue Cochrane a tenté de trancher ce débat en analysant les données de 45.353 enfants de 71 essais cliniques différents. Après s'être concentré sur les 13 études les plus fiables, cette révision conclut qu'il n'y a pas de différences statistiquement significatives concernant les épisodes palustres entre les enfants supplémentés par rapport aux enfant ayant reçu un placebo (RR = 0,99 ; IC 95 % (0,9; 1,09). Nonobstant, cette revue décrit un risque de paludisme plus élevé chez les enfants supplémentés, en l'absence de surveillance épidémiologique ou de traitement. Une évaluation de l'augmentation du risque en prenant en compte les niveaux de fer de départ, mesurés par la ferritine, reste également à faire.

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

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

3. Effet du paludisme gestationnel et du TPI sur le paludisme de l'enfant

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

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

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

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

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

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

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

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

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

de plomb très élevés. Des niveaux élevés de plomb sont associés à un risque accru d'anémie et à des troubles neurologiques , symptomatologie également présente dans le paludisme. Ceci est d'autant plus préoccupant que la pathologie se concentre aussi dans la tranche d'âge de 12 à 36 mois, période particulièrement délicate pour les enfants impaludés . Enfin, Nriagu avait trouvé en 2008 une effet négatif significatif du paludisme sur les niveaux de plomb , alors que la prévalence de niveaux de plomb élevés en Afrique de l'Ouest est très importante . Pour ces raisons, nous voulions déterminer la nature de l'association entre les niveaux de plomb et le risque palustre tout en considérant d'autres facteurs de risque de paludisme.

III. Résultats

1. Effet des niveaux de fer sur le paludisme gestationnel

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

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

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

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

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

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

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

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

IC 95 % (1,95 ; 3,96) ; valeur p < 0,001) et à une parasitémie par *P. falciparum* plus élevée (estimateur beta = 0,38 ; IC 95 % (0,29 ; 0,47) ; valeur p < 0,001).). Plus précisement, ces modèles comprennent les résultats de 746 gouttes épaisses et de 746 frottis sanguins de 329 enfants. Egalement, les enfants carencés en fer étaient significativement à moindre risque d'avoir une goutte épaisse positive et une parasitémie élevée (valeur p < 0,001 dans les deux cas). En parallèle, la présence d'un processus inflammatoire actif était associée à un risque accru d'avoir une goutte épaisse positive (OR ajusté = 4,37 ; IC 95 % (2,20 ; 8,65) ; valeur p < 0,001) et une parasitémie élevée (estimateur beta = 0,77 ; IC 95 % (0,53 ; 1,01) ; valeur p < 0,001).

Par ailleurs, des niveaux de folate maternels élevés et la présence d'helminthes chez la mère à l'accouchement étaient significativement associés à un risque accru d'avoir une parasitémie élevée pendant la première année de vie (estimateur beta = 0,34 ; IC 95 % (0,01 ; 0,66) ; valeur p = 0,04, et estimateur beta = 0,88 ; IC 95 % (0,20 ; 1,57) ; valeur p = 0,03, respectivement). Un statut socio-économique bas était aussi lié à une parasitémie élevée (estimateur beta = 0,12 ; IC 95 % (0,01 ; 0,23) ; valeur p = 0,03). Le volume des précipitations, indicateur du risque anophélien, était marginalement associé à un risque élevé de paludisme (OR ajusté = 1,06 ; IC 95% (0,99 ; 1,11) ; valeur p = 0,06) et à une parasitémie plus importante (estimateur beta = 0,03 ; IC 95% (-0,00 ; 0,06) ; valeur p=0,06).

Finalement, les enfants avec des niveaux de ferritine dans les deux derniers quartiles étaient significativement plus à risque de paludisme.

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

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

A 12 mois, 25 des 203 enfants pour qui les niveaux de plomb avaient été évalués (12,5 %), avaient une goutte épaisse positive, avec une parasitémie moyenne de 13460 parasites / µl. Les niveaux de plomb élevés sont définis par le CDC comme des niveaux de plomb sanguin > 5 µg / dL. Trente-

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

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

IV. Discussion

1. Effet des niveaux de fer sur le paludisme gestationnel

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

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

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

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

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

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

Le paludisme gestationnel étant connu comme influençant l'état de santé de l'enfant[10], il est plausible qu'une intervention préventive chez la mère ait également un effet sur le paludisme de l'enfant. Nos résultats sont de ce point de vue cohérents avec la littérature. Borgella et al. ont trouvé que les infections pendant le dernier trimestre de grossesse étaient associées à un risque

accru d'infection (OR = 4,2 ; CI 95 % (1,6; 10,5) ; valeur p = 0,003) ainsi que d'épisode palustre (OR = 4,6 ; CI 95 % (1,7; 12,5) ; valeur p = 0,003) . En outre, Huynh et al. avaient décrit que le calendrier du TPI et les infections au premier trimestre de grossesse étaient liés à un plus grand risque de petit poids à la naissance (-98,5 g; valeur p = 0,03) . Par contre, Harrington avait trouvé en Tanzanie que le TPI était associé à des épisodes palustres plus précoces parmi les enfants issus d'un placenta infecté . Néanmoins, on retrouve une résistance très importante à la SP dans le Nord-Est de la Tanzanie, et la même équipe a déjà montré que, dans cette région, le TPI se révèle inefficace . Dans cette population, le TPI est associé à une grande fréquence d'allèles de résistance à la SP, à une densité parasitaire plus importante. Ces arguments renforcent indirectement l'hypothèse du processus de tolérance immunitaire *in utero*. Dans tous les cas, le fait que l'augmentation de la durée totale du TPI (par espacement ou ajout de nouvelles prises) ait un effet protecteur sur le paludisme de l'enfant est plutôt rassurant à la lumière des nouvelles recommandations de l'OMS en faveur d'un renforcement du rythme d'administration de la SP.

En parallèle, nous avons trouvé une association très significative entre les niveaux de fer et le risque palustre (prévalence et densité parasitaire) pendant toute la première année de vie en prenant en compte d'autres facteurs de risque environnementaux, socio-économiques, et obstétricaux. La carence en fer avait en particulier un effet protecteur pendant tout le suivi. Plus précisément, les enfants avec de faibles niveaux de fer, dans le premier quartile de l'échantillon, étaient significativement à moindre risque d'épisodes palustres et avaient une densité parasitaire significativement plus basse que les autres.

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

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

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

3. Effet des niveaux de plomb sur le risque palustre

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

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

V. Conclusion

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

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

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

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

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

VI. Perspectives

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

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

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

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

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

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

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

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I. Introduction

I. 1. The global burden of disease in Africa

The global burden of disease in the African continent is mainly driven by infectious diseases and nutritional deficiencies, the pregnant women and the children under 5 years being the most vulnerable groups in the population. In the African region communicable, maternal and nutritional conditions gather the largest proportion of the crude death rate by broad cause group between 2000 and 2012 (Figure 1). More precisely, in 2012, out of the 1000 deaths per 100,000 people, approximately 60% were due to communicable, maternal and/or nutritional conditions, whereas communicable diseases gathered 30% and injuries 10%, respectively.

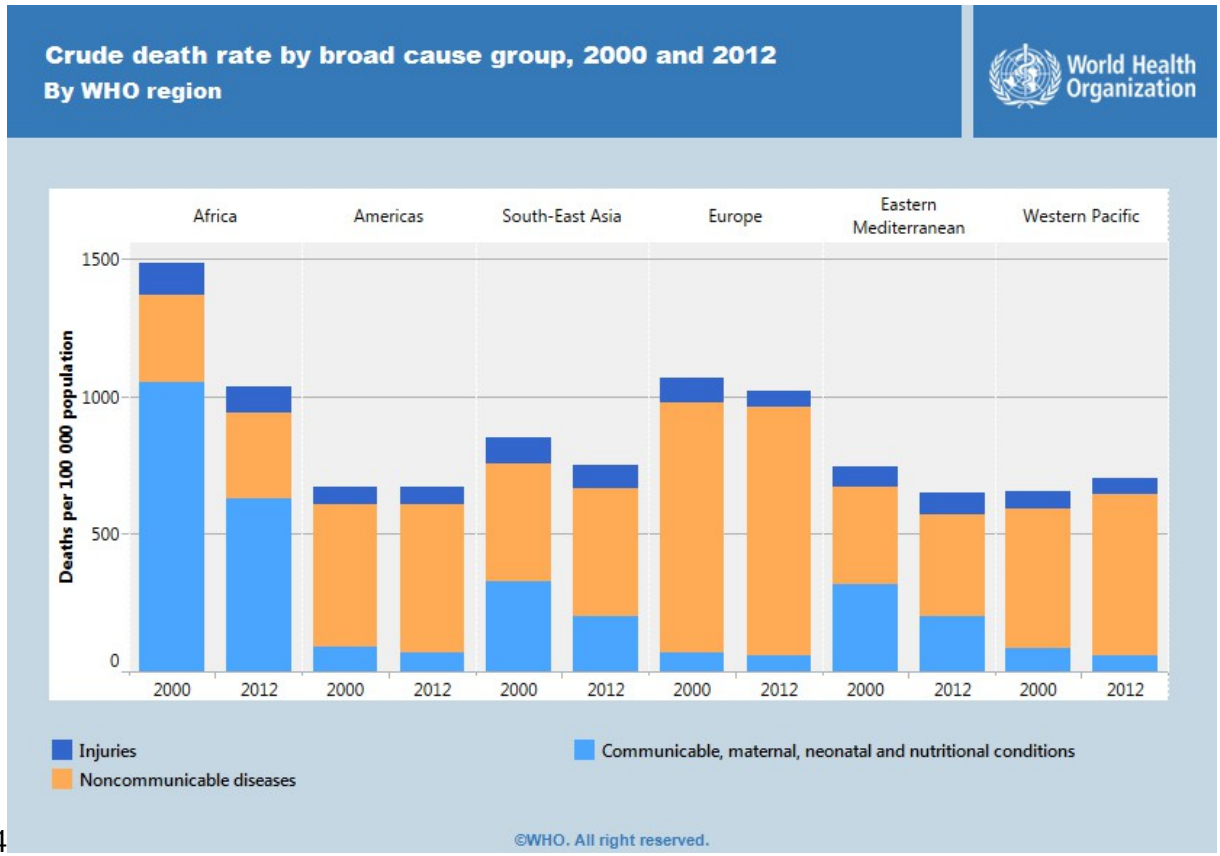


Figure 1. Crude death rate by broad cause group between 2000 and 2012, WHO 2013.

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

combat HIV/AIDS, malaria, and other diseases). To be most effective, public health strategies need to target the main causes of disease underlying the impaired health status of pregnant women and children.

I. 2. The burden of disease in Benin

In Benin, the three risk factors that account for most of the disease burden (in disability-adjusted life years (DALYs)) are childhood underweight, household air pollution from solid fuels, and iron deficiency (defined by WHO as serum ferritin levels<15µg/l) (Figure 2). The leading risk factors for the burden of diseases in children under 5 and adults aged 15-49 years were childhood underweight and iron deficiency, respectively, in 2010.

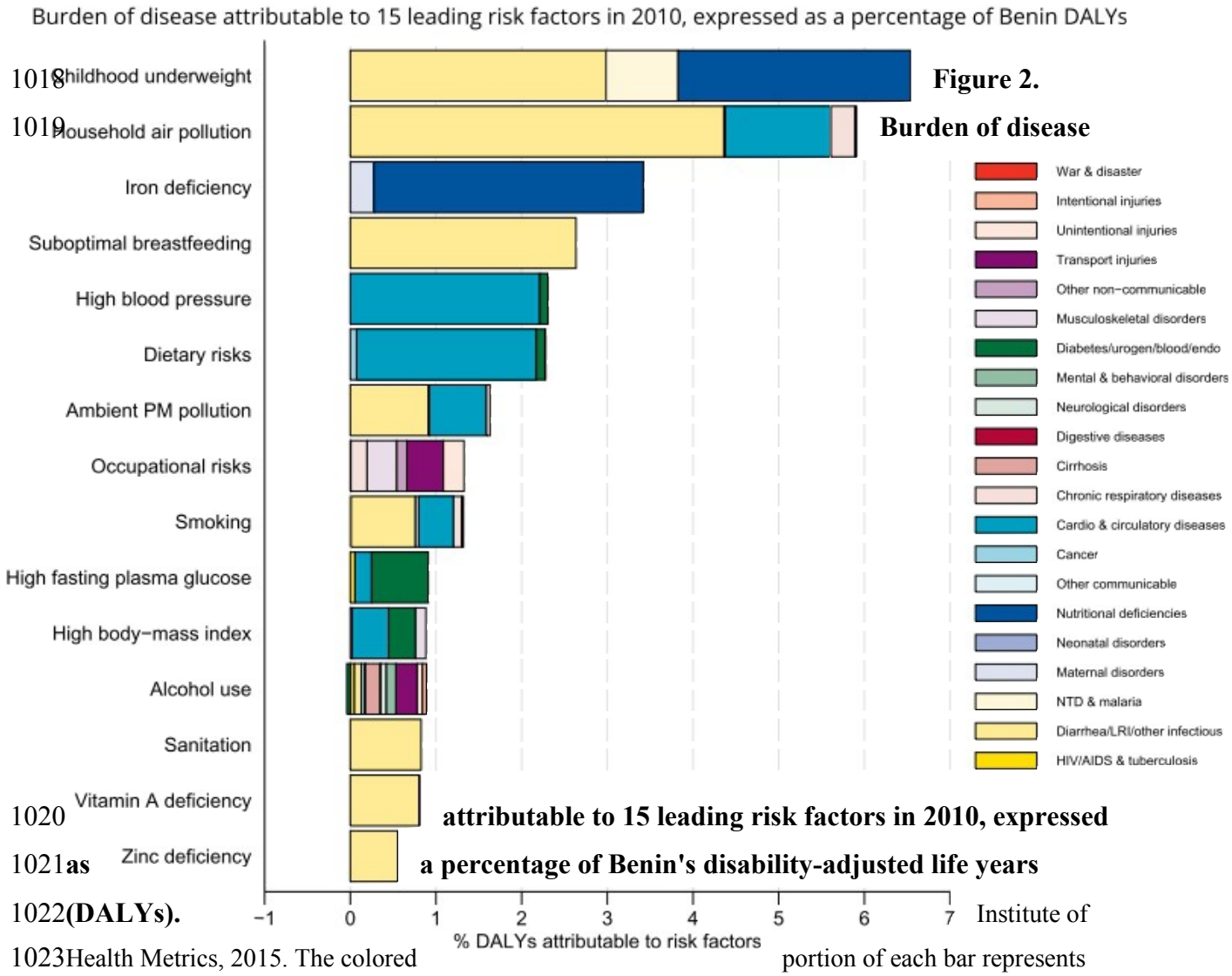


Figure 2. Burden of disease attributable to 15 leading risk factors in 2010, expressed as a percentage of Benin's disability-adjusted life years (DALYs). Institute of Health Metrics, 2015. The colored portion of each bar represents

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

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

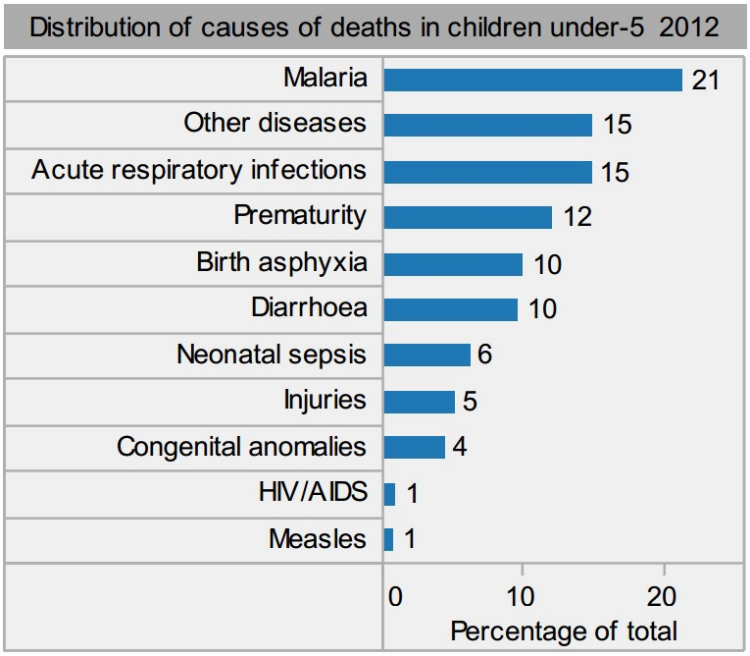


Figure 3. Distribution of causes of deaths in children under 5 years in Benin (2012). WHO, 2014.

Therefore, not only globally but also in Benin do nutritional deficiencies and malaria lead morbidity and mortality rates in children under 5 years. For these reasons, substantial efforts have been made by to fight these diseases in Benin.

For further knowledge, information on malaria physiopathology is explained in Box 1. Complementary information about the epidemiology of malaria in Benin is presented in Box 2.

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.

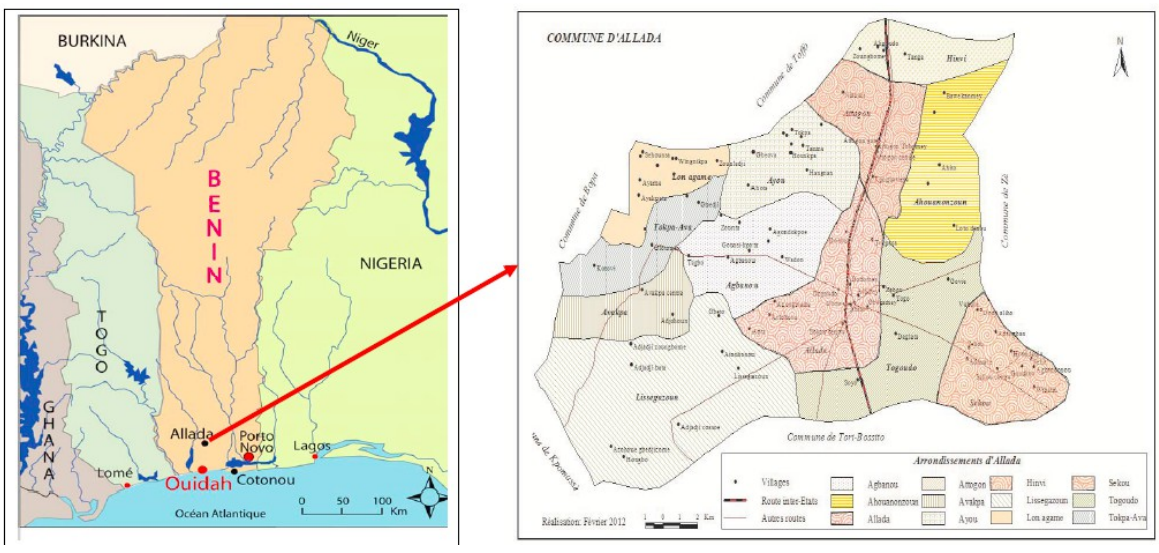


Figure 4. Map and plan of the disctric of Allada. Source: Institut Géographique National du Bénin.

I. 3. Preventive strategies to tackle the disease burden during pregnancy and infancy in Benin

As stated previously, in Benin the main contributors to disease burden during infancy are malaria and nutritional deficiencies.

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

With regard to nutritional diseases, underweight and iron-deficiency anemia are the main conditions contributing to enhance nutritional deficiencies.

Underweight is significantly linked to low birth-weight (LBW, defined as birth-weight<2500g), whose rates in Benin reached 13% in 2012. Indeed, infants with lower birth weights are likely to remain shorter and lighter throughout childhood compared to infants without LBW, especially those having experienced intra-uterine growth retardation (IUGR, defined as birth-weight below the 10^{th} percentile of a reference weight distribution according to gestational age). In addition, LBW is significantly associated to increased morbidity and mortality. Furthermore, LBW and malnutrition have a synergistic relationship with infectious diseases.

From the epidemiological perspective, LBW is correlated with pregnancy associated malaria (PAM), low maternal body-mass index (BMI), and maternal micronutrient deficiencies. Therefore, interventions during pregnancy to fight LBW include the prevention of PAM, low BMI and maternal micronutrient deficiencies. To prevent the consequences of PAM, the Ministry of Health implements an intermittent preventive treatment in pregnancy (IPTp) against malaria. This intervention consists in 1500/75 mg of sulphadoxine-pyrimethamine (SP). Usually, it is joint to the anti-helminth parasitic preventive treatment of 600 mg of albendazole, although other treatments are available (appendix 3). Thereby Benin follows

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

recommends supplements of 200 mg of ferrous sulphate and 5 mg of folate given daily until 145 days after delivery.

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

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

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

II. State of the art

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

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

II.1.1.a. Epidemiological evidence

Pregnancy associated malaria (PAM) is defined as peripheral or placental infection by *Plasmodium*, and it constitutes a stake of interest for infant health as its consequences may attain 125 million pregnancies at risk of malaria infection every year. More precisely it is estimated that 32 million women become pregnant every year in Sub-Saharan Africa endemic countries. The prevalence of malaria in pregnancy is influenced by transmission, the immunity of the mother and protective measures. The main protective interventions against PAM are insecticide-treated nets (ITNs) and intermittent preventive treatment (IPT). IPT is a widespread preventive strategy to fight malaria consisting in the administration of a curative dose of an effective anti-malarial drug, regardless of the presence of *Plasmodium* in the blood, to prevent the effects of the disease. A landmark review gathering evidence on PAM between 1985 and 2000 in Sub-Saharan Africa stated a median prevalence of PAM of 27.8% among all gravidae. In low transmission African settings the median prevalence peripheral infection was 13.7% and placental malaria median prevalence was 6.7%. In general, recent studies report a significant decline in prevalence following IPTp implementation since the beginning of the XXI century. A systematic review and meta-analysis of trials determining whether regimens containing 3 or more doses of SP for IPTp were associated with a higher birth weight or lower risk of LBW than standard 2-dose regimens showed that the [24][3]-dose group

1139had less placental malaria (RR=0.51; 95% CI (0.38; 0.68) in 6 trials, 63 vs 32 per 1000;
1140absolute risk reduction, 31 per 1000 (95%CI (20; 39)). However, the augmented efficacy
1141related to higher doses is mostly observed in the case of clinical trials rather than in studies
1142issued from public health program implementations. Finally, the additional protection of the
1143joint use of ITNs with IPTp-SP is significant only in certain trials, but reported ITN use
1144ranges from 5% to 25%, and it might not be sufficient to show an effect.

1145In short the prevalence of PAM has evolved according to transmission and protective
1146measures like IPTp or ITN use. Further elements like gestation and the moment of infection
1147during pregnancy have shown to influence its pathologic consequences as well. Because
1148immunity develops during the first pregnancy, primigravidae are especially at higher risk for
1149PAM.

1150Finally the timing of high parasitemia infections during pregnancy entails different effects on
1151PAM outcomes like anemia or LBW. Therefore, the administration of IPTp at different
1152moments determines different protection patterns for the infant.

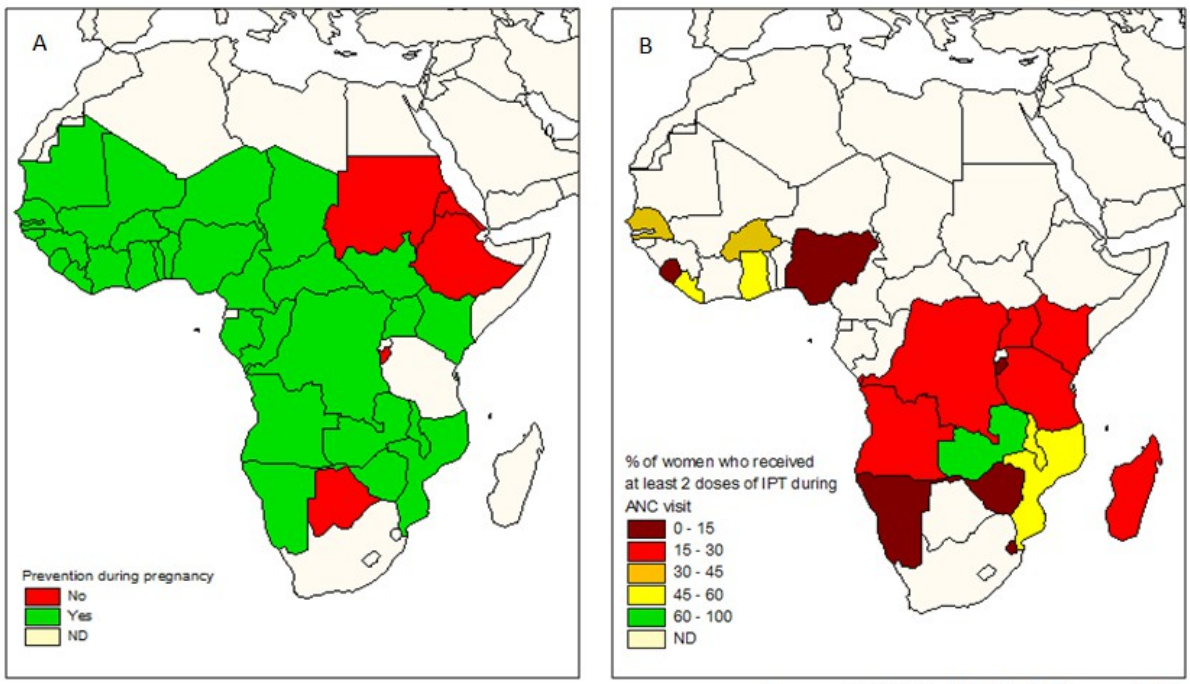
1153As a result of these concurrent realities, we have to consider other determinants of PAM, such
1154as transmission, IPTp regime and gestity, to better understand the shades of the influence of
1155IPTp on PAM.

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

1157WHO recommends in areas of moderate to high malaria transmission, IPTp with SP for all
1158pregnant women as early as possible in the second trimester, and at each scheduled antenatal
1159care visit at least one month apart. The different IPTp regimes implemented in the African
1160region are described in Figure 4.

1161

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



Source: WHO World Malaria Report 2013

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

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

Cohort	Study design and simple 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%
Lambarené[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.

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

Problems related to reduced compliance with drug regimes and the increasing resistance to anti-malaria drugs bring up 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 by day 14 in children, and 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. However, studies published more recently display contradictory results. A study in Malawi, where there is a strong fixation of the resistant quintuple mutant (mutations at *dhfr* codons 51, 59, and 108 and *dhps* codons 437 and 540), showed that the number of IPTp doses has a protective effect on birth outcomes but not on placental infection. More concretely, there were significantly less small for gestational age (SGA) rates in offspring of primigravid women having received ≥2 doses of SP compared to 0-1 doses even if peripheral parasitemia 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 the odds of PM, LBW or maternal anemia. Furthermore, it was associated with increased odds of fetal anemia and severe malaria among the offspring (AOR=2.31). IPTp in this setting was associated with an increased risk of severe malaria overall. Nevertheless, a recent longitudinal study showed 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. In conclusion, evidence on the present

efficacy of SP-IPTp regimes is inaccurate but resistance to SP is spreading. And close monitoring of its efficacy is necessary to determine if or when the treatment failure of SP-IPTp detected by some recent studies is generalized at the population level and, in this case, a switch to other drug regimes would become necessary.

Furthermore, effective IPTp diminishes PM and malaria associated morbidity like LBW, pre-term delivery, IUGR and perinatal mortality in areas where resistance to SP is not highly significant. Even the Malaria Policy Advisory Committee (MPAC) concluded that there is currently insufficient data to determine at what level of resistance IPTp-SP should be interrupted in the absence of an established and effective alternative. 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* are neither elucidated so far. Further evidence lacks as well on the importance of the timing of infection during pregnancy and infant malaria morbidity for instance. There is some evidence that earlier administration of IPTp has a positive effect on birth outcomes like LBW, nevertheless, it seems that later dosing provides a better protection at delivery. This is one reason because of which the administration of 3 doses instead of 2 shows better clinical outcomes. In addition, the implementation of different IPTp regimes, in the context of different resistance patterns, entails novel stakes to the question regarding the adequate IPTp policy according to the transmission and resistance setting. For instance, intermittent screening and testing (IST) has been applied successfully in an area of moderately high malaria transmission in Ghana. 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. Currently, the DHA-PQ IST is a proposed alternative to IPTp in areas with substantial resistance against IPTp regimes. However, at present conclusive evidence on IST efficacy is lacking in African regions and

further efficacy studies should be conducted.

The evaluation of its efficacy in other transmission settings is necessary to ascertain its utility as an effective tool for the control of PAM.

II.1.2. Effect of iron levels on PAM

II.1.2.a. Iron markers

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

The joint WHO-CDC Technical Consultation for iron assessment selected 5 different indicators as good iron markers: hemoglobin, mean cell volume (MCV), (sTfR) concentration, serum ferritin concentration, and red cell protoporphyrin (measured by the zinc protoporphyrin/hemoglobin ratio (ZPP:H)). Hemoglobin is deeply useful in the monitoring of health status and its determination is easy to realize on the field. Although it is a basic fundamental haematological indicator, it is not specific as an iron marker because of the multiple causes of anaemia and the physiological variations with regard to sex, age or ethnicity. Therefore, it can be misleading for the extrapolation of conclusive results. Mean cell volume accuracy is limited in the context of thalassemia and malaria as inflammation serum transferrin receptor modifies significantly its values. Due to its physiopathological pathway, serum transferrin receptor is also influenced by the haemolysis of malaria, and its determination method is not always standardized nor cost-effective.

Serum ferritin is a precise indicator of iron storages in healthy individuals and it can be corrected according to other inflammation proteins. It provides further information as it also shows different patterns of behaviour depending on the aetiology of anaemia. In an iron supplementation study in children, Doherty et al. compared the erythrocyte incorporation of oral iron supplement in 37 Gambian children 8 to 36 months old with anaemia after malaria treatment, to supplemented control children with IDA but no recent malaria. The non-malaria control children showed progressively increased serum ferritin whereas the post-malarial children showed decreased serum ferritin levels. Serum ferritin levels became similar in both groups only by day 15 and 30. This is thought to be due to the normalization of the immune response and to the normalization of the acute phase proteins following the malaria treatment. Indeed serum ferritin is an acute phase protein. Hence, serum ferritin is either corrected upon inflammation (with correction factors according to C-reactive protein (CRP) or α-1-glycoprotein (AGP) levels), or samples with high acute inflammation proteins are systematically excluded. Nevertheless the exclusion of samples with increased inflammation might entail a subsequent bias in the context of malaria, as samples with high ferritin would be systematically excluded as well. Despite its limited accuracy in case of inflammation, ferritin is a consistent extended iron marker.

Along with ferritin, ZPP:H ratio is the most frequently used indicator for iron assessment. The chelation of ferrous iron by protoporphyrin is the final step for the heme synthesis. In iron deficiency zinc is chelated as iron is not available and ZPP formation is decreased. In the iron-deficient parasitized RBC, the increased ZPP could bind to heme crystals, and inhibit the formation of hemozoin. Longstanding inflammation processes, thalassaemia, and asymptomatic *P. falciparum* parasitemia might also show elevated ZPP:H ratios, and consequently be erroneously associated to iron deficiency. In addition there is no standardized corrections applicable to ZPP:H ratios in the context of long-term inflammation processes.

Finally high lead levels interfere with ZPP:H, and polluted regions frequently overlap with malaria endemic settings. However, the impact of inflammation on ZPP:H is not as important as on serum ferritin.

A novel marker has recently emerged as an alternative indicator: hepcidin. Hepcidin is a peptide hormone, which plays a crucial role in iron regulation and is determinant in the malaria infection process. Hepcidin binds ferroportin, it increases in response to inflammation and blocks iron entry into the plasma. It has been proposed as a good marker for iron levels, especially because it might be up-regulated after malaria episodes compared to other markers of iron-deficiency. Therefore, a priori, it might permit to distinguish between iron-deficiency and malaria related anaemia. However, hepcidin shows a non-linear association with anaemia in the context of malaria albeit its significant association with parasitemia in children. Furthermore, in Kenya it was increased on admission at hospital for *P. falciparum* malaria and was significantly associated with parasite density, but hepcidin levels were very low in severe malaria anaemia. In addition, its accuracy as an iron marker has been recently questioned as it has been shown that it is associated with the anti-inflammatory response but not with iron or anaemic status among malarial Nigerian children. Hence, further studies with more statistical power should be encouraged to ascertain its utility as an iron marker.

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

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

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

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

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, these studies report iron levels only at enrolment, at delivery, or both, and the limited sample sizes may be insufficient to show a statistically significant effect.

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.

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

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

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

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

Congenital malaria is defined as the presence of asexual parasites in the cord blood or in the peripheral blood during the first week of life. It is the result of transplacental transmission of parasites just before or during delivery. Congenital malaria rates range between 0,83-5,96% of total births in recent epidemiological studies. Nevertheless, the introduction of molecular techniques has increased the detection of cord blood parasitemia raising prevalence rates up to 33%. Although it might entail clinical important consequences in some cases and should be considered in the differential diagnostic of neonatal fever in endemic countries, congenital malaria does not seem to constitute an epidemic emergency at present. Nevertheless, we should 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

duration of exposure to the parasite *in utero* determine susceptibility to malaria, infections occurring during the 3rd trimester are associated with increased risk of infection and clinical malaria during the first year of life in another study in Mono (Benin). In parallel, there is also a first scientific evidence on the fact that HLA-G polymorphisms could be associated with different malaria susceptibility.

But the effect of PAM may entail consequences for the morbidity and mortality of the infant also in a broad manner. Indeed, both acute placental malaria and cord blood parasitemia have been found associated with increased mortality. Moreover, placental malaria was a significant risk factor for mortality in general during the first year of life in a study in Malawi. In Mozambique infant mortality was also significantly associated with malaria infection of the placenta (p-value<0.012) after adjustment for HIV status, LBW, maternal clinical malaria during pregnancy, fetal anemia and IPTp regime. And mortality risk was significantly higher (odds ratio (OR)=5.08) for infants issued of acute infection of the placenta at delivery.

Placental malaria was also 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.

Even if complete explanation of the physiopathology of PAM has not been found so far to our knowledge, *in utero* exposure to malaria might be correlated with placental sequestration of erythrocytes, and the immune tolerance process might depend on the type of malaria antigen in contact with the foetus, the amount and the duration of the exposure, and the moment of exposure during pregnancy. However these parameters are modified by the introduction of intermittent preventive treatment in pregnancy (IPTp). Indeed, intermittent preventive treatment in pregnancy modifies parasite exposure to the fetus. Hence, IPTp may introduce substantial changes in the epidemiologic pattern of malaria in infants, possibly as the result of an ongoing process of immune tolerance to antigens *in utero*. However, little evidence exists

on the subject at present.

II.2.2. Effect of iron on malaria in infants

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

Observational studies display information reflecting the association between iron and malaria based on the real circumstances of the field, but accurate iron monitoring is not commonly realized on a systematic basis in this context. Clinical trials focus rather on the effect of supplements and investigate the possible consequences for malaria outcomes of the iron supplementation policy, but their methodological protective constraints do not reflect the epidemiological reality of malaria endemic settings. Indeed, both approaches assemble different but important information and, therefore, both should be considered for the analysis of the iron-malaria link. The results of the main studies on the malaria-iron relationship in infants 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<11g/dl Serum iron concentration<4.48µmol/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	minum 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 enrolement	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)	Non significative 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<.001) 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	Compared to placebo: All malaria episodes: Zinc group: AHR= 0.99 (CI 0.82; 1.18) Multi-nutriments without zinc: AHR=1.04 (CI 0.87; 1.23) Multinutriments with zinc: AHR=1.14 (CI 0.96; 1.35) First malaria episodes: Zinc group: AHR= 1.12 (CI 0.86; 1.44) Multi-nutriments without zinc: AHR=1.35 (CI 1.05; 1.73) Multinutriments with zinc: AHR=1.38 (CI 1.07; 1.77) Number of episodes with versus without multinutriments Iron deficient: HR=1.41 (1.09; 1.82) Iron replete: HR=0.93 (0.77; 1.13)	*Difference relative to placebo (95%CI), Hemoglobin concentration (g/l) Micronutrients without zinc: 106.6 (10.7) *2.6 (0.0; 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 (0.05) *24.5 (14.8; 36.2) Micronutrients with zinc: 57.2 (0.05) *24.6 (14.8; 36.3) without inflammation: Micronutrients without zinc: 43.9 (0.05) *19.5 (11.3; 28.6) Micronutrients with zinc: 51.1 (0.05) *26.7
Brong-Ahafo	Ghana	2010	double blind, cluster-randomized trial	perennial with seasonal peaks	1958	6 months	6 to 35 months	ID: Splasma ferritin concentration <12 µg/L	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 0.65; 1.05) RR for iron replete children without inflammation=0.92 (CI 0.81; 1.06)	
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	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)	Iron versus placebo or no treatment, iron plus folic acid versus placebo or no treatmen, 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

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

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

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

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

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

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

Because of these a priori contradictory results of the studies, a Cochrane review of 2011 analysed 71 trials collecting evidence on 45,353 children. For the 13 trials selected, the Cochrane review concluded to an absence of significant differences in clinical malaria rates between iron and placebo (RR=0.99, 95%CI (0.9; 1.09)). No statistical differences were found neither among supplemented infants (children<2years) (RR=0.94, 95%CI (0.82; 1.09)) nor for severe malaria (RR=0.91, 95%CI (0.76; 1.08)) compared to placebo. Furthermore, no statistical difference was found among non-anemic children at baseline (RR=0.97, 95%CI (0.86; 1.09)). However, analyses on iron deficiency defined by ferritin were not realized. Even if it is difficult to screen children for iron status at the population level, information on the effect of iron deficiency is relevant to develop useful supplement strategies based on scientific accurate evidence. Finally, this Cochrane meta-analysis describes increased risk for clinical malaria among iron or iron plus folic acid supplemented children in the absence of malaria surveillance and treatment.

Beyond clinical malaria, it is necessary to consider also malaria mortality to capture broader aspects of the iron-malaria association. In the context of the clinical trial with iron supplements in Pemba, mortality due to malaria was higher (although not significantly higher) among supplemented children compared to placebo (RR=1.08, 95%CI (0.84; 1.40)). Among children supplemented with iron and folic acid, there was a significant increased risk for cerebral malaria as a cause of death compared to placebo (RR=1.70, 95%CI (1.08; 2.68)). The iron and folic acid supplemented children were 12% more likely to suffer an adverse event resulting in hospitalisation or death (95%CI (2; 23)) compared to placebo and all-cause mortality was also significantly higher (OR= 1.61, 95%CI (1.03; 2.52)). Iron deficiency and moderate anaemia at baseline were significantly associated to lower rate of adverse events (death or severe morbidity leading to admission) among supplemented children compared to placebo. Further extensive studies on the impact of iron supplements on malaria attributable

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

III. Objectives

In Benin, the prevalence of anemia during pregnancy is over 60%. The main causes of anemia in pregnancy are malaria and helminth infections. 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 recommends supplements of 200 mg of ferrous sulphate and 5 mg of folate given daily until 45 days after delivery.

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

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

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

in infants, and IPTp has an impact on secondary malaria outcomes (such as LBW and anaemia). Hence, our second objective was **to investigate the possible impact of IPTp on malaria in infants during the first year of life**.

Finally, a research group working on the same cohort found very high rates of elevated blood lead levels in the infants. Both malaria and elevated lead levels have a severe impact on the infant health. In addition Nriagu had found a significant effect of malaria on the children lead levels in different areas of Nigeria. Therefore, our third objective was **to assess the possible effect of elevated lead levels on malaria in infants**, as their possible association may have severe long-term implications for the development of the children. Indeed, no published study exists on lead levels in Benin, and in particular, on the effects of lead levels on malaria risk in infants.

IV. Methods

To investigate our objectives, we conducted our research in the context of the clinical trial MiPPAD and a nested study APEC.

The clinical trial MiPPAD (Malaria in pregnancy preventive alternative drugs, http://clinicaltrials.gov/ct2/show/NCT00811421) was conceived to compare the efficacy and safety of IPTp with SP (1500/75 mg per dose) and mefloquine (15 mg/kg taken either in simple or split intake).

The study APEC (Anemia in pregnancy: etiology and consequences) was a nested study to MiPPAD that analysed parameters relevant to the anemia status of both the pregnant women and infants.

More precisely, in the context of both studies in Benin, 1005 pregnant women and 400 of their offspring (200 born to mothers with anemia at delivery, and 200 born to mothers without anemia at delivery) were followed through pregnancy and the first year of life, respectively.

The APEC study was conducted in three maternity clinics in the district of Allada, between January 2010 and May 2012. 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.

The eligibility criteria included no intake of IPTp, iron, folic acid, vitamin B12, or anti-helminthic treatment. All women were offered confidential pre-test HIV counselling and thereafter informed consent was obtained.

IV. 1. Cohort follow-up methods

Clinical and biological follow-up:

During follow-up, socio-demographic, economic, clinical and biological data were collected in mothers at 1st antenatal clinical visit (ANC), 2nd ANC and delivery. The same data were also recorded in infants at birth, 6, 9 and 12 months of life. In case of sickness, both pregnant

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

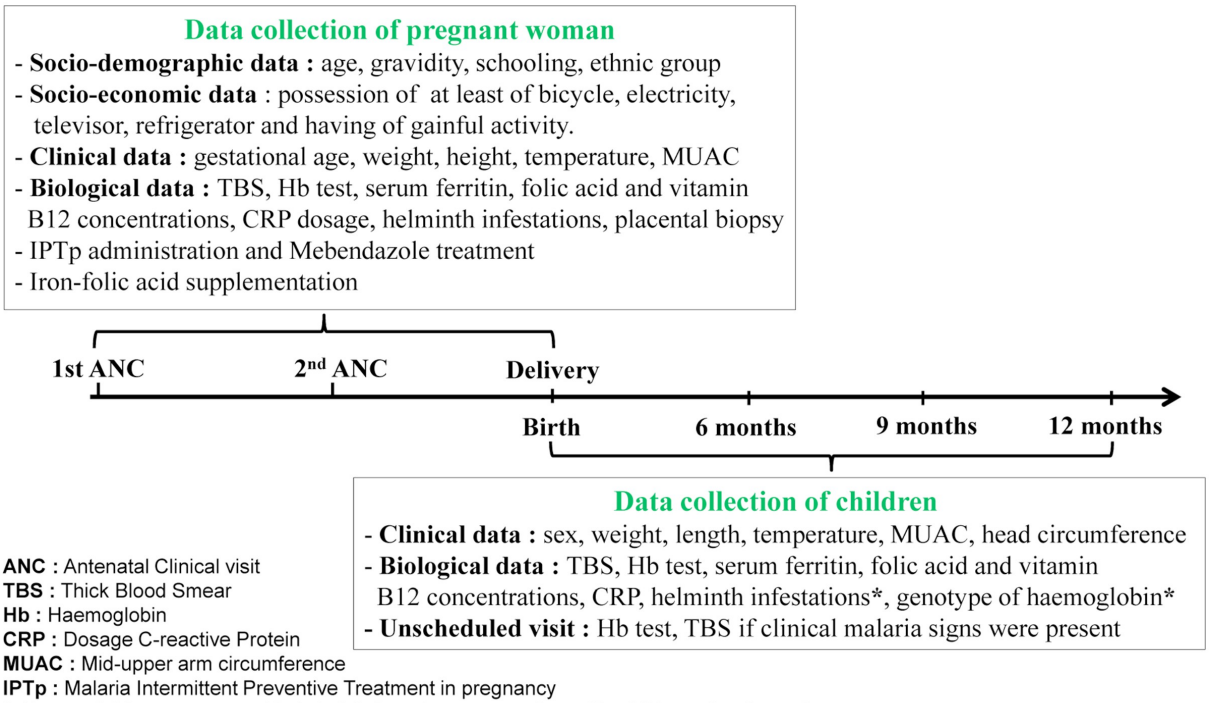


Figure 6: Clinical and biological exams during the follow-up through pregnancy and infancy. (Figure realized by M. Accrombessi)

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

At birth, newborn’s sex, weight, length, head circumference and axillary temperature were

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

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

On unscheduled visits, Hb dosages and thick blood smears were performed in infants with clinical signs of malaria (history of fever in the last 24 hours or temperature $\frac{24}{12}$ 37.5°C and pallor).

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

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

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

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

The Lambaréné technique was used to analyse peripheral malaria infection in blood smears. It consists of spreading a calibrated 10 µl amount of blood on a slide's rectangular area of 1.8 cm^2 (1.8 x 1 cm). The slide was stained with Giemsa and read at a magnification of 1,000 × with an oil immersion lens. A multiplication factor was applied to the average parasitemia/ field to determine the number of parasites/µl. The Lambaréné method detection threshold has been estimated to be 5 parasites/µL.

Placental biopsies (2.5 x 2.5 cm3), collected at delivery for histology assessment, were immediately put in 50 ml of 10% buffered formalin. It was then stored at 4°C in a refrigerator until the placental tissue was processed at the pathology department. The maximum delay before fixation was of 5 days. Placental malaria infection was defined as the presence of parasites with /without pigment or pigment confined to fibrin in the histological examination. Placental histology was examined without knowledge of the peripheral blood smears results. In addition, an external quality control was made on 100% of positive slide and 10% of negative slide in reference laboratory to Barcelona Centre for International Health Research (CRESIB), Hospital Clínic-Universitat de Barcelona. Infestations by helminths were assessed by using the Kato-Katz concentration method (Vestergaard Frandsen, Lausanne, Switzerland).

Environmental data: As no entomological data was 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 prior to the two weeks before the consultation.

Ethics statement

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

IV. 2. Cohort follow-up

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

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

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

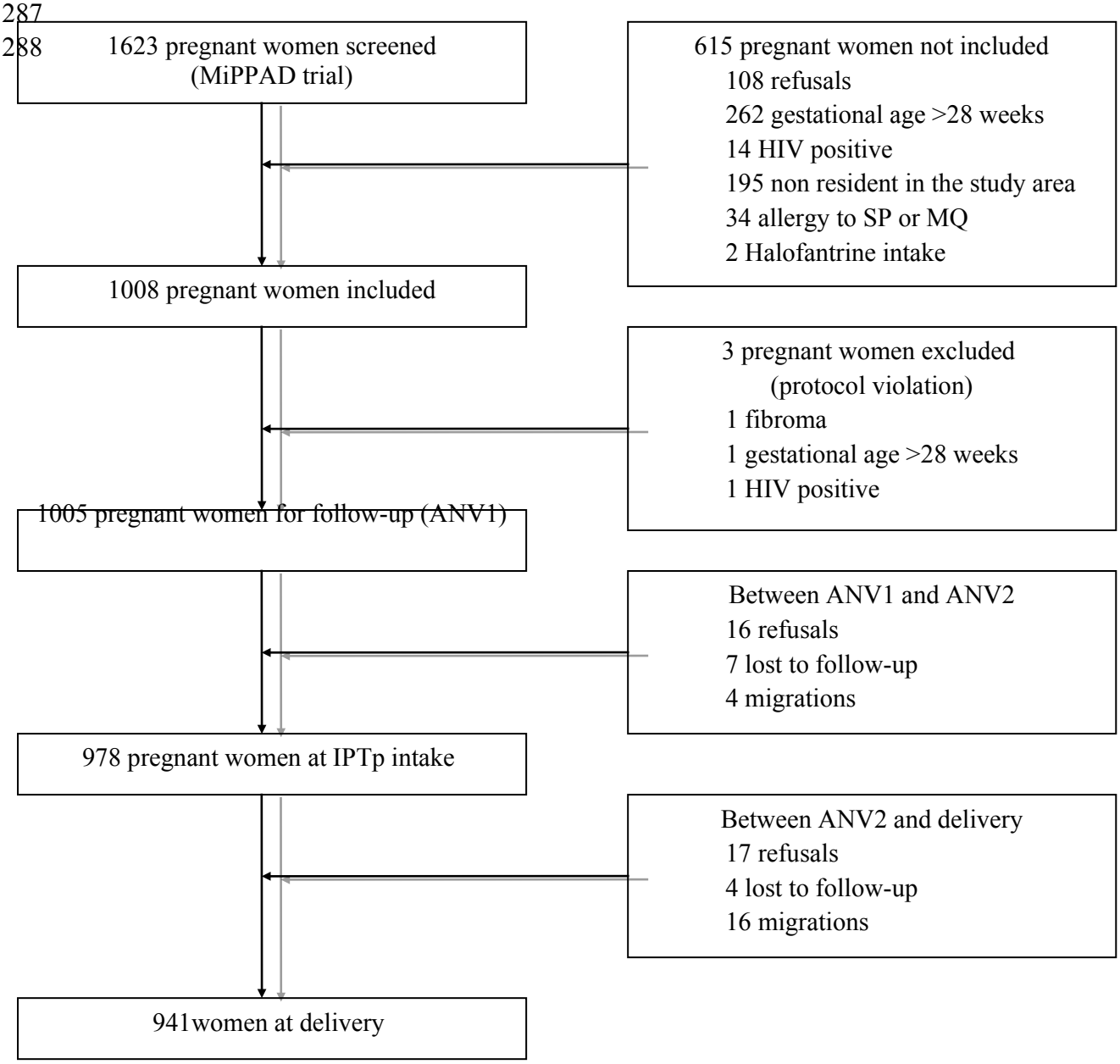


Figure 7: Follow-up of pregnant women

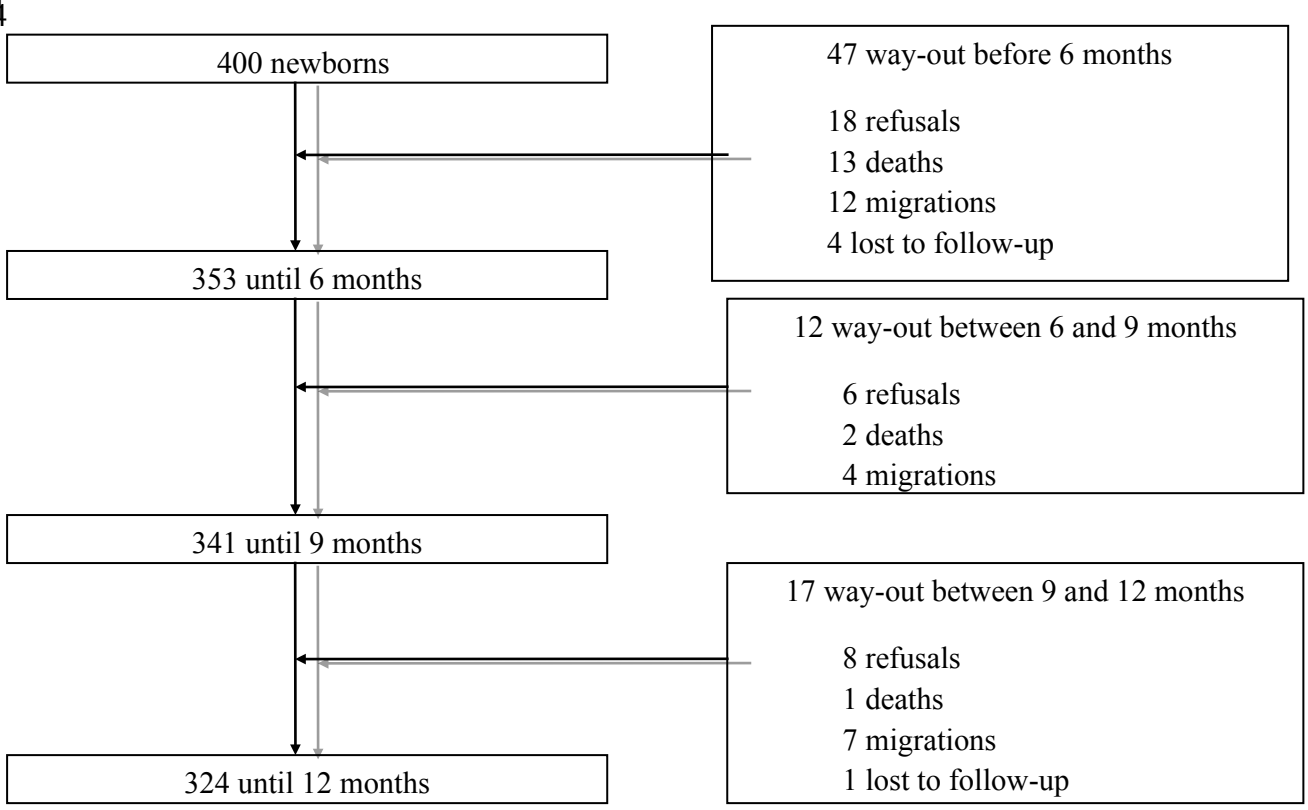


Figure 8: Follow-up of infants

IV. 3. Definitions

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

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

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

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.

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 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.

ID was then defined as corrected serum ferritin <15 µg/l in pregnant women and corrected serum ferritin concentration <12 µg/l in infants. 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 B_{12} 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.

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

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

IV. 4. Statistical analyses

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

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

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

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

Socio-economic items (home possession of latrines, electricity, a refrigerator, a television, a vehicle with at least two 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 was kept as the final socio-economic index.

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

Thereafter, two 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-values<0.2 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. Preliminary fixed effects analyses were realized using the maximum likelihood method, and variance components were estimated using the restricted maximum likelihood method. However, for both the analysis of the possibility of a positive blood smear and for the analysis of parasite density, random coefficient 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, random intercept was applied in both cases at the individual level as the effect of the variables is correlated within the women. Random slope was applied to gestational age as the effect of gestational age might 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: socio-economic status and rainfall in the case of malarial indicators, and BMI in the case of LBW. Manual backward selection procedure was performed and statistical significance was set at $P < 0.05$. The presented p-values and the significance threshold were two-sided.

In the infant follow-up, univariate analysis was conducted to assess the association of all variables with positive smear and the infant peripheral parasitaemia using multilevel models with a random intercept at the individual level. More precisely, we used the following co-variables: sex, low birthweight ((LBW), weight < 2500 g), preterm birth (gestational age < 37 weeks), fever (temperature 37.5°C), inflammation syndrome, placental malaria status, age (months), ethnic group, socio-economic index, gestational age at birth (weeks), maternal hemoglobin at delivery, maternal anaemia at delivery, hemoglobin, iron levels, folic acid, vitaminB12, folic acid and vitaminB12 deficiencies, IPTp regime, IPTp interval length (number of days between IPTp doses), IPTp timing, and Kato-Katz positivity.

Thereafter, two 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-values<0.2 in univariate analysis were included in the multilevel models. Preliminary fixed effects analyses were realized using the maximum likelihood method, and variance components were estimated using the restricted maximum likelihood method. However, for both the analysis of the possibility of a positive blood smear and for the analysis of parasite density, random coefficient models were used as they were statistically better than fixed effects according to AIC and BIC criteria. More precisely, random intercept was applied in both cases at the individual level as the effect of the variables is correlated within the infant. Random slope was applied to age as the effect of age might

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

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

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. Manual backward selection procedure was performed and statistical significance was set at $P < 0.05$. The presented p-values and the significance threshold were two-sided.

V. Results

V.I. LITERATURE REVIEW

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

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

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

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

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

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

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

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

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

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

With regard to control strategies, 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. Still, the influence of different IPTp regimes on malaria morbidity in infants remains a question for further research.

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

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

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

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

MALARIA JOURNAL

REVIEW Open Access

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

Violeta Moya-Alvarez[1,2,3*], Rosa Abellana[4] 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 preconceptual 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,

* Correspondence: vmoyaalvarez@gmail.com
[1]Institut de Recherche pour le Développement, UMR 216 Mère et enfant face aux infections tropicales, Faculté de Pharmacie Paris Descartes, 4 Avenue de l'Observatoire, 75270 Paris, France
[2]Université Pierre et Marie Curie (Paris 6), Centre Biomédical des Cordeliers, 15, rue de l'Ecole de Médecine, 75006 Paris, France
Full list of author information is available at the end of the article

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 simple 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*** Multigravidae: PM+: AOR =1.59, (1.16–2.17) PM-:AOR=0.67, (0.50–0.91)	PM+ ≈20%; PM-≈10%
Lambarené [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 [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	

Table. Influence of maternal parasitemia on malaria in infants (Continued)

Tori Bossito [illegible] (Benin)	Infant cohort follow-up (550)	2007-2008	Perennial with seasonal peaks (400 infective mosquito bites each year)	SP	AL	11%	0.85%	12 months	PM+: 3.4 (4.83) PM-: 4.5 (4.85)	TM AOR=2.13 (1.24-3.67) No TM AOR=1.18 (0.60-2.33)	22.3%
Mono (24) (Benin)	Mother and infant cohort follow-up (218)	2008-2010	Mesoendemic (1.35 bites/ person/year)	SP	Quinine or SP	32.7%		12 months	PAM+: 3.62 (3.5-3.89) PAM-: 3.6 [illegible]	PAM during the 3rd trimester of pregnancy: AOR=4.6 (1.2-17.2); 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 (AHR=3.1 [illegible]).

***Analysis of the effect of IPTp on parasitemia of the offspring was performed for 882 women of this cohort. Among them, 2.6% received no IPTp, 43% one dose, and 54.4% two or more doses.

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 neurocognitive 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 is 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

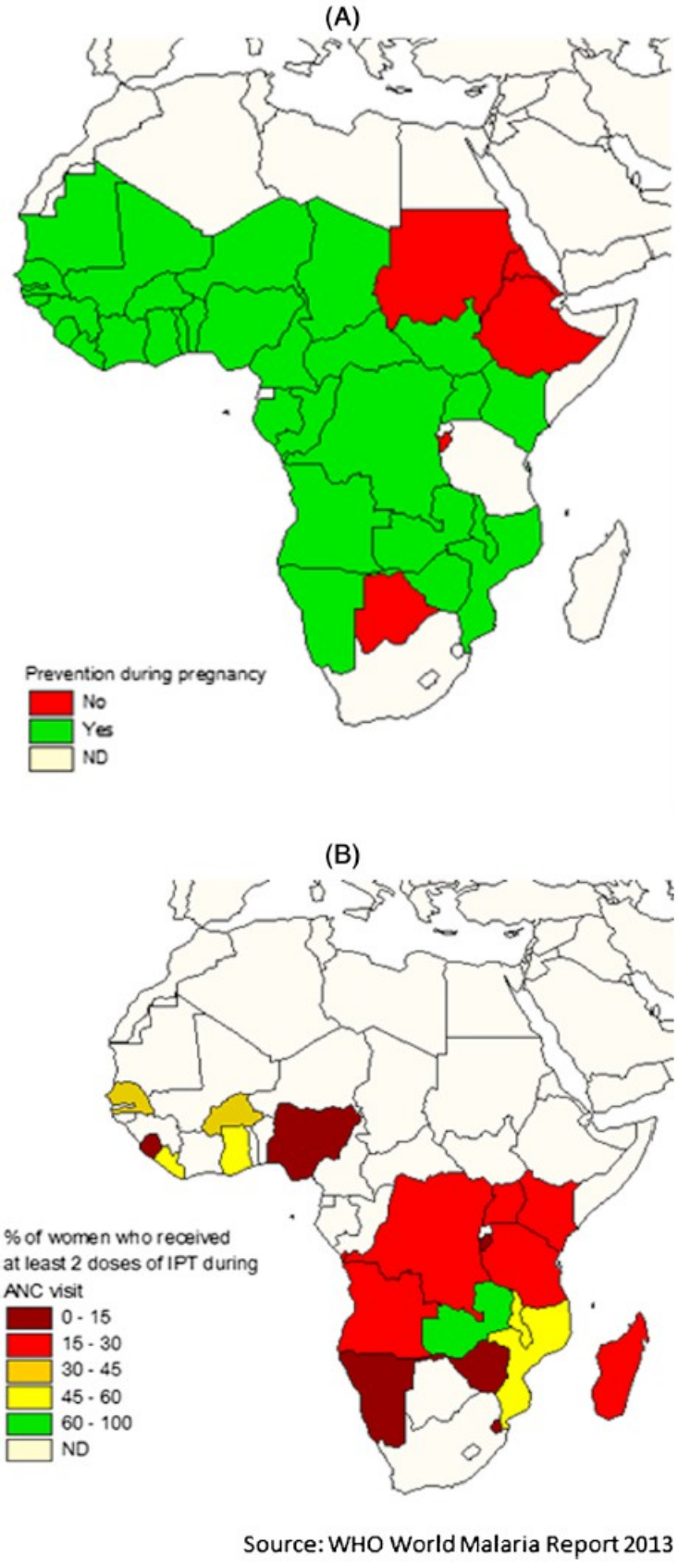


Figure 1 Intermittent Preventive Treatment in pregnancy in Africa. Source: World Malaria report 2013. WHO publications 2013. **A.** Implementation of intermittent preventive treatment in pregnancy in Africa. **B.** The percentages of women having received at least 2 doses of IPTp are approximated data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

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Author details

[1]Institut de Recherche pour le Développement, UMR 216 Mère et enfant face aux infections tropicales, Faculté de Pharmacie Paris Descartes, 4 Avenue de l'Observatoire, 75270 Paris, France. [2]Université Pierre et Marie Curie (Paris 6), Centre Biomédical des Cordeliers, 15, rue de l'Ecole de Médecine, 75006 Paris, France. [3]Réseau doctoral de l'Ecole des Hautes Etudes en Santé Publique, Avenue du Professeur Léon-Bernard, CS 74312-35043 Rennes, France. [4]Departament de Salut Pública, Facultat de Medicina, Casanova 143, 08036 Barcelona, Spain.

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

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

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

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

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

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

Article under review in *Nutrition reviews*:

Malaria and iron levels: the dangerous liaisons?

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

Abstract: Malaria is the disease with the highest infant morbidity and mortality (WHO estimates 207 million cases and 627,000 deaths in 2012), and it raises the burden of anaemia in low-income countries, where 40% of children are anaemic according to WHO estimates. Anaemia compromises immunity, and iron deficiency anaemia (IDA) has long-term permanent neuro-cognitive consequences. However iron has been pointed out as an important co-factor for *Plasmodium falciparum*, the main parasite responsible for malaria, raising fears that current iron supplementation policies might be harmful. Albeit the complexity of the effect of iron levels on malaria risk, an accurate review clarifying their epidemiological association and assessing the different novelties on iron markers has yet to be conducted. Observational studies describe a certain protection for malaria risk among iron deficient children, and ancient clinical trials report increased susceptibility to clinical malaria among iron supplemented children. Nevertheless neither recent clinical trials with important malaria monitoring and protective measures, nor the Cochrane review show significant increase for malaria risk among iron supplemented children. Evidence on the effect of iron levels on malaria risk is subject to limitations, such as the interference of protective measures, and the lack of homogenous iron markers and haematological indicators. The effect of the previous haematological and infectious health status, including the chronicity of iron deficiency and the possible threshold effect of iron levels, needs to be investigated in the context of a gold standard combination of iron markers taking into account both parasitological and clinical malaria outcomes. Further epidemiological elements, such as age of the children, immunity status, hemoglobinopathies, or the transmission setting should be considered as well. Finally,

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

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

Abstract.

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

Key words. Iron, malaria, iron supplements, iron indicators, anaemia.

Introduction

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

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

Methods: Search strategy and selection criteria

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

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

Malaria and iron levels: evidence from epidemiological studies

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

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

Malaria indicators and iron: epidemiological evidence

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

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

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

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

iron deficient children (Adjusted incidence rate-ratio (IRR)= 0.7 (95%CI 0.51;0.99) with ferritin<12µg/ml and transferrin saturation<10%) [22]. Furthermore, iron status was inversely correlated with malaria-specific immunoglobulins. Similar results were found in an observational cohort study in Tanzania [23] among children between birth and 3 years. Iron deficiency (defined by ferritin concentration corrected on CRP) was also associated with a significant protection with regard to lower odds of malaria parasitemia (OR=0.15 (95%CI 0.12;0.19)), lower odds of hyperparasitemia (parasites>2500/200 white blood cells (OR=0.04 (95%CI 0.02;0.07)), and lower odds of severe malaria (OR=0.25 (95%CI 0.14;0.46)) after adjustment for possible confounders. In a pioneer randomized placebo controlled trial in Tanzania in 1995 in infants between 8 and 24 weeks of age, no increased susceptibility to malaria was observed among iron supplemented children with regard to first or only malaria episode compared to placebo (protective efficacy (PE)= 12.8% (CI -12.8;32.5) [24]. Albeit this first reassuring result, supplementation effects on children health status had to be re-evaluated after the Pemba trial. In 2002-2003 a randomised, double blind, placebo-controlled trial, gathered medical evidence on all-cause morbidity and mortality among over 24,000 children up to 35 months daily supplemented with folic acid and iron, iron, folic acid, zinc or placebo10 in Pemba, Tanzania. In the same cohort, a sub-study among 2413 children addressed the impact of supplements on haematological status, zinc, malaria prevalence, and infectious disease morbidity. Combined groups of supplemented children had significant higher risk for serious clinical events resulting from malaria compared to placebo (RR=1.16, CI 1.02; 1.32). Malaria related hospital admissions were also significantly higher (RR=1.18, (95%CI 1.02; 1.36)) among supplemented children. In the case of cerebral malaria, the RR of the iron and folic acid group, was also significant compared to placebo (RR=1.22 (CI 1.02; 1.46). In addition another deeply relevant aspect of the malaria-iron association was first raised up: the importance of the iron levels at baseline. Iron-deficient children at baseline, defined by zinc protoporphyrin>80 µmol/molhaeme, had a reduced risk of malaria-related adverse events when supplemented compared to placebo (RR=0.56, 95%CI 0.32; 0.97). Due to the increased

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

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

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

Because of these a priori contradictory results of the studies, a Cochrane review of 2011 analysed 71 trials collecting evidence on 45,353 children [28]. For the 13 trials selected, the Cochrane review concluded to an absence of significant differences in clinical malaria rates between iron and placebo (RR=0.99, 95%CI 0.9; 1.09). No statistical differences were found neither among supplemented infants (children<2years) (RR=0.94 (95%CI 0.82; 1.09) nor for severe malaria (RR=0.91 (95%CI 0.76; 1.08)) compared to placebo. Furthermore, no statistical difference was found among non-anaemic children at baseline (RR=0.97 (95%CI 0.86; 1.09). However, analyses on iron deficiency defined by ferritin were not realized. Even if it is difficult to screen children for iron status at the population level, information on the effect of iron deficiency is relevant to

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

Beyond clinical malaria, it is necessary to consider also malaria mortality to capture broader aspects of the iron-malaria association. In the context of the clinical trial with iron supplements in Pemba, mortality due to malaria was higher (although not significantly) among supplemented children compared to placebo (RR=1.08, (95%CI 0.84; 1.40)). Among children supplemented with iron and folic acid, there was also a significant increased risk for cerebral malaria as a cause of death compared to placebo (RR=1.70, 95%CI 1.08; 2.68). The iron and folic acid supplemented children were 12% more likely to suffer an adverse event resulting in hospitalisation or death (95%CI 2;23) compared to placebo and all-cause mortality was also significantly higher: OR= 1.61 (95%CI 1.03; 2.52). Iron deficiency and moderate anaemia at baseline were significantly associated to lower rate of adverse events (death or severe morbidity leading to admission) among supplemented children compared to placebo. Further extensive studies on the impact of iron supplements on mortality to malaria are scarce due to the difficulty of attributing correctly the cause of death in endemic settings and, hence, it is difficult to accurately assess the interaction between malaria and infection with regard to mortality. In addition more statistical power is needed as iron measures are rare and death is also a rare event.

In a good attempt to clarify finally the conundrum, the Cochrane meta-analysis[28] on the impact on iron supplements addressed certainly this question but did not provide a definite answer. In this review, the relative risk for all-cause mortality was not estimable. However, it was capable of displaying useful information with regard to transmission settings. Mortality was not significantly different between hyper- and holo-endemic areas (Risk difference= 1.93 per 1000 children (95% CI -1.78; 5.64).

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

protection for malaria risk among iron deficient children. In parallel, meaningful ancient studies report increased susceptibility to clinical malaria among iron supplemented children[7,8], and so does the Pemba trial, which has a considerable statistical power. However, other recent clinical trials with important malaria monitoring and protective measures, show no significant increase for malaria risk among iron supplemented children[26,27] and neither does the Cochrane review[28]. Albeit the absence of overall significance, the cross-sectional studies in Tanzania report also significant earlier malaria among supplemented children[26].

Evidence on the effect of iron levels on malaria risk is subject to certain limitations, such as methodological study constraints, homogenous measurement of iron and haematological indicators, the effect of different transmission patterns, and further possible confounders. In effect, statistical limitations are inherent to ethical research studies. Clinical trials display results based on intensively monitored parameters. In most of them prophylactic protection by ITNs or preventive treatment for malaria is more frequent among enrolled patients than in observational studies, and treatment is also given as soon as a case is confirmed. As a consequence, it is difficult to disentangle the possible protective effect of IDA from the protection given by protective measures, especially in the case of severe malaria or hyperparasitemia in clinical trials. Preventive measures reduce the number and the severity of malaria episodes and, hence, statistical power decreases as does the force of the association. The dimension of the association, or its absence, should be ideally assessed in the conditions in which population undergo the malaria burden and the nutritional interventions. Nevertheless, accurate iron monitoring is not realized systematically and malaria episodes are not always captured by demographic or surveillance data. In addition, observational studies that do not provide treatment are unethical in malaria endemic countries with limited access to health care. However, surveillance data or data issue of demographic surveys may be useful to get a basic idea on malaria risk and haematological indicators.

With regard to the epidemiological indicators, malaria infection outcomes (clinical malaria and

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

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

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

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

conundrum.

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

In order to better discern the importance of iron levels for malaria morbidity and mortality, the determination of iron levels requires precision and consensus among researchers. The understanding of the nature and the meaning of the different iron and haematological markers is necessary as the definition of common indicators might enable the extrapolation of results and improve their interpretation. Therefore, it becomes a prerequisite to remind briefly the physiopathology of iron involved in the *P. falciparum* infection process.

Iron has multiple effects on malaria physiopathology: it interacts with the host's immunity but also with the parasite. With regard to the host immunity, iron interferes with zinc and with the inducible nitric oxide synthase (iNOS). In parallel, the host inflammation process increases hepcidin, a hormone regulating iron disposal in plasma, in order to block iron absorption. Thus it was first reported that by inhibiting the absorption of zinc, iron would alter the immune response to infection [31], but recent studies describe no improvement in infection outcomes in zinc supplemented children [26]. In addition, iron inhibits the synthesis of nitric oxide, an anti-infectious agent [32], even if the subsequent consequences for malaria are not fully understood.

At the host level the interaction of iron and *P. falciparum* is also significantly determined by the NTBI, involved in parasite metabolism. Hepatocytes take up faster NTBI than transferrin-bound iron [33] and, in animal models, the supply of iron contributes to the penetration of hepatocytes by *Plasmodium* and stimulates their growth to merozoites [34]. Furthermore NTBI is involved in parasite sequestration of malaria-infected erythrocytes in the capillaries of the brain and intestine through up-regulation of ICAM-1 and is thus linked to severe malaria [14–16].

The biological indicators reflect the different pathways in which iron interferes with malaria infection, and their choice as iron markers in research studies are crucial to determine the meaning of the results. The joint WHO-CDC Technical Consultation for iron assessment selected 5

different indicators as good iron markers: haemoglobin, mean cell volume (MCV), (sTfR) concentration, serum ferritin concentration, and red cell protoporphyrin (measured by the zinc protoporphyrin/haemoglobin ratio (ZPP:H) [35,36]). Table 2 summarizes the main characteristics of these markers. Haemoglobin is deeply useful in the monitoring of health status and its determination is easy to realize on the field. Although it is a basic fundamental haematological indicator, it is not specific as an iron marker because of the multiple causes of anaemia and the physiological variations with regard to sex, age or ethnicity. Therefore, it can be misleading for the extrapolation of conclusive results. Mean cell volume accuracy is limited in the context of thalassemia and malaria as inflammation serum transferrin receptor modifies significantly its values. Due to its physiopathological pathway, serum transferrin receptor is also influenced by the haemolysis of malaria, and its determination method is not always standardized nor cost-effective [37].

Serum ferritin is a precise indicator of iron storages in healthy individuals and it can be corrected according to other inflammation proteins. It provides further information as it also shows different patterns of behaviour depending on the aetiology of anaemia [16]. In an iron supplementation study, Doherty et al. compared the erythrocyte incorporation of oral iron supplement in 37 Gambian children 8 to 36 months old with anaemia after malaria treatment, to supplemented control children with IDA but no recent malaria [38]. The non-malaria control children showed progressively increased serum ferritin whereas the post-malarial children showed decreased serum ferritin levels. Serum ferritin levels became similar in both groups only by day 15 and 30. This is thought to be due to the normalization of the immune response following the malaria treatment [16]. Indeed, serum ferritin is an acute phase protein. Hence, serum ferritin is either corrected upon inflammation (with correction factors according to C-reactive protein (CRP) or α-1-glycoprotein (AGP) levels), or samples with high acute inflammation proteins are systematically excluded. Nevertheless the exclusion of samples with increased inflammation might entail a subsequent bias in the context of malaria, as samples with high ferritin would be systematically

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

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

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

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

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

Conclusion

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

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

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

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

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

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

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

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

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Tables:

V. Results

Table 1. 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<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 51±1.4
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	minum 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 enrolement	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)	Non significative 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<.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	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
Handzeri	Tanzania	2008-2009	randomised placebo-controlled trial	intense	612	median follow-up 331 days	6-60 months	ID: plasma ferritin concentration <12 µg/L	Compared to placebo: All malaria episodes: Zinc group: AHR=0.99 (CI 0.82; 1.18) Multi-nutriments without zinc: AHR=1.04 (CI 0.87; 1.23) Multinutriments with zinc: AHR=1.14 (CI 0.96; 1.35) First malaria episodes: Zinc group: AHR= 1.12 (CI 0.86; 1.44) Multi-nutriments without zinc: AHR=1.35 (CI 1.05; 1.73) Multinutriments with zinc: AHR=1.38 (CI 1.07; 1.77) Number of episodes with versus without multinutriments Iron deficient: HR=1.41 (1.09; 1.82) Iron replete: HR=0.93 (0.77; 1.13)	*Difference relative to placebo (95%CI), Hemoglobin concentration (g/l) Micronutrients without zinc: 106.6 (10.7) *2.6 (0.0; 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 (0.05) *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 (0.05) *19.5 (11.3; 28.6) Micronutrients with zinc: 51.1 (0.03) *26.7
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	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)	
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	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)	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

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

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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V.II. ARTICLES REPORTING ORIGINAL RESULTS

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

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

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

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

= 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) and all-cause outpatient attendances during pregnancy (850/557.8 outpatients visits PYAR in the SP group and 1,480/1,110.1 visits PYAR in the MQ group (RR= 0.86, 95%CI (0.78; 0.95), p-value =0.003). In the MiPPAD study there were no differences in the prevalence of placental infection and adverse pregnancy outcomes between groups. In conclusion women taking MQ IPTp (15 mg/kg) in the context of long lasting insecticide treated nets had similar prevalence rates of low birth weight as those taking SP IPTp. MQ recipients had less clinical malaria than SP recipients, and the pregnancy outcomes and safety profile were similar. The conclusions of the MiPPAD trial do not support a change in the current IPTp policy.

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

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

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

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

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

MAJOR ARTICLE

Does Iron Increase the Risk of Malaria in Pregnancy?

Violeta Moya-Alvarez,[1,2,3,4] Gilles Cottrell,[1,4] Smaila Ouédraogo,[4,5,6,7] Manfred Accrombessi,[2,4] Achille Massougbodgi,[8] and Michel Cot[1,4]

[1]MERIT- Mère et Enfant Face aux Infections Tropicales, Institut de Recherche pour le Développement, Paris, France; [2]Université Pierre et Marie Curie, Paris, France; [3]Réseau Doctoral de l'Ecole des Hautes Etudes en Santé Publique, Rennes, France; [4]Université Paris Descartes, Sorbonne Paris Cité, France; [5]MERIT- Mère et Enfant Face aux Infections Tropicales, Institut de Recherche pour le Développement, Cotonou, Benin; [6]Unité de Formation et de Recherche en Sciences de la Santé, Université de Ouagadougou, Burkina Faso; [7]Public Health Department, Centre Hospitalier Universitaire Yalgado Ouédraogo, Ouagadougou, Burkina Faso; and [8]Faculté des Sciences de la Santé, Université d'Abomey Calavi, Cotonou, Benin

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 $\log_{10}$ 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 socioeconomic 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

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Correspondence: Violeta Moya-Alvarez, MD, MPH, IRD/UMR 216 – MERIT, Université Paris Descartes, 4 avenue de l'Observatoire, 75270 Paris cedex 06, France (vmoyaalvarez@gmail.com).

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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

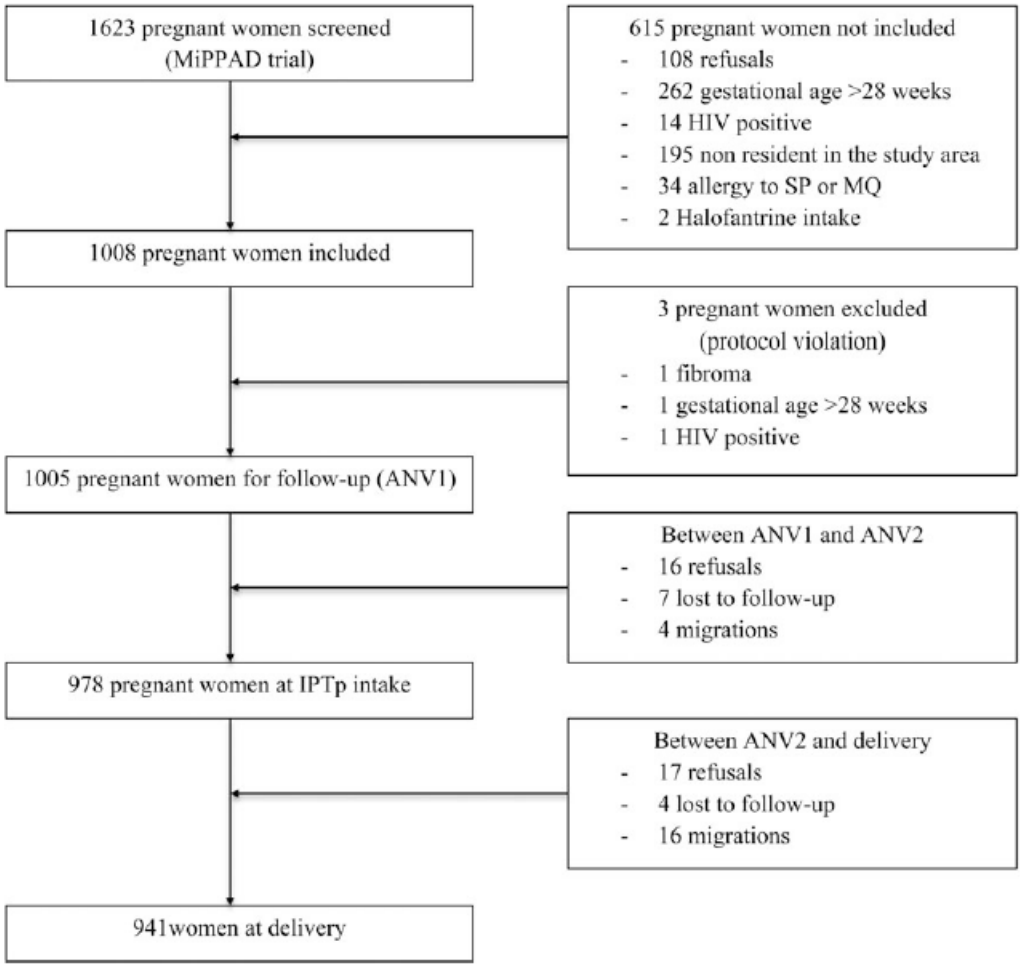

Figure 1. Study profile. Abbreviations: ANV, antenatal visit; HIV, human immunodeficiency virus; IPTp, intermittent preventive treatment; MiPPAD, Malaria in Pregnancy Preventive Alternative Drugs; MQ, mefloquine; SP, sulphadoxine-pyrimethamine.

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 B_{12} 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

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 primigravid, secundigravid, and multigravid 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 primigravid, 13.4% of secundigravid, and 6% of multigravid women ($P < .01$). The proportion of LBW was also inversely correlated with gravidity: 18%, 10.7%, and 7.5% for primi-, secundi-, and multigravid 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.

Table 2. Indicators of Malaria, Folate, and Iron Indicators During Pregnancy[a]

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%)
Plasmodium falciparum 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.

[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.

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 ($\log_{10}$ 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)	*P* 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.

[a]Random 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.

[a]Analysis 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.

[a] Analysis 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.

[a] Random intercept at the individual level and random slope for gestational age. Analysis on 1605 blood smears from 747 women.

were reported to be insufficient for assessing the potential for an 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 cohort 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 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 an increased number of clinical episodes [29–31]. Consequently, the association of high iron with placental malaria might contribute to enhance its consequences 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 [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 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 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. Although iron supplementation trials do not show augmented malaria morbidity associated with iron supplements, ID is correlated with lower odds of malarial episodes [25]. Iron deficiency was statistically linked to reduced risk of placental malaria in Tanzania [17]. Ferritin was also higher among placenta-infected mothers in Gabon [33] and zinc protoporphyrin in Malawi [34], but these differences were not statistically significant. 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 difference in placental malaria associated with iron supplementation depending on sickle cell genotype [25]. However, as stated previously, these studies report iron levels only at enrollment, 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 [37]. Furthermore, the changes in iron metabolism during a malaria infection may modulate susceptibility to coinfections [37]. In addition, iron inhibits the synthesis of nitric oxide by inhibiting the expression of inducible nitric oxide synthase and thereby interferes with macrophage-mediated cytotoxicity 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-Gonçalves 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 inflammation on ferritin values (ferritin is an acute phase protein), we corrected ferritin upon inflammation (with correction factors according to CRP). Then, we included systematically inflammation in the statistical models to capture its independent association with malarial risk.

Another important finding of our study is the association between folate levels and PAM outcomes. High folate was correlated with reduced risk of malarial episodes, parasite density, and PM. Folate is an important cofactor used by (1) *P falciparum* in DNA synthesis and methylation and (2) mRNA translation. 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 preventive treatment in pregnancy clears *Plasmodium* parasites and has a prophylactic effect on malarial episodes. Intermittent preventive 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

according to gestational age. For these reasons, it is important to 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.

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V.II.2 Association of iron levels and interval length between IPTp

doses on malaria in infants during the first year of life

Summary of the article: As already explained in the « State of art » section, epidemiological studies have reported an increased malarial risk in infancy associated with high iron levels. This aspect is of special concern in Benin, because malaria is the first cause of mortality of infants under 5 years, and no national guidelines exist at present regarding iron supplements in infancy.

To investigate the effect of iron on malaria risk during the first year of life, we used the data of 400 infants (200 born of the anemic and 200 born of non anemic mothers) included in the APEC (Anaemia in Pregnancy: Etiology and Consequences) study. In addition to the mother's follow-up, clinical data of the infants were collected during systematic visits at 6, 9, and 12 months in three clinics in the district of Allada (Allada, Attogon, Sékou). In case of sickness infants were accurately examined in unscheduled visits and, if necessary, treated according to the Beninese Ministry Health guidelines. In the unscheduled visits clinical and biological exams were realized following the same protocol as systematic visits, i.e., anthropometric measures, and an extensive clinical examination were realized. In addition, 8 ml of venous blood (4ml in a dry tube and 4ml in an edta tube) were collected at each visit. Haemoglobin, serum ferritin, CRP, vitamin B12, and folate levels were assessed. A container was also given to the women to collect stools to examine the presence of intestinal helminths in the infants. These containers were collected the following day by the study nurses within the first 6 hours after emission.

During the first year of life, 40% of the infants had at least one malarial episode, with a range of 0-4 positive smears. Offspring of mothers with longer IPTp protection (number of days between IPTp doses) were significantly less likely to both have a positive smear (adjusted

odds ratio (aOR)=0.87, p-value=0.04) and high *P. falciparum* parasite density (beta estimate=-0.06, p-value<0.001) during the entire follow-up period. Iron levels (measured by the log of ferritin corrected on inflammation) were significantly associated with the risk of a positive blood smear (aOR=2.77, p-value<0.001) and *P. falciparum* parasitaemia (beta estimate=0.38, p-value<0.001). In multilevel model analysis, infants with iron levels in the lowest quartile were significantly less likely to have a positive blood smear during the first year of life (p-value<0.001), and the risk increases with higher iron levels.

We were surprised that the interval length between IPTp doses (i. e. the number of days between doses) was associated with malarial risk and not with PAM. However, PAM might not be symptomatic enough in the women of our cohort to make them go consult to the clinics. Therefore, we might have lost valuable information during the mother's follow-up and, hence, our data might not have enough power to show an effect. Nevertheless, it is coherent that, knowing that the interval length between IPTp doses modifies the time of exposure of the foetus to *Plasmodium*, it might have an effect of malaria in infants due to a possible immune tolerance process.

Similarly to the mother's case, iron levels in infants were significantly associated with increased malaria risk during the first year of life. Furthermore, our results suggest the existence of dose effect of iron levels on malaria risk. Because of these results and the previous literature on the topic, we think that additional epidemiological studies are required to evaluate the effect of different doses of iron supplements on the infant health outcomes. In addition, the comparison of cohorts in which iron is given with preventive purpose versus iron given for the treatment or anaemia or iron deficiency (ID) is also interesting. Finally, public policies should be encouraged to increase the observance of IPTp as it has a protective effect not only in mothers but also in their offspring.

What's known on this subject: Malaria and iron deficiency are the main causes of anemia in

infants. The benefits of iron supplements are questioned in malaria settings, but no longitudinal data exist. Moreover, the influence of IPTp on malaria in infants has seldom been analysed.

What this study adds: We show that elevated iron levels and short interval between IPTp doses are associated with increased risk of malaria and *P.falciparum* density in a longitudinal prospective cohort during infancy in the context of ITN use, considering environmental, clinical and obstetric risk factors.

Article under review in *"Pediatrics"*

The effect of iron levels and IPTp on malaria risk in infants: a prospective cohort study in Benin

Violeta Moya-Alvarez, Gilles Cottrell, Smaila Ouédraogo, Manfred Accrombessi, Achille Massougbodgi, Michel Cot

Abstract:

Background: In areas with high malaria and anaemia rates, intermittent preventive treatment in pregnancy (IPTp) and iron supplements are recommended by WHO. However, studies have set into question the inviolability of the benefits of iron supplementation in the context of malaria. In addition, pregnancy-associated malaria (PAM) has been found to be associated to malaria in infants, but epidemiological studies do seldom analyse the influence of IPTp. We investigated the effect of IPTp and iron levels during the first year of life on malarial risk.

Methods: We followed 400 women and their offspring between January 2010 and May 2012 in Allada (Benin). Environmental, obstetric and numerous clinical maternal and infant risk factors were considered.

Results: In multilevel models, offspring of mothers with longer IPTp protection were significantly less likely to both have a positive smear (adjusted odds ratio (aOR)=0.87,p-value=0.04) and high P.falciparum parasitaemia (beta-estimate=-0.06,p-value<0.001). Iron levels were significantly associated with the risk of a positive blood smear (aOR=2.77,p-value<0.001) and P.falciparum parasitaemia (beta-estimate=0.38,p-value<0.001). Infants with iron levels in the lowest quartile were less likely to have a positive blood smear (p-value<0.001), and the risk increased with higher iron levels.

Conclusion: Our results appeal for additional evaluation of different doses of iron supplements on the infant health outcomes. Thus, the comparison of cohorts in which iron is

given with preventive purpose versus iron given for treatment is also required. Finally, the observance of IPTp should be encouraged as it has a protective effect not only in mothers but also in their offspring.

Body of the article:

Introduction

Infant health morbidity in Sub-Saharan Africa is mainly driven by infectious diseases and nutritional deficiencies [1]. Indeed, malaria and anaemia (mainly due to iron deficiency) are two leading pathologies contributing to enhance the disease burden among African infants [2]. In 2013, malaria was responsible for an estimated 198 million cases and an estimated 584 000 deaths [3]. In addition, malaria causes anaemia, which is the second leading cause of disability [4] and entails severe consequences for the development of the children [5]. Moreover, both diseases harm mainly children under 5 years of age. For these reasons, public health strategies have been developed to tackle both diseases simultaneously.

To tackle anaemia WHO recommends the administration of 12.5 mg iron and 50µg folic acid daily between 6 and 12 months [6]. However, in Benin this policy has not been implemented so far, and, in general, Beninese paediatricians give a preventive treatment consisting in 10 mg/kg and day during 2 months every 6 months starting at 6 months of age until 5 years of age. With regard to malaria, the present WHO recommendations for the control of malaria are the use of insecticide treated nets (ITNs) and/or indoor residual spraying (IRS) for vector control, and prompt access to diagnostic testing of suspected malaria and treatment of confirmed cases.

Albeit the large implementation of these interventions, epidemiological studies have set into question the inviolability of the benefits of iron supplementation in the context of malaria-endemic countries [7], and iron deficiency has been associated to reduced malaria odds among pregnant women and infants [8,9]. However, in a recent meta-analysis of the association between malaria and iron status or supplementation in children, data were reported to be insufficient for assessing the potential of an increased risk of *P.falciparum* infection [9].

Indeed, evidence on the iron-malaria association lacks from prospective cohorts during infancy.

In parallel, PAM is significantly associated to malaria in infants [10], but epidemiological studies do seldom analyse its influence. Therefore, we investigated the effect of iron levels during the first year of life on malarial risk in infants taking into account complementary information on PAM, IPTp, environmental, socio-economic, and clinical indicators and co-morbidities to better understand malaria risk factors in the context of the present malaria control strategies.

Materials and methods

A prospective cohort of 400 infants was followed from birth to 12 months of age in the context of the APEC study (Anaemia in Pregnancy: Etiology and Consequences). APEC study is an ancillary survey nested within the MiPPAD trial in Benin (Malaria in Pregnancy Preventive Alternative Drugs “http://clinicaltrials.gov/ct2/show/NCT00811421”). This study was conducted in three clinics in the district of Allada, between January 2010 and May 2012. 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.

Complete details of MiPPAD are presented elsewhere [11], but, briefly, MiPPAD was a randomized trial comparing the efficacy and safety of IPTp with SP (1,500/75 mg per dose) and mefloquine (15 mg/kg per dose). At delivery placenta was examined in order to analyse *P. falciparum* parasite infestation. Clinical data of the infants were collected during systematic visits at 6, 9, and 12 months. In case of sickness infants were accurately examined in unscheduled visits and, if necessary, treated according to Beninese guidelines. In the unscheduled visits clinical and biological exams were realized following the same protocol as systematic visits. All drugs prescribed to the infants during the follow-up were free of charge.

For the purpose of the APEC sub-study, anthropometric measures, and an extensive clinical examination were realized during the visits. In addition, 8 mL of venous blood were collected at each visit. Haemoglobin, serum ferritin, CRP, vitamin B12, and folate levels were assessed. A container was also given to the women to collect stools that were collected the following day by the study nurses within the first 6 hours after emission. Microbiological exams were realized as follows: Lambaréné technique was used to assess malaria infection [12]. Helminthic infestations were assessed using the Kato-Katz concentration method (VestergaardFrandsen kit®). In case of inflammation (CRP>5mg/l) serum ferritin was adjusted following the corrections recommended by Thurnham et al. in their meta-analysis [13] to avoid the extrinsic effect of inflammation on serum ferritin levels.

We used rain quantity as a surrogate for the risk of exposure to anopheline bites. Because of the anopheline timeliness, rainfall quantity was calculated as the mean rain volume of the 7 days prior to the two weeks before the consultation. It was independently assessed for each visit and each health centre of the study.

Socio-economic status was assessed using a socio-economic index. The socio-economic index was created in a two-step process. First a multiple correspondence analysis of socio-economic items was performed. Then the first principal axis was used as an overall socio-economic index in the further regression analysis.

Data were double entered and analysed with ACCESS 2003, and STATA 12.0 Software (Stata Corp, College Station, TX, USA). Then 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-square and Kruskal-Wallis tests were used in the univariate analyses. For time-dependent variables, univariate analyses were realized using a random intercept model at the infant level. Thereafter, all variables with P values<0.2 were included in either a logistic or a linear 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

parasites/mm3 (95% CI= 45882.41; 263310.7) at 9 months, and 7919.5 parasites/mm3 (95% CI= 26019.96; 136360.9) at 12 months.

In parallel, the mean haemoglobin values increased slightly, though not significantly, through the follow-up (102.1 g/l, 102.9 g/l, and 103.6 g/l at the 6, 9, and 12 month systematic visits, respectively).

Iron indicators decreased through the follow-up. The mean ferritin levels decreased after the 6 month visit from 605 µg/l (95% CI= 508; 702) to 455 µg/l (95% CI= 384; 526) at 9 months, and then decreased again until 436 µg/l (95% CI= 350; 522) at 12 months. Iron deficiency increased in parallel from 16% at 6 months, to 29% at 9 months, and up to 34% at 12 months.

During the first year of life malaria rates and *P. falciparum* parasitaemia were determined by clinical, environmental and socio-economic factors, but pregnancy related aspects did also influence significantly the malarial outcomes of the infant during the entire follow-up. The risk factors for malaria and *P.falciparum* parasite density are presented in Table 3 and Table 4, respectively.

There were no statistical differences in the number of positive smears or *P. falciparum* density during the first year of life depending neither on the placental malarial status nor on the intermittent preventive treatment in pregnancy (IPTp) regime of the mothers (either sulphadoxine-pyrimethamine (SP) IPTp or mefloquine (MQ)). Nevertheless, the time interval between IPTp doses of the mothers (number of days between IPTp doses), i.e. the period during which the mothers were protected against malaria, was significantly associated with the risk of malarial infection of the infant during the first year of life. Infants born to mothers who had longer IPTp protection were significantly less likely to both have a positive smear (adjusted odds ratio (aOR)=0.87, 95% CI= 0.76; 0.99, p-value=0.04) and high *P. falciparum* parasite density (beta-estimate=-0.06, 95% CI= -0.10; -0.01, p-value<0.001) during the entire follow-up. Higher maternal folate levels and helminth infection at delivery were also significantly linked to increased parasite density during the first year of life (beta-

2887estimate=0.34, 95% CI=0.01; 0.66, p-value=0.04, and beta-estimate=0.88, 95% CI= 0.20;
28881.57, p-value=0.03, respectively).
2889The clinical and nutritional status of the infant was also correlated with malarial risk. Iron
2890levels (log of ferritin corrected on inflammation) were significantly associated with the risk of
2891a positive blood smear (aOR=2.77, 95% CI= 1.95; 3.96, p-value<0.001) and *P. falciparum*
2892parasite density (beta-estimate=0.38, 95% CI= 0.29; 0.47, p-value<0.001) during the first year
2893of life. Infants with iron deficiency were significantly less likely to have a positive blood
2894smear and a high *P. falciparum* density (p-value=0.01 in both cases). In parallel, ongoing
2895inflammatory status of the infant (CRP>5mg/l) was significantly associated to an increased
2896risk of positive blood smear (aOR=4.37, 95% CI= 2.20; 8.65, p-value<0.001) and to a higher
2897*P. falciparum* parasite density (beta-estimate=0.77, 95% CI= 0.53; 1.01, p-value<0.001). The
2898presence of other parasites such as intestinal helminths was not significantly associated with
2899increased malaria risk. There were no statistical differences in malaria risk between the
2900different age periods of the follow-up.
2901The rain volume (representing the anopheline risk) was marginally associated to increased
2902malaria risk with regard to both increased risk of a positive smear (aOR=1.06, p-value=0.06),
2903and to increased *P.falciparum* parasitaemia (beta-estimate=0.03, p-value=0.06).
2904Finally, we investigated further the differences in malarial risk factors considering the
2905different quartiles of iron levels in infants to evaluate the possible different effects of iron on
2906malaria risk depending on the different levels of iron. Indeed, infants with iron levels in the
2907three upper quartiles had significantly higher risk of having malaria during the first year of
2908life (table 5). Infants with iron levels in the upper quartiles had significantly higher *P.*
2909*falciparum* parasite density.

2910**Discussion**

2911In this study, we evidenced the influence of two important factors related with malaria
2912infection during the first year of life, time duration between two IPTp doses and iron levels.

More precisely, we found that the time period between IPTp doses (number of days) 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. High iron levels also have 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 offspring [10]. In a recent follow-up of a mother-child cohort in Benin, Borgella et al. showed that 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 episode (OR=4.6 95% CI (1.7; 12.5), p-value=0.003), assuming the period covered by IPTp (2nd trimester of pregnancy) was at low risk for malaria infection [14]. In addition, Huynh found IPTp calendar was associated with consequences of malaria such as LBW and anaemia [15]. Considering that PAM has a significant effect on malaria in infants and that IPTp, by preventing new infections, has an impact on secondary malaria outcomes, such as LBW and anaemia, we think that albeit their novelty our results are coherent with the existing literature. A single discordant study in Tanzania found that IPTp could be associated with an overall increase of severe malaria and earlier first malaria episodes in the offspring [16]. Such paradoxical results could be explained by the high level of resistance to SP in this area of Tanzania [17,18]. Indeed, Dechavanne found increased susceptibility of infants to *P. falciparum* parasites with antigens to which they were previously exposed in utero [19], suggesting the existence of an in utero ongoing immune tolerance process. However, no evidence exists at present on its concrete physiopathological pathways.

At present an adjustment of IPTp calendar to enhance protection is already ongoing in Benin. In effect, Benin is setting a 3rd dose in the IPTp regime to implement the WHO new recommendations.

Another important result of our study is the significant association of iron levels with malaria risk. We have assessed the influence of iron levels on malarial risk with regard to the possibility of having a positive blood smear and *P.falciparum* parasite density throughout the first year of life in a prospective longitudinal cohort, considering environmental, socio-economic, and PAM factors. Iron levels, measured by ferritin corrected for inflammation, a consistent indicator of iron [20,21], were significantly associated with malarial episodes and *P.falciparum* parasitaemia. Furthermore, this association was significant even after adjustment on inflammatory status. Iron deficiency was associated to a significant protection through the entire follow-up. More precisely, infants with iron levels in the first quartile seemed to be significantly protected against malaria. Indeed, iron deficiency has frequently been linked to a certain protection against malaria [9]. 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 [9] no significant difference in clinical malaria episodes was detected between children supplemented with iron alone and those receiving a placebo (risk ratio (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 high iron levels was observed in trials that did not provide malaria surveillance and treatment, and the risk of malaria and parasitaemia was higher with high iron levels (RR=1.13, 95% CI (1.01; 1.26) [9]. Furthermore, in numerous studies included in the meta-analysis, iron was seldom determined longitudinally.

Malaria physiopathology could explain the increased malarial risk associated with elevated iron levels. In effect, iron inhibits the synthesis of nitric oxide by inhibiting the expression of inducible nitric oxide synthase (iNOS) at the host level, and thereby interferes with macrophage-mediated cytotoxicity against *Plasmodium* [22]. Furthermore, non-transferrine bound iron (NTBI) is associated with the severity of malaria [23–25], and *Plasmodium* has the capacity of acquiring iron in a transferrin-independent pathway [26].

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, of iron deficiency, and of iron deficiency anaemia [27]. 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.

Complementary findings of our study are the significant impact of maternal folate levels and helminths at delivery on *P. falciparum* parasite density of the infant. Indeed, folate is an important cofactor used by *P.falciparum* in DNA synthesis and methylation, and mRNA translation. Therefore, high folate levels could enhance the immune tolerance process undergoing PAM. With regard to the significant association between maternal helminths and increased *P. falciparum* parasite density, this was already described In Uganda, where Ndibazza et al. suggested this could be due to hyporesponsiveness of T-cells of the of the infants previously exposed in utero to parasites [28]. Therefore, further epidemiological evidence could be useful to analyse the extent of the immune tolerance process undergoing in utero.

Conclusion

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 and PAM preventive interventions have a significant influence on malaria in infants as well. In our study, long IPTp interval is associated to reduced malaria risk in infants during the first year of life. Public policies should be encouraged to increase the observance of IPTp as it has a protective effect not only in mothers but also in their offspring, as it has been recently recommended by the WHO [29]. Malaria risk during the first year of life is also associated with high ferritin levels in a prospective longitudinal cohort considering complementary risk factors. Our data also suggest that malaria risk increases with higher ferritin levels. Indeed, the interaction between iron and malaria is complex because of the iron requirements during infancy and the fact that iron

contributes to the parasite growth. These results appeal for additional epidemiological studies to evaluate the effect of different doses of iron supplements on the infant infectious and haematological outcomes. Complementary interventional data are needed to determine the benefits and risks of differently dosed iron supplements, in order to ascertain their impact on infant health in malaria-endemic regions. Finally, the epidemiological comparison of cohorts in which iron is given as preventive intervention and cohorts in which iron is given solely on the purpose of treatment for anaemia or ID should also be analysed.

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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)
***P. falciparum* infected placenta (%)**	10.9 (7.8; 13.9)
Haemoglobine (g/l)	139 (136.9; 141)

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)
***P. falciparum* infection (%)**	12.06 (8.45; 15.68)	12.00 (8.28; 15.52)	12.34 (8.70; 15.99)
Parasite density (nb/mm3)	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% Confindence 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-valu
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.

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.

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 *P.falciparum* 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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V.II.3 Other factors associated with malaria risk during infancy: the case of lead

Summary of the article: As said in the "State of art" section, simultaneously to our study in the same cohort another epidemiological project was evaluating the effect of lead on the neurocognitive development in children. Our colleagues found out lead levels were particularly high in the infants of our cohort. Nriagu had found in Nigeria that malaria had a significant effect on lead levels in univariate analysis. In addition, elevated blood lead levels (BLL) carry a significant burden of disease in Western Africa and malaria is the first cause of infant mortality in Benin. Therefore, we aimed at assessing the possible association of lead levels with malaria risk considering other major malarial risk factors.

Elevated lead levels have severe harmful effects on infant health. They are associated with impaired neurocognitive development, anemia (due to either disruption of heme synthesis or hemolysis), and renal and gastro-intestinal effects. Although high blood lead levels (BLL) (BLL >100 µg/dl) can entail acute neurologic symptoms, such as ataxia, hyperirritability, convulsions, coma, and death, BLL as low as 10 µg/dl have been also correlated with poor neurocognitive outcomes and behavioral disorders. Indeed, the Center for Disease Control (CDC) reduced the reference level of blood lead from 10 µg/dl to 5 µg/dl in 2012. This is of special concern in young children as neuro-cognitive impairment has been found to be associated with the degree of exposure to lead between the ages of 12 and 36 months. Albeit the severe impact of elevated lead levels on infant health, epidemiological studies of lead levels in Sub-Saharan Africa are limited. Data from the few existing studies, published in a systematic review on BLL among Sub-Saharan children, suggest an alarming burden of disease. This review reported a BLL weighted mean of 13.1 µg/dl which increases up to 16.2

µg/dl considering solely studies with robust quality BLL analyses. In addition, the prevalence of BLL >10 µg/dl ranged from 7.0% to 70.9% in six of the studies reviewed. Recent mass level intoxications reported in Senegal and Nigeria further raise the public health concern about lead levels in West Africa. Notwithstanding these concerns, infectious diseases, mainly malaria, lead the disease burden in West Africa. In Benin, malaria is the main cause of mortality among children less than 5 years and there were over 1.5 million cases in 2012. Both malaria and lead poisoning can have severe hematologic and neurologic symptoms on children and development disruptions. Because of the recent evidence on 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 neurocognitive impairment of infants exposed to *Plasmodia in utero*.

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

What's known on this subject: Malaria and elevated lead levels overlap geographically in West Africa. Both entail anemia and impaired neuro-cognitive development and their effect is

particularly severe in infants. Albeit the effect of malaria on lead levels found by Nriagu in Nigeria, no evidence exists at present on the effect of lead levels on malaria risk.

What this study adds: We show that the rate of elevated blood lead levels is very high in Benin. In addition, we show that elevated lead levels give a certain protection to infants with regard to the malaria risk, possibly due to a toxic effect of lead on *Plasmodium*. Furthermore, we show that even in the context of high lead levels, iron levels are still significantly associated to increased malaria risk.

Article under review in *Plos One*:

Elevated blood lead levels are associated with reduced risk of malaria in Beninese infants

Violeta Moya-Alvarez, Michael Osei Mireku, Pierre Ayotte, Michel Cot, and Florence Bodeau-Livinec.

Abstract

Introduction: Elevated blood lead levels (BLL) and malaria carry an important burden of disease in West Africa. Both diseases might cause anemia and they might entail long-term consequences for the development and the health status of the child. Albeit the significant impact of malaria on lead levels described in Nigeria, no evaluation of the effect of elevated BLL on malaria risk has been investigated so far.

Materials and methods: Between 2010 and 2012, 203 Beninese infants were followed during the first year of life through three systematic visits at 6, 9, and 12 months, and emergency unscheduled visits to evaluate their health status and gather clinical, microbiological and hematological data. Blood lead levels were assessed at 12 months.

Results: At 12 months, the mean BLL of infants was 7.41 µg/dl (CI: 65.2; 83), and 128 infants (63%) had elevated blood lead levels, defined by the CDC as BLL>5 µg/dl. Lead poisoning, defined as BLL>10 µg/dl, was found in 39 infants (19%). Twenty-five infants (12.5%) had a positive blood smear at 12 months and 144 infants were anemic (71%, Hb<110 g/l). Elevated blood lead levels were significantly associated with reduced risk of a positive blood smear (aOR=0.98, p-value=0.02) and P. falciparum parasite density (beta-estimate=-0.003, Pvalue=0.048) in logistic and linear regression multivariate models, respectively adjusted on clinical and environmental indicators.

Conclusion: Our study shows for the first time that BLL are negatively associated with

malarial risk considering other risk factors. Malaria is the main cause of mortality for infants under 5 years worldwide, and lead poisoning is the 6th most important contributor to the global burden of diseases measured in disability adjusted life years (DALYs) according to the Institute of Health Metrics. In conclusion, environmental factors, such as lead levels, need to be considered in the debate about iron supplements in malaria endemic countries.

Body of the article

Introduction

Elevated lead levels have severe harmful effects on infant health. Symptoms related to toxicity occur from mid to high levels of exposure and they depend on the amount of lead in the blood and tissues. High lead levels are associated with impaired neurocognitive development, anemia (due to either disruption of heme synthesis or hemolysis [1]), and renal and gastro-intestinal effects [2]. Although high blood lead levels (BLL) (BLL >100 µg/dl) can entail acute neurologic symptoms, such as ataxia, hyperirritability, convulsions, coma, and death, BLL as low as 10 µg/dl have been also correlated with poor neurocognitive outcomes and behavioral disorders [3,4]. This is of special concern in young children as neuro-cognitive impairment has been found to be associated with the degree of exposure to lead between the ages of 12 and 36 months [5]. Indeed, the Center for Disease Control (CDC) reduced the reference level of blood lead from 10 µg/dl to 5 µg/dl [6] in 2012.

Albeit the severe impact of elevated lead levels on infant health, epidemiological studies of lead levels in Sub-Saharan Africa are limited. Data from the few existing studies, published in a systematic review on BLL among Sub-Saharan children, suggest an alarming burden of elevated BLL. This review reported a BLL weighted mean of 13.1 µg/dl, which increased up to 16.2 µg/dl considering solely studies with robust quality BLL analyses [7]. In addition, the prevalence of BLL >10 µg/dl exceeded 44% in all cases reviewed, with a maximum of 70.9% in Nigeria. Only one study in Kenya reported a relatively low prevalence (7%). Recent mass level intoxications reported in Senegal and Nigeria [8] further raise the public health concern about lead exposure in West Africa.

In addition, malaria and lead poisoning overlap geographically. Indeed, infectious diseases,

mainly malaria, dominate the disease burden in West Africa [9]. In Benin, malaria is the main cause of mortality among children less than 5 years and there were over 1.5 million cases in 2012 [10]. As already explained, both malaria and lead poisoning can have severe hematologic and neurologic symptoms on children and their development. Malaria and lead poisoning may not only overlap, but they have major impact on the health of children, especially those under 5 years. Consequently, their possible association may have an effect on one of the most vulnerable age groups in the population, and it could have severe long-term implications for the development of the children. Furthermore, Nriagu found a significant effect in univariate analysis of malaria on the children lead levels in different areas of Nigeria [11]. However, no evidence exists at present on the possible joint effect of lead and *P.falciparum*. To our knowledge, no published study exists on lead levels in Benin, and in particular, on the effects of lead levels on malaria risk in infants. Therefore we aim at analyzing the effect of lead levels on malaria risk with regard to both the possibility of having a positive smear and their effect on *P.falciparum* parasite density taking into account hematological and parasitological factors.

Materials and methods

Data used in this study were obtained from two-hundred and three infants who were followed from birth until 12 months of age in two embedded studies: the APEC study (Anemia in Pregnancy: Etiology and Consequences) and the TOVI study. More precisely, a prospective cohort of 400 infants was followed from birth to 12 months of age in the context of the APEC study (Anaemia in Pregnancy: Etiology and Consequences). APEC study is an ancillary survey nested within the MiPPAD study in Benin (Malaria in Pregnancy Preventive Alternative Drugs “http://clinicaltrials.gov/ct2/show/NCT00811421”). This study was conducted in three clinics in the district of Allada (Allada, Attogon, Sékou), between January 2010 and May 2012. 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. Complete details of MiPPAD are presented elsewhere [12], but, briefly, MiPPAD was a randomized trial comparing the efficacy and safety of intermittent preventive treatment in pregnancy (IPTp) with sulphadoxine pyrimethamine (SP) (1,500/75 mg per dose) and mefloquine (15 mg/kg per dose). At delivery placenta was examined in order to analyse *P. falciparum* parasite infestation. All live born children of recruited pregnant women who survived to 12 months were invited for neurocognitive assessment in the TOVI study, which evaluated the children for cognitive and motor functions using the Mullen Scales of Early Learning as well as their lead levels at 12 months [13]. The 203 infants of the sample correspond to the infants for whom data at 12 months include a complete follow-up of lead and malaria indicators.

Clinical data of the infants were collected at systematic visits at 6, 9, and 12 months. After delivery, in any case of sickness, infants were accurately examined and when necessary, treated in unscheduled visits. All drugs prescribed to the infants during the follow-up were free of charge. During the visits, anthropometric measures and clinical examination were realized. Eight milliliters of venous blood were collected at each visit. Hemoglobin, serum ferritin, CRP, vitamin B12, and folate levels were thereby assessed. At 12 months, lead levels were also determined. A container was also given to the women to collect stools to examine the presence of intestinal helminths in the infants. Microbiological exams were realized as follows: Lambaréné technique was used to assess malaria infection on thick blood smears [14]. To assess parasite density (in parasites/µL), a multiplication factor was applied to the average parasitaemia/field. Helminthic infestations were assessed using the Kato-Katz concentration method (VestergaardFrandsen kit®). Iron deficiency is defined according to WHO standards by serum ferritin levels<15µg/l [15].

With regard to BLL, eight milliliters (ml) of venous blood were obtained from each participant, of which 4 ml were collected into a tube containing dipotassium EDTA and 4 ml into an iron-free dry tube. Blood samples were analysed at the Centre de Toxicologie, Institut National de Santé Publique du Québec (Québec, Canada), by inductively coupled plasma mass spectrometry (ICP-MS; Perkin Elmer Sciex Elan DRC II ICP-MS instrument) prior to 20 fold dilution in amonia 0.5% v/v and 0.1 % v/v surfactant Triton-X. The limit of detection for blood sample analysis was 0.2 µg/l.

Because of the anopheline breeding cycle, the mean rainfall of the 7 days prior to the two weeks before the consultation was calculated. It was independently assessed for each health centre of the district of Allada.

Socio-economic status was assessed using a socio-economic index created in a two-step process. First all socio-economic items (home possession of latrines, electricity, a refrigerator, a television, a vehicle with at least two wheels, being married, and working outside the home) were plotted into a multiple correspondence analysis. Then, two predictors were created to synthesize the information, and as the first captured the large majority of the information, it was withheld as the socio-economic index.

Statistical analysis

Data were double entered and analysed with ACCESS2003 and STATA12.0 softwares for Windows (Stata Corp, College Station, TX, USA). Univariate analysis was realized to assess the association of all variables with either the infant positive smear or peripheral *P.falciparum* density at the moment of lead assessment (at 12 months of age). Thereafter, all variables with P values<0.2 were included in a multivariate model regression. Logistic regression was used to evaluate the determinants associated with a positive blood smear. Linear regression was used for the multivariate analysis of *P.falciparum* parasite density. Socio-economic status was

forced into the model because of its known association with lead levels according to the literature [11]. The statistical significance in the final multivariate models was set to $P<0.05$.

Ethical considerations

This study and the consent procedure regarding the women and their offspring were approved by the Ethics Committee of the Faculty of Medicine of Cotonou, Benin. It was explained in local language to the participant and her voluntary written consent was obtained and recorded in the clinic files before enrolment. In case the woman could not read, an impartial witness was included in the process. In the case of the inclusion of minor women, both their consent and the consent from the parents or legal guardians were obtained. Women were free to interrupt their participation at any time of the study.

Results

The BLL of 203 infants included in the APEC-cohort were obtained at the 12-month visit between April 2011 and May 2012. During the 12-month follow-up 84 infants (42%) had at least one malarial episode. More precisely, 60.25% of infants had no positive blood smear during the entire follow-up period, 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 main malarial and hematological indicators as well as lead levels are presented in table 1. At the moment of lead assessment, 25 out of 200 (12.5%) of the infants had a positive blood smear, with a mean parasite density of 13460 (CI:2775; 24145). Lead levels were high overall. The mean BLL of infants was 7.41 µg/dl (CI: 65.2;83), and 128 infants (63%) had elevated blood lead levels, defined by the CDC as BLL>5 µg/dl. Lead poisoning, defined as BLL>10 µg/dl, was found in 39 infants (19%). With regard to the hematological indicators, 144 infants were anaemic (71%, Hb<110 g/l), and 85 were iron deficient (42%, CRP-corrected serum ferritin (SF) <15 µg/l). The mean and median hemoglobin and ferritin values were 101.69 g/l and 104 g/l (CI: 99.51; 103.86),

and 571 mg/l and 201.5 mg/l (CI: 429.67; 712.34), respectively.

At 12 months, ferritine, folate, and CRP levels as well as rain quantity and low socio-economic status were associated in univariate analysis with increased malaria risk with regard to both the risk of having a positive smear and P. falciparum parasite density. In parallel, hemoglobin and lead levels were inversely correlated with malaria risk in univariate analysis. In univariate and multivariate analyses, girls were significantly less likely to have high P. falciparum parasitemia but there were no statistical differences in malaria risk between boys and girls with regard to the probability of having a positive smear. There were no statistical significant differences in malarial, lead, or hematologic indicators depending on the health care centre. Table 2 and table 3 describe risk factors associated with the possibility of having a positive blood smear and high P.falciparum parasitaemia with lead levels as a continuous variable, whereas table 4 and table 5 include the possibility of having elevated BLL.

In multivariate analysis, high lead levels were significantly associated with reduced risk of a positive blood smear (p-value=0.02) and P. falciparum parasite density (p-value=0.048) in logistic and linear regression models, respectively. More precisely, no positive blood smear was found among infants with lead poisoning, and infants with elevated BLL (table 4) were significantly less likely to have a positive blood smear and a high P. falciparum density (aOR=0.38 95% CI (0.15; 0.99), and Coefficient=-0.44, p-value=0.03, respectively). Factors associated with increased malaria risk include high iron and folate levels and ongoing inflammatory process. In effect, elevated ferritin levels (log of ferritin corrected on inflammation) were associated with increased risk of a positive blood smear (aOR=2.46 (1.01; 6.05), p-value=0.05). In addition, high folate levels were statistically associated to an increased P. falciparum parasite density (coefficient=0.0003, p-value=0.04).

Discussion

The high proportion of infants with elevated 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 [16], 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[11] (8.9 µg/dl). In Jos, Nigeria, another study reported an average BLL of 11.2 µg/dl (range: 9.1–13.3 µg/dl), and that 55% had BLLs above 10 µg/dl [17]. Indeed, the existing epidemiological evidence reveals the high prevalence of elevated BLL among African infants. However, there is very limited evidence on their effect on malaria. Nriagu et al described the inverse association of BLL and malaria in univariate analysis (pvalue=<0.001). Our results not only confirm this association in univariate analysis, but they are the first to evidence the significant effect of BLL on malarial risk. Furthermore, these results show that elevated BLL are also associated with reduced probability of a positive blood smear as well as reduced P.falciparum parasite density. As a consequence, epidemiological evidence in our study rejects the possible synergistic effect of lead on P.falciparum infection, but rather suggests a protective effect. Moreover, the high BLL present in our sample raise concern on their possible harmful consequences for the infant health.

The mechanism by which lead might influence malaria infection has not been elucidated so far. However, Nriagu postulated that there are multiple levels at which lead can modulate the specific host response to *Plasmodium* infection including alterations in heme synthesis,

immunoregulation, and iron metabolism.

Lead concentrates in red blood cells (RBC) in the context of lead poisoning [18]. The accumulation of lead in the RBC, the main nutrition source of *Plasmodium*, may inhibit the development of the parasite. Elevated intra-erythrocytic concentration of lead may interfere with the development from the ring form to the schizont stage and, consequently, lead exposure may be associated to reduced parasitemia in malaria-infected infants.

In addition, elevated BLL can exert a general effect on the immune regulatory function[19,20]. In this respect, both lead poisoning and malaria favor the cytokine response which, in turn, has an influence on the Th1/Th2 balance [21,22]. Indeed, a certain protection against severe malaria has been described as a consequence of the Th2 response following the alteration of the immune system induced by lead poisoning [23].

Alternatively, iron deficiency and hemoglobinopathies can foster the anti-parasite effect of lead in the context of the blood stages of P. falciparum. Indeed, iron deficiency may interfere with the proper use of iron by the parasite [24]. However, iron deficiency was not significantly correlated with malaria risk in our analyses. Finally, high intra-erythrocyte lead concentration can inhibit protein synthesis [25], and thereby interfere with the correct iron utilization by *Plasmodia* [24].

With regard to iron levels, high iron levels have already been associated with increased malaria morbidity [26]. This raises the concern on the iron supplements recommended by the WHO when anemia prevalence >40%, which is the case of Benin. Furthermore, published literature reports both iron and lead have a significant effect not only on malaria, but also on anemia. Indeed, strategies to tackle anemia should consider not only iron supplementation but public health policies should also imply the sources of elevated BLL.

Low folate levels are also associated to anemia. Nevertheless, high folate levels are associated

with high P.falciparum parasitemia as well. The significant role of high folate levels for increased risk of P. falciparum parasite density is coherent with the literature. Indeed, anti-folates are one of the most anti-malarial drugs used worldwide. Finally, boys displaying higher P. falciparum parasite density than girls has already been described[27], may be due to their higher exposure because of socio-behavioural habits.

Conclusion

Malaria is the main cause of mortality for infants under 5 years worldwide[10], and high iron levels have been associated to increased malaria risk[26]. Therefore, in the context of limited protection against malaria, iron supplements to fight anemia might entail some deleterious effect for P. falciparum infections. As a consequence, giving supplements to infants that do not need them might be harmful for their health status.

However, iron supplements are crucial to fight anemia especially in the context of elevated BLL[28]. This is of special relevance, as iron deficiency is associated to increased lead absorption[29]. Lead poisoning is the 6th most important contributor to the global burden of diseases measured in disability adjusted life years (DALYs) according to the Institute of Health Metrics, with Sub-Saharan African countries being predominantly responsible for the global DALYs[30]. Lead poisoning entails severe consequences for the development of the children and is associated with major health problems highly prevalent in West Africa, such as anemia, having an important impact on the infants and their communities. In addition, iron is essential for the neurocognitive development of the child brain.

In conclusion, environmental factors, such as lead levels, need to be considered in the debate about iron supplements in malaria endemic countries.

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Tables:

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%)
P.falciparum density (parasites/µL)	13460 (CI:2775; 24145)
Blood lead levels (µg/l)	74.1 (CI: 65.2;83)
Elevated blood lead levels (BLL>5 µg/dl)	128 (63.05%)
Lead poisoning levels (BLL>10 µg/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 µg/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 (µg/l)	0.98 (0.96; 0.99)	0.02
Ferritin levels (logartihm 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

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 ≥5 mg / ml)	0.72 (0.31; 1.14)	0.01
Ferritin levels (logartihm 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 (µg/l)	0.38 (0.15; 0.99)	0.048
Ferritin levels (logartihm 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 (logartihm 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

VI.1. Effect of preventive public health interventions during pregnancy on pregnancy associated malaria

IV.1.1. Effect of IPTp on PAM outcomes

PAM has not a single dimension. It entails different clinical manifestations that depend, as said in the second section, on transmission, immunity, and preventive strategies. Clinical malaria, high parasite density, LBW, and placental malaria are the main symptoms of the infection by *Plasmodium*. Therefore, a holistic analysis that includes every outcome issue of PAM, should lead us to contemplate the multiple different dimensions of the protective effect of IPTp.

IV.1.1.a. Effect of transmission and previous immunity

An initial approach of the results requires acknowledging the differences among women with regard to transmission and immunity. Differences in transmission have been estimated by rainfall, which is a useful tool to account for *anopheline* risk. Rainfall varies significantly throughout the follow-up period. Nevertheless, it is not associated with malaria risk during pregnancy nor with placental malaria or LBW. The possible effect of rain might be mitigated by the effect of previous immunity. Indeed, there is some evidence suggesting a certain protection for multiparous women because of the immunity due to previous gestations. In effect, in univariate analysis, gravidity was associated with the age of the mother, BMI, socio-economic status, number of positive blood smears, PM and LBW. In this respect, infants of primigravid women will be possibly at higher risk for subsequent malaria as a result of reduced antibody transfer, even if one study has shown the opposite results in women with no infected placenta. As shown in the results section, the mean of positive blood smears during pregnancy was significantly higher for primi- and secundigravidae than for multigravidae. In 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/µL, respectively) but then rose up to 3098.8, SD=31120.7 parasites/µL 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

during pregnancy

The individual particularities of each pregnant woman and the physiopathological evolution of iron levels within the different periods of pregnancy need to be considered in the analysis of the association of iron levels with malarial risk. Therefore, a well-defined and concrete statistical approach is necessary: the multilevel model analysis. Multi-level models are particularly suited to the statistical analyses of prospective cohorts with repeated measures at the individual level, as they can take into account the specificities of each individual at different time measures.

In order to treat the evolution of iron levels, we planned a multi-step statistical analysis. First, we assessed the association of iron with malaria risk by trimester, and high ferritin was significant in the observations of the different trimesters with regard to both the possibility of having a positive smear and also *P.falciparum* parasite density. Thereafter, in the context of the multilevel analysis we included a categorical variable to account for the specific pregnancy trimester of each observation and this variable was not significant. Ferritin levels may also differ from one trimester to the other also because women take iron supplements. Indeed, when we conducted the univariable analyses on the association between ferritin and the possibility of a positive blood smear or parasite density for each visit, the only case in which ferritin levels and a positive blood smear were not significantly associated (p-value=0.07), still borderline, was in the visit following the iron supplements. However, the association between ferritin levels and parasite density for this same visit, i.e. before iron intake, was statistically significant (p-value=0.003). Then we tested the link between ferritin levels and gestational age and their association was significant, possibly due to the timing of the supplements.

With regard to the specific characteristic inherent to the physiopathology of each individual, including 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*ln(likelihood) + 2*k, and BIC = -2*ln(likelihood) + ln(N)*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*ln(likelihood); the larger the value, the worse the fit. Complexity is measured positively, either by 2*k (AIC) or ln(N)*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.

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.

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

384811.2 µg/dl (range: 9.1–13.3 µg/dl), and that 55% had BLLs above 10 µg/dl. Indeed, the 3849existing epidemiological evidence reveals the high prevalence of elevated BLL among 3850African infants. However, there is very limited evidence on their effect on malaria. Nriagu et 3851al described the inverse association of BLL and malaria in univariate analysis (p-3852value=<0.001). Our results not only confirm this association in univariate analysis, but they 3853are the first to evidence the significant effect of BLL on malarial risk. More precisely, these 3854results show that elevated BLL are also associated with reduced probability of a positive 3855blood smear as well as reduced *P.falciparum* parasite density. As a consequence, 3856epidemiological evidence in our study rejects the possible sinergistic effect of lead on 3857*P.falciparum* infection, but confirms its significant protective effect. Moreover, the high BLL 3858present in our sample raise concern on their possible harmful consequences for the infant 3859health.

3860The mechanism by which lead might influence malaria infection has not been elucidated so 3861far. However, Nriagu postulated that there are multiple levels at which lead can modulate the 3862specific host response to *Plasmodium* infection including alterations in heme synthesis, 3863immunoregulation, and iron metabolism.

3864Lead concentrates in red blood cells (RBC) in the context of lead poisoning. The toxification 3865of the RBC, the main nutrition source of *Plasmodium*, may inhibit the development of the 3866parasite. More precisely, the elevated intra-erythrocytic concentration of lead may interfere 3867with the development from the ring form to the schizont stage and, consequently, lead 3868exposure may be associated to reduced parasitemia in malaria-infected infants.

3869In addition, EBLL can exert a general effect on the immune regulatory function. In this 3870respect, both lead poisoning and malaria favor the cytokine response which, in turn, has an 3871influence on the Th1/Th2 balance. Indeed, a certain protection against severe malaria has been 3872described as a consequence of the Th2 response following the alteration of the immune

system operated by lead poisoning.

Alternatively, iron deficiency and hemoglobinopathies can foster the anti-parasite effect of lead in the context of the blood stages of *P. falciparum*. Indeed, iron deficiency may interfere with the proper use of iron by the parasite. However, iron deficiency was not significantly correlated with malaria risk in our analyses. Finally, high intra-erythrocyte lead concentration can inhibit protein synthesis, and thereby interfere with the correct iron utilization by *Plasmodia*.

However, iron supplements are crucial to fight anemia especially in the context of elevated BLL. This is of special relevance, as iron deficiency is associated to increased lead absorption. Lead poisoning is the 6th most important contributor to the global burden of diseases measured in disability adjusted life years (DALYs) according to the Institute of Health Metrics, with Sub-Saharan African countries being predominantly responsible for the global DALYs. Lead poisoning entails severe consequences for the development of the children and is associated with major health problems highly prevalent in West Africa, such as anemia, having an important impact on the infants and their communities. In addition, iron is essential for the neurocognitive development of the child brain.

In conclusion, environmental factors, such as lead levels, need to be considered in the debate about iron supplements in malaria endemic countries.

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

VIII.1. The new WHO recommendations on IPTp in the context of increasing resistance

The effect on maternal and infant health of the extension by WHO of IPTp regime to a SP-dose at each ANV needs to be monitored. In theory LBW, prematurity and placental malaria but also malaria in infants should be carefully analysed to obtain an optimal timing and IPTp regime to optimize the protective effect of IPTp. However, operational research on the topic might be difficult to implement on the field, and the resulting data might be difficult to interpret. Protective strategies regarding iron levels should maybe start during the pre-conceptional period to better protect both mother and infant. Operational research on different preventive IPT strategies should also be continuously conducted, and cost effectiveness analysis for community-level IST interventions should be further investigated, considering as well that IST has no effect on sub-microscopic parasitemia, which might be troublesome when targeting the elimination.

In addition, as there is evidence of increased infant susceptibility to parasites carrying antigens to which they were previously exposed while *in utero*, further research should also tempt to explain the ongoing immune process. Furthermore, the role of protective maternal antibodies has not yet been clarified. 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.

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.

VIII.2. Iron supplements in malaria endemic settings

The significant association between iron levels and malarial risk in both pregnant women and infants appeals for additional epidemiological studies. Furthermore, the possible dose effect of iron levels for malaria risk, advocates for the evaluation of the effect of different doses of iron supplements on the infant infectious and haematological outcomes. Complementary interventional data are needed to determine the benefits and risks of differently dosed iron supplements, in order to ascertain their impact on infant health in malaria-endemic regions. Finally, the epidemiological comparison of cohorts in which iron is given as preventive intervention and cohorts in which iron is given solely on the purpose of treatment for anaemia or ID should be also analysed.

With regard to the difficulty of finding a gold standard for iron levels evaluation, a complete combination of iron markers is desirable. This evaluation should at least include the markers recommended by the joint WHO-CDC Technical Consultation for anaemia assessment (hemoglobin, mean cell volume (MCV), serum transferrin receptor (sTFR) concentration, serum ferritin concentration, and red cell protoporphyrin (measured by the zinc protoporphyrin/hemoglobin ratio (ZPP:H)) in addition to hepcidin, haptoglobin and inflammation indicators (C-reactive protein (CRP) and alpha-1-acid glycoprotein (AGP). The index sTfR/log ferritin adjusted on CRP has also been recommended.

In parallel, the association of iron with malaria risk might be different depending on malaria transmission patterns. Indeed, the dose-dependent effect, might be modified by the differences in the prevalence of *Plasmodium falciparum*, and this notable aspect has not been evaluated so far in clinical trials.

Finally, if women had sufficient pre-conceptual iron storages, iron supplements might not be necessary during pregnancy and the supplementary risk of adding iron, which could be used as a growing factor by the parasite, would not be necessary during that critical period.

In infants, iron storages depend on the mothers', but other strategies like delayed cord

clamping can also contribute to increase them.

In any case, sufficient iron levels are crucial for both the mother and the infant, and they need to be reached in every possible manner. Therefore, malaria control interventions should be optimized to better ensure a minimal infective risk during pregnancy and infancy.

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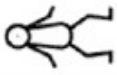
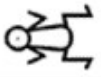
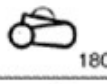
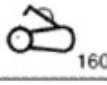
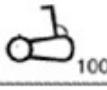
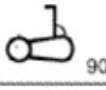
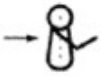
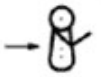
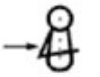
4491

X. Appendix

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							

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	
Plantar surface	Heel-toe 40-50 mm: –1 <40 mm: –2	>50 mm, no crease	Faint red marks	Anterior transverse crease only	Creases anterior ⅔	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	
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	
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	
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	

Maturity Rating

Score	Weeks
-10	20
-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

Appendix 2: Further details of the study APEC

The preparation of the APEC project started in 2008. Nineteen people (2 medical doctors, 8 nurses, 5 lab technicians and 4 supporting agents) formed the APEC-MiPPAD research team. The study implied people from 33 different villages of the Allada region. Even if the follow-up took place in three maternities (Sékou, Allada, and Attogon), the laboratory was in the centre of Sékou. In APEC the first woman was recruited on the 15th January 2009 and the last one delivered on the 10th January 2012.

In case of illness each case has been managed according to Beninese guidelines.

Uncomplicated malaria has been treated in the different maternities and complicated malaria cases have been refered either to the Hospital of Calavi, to the Hôpital de la mère et de l'enfant Lagune, or to the Centre hospitalier universitaire-Hubert Koutoukou Maga in Cotonou, where they have received quinine.

In case of severe anemia the patients have been transfused and detected and treated the cause of anemia.

With regard to the lost-of follow-up, every trimester the entire cohort was controlled. A case was determined to be lost of follow-up when there were no news of the mother or the infant for longer than 3 visits. Each case was documented and the cause was also determined. In case of death, the date, the cause, and the previous treatments were also investigated.

HIV rapid tests were proposed to the pregnant women after HIV counselling at the 1st ANC visit. The tests realized were Determine® HIV-1/2 (Abbott Determine Kit HIV 1 and 2 package insert) et Bioline (SD Bioline Kit HIV 1 and 2 3.0 package insert). When the result was positive, women were sent to the Hospital of Allada for an ELISA confirmation. In case of confirmation of the diagnostic, women were treated and followed according to the Beninese 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:

$\frac{35}{17}$ more than 1000 parasites/ field: count 0.5 field

$\frac{35}{17}$ 100 to 999 parasites/ field: count 1 field

$\frac{35}{17}$ 10 to 99 parasites/ field: count 10 fields

$\frac{35}{17}$ 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.

CRP was determined by a qualitative-semiquantitative kit (Cypress Diagnostic). It is a suspension of latex polystyrene particules covered with a specific anti-serum of IgG fraction anti-human CRP. The test is positive is the concentration is equal or higher than 6 mg/l. The sensibility of the test is 95,6% and the specificity 96,2%.

Helminth infections were analysed using the Kato-Katz technique. It consists in the examination of a calibrated film of fecale substance previously impregnated in a chemical solution. It can detect also eggs, especially ankylostome eggs, as well as the intensity of helminth 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 od 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 trismester on month appart. 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.

Cette thèse n'aurait pas eu lieu sans l'aide des bailleurs suivants :

L'Ecole des Hautes Etudes en Santé Publique (EHESP) a financé la majorité de mon contrat doctoral, ainsi que les déplacements pour les Congrès de l'ASTMH en 2014 et 2015. Egalement, l'EHESP a financé les deux stages que j'ai réalisés au cours de ma thèse à la SMRU.

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Je remercie vivement toutes ces institutions ainsi que les personnes qui y travaillent pour leur aide pendant ces années.

A ces mots, il partit, et le laissa là, avec, dans le coeur, des pensées qui ne devaient pas se réaliser : Agamemnon se disait qu'il prendrait la ville de Priam ce jour-là, l'insensé, et il ignorait les desseins de Zeus, qui devait encore infliger bien des douleurs et des gémissements aux Troyens et aux Danaens, en de rudes mêlées. Il s'éveilla de son sommeil, et la voix divine s'écoula autour de lui.