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## Mucosal lactoferrin response to genital tract infections is associated with iron and nutritional biomarkers in young Burkinabé women

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### Abstract

**BACKGROUND/OBJECTIVES:** The iron-binding affinity of vaginal lactoferrin (Lf) reduces iron available to genital pathogens. We describe host reproductive, nutritional, infection and iron biomarker profiles affecting vaginal Lf concentration in young nulliparous and primigravid women in Burkina Faso.

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#### Author contributions

BJ B was the Principal Investigator for the main RCT on iron supplementation and wrote this paper; SR was co-author and study statistician; LB conceived the study and was joint co-author; SG was the clinical and field co-ordinator for the research; Lf assays were conducted in Burkina Faso by SD; hepcidin by DS and AG-M in the Netherlands and microbiome by AN and CS at Northumbria University, UK. AZ and SO were responsible for data management and safety monitoring; UDA and HT were members of the Scientific Advisory Committee and reviewed the paper. All authors agree to be accountable for their work.

#### Conflicts of interest

Dorine Swinkels is Medical Director of the “Hepcidinanalysis.com” initiative ([www.hepcidinanalysis.com](http://www.hepcidinanalysis.com)), which aims to serve the scientific, medical and pharmaceutical communities with high-quality hepcidin measurements. Other authors declared no conflicts of interest.

**SUBJECTS/METHODS:** Vaginal eluates from women who had participated in a randomized, controlled periconceptional iron supplementation trial were used to measure Lf using a competitive double sandwich ELISA. For this analysis samples from both trial arms were combined and pregnant and non-pregnant cohorts compared. Following randomization Lf was measured after 18 months (end assessment) for women remaining non-pregnant, and at two antenatal visits for those becoming pregnant. Associations between log Lf levels and demographic, anthropometric, infection and iron biomarker variables were assessed using linear mixed models.

**RESULTS:** Lf samples were available for 712 non-pregnant women at end assessment and for 303 women seen at an antenatal visit. Lf concentrations of pregnant women were comparable to those of non-pregnant, sexually active women. Lf concentration increased with mid-upper-arm-circumference, (P=0.047), body mass index, (P=0.018), *Trichomonas vaginalis* (P<0.001) infection, bacterial vaginosis (P<0.001), serum C-reactive protein (P=0.048) and with microbiota community state types III/IV. Adjusted Lf concentration was positively associated with serum hepcidin (P=0.047), serum ferritin (P=0.018) and total body iron stores (P= 0.042). There was evidence that some women maintained persistently high or low Lf concentrations from before, and through, pregnancy.

**CONCLUSION:** Lf concentrations increased with genital infection, higher BMI, MUAC, body iron stores and hepcidin suggesting nutritional and iron status influence homeostatic mechanisms controlling vaginal Lf responses.

## INTRODUCTION

An essential role of human lactoferrin (Lf) is to prevent accumulation of free iron at mucosal sites. Lf demonstrates a bacteriostatic effect related to its iron binding affinity<sup>1</sup> as well as antimicrobial activity that reduces bacterial virulence,<sup>2</sup> inhibitory effects on bacterial adhesion and cell invasion, and ability to induce bacterial lysis.<sup>3,4</sup> Lf is expressed in the genital tract as part of the innate immune system, active against common genital tract infections such as *Trichomonas (T) vaginalis*, bacterial vaginosis (BV) related species, and *candida spp*. Iron is an essential nutrient for many genital tract bacteria,<sup>5</sup> which have developed specialised mechanisms for obtaining iron from host tissues and extracellular fluid.<sup>6</sup> Iron is readily available from heme during menses, while transferrin and ferritin are transudated across the vagina lamina propria.<sup>7</sup> Lf concentrations rise with genital infection<sup>8,9</sup> because neutrophils release Lf from their secondary compartment to sites of infection. Although Lf binds and reduces iron availability, Lf-bound iron may itself become an iron source for common bacteria such as *Gardnerella vaginalis*.<sup>10</sup> In systemic infection, proteins involved in iron homeostasis are regulated at the macrophage level during inflammation<sup>11</sup> and the interaction between ferroportin and hepcidin ensures that circulatory iron is related to host requirements. A local hepcidin response to genital tract inflammation has not been described. Independent of inflammation, Lf gene expression is also regulated by estrogen, which controls Lf produced constitutively in vaginal epithelial cells.<sup>3,12</sup> Vaginal Lf concentrations vary over the menstrual cycle with estrogen surges<sup>13</sup> and plasma Lf was reported higher in pregnancy.<sup>14</sup>

Given the lack of studies characterising Lf profiles in women of reproductive age, we sought to describe factors affecting Lf concentration in a young, largely adolescent population

participating in a periconceptional trial to investigate safety of weekly iron supplementation in rural Burkina Faso. Results of this randomized trial have been reported<sup>15,16</sup> including our finding of an effect modifier of nutritional status for BV infection. The aims of the present sub-study were (1) to measure and compare Lf concentrations from vaginal eluates in nulliparae and primigravidae; (2) to estimate the effects of participant characteristics and nutritional status, measured by body mass index (BMI) and mid-upper arm circumference (MUAC) on vaginal Lf concentration; (3) to estimate the effects of vaginal infections (BV, *T. vaginalis*, microbiota profiles) and (4) to compare systemic iron and inflammation biomarker concentrations, body iron stores and hepcidin levels with vaginal Lf concentrations in order to determine if Lf, a vaginal mucosal biomarker of infection, was associated with systemic iron status and homeostasis.

## MATERIALS AND METHODS

Trial registration number [NCT01210040](https://clinicaltrials.gov/ct2/show/study/NCT01210040). Registered with [Clinicaltrials.gov](https://clinicaltrials.gov) on 27 September 2010. The main trial recruited young, healthy, nulliparous, non-pregnant women aged 15-24 years from 30 rural villages.<sup>17</sup> Adolescents (<20 years) comprised 93% of the sample. Human immunodeficiency virus prevalence is low (<2%).<sup>18</sup> After enrolment individuals were randomized to receive either a capsule containing ferrous gluconate (60mg elemental iron) and folic acid (2.8mg), or an identical capsule containing folic acid alone (2.8mg), as recommended by the World Health Organization, and investigators and recipients were blinded to allocation.<sup>19</sup> Field workers dispensed supplements at weekly home visits, and referred women with reported vaginal discharge or generalised symptoms for free treatment at their nearest Health Centre. Participants who became pregnant before the end assessment at 18 months entered the pregnant cohort, with the main trial outcomes determined at the first antenatal visit (ANC1). Thereafter, weekly supplements ceased and all pregnant women regardless of trial allocation received routine daily antenatal iron supplementation (60 mg elemental iron, 400µg folic acid daily). In the primary trial analyses, weekly iron supplementation over an 18 month period did not significantly improve iron status, probably because the iron was not absorbed due to the effects of chronic malaria infection,<sup>15</sup> thus the two trial arms are pooled for the analyses presented here.

All subjects gave written informed consent in accordance with the Declaration of Helsinki. Individual and guardian written consents for minors were obtained from all non-pregnant women at recruitment with re-consent taken at entry to the pregnancy cohort. The study protocol and subsequent amendments were approved by the Liverpool School of Tropical Medicine, UK, Research Ethics Committee (LSTM/REC), the Institutional Review Board of the Institute of Tropical Medicine, Antwerp, Belgium (IRB/ITM), the Antwerp University Hospital Ethics Committee (EC/UZA), the Institutional Ethics Committee of Centre Muraz (Comité d'Ethique Institutionnel du Centre Muraz, CEI/CM); and the National Ethics Committee (Comité Ethique pour la Recherche en Santé, CERS) in Burkina Faso.

At enrolment demographic data were recorded and height (nearest mm), weight (nearest 100 g), and mid-upper arm circumference (mm; MUAC) measured in duplicate. Lf, BV and *T. vaginalis* were assessed after 18 months at the end assessment of non-pregnant women and twice during pregnancy at an early/late antenatal visit (ANC<sup>1/2</sup>). Women were requested to

provide self-taken vaginal samples using cotton-tipped swabs that were returned to sterile, sealed tubes (Pro bact transport swabs, Technical Services Consultants, Lancs, UK) and kept cool until brought to the laboratory within two to four hours. Samples were not requested during menses. Reported discharge was recorded. One swab was used for a BV Gram stain and a second for measurement of vaginal pH (pH indicator sticks ranged from 3.6-6.1), as previously described.<sup>16</sup> Gram stains were scored using Nugent criteria with 7-10 indicating BV, 4-6 intermediate, and 0-3 normal flora. Duplicate swabs were retained for preparation of vaginal fluid eluates for Lf assays, microbiota and *T. vaginalis* Polymerase Chain Reaction (PCR) assays.<sup>16</sup> For microbiota, bacterial profiling of the variable region 4 (V4) of the 16S rRNA gene was performed by NU-OMICS (Northumbria University, UK) based on the Schloss wet-lab MiSeq procedure.<sup>20</sup>

Each tube containing a swab for vaginal eluate was weighed before and after sampling and the weight difference between initial and final weights was recorded. On laboratory arrival 5mls phosphate buffered saline was added to the tube which was shaken for five minutes on high speed, before pipetting and freezing at  $-20^{\circ}\text{C}$ . Lf concentration was measured by a two-site enzyme-linked immunosorbent assay (double antibody sandwich ELISA) specific to human Lf (Kamiya Biomedical Co, KT-489, Seattle, US). The intra-assay coefficient of variation was 9.7%. Duplicate samples were processed and analyzed independently. The derived mean sample weight was 0.033g, although weights were highly variable and 18% were negative. Residuals from a linear regression of the difference in estimated weights between the repeat samples against the difference in Lf concentration suggested a sample weight measurement error (standard deviation) of 0.035g. Temporal variation in derived weights suggested a batch or seasonal effect and operator differences were not discerned. Mean Lf values were therefore normalised by mean weight for type of visit (allowing for pregnancy effects) derived from a linear model which included month as a covariate. The denominators used were ANC1: 0.038g; ANC2: 0.048g; end assessment: 0.038g. A dilution series was used and values above the ELISA assay range were assigned a nominal high value of 104  $\mu\text{g/ml}$ , and those with no Lf detected were assumed to have no fluid collected if the duplicate sample recorded a detectable level.

Hemoglobin was measured (Sysmex automated analyser) on fresh whole blood. Cut-off points for anaemia were  $<12\text{g/dl}$  for non-pregnant and  $<11\text{g/dl}$  for pregnant women. Methods for assessing iron biomarkers and C-reactive protein (CRP) have been previously reported.<sup>21</sup> Briefly, plasma ferritin and transferrin receptor (sTfR) were measured in duplicate by ELISA (Spectro Ferritin S-22 and TFC 94 Transferrin Receptor, RAMCO Laboratories Inc, Texas, US). CRP was assessed by ELISA (EU59131, IBL International, GMBH, Hamburg, Germany) at the Nanoro Research Laboratories, with an upper limit of the normal range in non-pregnant individuals between 5 and 8  $\text{mg/l}$ . Iron deficiency was estimated using a ratio of sTfR  $\mu\text{g/ml}$  to  $\log_{10}$  ferritin  $>5.6$ .<sup>21</sup> Body iron stores (BIS) ( $\text{mg/kg}$ ) were calculated using the equation derived by Cook et al<sup>22</sup>:  $\text{body iron (mg/kg)} = -[\log_{10}(1000 \times \text{sTfR/ferritin}) - 2.8229]/0.1207$ , using adjusted ferritin based on an internal regression correction approach allowing for inflammation as described by Namaste et al.,<sup>23</sup> based on all the trial data.<sup>21</sup> Serum hepcidin was measured by competitive ELISA assay as previously described.<sup>24</sup>

## Statistical analysis

No differences in vaginal infections<sup>16</sup> or Lf levels were detected by study arm in the formal intention to treat (ITT) analysis (Supplementary file Table 1), and trial arms were pooled in all analyses presented here. Two primary analysis datasets were constructed: 1) pregnant women with Lf assessed at either ANC1 or ANC2 (303/315 with an ANC visit); and 2) women who remained non-pregnant at end assessment (712/819 of this cohort), (Figure 1). Women with uncertain pregnancy status at end assessment were excluded from this data set. A subset of 53/73 women first identified as pregnant at, or within 6 months, of end assessment were screened again for Lf at ANC1 and ANC2 and are considered in a separate longitudinal analysis.

The contribution of each source of variation in Lf concentration was assessed using a linear random effects model with terms for participant and visit (within participant), the residual variance being that between the duplicate samples taken at the same time from the same woman. Means of the log(Lf) values derived from repeat samples collected at the same visit were used for further analysis. The associations between log(Lf) levels and demographic, anthropometric, infection and iron biomarker variables were assessed using a linear mixed model, pooling all visits with a random effect term to allow for correlations between levels in the same individuals. A combined (non-pregnant and pregnant) regression slope estimate was used. As nutritional status was *a priori* identified as a potential confounder, a second model was fitted which included MUAC, a surrogate for nutritional status, as a covariate. Additional models considered interaction terms between visit and the independent variable: as none of these approached statistical significance ( $P > 0.05$ ) they are not considered further. Statistical analyses were conducted in the R statistical environment version 3.3.1.<sup>25</sup>

## RESULTS

Lf samples were available for 712 non-pregnant women at end assessment (after up to 18 months weekly iron supplementation) and for 303 women seen at ANC1 or ANC2 who had become pregnant after randomization. Lf assays were conducted for 271 at ANC1 and 241 at ANC2, providing a total of 1224 samples for Lf assay (Figure 1). At ANC1 median and interquartile range (IQR) gestational age was 18 (14-22) weeks and at ANC2, 34 (33-35) weeks. Baseline characteristics of the non-pregnant and pregnant women are shown in Table 1 for those with an Lf measurement. Women who became pregnant were primigravidae, older and more sexually active at baseline than women who remained non-pregnant. At ANC1 prevalence of iron deficiency was 11.3% in pregnant women, and at end assessment 20.3% in non-pregnant women.

Mean Lf concentration for pregnant women was 806 (65-4386)  $\mu\text{g/ml}$  ( $n=271$ ) and for non-pregnant 201 (66-936)  $\mu\text{g/ml}$  ( $n=712$ ). Lf distributions are shown in Supplementary file, Figure S1. Lf concentration did not differ by menarcheal status, therefore both menarcheal groups were pooled in further analyses of the non-pregnant cohort (Supplementary file, Figure S2). Pregnant women had higher Lf levels (difference in  $\log_{10}$  Lf = 0.45, 95% CI, 0.33 - 0.57,  $P < 0.0001$ ) than non-pregnant. Concentrations did not differ between ANC1 and

ANC2 (Supplementary file, Figure S2). Mean Lf concentration in pregnancy was similar to that of sexually active non-pregnant women and was independent of gestation (Figure 2).

There was large variation in  $\log_{10}$  Lf values between individuals ranging over six orders of magnitude. The within patient/visit estimated assay standard deviation (SD) in  $\log_{10}$  Lf was 0.47 and the interclass correlation coefficient (ICC) for replicate measurements was 0.90, indicating 90% of variation was due to true differences between women or visits. The between visit (within woman) variation SD was 0.65, and the between women SD was 0.75, giving a total SD between independent assessments of 1.00. The within patient ICC was 0.57, indicating that Lf values were strongly correlated over time, with individuals having levels that persisted. This is illustrated in Figure 3 which shows the longitudinal trends in Lf concentration for the 51 women assessed at end assessment and who were identified by urine screening at that time as in very early pregnancy and then who later provided additional samples as part of the pregnant cohort at ANC1 and/or ANC2.

Some 37% of non-pregnant women had a baseline body mass index (BMI)  $< 18.5 \text{ kg/m}^2$  (Table 1). Across both pregnant and non-pregnant women, Lf concentrations increased with MUAC ( $P=0.047$ ) and BMI ( $P=0.018$ ), (Table 2). The effect of sexual activity on Lf concentration in non-pregnant women remained after adjusting for MUAC ( $P=0.005$ ) (Table 2). There was no Lf association with calendar age or reproductive age.

Prevalence of BV was 11% and *T. vaginalis* 4% at the non-pregnant end assessment and 7% and 13% respectively at ANC1. Lf concentration by infection category is shown in Supplementary file 1 Figure S3. Lf levels were 6.6-fold higher in all women with BV (difference in  $\log_{10}$  Lf = 0.82, 0.63 - 1.00,  $P < 0.001$ ), 11.5-fold higher with *T. vaginalis* (difference in  $\log_{10}$  Lf = 1.06, 0.85 - 1.26,  $P < 0.001$ ), and 1.8-fold higher with vaginal discharge (difference in  $\log_{10}$  Lf = 0.26, 0.07 - 0.45,  $P = 0.006$ ), (Table 2). Receiving an antifungal prescription, predominantly for a vaginal infection, during the six months prior to assessment, was associated with higher Lf values ( $< 0.001$ ). Antibiotic prescriptions, mainly for respiratory, gastrointestinal and localised infections, showed a borderline significant association ( $P = 0.066$ ). Infection associations with Lf did not differ significantly after adjusting for MUAC.

Three microbiota vaginal Community State Types (CSTs) were matched: CST I (40.4%), III (25.8%) and IV (33.8%), dominated respectively by *Lactobacillus (L) crispatus*, *L. iners* and a mixed community with reduced lactobacilli. The 15 bacterial genera associated with each CST category are shown in Figure 4. Log Lf concentration differences for CST III and IV compared to CST I were 0.59, 0.46 - 0.73, and 0.58, 0.46 - 0.71, respectively ( $P < 0.001$ ). (Supplementary file, Figure S4)

There were significant positive trends towards increasing Lf concentration, adjusted for visit and MUAC, with serum ferritin ( $P = 0.018$ ), serum hepcidin ( $P=0.047$ ) and total body iron stores ( $P=0.042$ ), with a borderline association with serum CRP concentration ( $P=0.048$ ), (Table 2). ZPP and sTfR adjusted for MUAC were not significantly associated with Lf concentration.

## DISCUSSION

Both nutritional and iron biomarker correlates with Lf concentration were identified in this analysis. The higher Lf in pregnancy probably relates to exposure to sexually transmitted infections such as *T. vaginalis* consequent to regular sexual activity, as well as to higher estrogen levels.<sup>26</sup> No standardized methodology is available for measuring Lf in vaginal fluid and no pre-determined cut-offs are available to denote high and low values.<sup>27,28</sup> For specimen collection, self-taken swabs were acceptable to participants. Although swabs/tubes were weighed to allow adjustment for the volume of vaginal fluid sampled, this did not account for all variation between mean weights, which could be affected by hydration and seasonal effects. Normalisation therefore used mean rather than individual sample weights. Despite this the assay was sufficiently sensitive to detect differences associated with participant and clinical characteristics. Lf concentrations showed a wide range in values but longitudinal profiling and analysis of the components of variation indicated women with persistently high or low Lf concentrations, maintained from before, and through, pregnancy. They may represent hyper- or hypo- responsive cohorts since BV and *T. vaginalis* infections were identified in both categories (Figure 3). Infection was the main factor affecting Lf concentration, but it remains unclear whether these higher Lf concentrations are helpful or detrimental. Lf is known to be a potent inhibitor of *Candida albicans*<sup>29</sup> and synergistically enhances the effects of the azole class of antifungal agents.<sup>30</sup> We report higher Lf concentrations in CSTs III/IV, conditions in which healthy lactobacilli are generally reduced but *L. Iners* survives. Unlike other lactobacilli, *L. iners* requires iron for growth. It probably utilises iron released from erythrocyte destruction by *Gardnerella vaginalis* and flourishes during menses.<sup>31</sup> Its role in maintenance of iron homeostasis during infection warrants further research.

Adjustments were made for nutritional status using MUAC, which unlike BMI does not change with gestation in normal pregnancies, and mean MUAC was comparable for non-pregnant and pregnant women. Nutritional status is important for mucosal immunity, which may be affected by states of under- or over-nutrition. Serum Lf concentration positively correlates with insulin resistance in obese women.<sup>32</sup> Leptin, an adipocytokine produced by epithelial cells and adipose tissue, has a role in mucosal defences possibly mediated by expression of IL23.<sup>33</sup> The significant positive association of BMI and MUAC with vaginal Lf is consistent with leptin influence on constitutive Lf production in young women who are still maturing.

We have shown a positive association between serum hepcidin and vaginal Lf concentration. These results may indicate a simple association of inflammation with these markers, but increased hepcidin would still impact on iron homeostasis. Hepcidin is produced by hepatocytes, but may also be locally synthesised by myeloid cells in response to pathogens, and has been observed in neutrophils migrating to tissue sites of infection.<sup>34</sup> There are few prior reports on hepcidin response following local, as opposed to systemic infection other than in the gastro-intestinal tract during colitis,<sup>35</sup> and in viral keratitis.<sup>36</sup> The mechanisms are unclear but *in vivo* studies implicate host induction of local acute phase response proteins and evidence of a host-imposed metal ion limited environment.<sup>37</sup> Hepcidin expression is inhibited in states of iron deficiency. Host nutritional and iron status may

influence the homeostatic mechanisms controlling vaginal Lf expression. Vaginal Lf concentrations were increased in better nourished women, yet we have previously reported that at baseline, women who were iron deficient were more likely to have normal vaginal flora.<sup>16</sup> In conclusion Lf concentrations increased with genital infection, higher BMI, MUAC, body iron stores and hepcidin suggesting nutritional and iron status influence homeostatic mechanisms controlling vaginal Lf responses. Further research is needed to elucidate whether genital infections induce a hepcidin response which could influence iron availability to vaginal bacteria and fungi. The figure S5 in Supplementary file 2 outlines potential pathways that could be explored.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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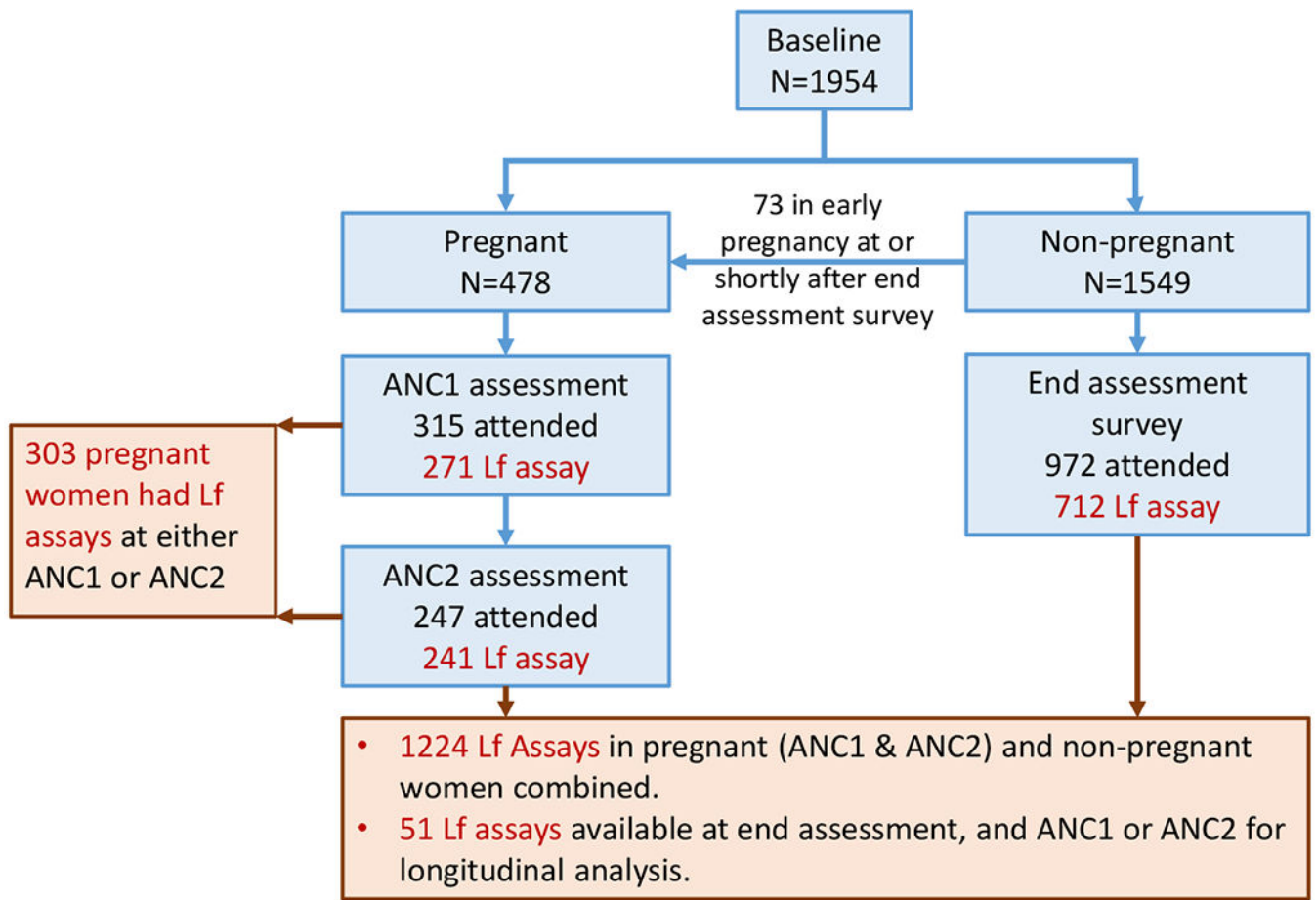
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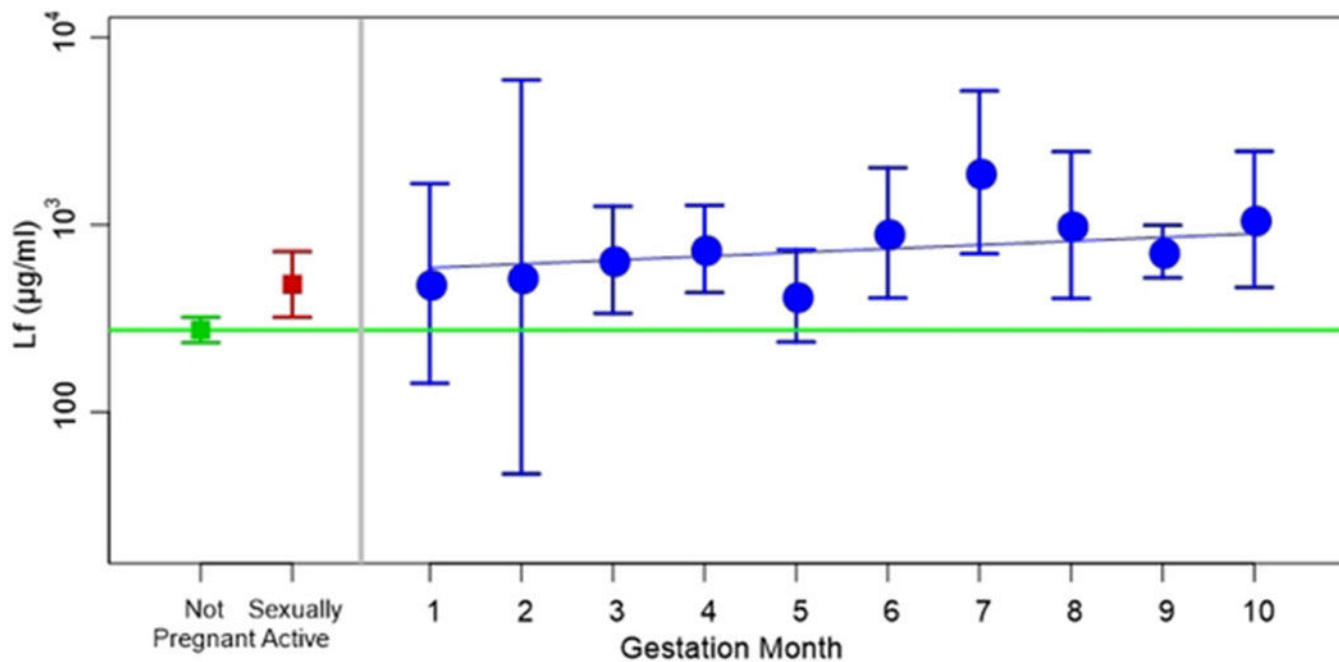


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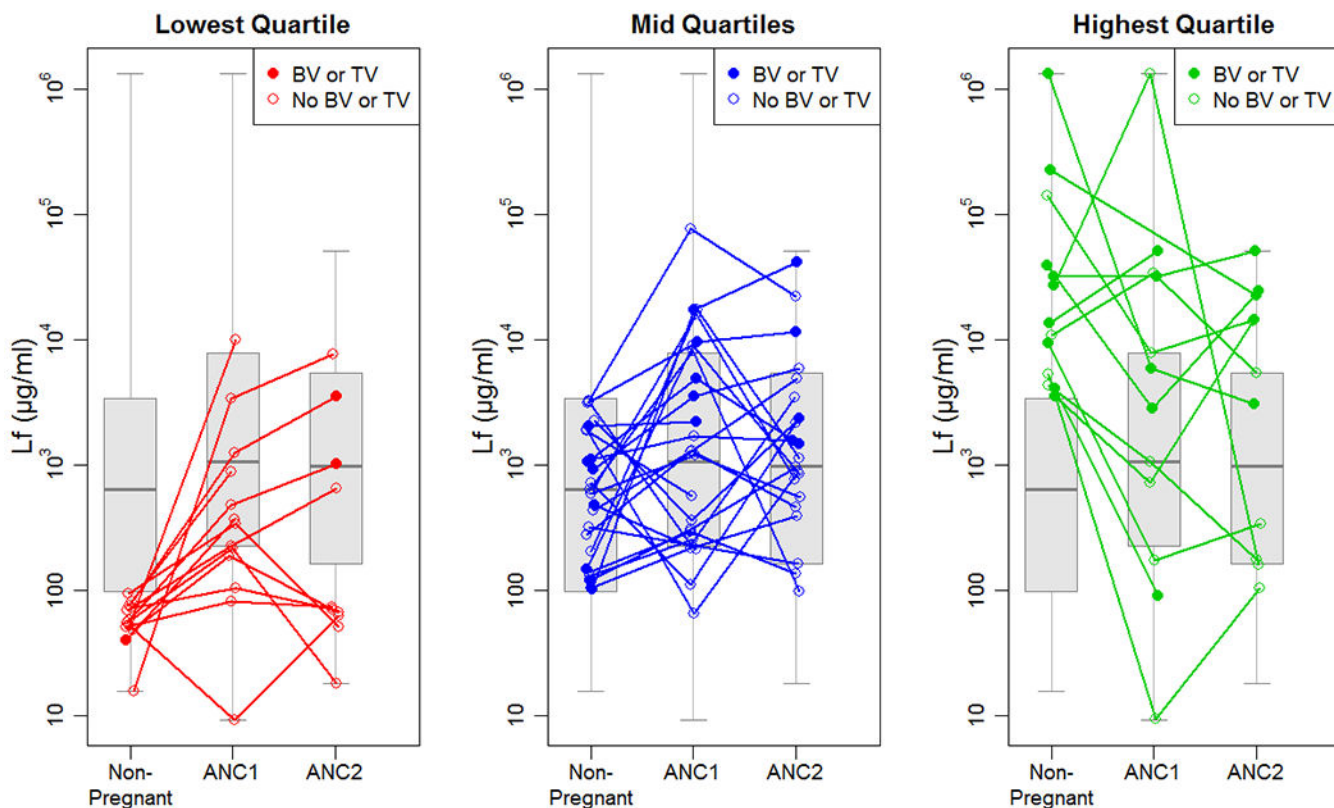


**FIGURE 1.** Flow diagram indicating non-pregnant and pregnant study numbers and sample sizes for Lf assays.

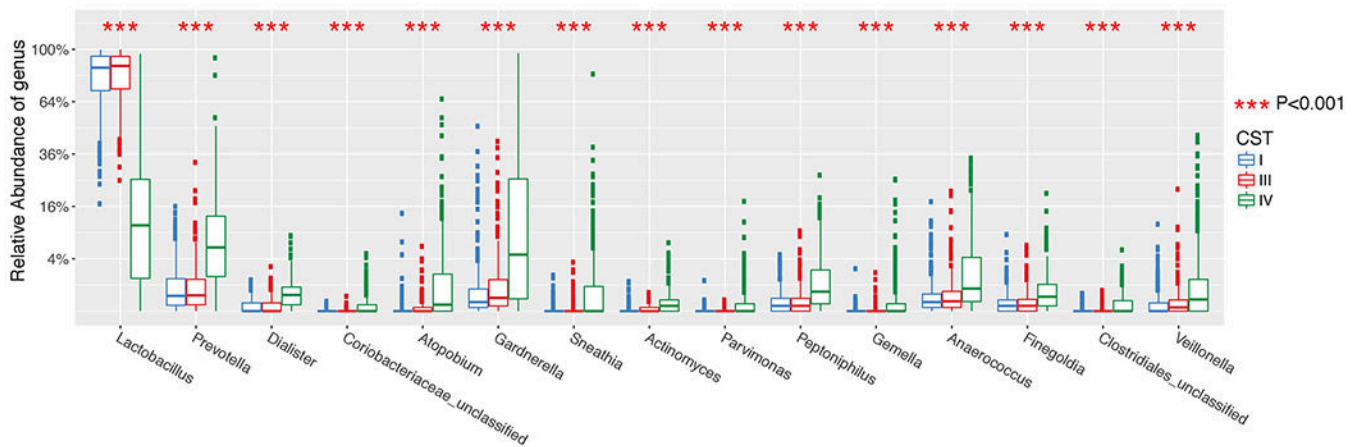


**FIGURE 2. Lf concentration (µg/ml) by gestation and in non-pregnant and sexually active women**

Gestation determined by ultrasound at ANC1 in the pregnant cohort. Mean with 95% CI for each lunar month of gestation plus values for the non-pregnant group (Non-pregnant) and the subset of non-pregnant women who were known to be sexually active at the baseline assessment. Horizontal green line is non-pregnant mean; blue line is linear regression line of log (Lf) v gestation with a random effect to allow for correlations within participants. The slope is non-significant, P=0.14.



**FIGURE 3. Longitudinal Lf concentration (µg/ml) in 51 women seen whilst non-pregnant (at end assessment) and whilst pregnant (at ANC1 and/or ANC2)**  
 Lines join the measurements on the same woman at the three time points. Panels and colours indicate the non-pregnant Lf quartile: low, intermediate and high levels at the first assessment time. Closed circle: BV or *T. vaginalis* infection at time of visit; open circle: no infection. Data are offset slightly for clarity. Shaded boxplots are included showing the full cohort range (median, IQR, min, max).



**FIGURE 4. Taxonomic abundance plots of bacteria associated with CST I, III and IV**  
 Boxplots show the 15 genera most associated with CST differences and are plotted in order of significance. The centre line denotes the median, the boxes cover the 25th and 75th percentiles, and the whiskers extend to the most extreme data point, which is no more than 1.5 times the length of the box away from the box. Points outside the whiskers represent outlier samples. Statistical comparisons based on the Kruskal-Wallis test and all P-values are adjusted for multiple comparisons using the false discovery rate algorithm, where \*\*\* denotes FDR  $P < 0.001$ .

TABLE 1

Baseline characteristics and follow-up iron biomarkers in women who became pregnant (at ANC1 or ANC2) or remained non-pregnant (at end assessment)

Variable <sup>1</sup>	Baseline Became pregnant n=303	Baseline Remained non-pregnant n=712
<b>Demographics and medical history</b>		
Mean age at visit, years	17.0 (16.0-18.0)	16.0 (15.0-17.0)
Menarcheal at baseline (%)	282/303 (93.1)	544/712 (76.4)
Mean reproductive age (if menarcheal), years	3.0 (2.0-4.0) [1 missing]	3.0 (2.0-4.0)
Education (%)	No Schooling	199/302 (65.9)
	Primary	55/303 (18.2)
	Lower/higher Secondary	48/302 (15.9)
Drinks Alcohol (%)	166/303 (54.8)	258/712 (36.2)
Sexually Active (%)	113/303 (37.3)	97/711 (13.6)
<b>Anthropometry</b>		
Mean MUAC, cm	24.0 (22.7-25.2)	23.0 (21.7-24.5)
MUAC <21cm	16/303 (5.3)	107/712 (15.0)
Mean BMI, kg/m <sup>2</sup>	20.0 (18.8-21.0)	19.1 (17.8-20.4)
BMI<18.5 kg/m <sup>2</sup>	61/303 (20.1)	262/712 (36.8)
<b>Anaemia and iron biomarkers</b>		
	ANC1 <sup>2</sup>	End Assessment <sup>3</sup>
Pregnant Hb <11 g/dl; non-pregnant Hb <12 g/dl	209/303 (69.0)	307/709 (43.3)
Mean ferritin, µg/l	97.0 (50.2-175.8) [1 missing]	52.0 (28.0-91.5) [4 missing]
Mean adjusted ferritin, µg/l <sup>4</sup>	46.9 (26.4-88.1) [1 missing]	37.2 (18.8-65.1) [4 missing]
Mean sTfR, µg/ml	3.0 (2.3-4.1) [2 missing]	3.7 (2.8-5.2) [6 missing]
Ratio sTfR(µg/ml)/log <sub>10</sub> ferritin(µg/l) >5.6, (%) <sup>5</sup>	34/301 (11.3)	143/706 (20.3)
Mean ZPP, µmol/mol heme	102 (87-120) [24 missing]	93 (78-115) [2 missing]
Mean body iron stores, mg/kg <sup>4</sup>	5.9 (3.8-8.6) [2 missing]	5.1 (2.3-7.4) [6 missing]
Mean hepcidin, µmol/ml	3.3 (1.1-7.8) [4 missing]	3.2 (1.3-6.2) [6 missing]
Mean CRP, mg/l	4.4 (1.2-13.0) [3 missing]	0.6 (0.2-1.5) [5 missing]

MUAC: Mid-Upper Arm Circumference; BMI: body mass index; sTfR: serum transferrin receptor; CRP: C-reactive protein; ANC: Antenatal Clinic visit; Hb: hemoglobin

<sup>1</sup> Brackets: interquartile range, or %

<sup>2</sup> Includes pregnancies identified at, or shortly after end assessment

<sup>3</sup> Menarcheal n= 621; non-menarcheal n = 91

<sup>4</sup> Ferritin estimated using an internal regression correction approach allowing for inflammation based on CRP as described by Namaste *et al.*<sup>23</sup>

<sup>5</sup>Ratio sTfR/log<sub>10</sub> ferritin provides estimate of proportion iron deficient. 21

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TABLE 2

Effect estimates of demographic, anthropometric, infection, and iron biomarkers with vaginal Lf concentration

Parameter	Adjusted for visit <sup>1</sup>				Adjusted for visit and MUAC <sup>2</sup>		
	N <sup>3</sup>	P visit <sup>4</sup>	Effect (95%CI)	P <sup>5</sup>	P visit <sup>4</sup>	Effect (95%CI)	P <sup>5</sup>
Reproductive age (per year)	1188	<0.001	0.01 (-0.02-0.05)	0.40	<0.001	0.01 (-0.03-0.04)	0.67
Sexually active <sup>6</sup> (yes)	711	NA	0.28 (0.08-0.48)	0.005	NA	0.24 (0.04-0.44)	0.019
Alcohol (yes)	1212	<0.001	-0.02 (-0.14-0.09)	0.71	<0.001	-0.01 (-0.13-0.10)	0.81
BMI (per kg/m <sup>2</sup> )	1224	<0.001	0.03 (0.01-0.06)	0.018	NA	NA	NA
MUAC (per cm)	1224	<0.001	0.03 (0.00-0.06)	0.047	NA	NA	NA
No schooling (yes)	1219	<0.001	-0.01 (-0.13-0.11)	0.83	<0.001	-0.01 (-0.12 - 0.0)	0.93
Nugent scores:							
	0-3	1146	<0.001	reference	<0.001	reference	<0.001
	4-6	NA	NA	0.41 (0.23-0.59)	NA	0.42 (0.24-0.60)	NA
	7-10	NA	NA	0.82 (0.63-1.00)	NA	0.81 (0.62-1.00)	NA
<i>T. vaginalis</i> (yes)	1215	<0.001	1.06 (0.85-1.26)	<0.001	<0.001	1.06 (0.86-1.26)	<0.001
Vaginal discharge (yes)	1221	<0.001	0.26 (0.07-0.45)	0.006	<0.001	0.26 (0.08-0.45)	0.006
Antibiotic use (yes)	1224	<0.001	0.12 (-0.01-0.24)	0.066	<0.001	0.11 (-0.01- 0.24)	0.075
Antifungal use (yes)	1224	<0.001	0.49 (0.25-0.73)	<0.001	<0.001	0.48 (0.24-0.72)	<0.001
Adjusted ferritin, µg/l <sup>7</sup>	1216	<0.001	0.07 (0.01 - 0.13)	0.021	<0.001	0.07 (0.01 - 0.13)	0.018
sTfR, µg/ml	1216	<0.001	-0.01 (-0.15- 0.13)	0.86	<0.001	0.00 (-0.14-0.14)	0.99
ZPP, µmol/mol heme	1216	<0.001	-0.03 (-0.18-0.12)	0.73	<0.001	-0.02 (-0.17-0.13)	0.82
Ratio sTfR (µg/ml) /log <sub>10</sub> ferritin (µg/l)	1213	<0.001	-0.08 (-0.18-0.02)	0.14	<0.001	-0.07 (-0.18-0.03)	0.16
Hepcidin, µmol/ml	1211	<0.001	0.05 (0.00-0.09)	0.040	<0.001	0.04 (0.00-0.09)	0.047
CRP, mg/l	1212	<0.001	0.03 (0.00-0.07)	0.062	<0.001	0.04 (0.00-0.07)	0.048
Hb, g/dl	1219	<0.001	-0.01 (-0.45-0.43)	0.96	<0.001	-0.06 (-0.49-0.38)	0.81
Body iron stores, mg/kg <sup>7</sup>	1213	<0.001	0.014 (0.001 - 0.028)	0.041	<0.001	0.014 (0.000 - 0.028)	0.042

BMI: body mass index; MUAC: Mid-Upper-Arm-Circumference; sTfR: serum transferrin receptor; ZPP: whole blood zinc protoporphyrin; CRP: C-reactive protein; NA: not applicable

<sup>1</sup> Combined data for Lf measurements at follow-up in non-pregnant (end assessment) or pregnant women (ANC1 and ANC2). Effect sizes are differences in Log<sub>10</sub> Lf per unit, or between groups, adjusting for visit, or visit and MUAC, as fixed factors, and participant as a random effect in a linear mixed model which includes all women and visits in a single analysis.

<sup>2</sup>Additional model adjusted also for MUAC assessed at ANC1 or end assessment.

<sup>3</sup>Number of Lf measurements included in the analysis across all visits after missing data excluded

<sup>4</sup>P<sub>visit</sub> : P-value associated with the visit effect.

<sup>5</sup>Significance of the effect of the variable itself – average across all visits.

<sup>6</sup>Assessed at baseline and related to Lf concentration measured for non-pregnant women at end assessment only.

<sup>7</sup>Ferritin estimated using an internal regression correction approach allowing for inflammation based on CRP as described by Namaste *et al.*<sup>23</sup>

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