Novel biomarkers in the pathogenesis of placental malaria in sub-Saharan Africa

Owens, S.

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### 6. PLACENTAL MALARIA AND MATERNAL SERUM DIHYDROEPIANDROSTERONE SULPHATE CONCENTRATIONS AT DELIVERY IN GHANA

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\textsuperscript{d}. Emma Kinderziekenhuis, Academic Medical Centre, University of Amsterdam, 1100 DD Amsterdam, The Netherlands

<table>
<thead>
<tr>
<th>Parity</th>
<th>Blood group</th>
<th>aPL concentration, expressed as MoM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>aCL IgG</td>
</tr>
<tr>
<td><strong>Primiparae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active</td>
<td>O</td>
<td>1.7 (1.0-1.8)</td>
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<td>Non-O</td>
<td>1.35 (1.3-1.95)</td>
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<tr>
<td>Past or no infection</td>
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<td>Non-O</td>
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<tr>
<td><strong>Multiparae</strong></td>
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<tr>
<td>Past or no infection</td>
<td>O</td>
<td>1.15 (1.0-1.7)</td>
</tr>
<tr>
<td></td>
<td>Non-O</td>
<td>1.3 (0.8-1.7)</td>
</tr>
</tbody>
</table>
Abstract

Background
It has recently been shown that the serum concentration of dihydroepiandrosterone sulphate (DHEAS), the most abundantly secreted human androgen, predicts resistance to P. falciparum infection in non-pregnant young adults. There are no published data on the role of DHEAS in pregnancy malaria.

Methods
A secondary analysis of stored sera from a previous cross-sectional study of placental malaria in Kumasi, Ghana was carried out. The aim was to assess the association between DHEAS concentrations, sampled at parturition and measured using enzyme-linked immunosorbent assay, histological changes of placental malaria and markers of malarial morbidity, in a cohort of 104 HIV-uninfected women.

Results
DHEAS concentration was associated with maternal age and parity. There was a weak association between DHEAS concentration and malaria infection in primiparae on stratified analysis only (P=0.062). There were no associations between DHEAS and maternal haemoglobin concentration or birth weight, even after adjustment for parity.

Conclusions
DHEAS is not a primary determinant of placental malaria susceptibility and morbidity in pregnant women. However, it may be a factor in the acquisition of pre-pregnancy malarial immunity and warrants further study especially in younger primiparae.

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DHEAS
pregnancy
malaria
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Plasmodium falciparum
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Keywords

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Introduction

Over 25 million pregnant women in sub-Saharan Africa are exposed to malaria infection every year (1). Pregnancy-associated malaria (PAM) results in substantial maternal and especially infant morbidity, contributing to 3-8% of all infant deaths globally (2) and 10000 maternal deaths through malarial anaemia in Africa alone (3). Susceptibility to PAM is a complex function of the unique ability of a subvariant class of malaria parasites to sequester in the placenta and the host’s specific immune response to these same parasite variants. This interplay occurs in the context of the profound immunological (4) and hormonal changes associated with pregnancy (5).

The role of hormonal factors in PAM susceptibility has been little studied (6). Malaria has been associated with reduced oestradiol production in later pregnancy (7) and with raised serum cortisol levels with placental malaria (8) (9) (10). Whether these changes reflect a non-specific endocrinological stress response to malaria, or whether altered hormone levels during pregnancy affect immune responses, thereby modulating susceptibility to malaria, is unknown. Further studies of the relationship between malaria and the major endocrinological changes of pregnancy are warranted.

Like cortisol, dehydroepiandrosterone sulphate (DHEAS) is steroid hormone produced by the adrenal cortex in concentrations which rise steeply during puberty, peak in early adulthood and decline thereafter (11). DHEAS has powerful immunomodulatory properties, and has been associated with immunity to a variety of infections (reviewed in (12)). In a longitudinal study of Kenyan males, resistance to malaria re-infection after antimalarial treatment increased during puberty and was predicted by the serum concentration of DHEAS, independently of age which was considered a proxy marker of cumulative parasite exposure (13). In another cross-sectional study of non-pregnant pubertal girls living in western Kenya, higher DHEAS concentration was associated with reduced P. falciparum parasite density, and with increased haemoglobin concentration, and these associations were also independent of age (14).

In addition to its immunological role, DHEAS is important in maintaining the maternal-placental-fetal endocrine system during pregnancy (15). The fetus utilises placental progesterone in order to synthesise steroids such as DHEAS, and supplies 19-carbon compounds to the placenta which then serve as precursors for maternal estrogens (5). This signaling method, which the fetus directs, controls important physiologic processes that affect fetal health, including utero-placental blood flow, and fetal adrenal gland function. By 20th week of gestation approximately 90% of estriol excretion can be accounted for by by DHEAS production by the fetal adrenal gland (16). The placenta must be extremely efficient in cleavage of the sulphate conjugates from the fetal blood stream, and placental sulphatase activity is rapid, and its deficiency is associated with low maternal estrogen excretion, which has clinical relevance (17). During normal pregnancy, concentrations of DHEAS fall progressively until term, when there is a temporary surge around delivery (18). PAM, which causes extensive placental pathology, may impair placental sulphatase activity (19), predisposing to fetal growth restriction. Yet no published studies have reported profiles of DHEAS with PAM. This is important, as low DHEAS levels may be a useful biomarker of fetal risk in this infection.
We carried out a cross-sectional study at delivery assessing serum DHEAS concentrations in Ghanaian women. The aim of the study was to determine if there was an association between placental malaria pathology, DHEAS concentrations and malarial morbidity. We hypothesised that raised DHEAS levels in pregnant women would be associated with reduced malaria infection of the placenta and with improved anthropometric markers of fetal malarial morbidity.

Methods

Study Design
A cross-sectional survey was carried out between April and June 2003 at the Labour Unit at the Komfo Anokye Teaching Hospital, Kumasi, Ghana. Kumasi is the second-largest city in Ghana, with a population of 1.2 million at the time that this study was conducted, (20). Located in the Ashanti region of central Ghana, the climate is semi-humid tropical, with peak rainfall between April and June and an intense perennial malaria transmission, the predominant parasite being P. falciparum (21).

The target population was gravid women delivering at the hospital during the study period and their babies. The current study was a component of an analysis of transplacental transfer of maternal measles antibody (MMA) in a malarial environment, on which the sample size calculation was based (22).

Enrolment of Subjects
Gravid women who delivered vaginally and consented to be enrolled were consecutively recruited at delivery in the labour ward. Those with hypertension (essential or pregnancy induced), twin delivery, or who had received a blood transfusion less than 24 h before delivery were excluded. At enrolment, basic demographic data and antenatal care were documented on a pre-prepared questionnaire. Information was obtained from each patient's antenatal health card, if available; participants who did not have such a card were questioned directly. Women were weighed on an electronic balance to the nearest 0.1kg. Mid-upper arm circumference (MUAC) was measured at the mid-point of the left arm using a paper tape measure. Gestational age was calculated from the estimated date of delivery (EDD) documented on the antenatal care when available, otherwise by maternal recall of last menstrual period (LMP).

Only babies delivered alive after the 24th week of gestation, and whose mothers gave consent, were recruited. Shortly after delivery, each baby was weighed on an electronic balance, and crown-heel length measured on an infantometer. The readings of each parameter were obtained and values recorded to the nearest 0.05kg and 0.5cm respectively. Each baby was examined and classified as appropriate, small or large for gestational age. Ponderal index was calculated from weight/length3 (Rohrer’s index).

Collection of Specimens
Maternal blood (5 mL) was obtained from a peripheral vein within 4 h of delivery. Cord blood (8 mL) was collected from a large vein on the fetal side of the placenta immediately after delivery. Sera were stored at -70°C within 8 h of collection, until assayed. Placental tissue samples (1 cm3) were obtained from an off-centre position and stored in 20 mL of 10% formaldehyde in phosphate buffer until processed for
histological examination. The placenta was weighed on an electronic balance to the nearest 0.05kg, after removing blood clots and cutting the cord close to its insertion (2-3cms).

**Malaria Diagnosis**
Paraffin-embedded sections (5µm thick) of the placental biopsy specimens were stained with hematoxylin-eosin and examined under light microscopy (×40) and under polarized light at the Pathology Department, University of Barcelona. Placental malaria infection was defined by the presence of parasites, malaria pigment and by the histological features of malaria. Classification was into non-infected, acute infection, chronic infection and past infection as previously described. In subsequent analyses, active infection included both acute and chronic infection. Thick and thin Giemsa-stained films were prepared with blood obtained from the cord and examined under light microscopy (×100) for malarial parasites at the laboratory in Komfo Anokye Teaching Hospital, and the number and species of parasites were measured against 200 white cells in the standard manner. A negative count was recorded if no parasites were seen in 100 fields from each blood film.

**Haemoglobin estimation**
Maternal and cord haemoglobin concentrations were measured with the HemoCue® (Angelolm, Sweden).

**DHEAS measurements**
DHEAS was assayed using a commercial DHEA-S solid enzyme-linked immunosorbent assay (ELISA) kit (DRG, US). The reference range for women <50-years given by the manufacturer was 40-217 µg/dl. There were no ranges given for pregnancy.

**HIV Testing**
Anonymous HIV testing was carried out on the maternal samples using a similar, but non-quantitative ELISA technique (Genscreen® HIV 1 / 2 version 2, Bio – Rad, France). Subjects who were HIV-positive were withdrawn from the study to avoid confounding of the primary analyses which were of the association between placental malaria, malarial outcomes and maternal DHEAS concentration at delivery.

**Statistical analysis**
Data entry was carried out in Excel (Microsoft) and analysis was carried out in STATA (version 9) (Statacorp). Dichotomous variables were assessed with chi-square or Fisher exact tests, with p values less than 0.05 considered statistically significant. Odds ratios (OR) and 95% confidence intervals (95% CI) were estimated using logistic regression where appropriate. Differences between means and standard errors (SE) were assessed by linear regression where data were normally distributed, or the Mann-Whitney/Wilcoxon Test where they were not. Multiple linear regression was used to analyse DHEAS and other factors associated with malarial outcomes. Interaction terms were used to adjust for effect modification in stratified analyses.
Ethical considerations
The study was approved by the ethical committees of the Liverpool School of Tropical Medicine and Kumasi University, prior to commencement of the fieldwork. All laboratory data, with the exception of haemoglobin measurements made at the bedside were anonymous. Codes linking results to individual patients were erased prior to data entry. Informed consent was taken for each mother in the study, and anonymous HIV testing was specifically discussed.

Results

There were 146 women who were eligible for enrolment, of whom 12 refused or left the hospital before consent could be obtained, 4 were excluded because of maternal hypertension, 2 because of congenital abnormalities and 2 because of sampling failure. We excluded the data from 3 HIV-positive women post hoc in order to avoid possible confounding from another maternal infection which is known to impact on placental function (23). Of the remaining 123 HIV-negative women who were recruited for the study, DHEAS and histological data were available for 104. There were no significant differences in demographic or biophysical profiles in women with and without data on DHEAS concentration or placental histology (data not shown).

Mean maternal age in this sample was 26.4 years (SE +/-0.65), 7% were classified as adolescent (<18-years) and 39% were primiparous (multiparity refers to all parities greater than one). The mean age of primiparous was 21.6 years (SE +/- 0.73) compared to 29.4 years (SE +/- 0.74) in multiparous (p<0.001). The median maternal MUAC was 27 cm (IQR 24.5-29.1) and 7% were malnourished (MUAC<23 cm).

Median neonatal gestational age was 39.1 weeks (IQR 37.1-40.4), and 22% were born preterm (<37 weeks gestation). Mean birth weight was 3010 g (SE +/- 40) and 7% had low birthweight (<2500g). Most women (93%) had documented attendance for at least one antenatal clinic.

Evidence of malaria infection was present in 33 placentas (32%) and there were no cases of cord parasitaemia. Of those with placental malaria, active infection was found in 18 (54%) and past infection in 15 (45%). There were 20 (50%) primiparous with placental malaria (all categories) compared with 13 (20%) multiparous (OR 3.92; 95% CI 1.46 – 9.35; p=0.002). Over half the subjects (53%) reported using antimalarial medication during pregnancy.

The overall male/female ratio in neonates was 1.08.

On univariate analysis, maternal serum DHEAS concentration at delivery was negatively associated with maternal age (R2=0.045; β -0.41; 95% CI -0.78, -0.04; p=0.030) and positively associated with primiparity (R2=0.047; β 5.62; 95% CI 0.64, 10.60; p=0.027). Mean DHEAS concentration in adolescents was 65.43 µg/dl (SE +/- 5.03) compared to 54.81 µg/dl (SE +/- 1.26) in non-adolescents (p=0.032). There was no association between DHEAS concentration and gestational age (p=0.680) or maternal MUAC (p=0.933), and no association between DHEAS concentration and placenta malaria (any infection, p=0.405; active infection, p= 0.438; past infection, p=0.626).
Table 1 summarises the data on DHEAS concentration by age, parity and gestational age, stratified by malaria infection (all categories). Among primiparae, placental malaria was weakly associated with lower DHEAS concentrations (p=0.067). However in multivariate analysis, adjusting for interaction with parity, there was no significant association between placental malaria and DHEAS concentration (R2=0.078; β -2.20; 95% CI -9.83, 5.43; p=0.569). Comparisons of DHEAS concentrations between infected and uninfected women in other stratified groups were not significant.

Table 2 summarises the results of multivariate regression analyses of DHEAS concentration on maternal haemoglobin concentration and birth weight, after adjustment for primiparity and malaria infection. In these models, reduced haemoglobin concentration was associated with active (p=0.014) and past (p=0.029) placental malaria infection. Birth weight was associated with primiparity (p=0.007) and marginally associated with past placental malaria infection (p=0.097). There was no association between DHEAS concentration and either parameter.

Discussion

In this secondary analysis of a cross-sectional study conducted in Kumasi, Ghana, maternal serum DHEAS concentrations measured at delivery were negatively associated with maternal age and were higher in primiparae compared to multiparae, reflecting the younger age of this group. There was a trend towards lower DHEAS concentrations with placental malaria infection, especially on stratification by parity, but this was not significant. There were no associations between DHEAS concentration and commonly used parameters of malarial morbidity in pregnancy – maternal haemoglobin concentration and birth weight, even after adjustment for parity.

To the best of our knowledge this is the first published study of DHEAS in pregnancy malaria. However in another preliminary multivariate analysis from Malawi, low DHEAS concentrations (<40 µg/dl) were associated with active placental infection (adjusted OR 2.72; 95% CI 1.02 – 7.30; P=0.047) (Senga and Brabin – personal communication; manuscript in preparation). We note that this latter study included a larger number and proportion of teenage mothers than does the current work.

In a cross-sectional study of 12 to 18-year old schoolgirls from western Kenya, DHEAS concentration was an independent, negative predictor of malaria parasite density in the parasitaemic girls who had entered puberty (14). As in an earlier longitudinal study of young Kenyan males (13), this relationship remained significant after adjustment for age. There was no association in pre-pubertal girls, and this group had lower DHEAS levels that were postulated to be below a critical protective threshold for malaria (14). Similarly to the present study there was no overall association between malaria prevalence and DHEAS level. In contrast to the present study, DHEAS level was positively associated with haemoglobin concentration in pubertal girls, even after adjustment for age and other determinants of haemoglobin concentration in this group (14).
Under conditions of stable transmission, adolescents, though at markedly reduced risk of severe malaria morbidity and mortality compared to young children continue to suffer frequent asymptomatic parasitaemia, periodic febrile illness and occasionally death (24). Adolescent and young adult women are more commonly parasitaemic than older women (25) and younger pregnant women have been reported to be more susceptible to pregnancy malaria in some settings, independently of parity (26). In both Kenyan studies (13, 14), DHEAS levels continued to be associated with resistance to malaria into young adulthood. These findings support the hypothesis that mature host androgen synthesis independently of age and cumulative malaria exposure, is necessary for the development and maintenance of pre-pregnancy malarial immunity, characterised by resistance to parasitaemia and protection against malarial morbidity. DHEAS may be causally linked with the expression of adult malaria resistance, through the up-regulated production of protective antimalarial immune responses. The full evaluation of this hypothesis will require longitudinal studies of the acquisition of malarial immunity, and the construction of experimental models of the effects of DHEAS on specific immune pathways.

DHEAS has potent immuno-activating properties which increase specific antibody responses and bolster NK cell number and function (reviewed in (27)); both mechanisms may contribute to malaria immunity. It was recently shown that $16\alpha$-bromoeiandrostone (EPI), an analogue of DHEAS, has anti-malarial activity against several strains of P. falciparum in vitro and against P. berghei in a mouse model (28). In a further experiment, plasma-compatible, low-micromolar concentrations of EPI induced exposure of phosphatidylserine on the surface of parasite ring-forms, a signal for phagocytic removal independent of opsonisation (29). Interestingly we have shown previously that elevated anti-phosphatidylserine antibody concentrations are a feature of the robust immune response to placental malaria which characterises multigravidae in endemic areas (30). This may indicate that anti-phospholipid antibodies and DHEAS are part of a common pathway in the mature immune response to malaria parasitisation, although they were not associated in this study possibly because of the small numbers (data not shown).

DHEAS is also a potent down-regulator of the pro-inflammatory cytokines tumour necrosis factor $\alpha$, interleukin-6 and interleukin-1 (reviewed in (27)) and may attenuate the deleterious consequences of parasite-associated proinflammatory cytokines on placental structure and function, and on maternal haemoglobin concentration.

In the absence of further data on DHEAS in PAM, comparable studies of the role of other hormones are of interest. In a study of 65 Gambian women living in an area of holoendemic malaria transmission, plasma oestradiol concentrations in mothers with malaria-pigmented placentae were significantly lower from 32 weeks of gestation onwards (7), suggesting a failure of placental endocrine function in association with chronic or recently resolved placental malaria. DHEAS was not measured in this study but is a substrate for the placental production of oestradiol through a feedback cascade and the action of placental $\alpha$-Sulfatase (15).

Several studies have examined the association between PAM and the archetypal adrenocortical hormone, cortisol. In Tanzania serum concentration of total cortisol was significantly higher in both nulliparae and multiparae with clinical malaria than in those without recorded malaria (9), while in another Kenyan study, serum cortisol
concentration was positively correlated with parasitaemia in primigravidae but not in multigravidae (10). In a prospective study in Gabon (31), cortisol concentrations were significantly higher in P. falciparum-infected women than in uninfected women at delivery and cortisol levels were also significantly correlated with the parasite load throughout pregnancy and delivery. These effects were not modified by gestational age at point of sampling. In eastern Sudan, cortisol concentrations were positively correlated with parasite counts in malaria-infected women, although there was no difference in cortisol concentrations between infected and non-infected women (32). It is thought DHEAS and other β-androstanes might counter the excessive down-regulation of immune activation attributed to the excess production of cortisol, which occurs during infections and stress (33).

There are limitations to this study which weaken its conclusions. Firstly, it was originally powered with another outcome in mind (22) and it is possible that a true association between DHEAS and placental malaria histology has been missed because the sample size was small. In the absence of comparable published studies on which to base meaningful power calculations, this is difficult to discern. However, as the first published study of DHEAS in placental malaria, the data provide an intriguing starting point from which to explore the endocrine interactions with pregnancy malaria further.

Parturition is a period of great hormonal flux and measurements taken at this time may not give an accurate representation of the subtleties of parasite-endocrine interactions. The fact that the present study was a secondary analysis based on a previous cross-sectional study made this unavoidable and further longitudinal studies are suggested. However such studies are difficult and expensive to undertake and opportunistic analyses of existing samples and data may provide useful insights in the interim on which such studies can be founded.

Finally in our study, we excluded women with hypertension which may have biased the results because hypertensive disorders of pregnancy could be associated with changes in placental endocrine function. However in several studies of pre-eclampsia there was no difference in DHEAS concentrations between affected and unaffected pregnancies (34).

Pregnancy-associated changes in hormonal balances can have profound effects on immunity to parasites and other infectious organisms. These have been little studied in the context of malaria and have been suggested as a critical area for new research (6). The present study opens up an original line of enquiry into the role of maternal androgens in the determination of host susceptibility to pregnancy malaria, which is based on an appreciation of possible immunological mechanisms. Larger and longitudinal studies might now explore these themes further, combining clinical, histological and immunological data in pursuit of a greater understanding of the pathogenesis of malaria in pregnancy, through which to inform better intervention strategies.
Abbreviations

DHEAS: dihydroepiandrosterone sulphate  
EDD: estimated date of delivery  
ELISA: enzyme-linked immunosorbent assay  
EPI: 16α-bromoepiandrosterone  
HIV: human immunodeficiency virus  
LMP: last menstrual period  
MMA: maternal measles antibody  
MUAC: mid-upper arm circumference  
NK cell: natural killer cell  
PAM: pregnancy-associated malaria  
OR: odds ratio  
SE: standard error

Competing interests

None declared

Authors’ contributions

SO conceived and designed the original study, collected the samples and demographic data, analysed the results and wrote the first draft of the manuscript. ES and NAN conceived the current study, suggested the analysis plan and revised the manuscript. JO supervised the placental histology and revised the manuscript. BB supervised the original study and edited the final manuscript.

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Table 1. Mean maternal serum dehydroepiandrosterone sulphate (DHEAS) concentrations at delivery, by maternal age, parity, gestational age and placental malaria infection

<table>
<thead>
<tr>
<th></th>
<th>Malaria uninfected</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DHEAS concentration (µg/dl)</td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>56.24 (1.65) [n=71]</td>
<td>54.00 (1.65) [n=33]</td>
</tr>
<tr>
<td>Maternal age</td>
<td></td>
<td></td>
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<tr>
<td>Adolescent</td>
<td>70.25 (4.99) [n=4]</td>
<td>59.00 (9.54) [n=3]</td>
</tr>
<tr>
<td>Adult</td>
<td>55.40 (1.68) [n=67]</td>
<td>53.50 (1.61) [n=30]</td>
</tr>
<tr>
<td>Parity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primiparous</td>
<td>62.43 (2.86) [n=20]</td>
<td>55.55 (2.25) [n=20] *</td>
</tr>
<tr>
<td>Multiparous</td>
<td>53.81 (1.92) [n=51]</td>
<td>51.62 (2.33) [n=13]</td>
</tr>
<tr>
<td>Gestational age</td>
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<tr>
<td>Preterm</td>
<td>51.40 (4.69) [n=10]</td>
<td>52.89 (3.63) [n=9]</td>
</tr>
<tr>
<td>Term</td>
<td>57.07 (1.72) [n=41]</td>
<td>51.69 (2.80) [n=13]</td>
</tr>
<tr>
<td>Post-term</td>
<td>58.40 (11.07) [n=5]</td>
<td>57.25 (6.21) [n=4]</td>
</tr>
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</table>

*Infected vs uninfected primiparae, p=0.067
Round parentheses – standard error
Square parentheses – sample size
Table 2. Multivariate linear regression analyses of the associations between dehydroepiandrosterone sulphate (DHEAS) (µg/dL) and haemoglobin levels (g/L) in pregnant women at delivery, and between DHEAS (µg/dL) and birth weight (g)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Maternal haemoglobin (g/L) (R²=0.151)</th>
<th>Regression coefficient (95% CI)</th>
<th>P</th>
<th>Birthweight (g) (R²=0.147)</th>
<th>Regression coefficient (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHEAS (µg/dl)</td>
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<td>0.01 (-0.27, 0.28)</td>
<td>0.969</td>
<td>-3.2 (-10.2, 3.7)</td>
<td>0.358</td>
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<td>Primiparity</td>
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<td>-6.24 (-13.69, 1.20)</td>
<td>0.099</td>
<td>-261.1 (-450.1, -72.1)</td>
<td>0.007</td>
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<td>Placental malaria</td>
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<td>Non-infected</td>
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<td>Reference</td>
<td>0.014</td>
<td>Reference</td>
<td>0.446</td>
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<tr>
<td>Active</td>
<td></td>
<td>-11.77 (-21.05, -2.48)</td>
<td></td>
<td>-91.7 (-329.4, 146.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Past</td>
<td></td>
<td>-11.06 (-20.94, -1.18)</td>
<td>0.029</td>
<td>-216.2 (-469.1, 37.0)</td>
<td>0.093</td>
<td></td>
</tr>
</tbody>
</table>