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ORIGINAL ARTICLE

Associations between maternal prenatal stress, methylation changes in *IGF1* and *IGF2*, and birth weight

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Maternal stress has been linked to low birth weight in newborns. One potential pathway involves epigenetic changes at candidate genes that may mediate the effects of prenatal maternal stress on birth weight. This relationship has been documented in stress-related genes, such as *NR3C1*. There is less literature exploring the effect of stress on growth-related genes. *IGF1* and *IGF2* have been implicated in fetal growth and development, though via different mechanisms as *IGF2* is under imprinting control. In this study, we tested for associations between prenatal stress, methylation of *IGF1* and *IGF2*, and birth weight. A total of 24 mother–newborn dyads in the Democratic Republic of Congo were enrolled. Ethnographic interviews were conducted with mothers at delivery to gather culturally relevant war-related and chronic stressors. DNA methylation data were generated from maternal venous, cord blood and placental tissue samples. Multivariate regressions were used to test for associations between stress measures, DNA methylation and birth weight in each of the three tissue types. We found an association between *IGF2* methylation in maternal blood and birth weight. Previous literature on the relationship between *IGF2* methylation and birth weight has focused on methylation at known differentially methylated regions in cord blood or placental samples. Our findings indicate there may be links between the maternal epigenome and low birth weight that rely on mechanisms outside known imprinting pathways. It thus may be important to consider the effect of maternal exposures and epigenetic profiles on birth weight even in the setting of maternally imprinted genes such as *IGF2*.

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Introduction

Low birth weight (LBW) in infants is associated with higher risks of mortality and morbidities which extend from the neonatal period all the way to adulthood, making LBW a major adverse perinatal outcome.[1] LBW infants are known to be at higher risk for infectious diseases, malnutrition, abnormal neurocognitive development in childhood and even chronic diseases such as hypertension and diabetes in adulthood.[2] In addition, across the world, LBW infants are significantly more likely to experience neonatal mortality than their normal birth weight counterparts.[3] A number of factors have been associated with a higher risk of LBW, with the majority related to maternal health or sociodemographic indicators.[4,5] For these reasons, birth weight is considered a valuable gauge of both maternal and child health. The associated morbidities combined with the well-documented evidence of racial disparities in the risk of developing LBW has made achieving adequate newborn birth weight a public health priority worldwide.[5,6]

One of the etiologic factors that has been connected to LBW is maternal stress.[7–9] This relationship has been linked to excessive glucocorticoid exposure during pregnancy from a dysregulated maternal hypothalamus–pituitary–adrenal (HPA) axis.[10] In an effort to understand the pathophysiologic effect of maternal stress, researchers have argued that the accumulation of stressful life events or 'allostatic load' of chronic stress triggers maladaptive physiologic responses during pregnancy. These responses in turn lead to adverse birth outcomes such as LBW.[11] The relationship between severe acute stressors experienced during pregnancy and lower birth weight has also been documented, leading other investigators to postulate that the mechanism by which stress leads to adverse birth outcomes is related to an acute effect on maternal energy intake and expenditure during pregnancy.[11]

Though the risks associated with differentially timed stress exposures of varying acuity are not yet fully understood, the relationship appears to be in line with the developmental origins of health and disease hypothesis, which posits that the intrauterine environment has the capacity to shape fetal, childhood and even adult disease or health.[12,13] Recently, epigenetic changes to gene expression have been proposed as a mechanism through which the intrauterine environment shapes postnatal phenotypes, even across generations.[14–16] In addition, there is evidence that perinatal exposures that alter offspring epigenetic marks through such mechanisms as DNA methylation or histone acetylation may be much more influential than exposures experienced through the offspring's life course.[17]

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The relationship between maternal stress and postnatal health outcomes via DNA methylation has been documented most robustly through studies of *NR3C1*. This gene, which encodes the glucocorticoid receptor, has been specifically implicated in newborn birth weight through cortisol-related pathways involving the HPA axis.[18] Our group has previously reported on the relationship between maternal stress, newborn birth weight and the methylation of *NR3C1* and several other genes in the HPA axis including *CRH, CRHBP* and *FKBP5*.[7,19]. Specifically, using culturally relevant measures of stress in a cohort of 24 mother–newborn dyads in the high conflict-zone of eastern Democratic Republic of Congo (DRC), we found that extreme maternal prenatal stress was significantly correlated with infant birth weight and methylation at the promoter region of *NR3C1*.[19] Based on these findings, we postulated that maternal prenatal stress might have epigenetic effects on genes implicated in fetal growth in addition to those implicated in the neuroendocrine stress response.

The insulin growth factor (IGF) system includes *IGF1* and *IGF2* as well as several other genes related to IGF-binding proteins. Though they are each expressed in various parts of the body, *IGF1* and *IGF2* are also both synthesized by the placenta, where they are involved in the regulation of fetal, placental and neonatal growth.[20] Importantly, they exert their effect through different mechanisms and at different times in fetal and neonatal development.[21,22] Deletion studies for both *IGF1* and *IGF2* have shown an association with altered or poor growth in animal models and infants.[23–25] However, these results have not been supported by all the existing literature. For instance, Zhang *et al.*[26] found lower levels of *IGF2* in both small-for-gestational-age (SGA) and large-for-gestational age infants, compared with appropriate-for-gestational-age infants.[26] These results suggest that *IGF2* in particular may not have a simple linear relationship with birth weight.

The regulatory mechanisms controlling these two genes are quite different. *IGF2* is known to be under genomic imprinting control. Imprinting results in monoallelic gene expression related to the specific parent-of-origin for the allele, a process at least in part controlled by DNA methylation at CpG dinucleotides in differentially methylated regions (DMRs).[27,28] *IGF2* is expressed solely from the paternal allele in most tissues, though there is some evidence it can be expressed from both the maternal and paternal allele in the liver[29] and some areas of the central nervous system.[30]

Though *IGF1* does not appear to be regulated through imprinting, there is evidence that its expression might also be modulated through epigenetic mechanisms like DNA methylation.[31] In addition, human studies have found associations between birth weight and differential methylation at both *IGF1*[32] and *IGF2*.[33,34] However, when reviewed systematically, the presence, strength and direction of associations between epigenetic changes in IGF-related genes and LBW or SGA infants are inconsistent across the literature[35] and thus require further study.

To our knowledge, there is no literature linking maternal stress to methylation differences at *IGF1*. Only a few groups report a relationship between maternal stress and *IGF2* expression and/or methylation despite the strength of the literature linking maternal stress and birth weight.[36–39] Notably, Mina *et al.*[36] discovered that increased maternal emotional distress correlated with increased placental mRNA expression of *IGF2*.[36] However, the relationship between maternal stress/distress, mRNA expression and *IGF2* methylation is not clear given the sparse inconsistent reports in the literature.[37–39] For instance, Vangeel *et al.*[37] found a positive association between *IGF2* methylation and maternal depression, anxiety and cortisol levels during pregnancy,[37] whereas Liu *et al.*[38] reported that *IGF2* DMR methylation did not appear to mediate the relationship between birth weight and maternal mood although *IGF2* methylation did differ significant by birth weight.[38] Furthermore, Heijman *et al.*'s[39] study of adults born to women pregnant during the Dutch Hunger Winter showed that these adults have *IGF2* methylation levels persistently lower through adulthood than same-sex siblings who were unexposed to famine.[39] These findings suggest a relationship between prenatal environmental stressors, birth weight and *IGF2* methylation that may persist throughout the life course but which is not yet fully understood.

Our cohort of women in the DRC represents a unique population of mothers facing extreme chronic and acute stressors and provides an opportunity to explore the epigenetic mechanisms by which prenatal stress affects fetal growth and development. This study aims to build on existing knowledge about the effects of *IGF1* and *IGF2* on newborn birth weight by exploring whether DNA methylation at these candidate genes in mothers or infants is associated with measures of extreme maternal stress and birth weight. We hypothesized that increased stress would be associated with changes in DNA methylation, which would in turn be associated with decreased birth weight (Fig. 1).

Method

Study participants and sample collection

Participants were recruited for this cross-sectional study from women delivering their babies at HEAL Africa Hospital in Goma in eastern DRC. Participants were enrolled immediately after giving birth. Stillbirths were excluded. Birth weight was collected once using a hospital scale. Subsequent cohort data collection by our group used an additional scale to create two birth weight measurements and these two sets of measurements showed a 97.4% correlation. Maternal venous whole blood

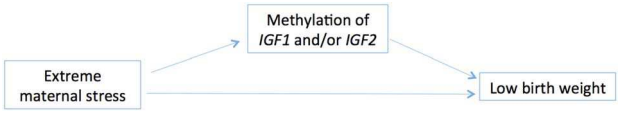

Fig. 1. Proposed model for associations of maternal stress, *IGF* methylation and birth weight.

samples were collected from 24 mothers who delivered between July and August of 2010. Umbilical cord blood and placental tissue samples were collected from these same 24 mothers' discarded placentas within several hours of delivery. All samples were collected into Vacutainer tubes and immediately processed on site for DNA extraction.[40] The study was approved by the Western Institutional Review Board, Olympia, WA (www.wirb.com, WIRB Project # 20100993), the University of Goma and by an ethical review committee at HEAL Africa hospital. Each mother gave oral consent. Documentation of written consent was waived by the WIRB and HEAL Africa Hospital because of the high level of illiteracy in the study population.

Sociocultural data

Detailed interviews were conducted with mothers within 24 h of birth to gather culturally relevant chronic and war-related stressors. This information was gathered via semi-structured oral history and ethnographic interview methods in the Congolese dialect of Swahili. This strategy allowed women to identify stressors that were relevant to their social and cultural norms. Interviews were then transcribed and coded for the presence of discrete stress-related items. Two composite measures of stress reflecting war trauma and chronic stress were constructed based on factor analysis as described previously by Kertes *et al.*[7] The war trauma measure captured 10 experiences such as 'refugee in the past,' 'raped in the past' and 'family killed in war' whereas the chronic stress measure was composed of 19 questions such as 'don't own a home,' 'pregnancy not wanted,' and history of emotional abuse.[7] Within the war trauma measure, three variables specific to personal experiences with rape (past rape, rape resulting in pregnancy, rape during current pregnancy) were pulled out and analyzed as a separate 'rape stress' measure. This was done because we showed previously that the impact of these three rape experiences eclipsed the effect of the war stress variables and accounted for 31% of birth weight variance in our cohort.[19] Thus, we analyzed three stress measures, war trauma, rape stress (with rape stress based on a subset of the war trauma questions), and chronic stress. Supplementary Material Table S1 lists the specific questions associated with each stress measure along with the number of women in our cohort who endorsed each particular stressor.

Epigenetic data and analyses

Genomic DNA was extracted on site using Qiagen QIAamp DNA Mini Kits (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions and eluted in two separate volumes of 200 µl. Methylation was assayed on the Illumina HumanMethylation 450 Bead Chip. Methylation data were filtered as described previously.[7] The final data set included 16 CpG sites for *IGF1* and 39 CpG sites for *IGF2*.

Principal component analysis (PCA) was performed on each tissue to summarize methylation across the CpG sites at each of the genes. In PCA, the first principal component (PC1) accounts for the largest portion of variation in the data set, followed by each subsequent PC. As PCA can be used to describe interrelated methylation signals of several CpG sites within a specific gene, the method has become widely used in many types of methylation studies.[19,41,42] Association was tested with the first two PCs in all tissues and genes as these PCs accounted for 40–50% of the variation in methylation in all cases. Supplementary Material Tables S2 and S3 provide a list of all the CpG sites included in the final data set, with a notation for which CpG sites made up PC1 and PC2 for *IGF1* and *IGF2*, respectively, in all three tissue sites. Location coordinates and primers are also provided. Measures of methylation in maternal and cord blood were corrected for cell composition using the Houseman method for the Illumina 450K chip,[43] as implemented in the minfi package for R.[44] Reference data sets were based on Houseman *et al.*[43] for venous blood and Bakulski *et al.*[45] for cord blood.

Statistical analyses

Multivariate regressions were performed to test for significant associations between the three types of stress measures (war trauma, rape stress, chronic stress), methylation PCs and birth weight in each of the three tissue types, accounting for cell type in maternal and cord blood. We conducted secondary regressions controlling for maternal age and education given the well-known relationship between these factors and birth weight.[46,47] We applied Bonferroni correction for all associations in this study by accounting for the three tissue types, three stress measures and the first two methylation PCs in each tissue, that is $P = 0.05/(3 \times 3 \times 2) = 0.0028$. All statistical analyses were conducted in JMP Genomics[48] and R.[49]

Results

Table 1 summarizes the mean number of variables within each of the three stress measures that were endorsed by the women in our cohort as well as the most commonly endorsed variable within each of the three stress measures. On average, women endorsed two war trauma variables and seven chronic stress variables. The most commonly endorsed war trauma stressor related to having been a refugee in the past (46%). Notably, a quarter of the women in our cohort endorsed rape at some point in the past. The most commonly experienced chronic stressor related to having no help with cleaning while pregnant (71% of women endorsed this). Supplementary Material Table S1 provides further information on the distribution of all questions related to these three stress measures in our cohort.

The mean age of women participating in our study was 26.9 years (S.D. = 5.6 years) and they had, on average, three children each. Fewer than half of the infants were female (42%). The mean infant birth weight was 3.2 kg (S.D. = 0.8 kg) but 20% of infants met WHO criteria for LBW (i.e. birth weight <2.5 kg). As we previously reported,[19] there was a strong correlation between each type of maternal stressor and birth weight,

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Table 1. *Distribution of endorsed stress variables*

Stressor (n = total number of variables)	War trauma (n = 10)	Rape stress (n = 3)	Chronic stress (n = 19)
Mean no. of variables endorsed	2.0	0.6	6.8
S.D.	2.9	1	6.3
Range	0–10	0–3	0–18
Most common stressor (% of women)	Refugee in the past (46%)	Raped in the past (25%)	No help cleaning during pregnancy (71%)

Table 2. *Summary of associations between maternal stress, IGF1/IGF2 methylation and birth weight*

Gene	Associated stress measure or birth weight	Tissue type	Associated methylation PC	β/adjusted R^2	P-value[a]
IGF1	War	Cord blood	PC2	−0.24/0.31	0.045
	Rape	Cord blood	PC2	−0.72/0.36	0.024
	Birth weight	Maternal blood	PC2	0.54/0.19	0.022
	War	Placenta	PC2	−1.58/0.14	0.040
	Rape	Placenta	PC2	−0.58/0.2	0.033
IGF2	Birth weight	Maternal blood	PC2	0.49/0.33	0.0027[b]
	War	Maternal blood	PC1	−0.38/0.53	0.019
	Rape	Maternal blood	PC1	−1.15/0.54	0.013
	Rape	Maternal blood	PC2	−1.09/0.17	0.031

[a] P-value needed after Bonferroni correction = 0.0028.
[b] When maternal age and education were sequentially added to this regression model, the P-value was attenuated to 0.0035 and 0.0478, respectively.

though the strongest effect was seen for chronic stress (P = 0.0003). We also found a positive association between maternal age and birth weight (P = 0.032).

A strong association was found between newborn birth weight and *IGF2* PC2 methylation in mother's blood (P = 0.0027) (Table 2; Fig. 2). Factor loading analysis showed that the CpG sites that significantly contributed to PC2 all loaded negatively onto PC2. This indicates that as *IGF2* methylation at the sites represented by PC2 increased, birth weight decreased. This significance remained even after Bonferroni correction for multiple testing (P-value of 0.0028 needed). The relationship between birth weight and *IGF2* methylation in maternal blood was attenuated when maternal age and education were sequentially added to the regressions resulting in P-values of 0.0035 and 0.048, respectively (Table 2).

There were several other interesting associations noted between war and rape stress, methylation at *IGF1* and *IGF2*, and birth weight that were also attenuated following correction for multiple testing (Table 2). For instance, war and rape stress both associated with *IGF1* methylation in cord blood and placenta, whereas war and rape stress associated with *IGF2* methylation in maternal blood. Within *IGF1*, all the associated CpG sites that made up the significant PCs loaded negatively in factor analysis, indicating an inverse relationship between these measures of maternal stress and DNA methylation at *IGF1*. Similarly, for *IGF2*, all the associations listed in Table 2 were only identified in maternal blood and relate to CpG sites that loaded negatively onto their respective PCs, again indicating a

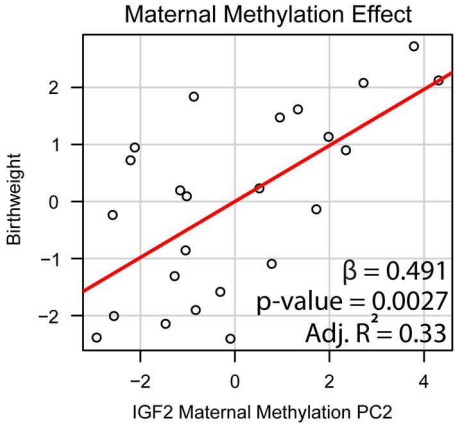

Fig. 2. Partial regression plot of the association between maternal *IGF2* methylation and birth weight. Though the β is positive leading to a positive slope to this line, the CpG sites associated with PC2 in maternal blood all loaded negatively onto PC2. Thus, the overall relationship can be assumed to be a negative one, that is as maternal methylation of *IGF2* increased at these CpG sites, birth weight decreased.

negative relationship between each stress measure and *IGF2* methylation in maternal blood.

It is also informative to note that associations were only identified with our measures of war and rape stress and not chronic stress.

Discussion

We identified a significant association between *IGF2* methylation in maternal blood and newborn birth weight (Fig. 2). *IGF2* expression is regulated primarily by several differentially methylated regions (DMRs) scattered throughout *IGF2* and the neighboring *H19*. As such, much of the research regarding the relationship between *IGF2* and birth weight has focused on methylation in cord blood or placental tissue at three main DMRs: DMR0, DMR2 and imprinting control region (ICR 1) located downstream of *IGF2* and just before the start of *H19*, which is involved in the silencing of the maternal *IGF2* allele.[50–52] For instance, several groups have noted lower *IGF2* DMR methylation in the cord blood of LBW or SGA infants compared with normal birth weight infants[33,38] or in conditions that precipitate fetal growth restriction, such as preeclampsia.[53] Similarly St-Pierre *et al.*[34] found that higher *IGF2* methylation at the DMR2 locus on the fetal side of the placenta was positively associated with greater birth weight, height and a larger head and thorax. St-Pierre *et al.*[34] also found that in blood from the maternal side of the placenta, methylation at two different *IGF2* loci in DMR0 positively correlated with both cord blood *IGF2* levels and higher birth weight.[34]

Not all groups have found this positive association between methylation at *IGF2* DMRs and birth weight. Some studies have documented the opposite relationship at DMR0 specifically[54,55] or no relationship between DMR0 methylation and infant ponderal index at birth.[37] What is consistent, however, is the focus on cord blood or placental tissue in these analyses and the findings of altered methylation at DMRs. Even the limited work on associations between maternal stress and *IGF2* methylation have examined methylation at DMRs and in particular DMR0.[37,39] Our study is unique in that we identify an association between birth weight and *IGF2* methylation in maternal blood outside known DMRs.

The Illumina HumanMethylation 450 Bead Chip primarily samples CpG sites in promoter regions. Our maternal *IGF2* methylation PC2 included a CpG site within a promoter region (cg23030069) but did not include any sites within the DMRs discussed above. These results suggest that *IGF2* methylation patterns in mothers may impact fetal growth, albeit through mechanisms different from known imprinting regulatory pathways involving DMRs that silence the maternally inherited allele in offspring. Our findings indicate it may be useful to explore *IGF2* methylation at promoter regions in pregnant women, and in particular the effect of methylation on *IGF2* expression in both mother and infant in the context of LBW.

In our analysis of the sampled *IGF1* CpG sites, we found a trend toward a negative association between war and rape stress and *IGF1* methylation in newborn cord blood and placental tissue (Table 2). Specifically, we found that these stress measures were associated with decreased methylation at several CpG sites outside known *IGF1* promoter regions, although this association was attenuated with Bonferroni correction. Though several studies have previously observed that *IGF1* appears to be differentially methylated by tissue type in infants with growth restriction, this body of work has focused on methylation at promoter regions.[31,56] Our findings suggest a pathway for a potential relationship between severe prenatal stress and epigenetic changes to *IGF1* that also varies by tissue type, but that may involve non-promoter regions. This conclusion is supported by recent studies that have found methylation at promoters is less variable over the lifespan than other regions, such as enhancers, suggesting that methylation at non-promoter regions may be more environmentally sensitive.[57,58] In addition, though the positive association between *IGF* serum levels in both mothers and infants and birth weight has been well documented in both animal[59] and human studies,[23,25] the literature reporting a relationship between neonatal growth measures and methylation in *IGF1*, particularly across tissue sites has been less consistent and at times, contradictory.[26,31,32,56] Although the significance of our results with *IGF1* methylation was attenuated following correction for multiple testing, Bonferroni correction is a very conservative method. Given the limited existing research on *IGF1* methylation in the context of maternal stress, we believe the multiple trends toward associations we found between *IGF1* methylation and war and rape stress as well as birth weight warrant discussion and follow-up in future studies.

The field of epigenetics offers the promise of better understanding the potential relationship between stressful environmental exposures and complicated multifactorial health outcomes. Our study is evidence that such exposures can be studied in meaningful ways even in resource-poor global settings by employing an ethnographic approach to identify culturally relevant stressors. Furthermore, though our population's stressors appear to be unique, it is important to recognize that war refugees are an increasingly prevalent population[60] and that sexual violence continues to be a serious widespread public health issue across the world.[61] Extreme maternal stress, especially as it relates to sexual violence, is thus a pervasive social determinant of perinatal health worldwide. We believe studies such as ours, which aim to elucidate how perinatal stress contributes to adverse child health outcomes, provide the rationale for targeted local and national policies to protect pregnant women from severe environmental stressors and support those who do experience them.

Limitations

The main limitation of our study is the small sample size, which may have contributed to the lack of significance after correction for multiple testing or increased the likelihood of false positive results. Thus, these results need to be replicated. However, the

existence of the discovered associations despite the small number of sampled mother–infant dyads argues for the potential importance of the relationship between maternal stress and methylation of insulin growth factors in understanding variation in birth weight. In addition, we utilized reference-based corrections for cell type whenever possible which lends confidence that the findings are due to true effects of prenatal stress on DNA methylation and not simply differences in cell composition.[45]

A second limitation relates to our methodology of collecting data at the time of birth, which precludes the documentation of additional contributing factors to LBW, including pre-pregnancy BMI, history of preeclampsia or gestational age. Acquiring prenatal medical history and early gestational age estimates in remote settings, and particularly in settings of social and political unrest where women may have no prenatal care before delivery, is often difficult or impossible. As a result, in these settings there is often reliance on indicators of health that can be measured at the time of delivery.[62] We therefore focused on two covariates previously associated with LBW, maternal age and education, which we felt could be documented with an adequate amount of accuracy.

Conclusions

We identify a significant negative association between methylation of *IGF2* in maternal blood and birth weight. This is the first study to report an association with *IGF2* methylation in maternal blood, which may be due to a prior focus on cord blood or placental methylation. Our findings suggest that links between the maternal epigenome of imprinted genes and LBW may exist that do not rely on mechanisms related to well-studied DMRs. As such, we believe it is important to consider the epigenetic effect of maternal environmental exposures on infant birth weight even in the setting of genes such as *IGF2* where the maternal allele is typically silenced.

Future studies should explore the effect of environmentally associated *IGF* methylation shifts on gene expression in order to better elucidate the complex relationship between maternal stress, birth weight and methylation at the *IGF* genes. In addition, our short list of candidate genes and CpG sites can be tested in other populations to determine the universality of these environmentally sensitive methylation changes. By examining these relationships in marginalized communities such as our cohort, we can ensure that potential hypothesis-building observations that may explain health disparities and increased risk of LBW worldwide do not go unexplored.

Acknowledgments

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Conflicts of Interest

None.

Ethical Standards

This observational study did not include any human experimentation but all procedures do comply with the Helsinki Declaration of 1975, as revised in 2008, and has been approved by the Western Institutional Review Board, Olympia, WA (www.wirb.com, WIRB project #20100993), the University of Goma and an ethical review committee at HEAL Africa Hospital. Each mother gave oral consent – documentation of written consent was waived by the WIRB and HEAL Africa Hospital because of the high level of illiteracy in the study population.

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S2040174417000800

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