ASSOCIATION BETWEEN SERUM ZINC LEVEL AND DYNAMICS OF GROUP B *STREPTOCOCCUS* COLONISATION AMONG PREGNANT WOMEN IN SOUTH AFRICA

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A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Public Health

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DECLARATION

I, Nisha Dhar declare that this research report is my own work. It is being submitted for the degree of Masters of Public Health in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

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13th day of June 2024

DEDICATION

I dedicate this research work to my son "Jai"

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS RESEARCH REPORT

Publication

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ABSTRACT

Background

Maternal colonisation by Group B *Streptococcus* (GBS) is a major risk factor for early onset invasive GBS disease among newborns. Zinc micronutrient plays a critical role in several biological processes that are essential to prevent bacterial colonisation and/or invasion. The aim of this study was to investigate the association of serum zinc levels with GBS rectovaginal new acquisition and clearance from 20 to 37+ weeks gestation in pregnant women.

Methods

Vaginal and rectal GBS colonisation was determined at 20–25 weeks (visit-1), followed by 3 subsequent visits at 5–6 weeks intervals, until 37–40 weeks gestation (visit-4). Serum was collected at visit-1 and visit-4 and serum zinc was estimated by inductively coupled plasma mass spectrometry. "New acquisition" group was defined as participants for whom GBS culture was negative at visit-1 and acquired GBS in one of the subsequent visits. Participants not colonised with GBS at any visits were categorized as GBS "persistently uncolonised". Participants who remained colonised throughout all study visits were defined as "persistently colonised" group. GBS "clearance group" included participants who were colonised at enrolment (visit-1) and in whom GBS colonisation cleared by last visit (visit-4).

Results

Participants in persistently un-colonised group had significantly higher zinc geometric mean concentration (GMC) at visit-1 compared with those who had new acquisition (20.18 µmol/L; 95%CI 17.99-22.64 vs 13.68 µmol/L; 95%CI 12.59-14.87, p=0.03). Higher zinc concentration in the persistently un-colonised group when compared with new acquisition group was significantly associated with lower odds of GBS rectovaginal acquisition [Odds ratio (OR) 0.15, p=0.001]. The lowest zinc threshold significantly associated with 45% lower

odds of new acquisition was ≥15 umol/L (27.2% in new acquisition vs 40.5% in persistently un-colonised; OR 0.55; 95%CI 0.31- 0.96; p=0.03). Furthermore, zinc concentration was higher among women in clearance group compared with those in persistently colonised group (20.03 µmol/L; 95%CI 16.54-24.27 vs 16.45 µmol/L; 95%CI 13.32-20.31, p=0.04).

Conclusion

There was an inverse association between serum zinc levels in pregnant women and odds of GBS acquisition in those not initially colonised. Zinc supplementation in early pregnancy could reduce risk of invasive GBS disease in their newborns.

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CPS:	Capsular polysaccharide
CRF:	Case report form
EOD:	Early onset disease
GBS:	Group B *Streptococcus*
GMC:	geometric mean concentration
HIC:	high-income countries
HIV:	Human Immunodeficiency Virus
HREC:	Human Research Ethics committee
IAP:	Intrapartum antibiotic prophylaxis
IQR:	interquartile range
LMIC:	low-and middle- income countries
NDoH:	National Department of Health
OR:	odds ratio
SD:	standard deviation
ST:	Sequence type
umol/L:	micromole per liter
VIDA:	The Vaccines and Infectious Diseases Analytics research unit
WHO:	World Health Organisation
Wits:	University of the Witwatersrand
95%CI:	95 percent confidence interval

1. INTRODUCTION

1.1 Background

Group B *Streptococcus* (GBS), also known as *Streptococcus agalactiae,* is one of the most common causes of severe invasive bacterial disease in neonates (1). Group B *Streptococcus* is a common commensal bacterium colonising gastrointestinal and genitourinary tracts of pregnant women (1). Vertical transmission of GBS from vaginally or rectally colonised pregnant women is the major risk factor associated with invasive GBS disease in the first seven days of life (i.e. early-onset invasive neonatal GBS disease; EOD) (2, 3). Invasive GBS disease in neonates manifests primarily as bacteremia, pneumonia or meningitis (3). About half of GBS meningitis survivors develop neurodevelopmental abnormalities such as mental retardation, blindness and deafness (4). Furthermore, ascending GBS infections from mother to foetus could also potentially result in stillbirth and preterm delivery (5-7). Colonisation with GBS has also been identified as an important risk factor for maternal invasive disease, which could be life threatening (8). Globally, approximately 18% of women are colonised with GBS, with the highest prevalence in the Caribbean region (35%) (2). Current strategies based on intrapartum antibiotic prophylaxis (IAP) during labour to women with GBS-colonisation at 35-37 weeks of gestational age has reduced EOD in high-income countries where implemented (9). Nevertheless, screening for GBS colonisation and use of IAP would be logistically difficult to implement in low- and middle- income countries (LMIC) (3, 9). Furthermore, IAP would not reduce risk of GBS associated preterm births and still births; and does not impact on invasive GBS disease beyond seven days of age (i.e. late-onset disease; LOD). Hence, investigation of alternative strategies targeted at prevention of recto-vaginal colonisation risks in pregnant women is required.

Maternal macro- and micronutrients' nutritional status is an important determinant of maternal, foetal, and infant health (10). Zinc is an essential micronutrient involved in host immunity through several mechanisms including inhibition of biofilm formation required for adherence and colonisation of bacteria on mucosal epithelia (11-13). In addition, zinc maintains epithelial integrity through restoring bacterial induced membrane permeability alterations; and aids in clearance of bacteria from epithelial surfaces (14-16). Furthermore, antimicrobial molecules including S100A-family proteins produced on mucosal surface upon interaction with bacterial pathogens participate in nutritional immunity via binding and sequestering zinc (17, 18). The effect of zinc deficiency in attenuated host resistance to bacterial infections is well-established (19-21). There is no published report on the role of zinc in modulating risk of GBS recto-vaginal colonisation in pregnant women. Group B *Streptococcus* forms biofilm under vaginal pH, which researchers have considered could be facilitating GBS colonisation, persistence and pathogenesis on vaginal mucosa (22-25). Zinc is a potent regulator of biofilm formation; biofilm establishment is associated with low zinc availability and repressed with exogenous zinc in various bacterial pathogens under *in vitro* conditions (11).

The hypothesis of this study was that zinc serum levels in pregnant women could be inversely associated with odds of GBS recto-vaginal colonisation and positively associated with colonization clearance during the latter half of pregnancy. The aim of this study was to investigate the association between serum zinc concentration and rectovaginal GBS dynamics from 20 weeks of gestational age in pregnant women.

1.2 Literature review

1.2.1 GBS epidemiology

Maternal colonisation of the genitourinary tract with GBS occurs in approximately 10%–40% of women worldwide (2), and is the major risk factor for EOD in newborns (26). Vertical transmission of GBS occurs to approximately 50% of newborns to women with recto-vaginal GBS colonisation. An estimated 1% to 2% of babies born to women colonised by GBS develop EOD (27). The estimated global incidence (per 1000 live births) of EOD is 0.41 (95% CI, 0.36–0.47), ranging from 0.32 (95% CI, 0.22–0.41) in Asia to 0.71 (95% CI, 0.24–1.18) in Africa. In the African region, Southern Africa has the highest incidence of EOD (1.07), with even higher incidence reported in Caribbean countries (1.47) (3). In South Africa, the incidence of EOD is approximately 1.41 (95% CI: 1.28–1.55) per 1000 live births (28). The case fatality risk of EOD is 10.0% (95% CI, 7.0%–12.0%); ranging from 5.0% (95% CI, 4.0%–7.0%) in high-income countries (HIC) to 27.0% (95% CI, 17.0%–37.0%) in African countries. Furthermore, approximately 1% and 4% of stillbirths in HIC and African countries, respectively, are associated with GBS (29). Also, pregnant women with recto-vaginal GBS colonisation have higher odds (1.85; 95% CI, 1.24–2.77) of preterm birth (7).

Group B *Streptococcus* is an encapsulated gram-positive bacterium covered with a sialic acid-rich capsular polysaccharide (CPS) (30). Based on CPS, it is classified into ten serotypes (Ia, Ib, and II–IX) (30). Globally, five serotypes (Ia, Ib, II, III, and V) are dominant accounting for 98% and 97% of all maternal GBS colonisation and infant invasive disease causing serotypes, respectively (2, 27). Group B *Streptococcus* has also been classified by sequence type (ST) based on allelic sequence profile present at seven different loci (31). Based on ST, majority of GBS human isolates belong to ST-1, ST-17, ST-19, or ST-23 (31). Sequence types 1, 23, and 19 are the dominant colonising strains (32). The prevalence of

pregnant women colonised with GBS vary by geographic regions; The Caribbean has the highest prevalence (35%), and the lowest is in Southern Asia (13%) and Eastern Asia (11%) (2). In Africa, the highest prevalence is in Southern Africa (29%) (2). In a longitudinal study from South Africa, the prevalence of GBS colonisation in pregnant women was 32.1%, 30.4%, 29.0% and 27.8% at 20–25 weeks, 26–30 weeks, 31–35 weeks, and 37+ weeks of gestational age, respectively (33). Decreases in the prevalence of GBS colonisation with increase in gestational age could likely be due to the influence of key hormones of third trimester pregnancy on cytokine responses in vaginal epithelial cells, thus affecting GBS colonisation (34). Serotypes Ia (23.7%), III (18.3%), and V (7.1%) were the most common serotypes associated with GBS colonisation in pregnant women (33). There is a high concordance (90.7%) in serotypes among GBS-colonised mother-newborn dyads and in relation to maternal colonising and EOD isolates (100%) (35, 36). In South Africa, serotypes Ia (28.2%), III (55.4%) and V (7.9%) are the most common invasive disease causing serotypes, which is similar to the proportional distribution of maternal recto-vaginal colonising serotypes (28).

1.2.2 Dynamics of GBS colonisation

In a longitudinal study conducted in South Africa on pregnant women at 20-25 weeks gestation, who were followed up in 5-6 weeks intervals until delivery, GBS recto-vaginal colonisation was found to be dynamic with women persistently, intermittently, transiently or never colonised (33). Among women who were found to be colonised at least once (49.7%) during the study, 27.8% were persistent carriers (colonised throughout the study period), 32.9% were transient carriers (colonised at one of the visits), and 39.3% were intermittently colonised (colonised at two or three visits) by any serotype (33). Serotype III was more likely to be associated with persistent colonization throughout the study (29%) than Ia (18%) or V

(6%) (33). Furthermore, 25.9% of women not colonised with GBS at baseline subsequently acquired GBS; and 46.6% women colonised with GBS at baseline cleared colonisation by the last visit (33). The prevalence of GBS between vaginal and rectal colonisation remained similar at each study time-point, however, the overall detection of GBS colonisation increased by approximately 10% across the four study time-points with both vaginal rectal swab for GBS culture (33).

1.2.3 Preventive strategies against GBS vertical transmission

The current recommendation by the Center for Disease Control and Prevention is that all pregnant women at 35 to 37 weeks' gestation be screened for rectovaginal GBS carriage to identify those eligible for IAP (37). Screening for GBS rectovaginal colonisation in pregnant women, coupled with successful implementation of IAP during labor targeted at GBS colonised pregnant women has lowered the incidence of EOD in HIC by 89% (38, 39). In USA, the incidence of EOD (per 1000 live births) in newborns declined from 1.7 in 1993 to 0.26 in 2010 following IAP implementation (40). In some countries, IAP is only offered to pregnant women with risk factors for EOD in their newborns', such as maternal fever or having a history of a previous child born with GBS disease (40). The implementation of IAP strategy targeted at GBS colonised women has not been adopted in LMIC or upper- middle income countries including South Africa, due to the cost of intervention, resources and logistics challenges associated with such a strategy (41, 42). In South Africa, IAP is recommended during labor to pregnant women with risk factors, however, only 11-26% of women with risk factors receive IAP (43).

1.2.4 Factors affecting GBS vaginal colonisation

The temporal changes in GBS colonisation could be influenced by several factors including vaginal pH and microbiome and host immune responses (44, 45). Shift of vaginal pH from acidic to neutral can enhance GBS adherence to vaginal epithelial cells, which is one of the most critical steps in successful GBS colonisation (44). The vaginal microbiome of healthy reproductive aged women is dominated by *Lactobacillus* species that produce lactic acid, which maintains the acidic vaginal pH (46). However, changes in the vaginal microbiota composition during pregnancy influences GBS colonisation risks (23). An inverse relationship between *Lactobacillus* density and GBS positivity is observed in women (23, 45). Other studies have demonstrated antagonistic activity of Lactobacillus against GBS adherence (47, 48). Whereas, *Candida albicans* and GBS cooperate with each other as both are frequently co-isolated in pregnant women (49, 50). Furthermore, host immune responses including the luminal mucus layer, vaginal epithelia, and immune cells in the vagina are important determinants of GBS vaginal colonization (45). Vaginal epithelial cells mediate GBS elimination through the recruitment of neutrophils, mast cells, and macrophages (45). In addition, multiple inflammatory cytokines and chemokines levels are associated with GBS colonisation persistence or clearance (45). Furthermore, metals are required cofactors for numerous fundamental processes that are essential at the host pathogen interface (51). Scarcity or toxicity of host produced metals including Zinc, Iron, Copper, Managanese restricts invading bacterial pathogens, a process known as “nutritional immunity” (51). A few studies report the antimicrobial role of copper and iron nutrition in the context of GBS infections (52, 53). The antimicrobial role of zinc against GBS is discussed in the following section.

1.2.5 Anti-microbial role of zinc

Zinc is a transition metal that serves as a co-factor for enzymes involved in critical cellular processes in both eukaryotes and prokaryotes (54). During the course of bacterial infection, pathogens acquire zinc from the host to colonise and cause disease (55). The host combats the pathogen invasion by producing factors that regulate zinc availability by either depleting zinc to starve the pathogen or accumulating zinc in excess to intoxicate the pathogen (55). These antagonistic host responses suppress growth of microbes in the host and inhibit the progression of infectious disease (56). Several studies have identified potential mechanisms through which elevated host zinc level could attenuate early stages of bacterial colonisation and pathogenicity (11-18, 57, 58). One of the mechanistic action of zinc is through reduction of hyaluronic acid capsule biosynthesis and biofilm formation (11). Formation of biofilm facilitates colonisation of a large variety of bacterial species by enhancing the ability of bacteria to adhere and persist on mucosal epithelial surfaces (25). Biofilm formation also facilitates survival and proliferation of pathogens by enhancing their resistance to host defenses and persistence under nutrient starvation conditions (25). Inhibition of biofilm formation in presence of zinc has been demonstrated against intestinal and respiratory pathogens including *Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus mutans, Streptococcus suis, Actinobacillus pleuropneumoniae, Staphylococcus aureus, Escherichia coli (E.coli), Salmonella typhimurium and Haemophilus parasuis* (11-13). Zinc also reportedly reduces epithelial adherence in *E.coli* through binding to structures with which the bacterium attaches to the epithelial lining (13, 57). In addition, zinc is critical for epithelial barrier integrity (14-16). *In vitro* studies show that zinc improves bacterial induced permeability alterations; and restores epithelial integrity and function, which clears the bacteria from the epithelial surface through non-specific barrier immune function (14-16). In *E. coli* infected mice, high zinc supplementation reduces translocation and entry of bacteria

through the disrupted epithelium (14). Furthermore, low bioavailability or low dietary levels of zinc in pregnant mice has been shown to result in altered gut microbiome composition (59, 60). Vaginal microbiome is an important factor considered to influence GBS colonisation (23). Furthermore, as part of host defense, a repertoire of antimicrobial molecules including S100A-family proteins are produced by vaginal epithelial cells into the overlying mucosal fluid upon interaction with bacterial pathogens, that participate in nutritional immunity via binding and sequestering zinc (17, 18). Some studies have also shown an association between zinc deficiency and impaired antibody responses to infection, which could also play an important role in the colonisation of bacteria (58).

1.3 Problem statement

Vertical transmission of GBS from pregnant women with vaginal or rectal colonisation is the major risk factor for invasive GBS disease in the first seven days of life (i.e. EOD). Maternal GBS colonisation could also result in preterm births and still births. The prevalence and new acquisition of GBS colonisation among pregnant women remains high. Currently, there are limited or no preventive strategies for reducing risks of GBS colonisation in low resource countries. Zinc micronutrient plays a critical role through several mechanisms that are essential for preventing bacterial colonisation, persistence and/or invasion within the host. While, the antibacterial role of zinc in host immunity is known, the significance of zinc nutrition against GBS colonisation acquisition or clearance in pregnant women remains unexplored.

1.4 Justification

This research aimed to evaluate the association of serum zinc concentration with rectovaginal GBS colonisation dynamics through gestation. This study was undertaken to

serve as an evidence for adoption and uptake of antenatal zinc supplements intervention in South Africa or elsewhere for prevention of GBS colonisation acquisition among pregnant women. The findings from this study warrant investigation of the effectiveness of zinc supplementation in pregnant women on their risk of being colonised by GBS.

1.5 Research question

What is the significance of zinc nutrition in GBS colonisation in pregnant women?

1.6 Aim

To elucidate association between serum zinc concentration and GBS colonisation dynamics among pregnant women cohort from 20 to ≥37 weeks of gestational age during 2010- 2011 in Johannesburg, South Africa.

1.7 Objectives

a. To determine the association of serum zinc concentration and odds of recto-vaginal GBS new acquisition in pregnant women.

b. To determine the association of serum zinc concentration and odds of recto-vaginal GBS colonisation clearance in pregnant women.

2. METHODS

2.1 Study design

This was a retrospective study that analysed data collected from a cohort study that aimed to identify GBS serological correlates of protection against GBS colonisation in pregnant women(33). The primary study was conducted in Soweto, South Africa by Wits- Vaccines and Infectious Diseases Analytics (VIDA) Research Unit.

2.2 Study site

In the primary study, participant recruitment and follow up was conducted at four local community antenatal clinics in Soweto (Pimville, Lillian Ngoyi, Diepkloof and Mofolo), a peri-urban township located in the Greater Johannesburg (33).

2.3 Study population

The study population was pregnant women aged 18–45 years not infected by human immunodeficiency virus and who attended community antenatal clinics during 2010-2011. Women were approached and informed regarding the study. Women who consented and were eligible for participation were allocated a unique identification number. Inclusion criteria included women 20-25 weeks of gestation age, who consented to study participation and were able to understand and comply with planned study procedures. Exclusion criteria included those with acute illness or a symptomatic vaginal discharge, those on antibiotic treatment in the previous two weeks, and/or to have a known or suspected condition in which clinical vaginal examination was contraindicated. A cohort of 661 participants was enrolled and all participants were followed-up in 5-6 weeks intervals until 37+ weeks of gestation (33). Of all the enrolled participants, 507 completed all four visits.

2.4 Sampling

All eligible and consenting participants recruited in the primary study were included for this study. Women with unknown status for GBS colonisation or serum zinc concentrations were excluded.

Sample size

For the current study, 507 participants who completed all four visits were included. It is estimated that at least a sample size of 500 pregnant women at 20-25 weeks of gestation age is required, to obtain sufficient numbers of mothers who become colonised during the course of the study. These numbers were based on the following assumptions. From previous and current studies, the number of new GBS acquisitions during pregnancy ranges from 8.5-25% (33, 61, 62). With a sample size of 500, and assuming a baseline colonisation rate of 30%, 350 participants will be un-colonised at baseline. With an assumed acquisition rate of 15%, approximately 50 cases of new acquisition and 250 un-colonised participants were expected, thus, resulting in a GBS new acquisition and un-colonised ratio of 1:5. Assuming 10% difference among normal zinc level (7.6 µmol/L) between GBS new acquisition and un-colonised, the sample size will have power of above 90%. Similarly, for colonisation clearance, of 30% (150/500) colonised at Visit-1, around 40% (60/150) will clear colonisation by last visit, thus resulting in a GBS clearance and persistent colonisation ratio of 1:1.5. Assuming 10% difference among normal zinc level (7.6 µmol/L) between GBS clearance and persistent colonisation, the sample size will yield power of above 90%.

2.5 Data collection

a) Demographic and baseline information

For all participants, demographic and other baseline characteristics in the primary study were collected using study case report form (CRF) by study research assistants for the following

variables: maternal age, parity, gravidity, previous abortion or stillbirth. This study retrieved the captured data from VIDA database.

b) Specimen collection

Vaginal and rectal swabs were collected for GBS culture starting at 20–25 weeks (visit-1), followed by 3 subsequent visits (visits 2–4) at 5–6 weekly intervals, until 37–40 weeks gestation (visit-4). Whole blood sample was collected from pregnant women at 20-25 weeks and 37+ weeks gestation and serum was stored at -70 degree celcius until analysis.

c) Processing of specimen

Vaginal and rectal swabs were processed in the primary study for isolation and conformation of GBS as described previously (33). Briefly, vaginal and rectal swabs were inoculated onto CHROMagar StrepB agar for GBS isolation and were confirmed by testing for Christie Atkinson Munch Petersen positive factor, inability to hydrolyze esculin, catalase negativity and group B antigen.

2.6 Measurement of zinc

Zinc level estimation in the serum samples was performed at Lancet laboratories in Johannesburg, South Africa during 2021 using inductively coupled plasma mass spectrometry (ICP-MS) with Lancet laboratories standard protocol. Briefly, samples were diluted 20-fold with a serum diluent. Appropriate internal standards and reference controls were used for generation of a calibration curve with r-squared value greater than 0.999. The zinc concentration was measured in triplicates, and the average was reported in µmol/L. The limit of quantitation was 0.31 µg/L (4.7 nmol/l or 0.005 umol/l). Serum zinc concentration of 7.6 µmol/L (50 µg/dL) was considered as lower cut off for normal zinc concentration in

pregnant women during second and third trimester as suggested by previous studies (63-65). Studies have shown that concentration of zinc in blood and/or plasma specimens determined using ICP-MS remain relatively constant during extended periods of storage at freezing temperatures (66).

2.7 Data Management

In the primary study, participant data collected on study-specific CRFs was entered into specially designed databases by data clerks. Internal monitoring and quality assurance was conducted on important variables. Edit checks were performed on the database. The data on zinc levels of participants was merged in the primary study database. For this study, validity check and variable range check of the primary database was done. Retrieved data was maintained in password-protected files.

2.8 Data analysis

Maternal demographics and baseline characteristics was a descriptive analysis that included frequency with percentages, geometric means with 95% confidence interval or median with inter-quartile range, as appropriate. In the initial parent study, based on the dynamics of GBS colonisation, women were grouped into four groups; persistently un-colonised, new acquisition, colonisation clearance and persistently colonised. The “New acquisition” group was defined as participants’ negative for GBS at visit-1 and who acquired GBS in subsequent visits. Participants not colonised with a GBS at any study visit were categorized as “persistently un-colonised” and were used as comparator group against new acquisition group for estimating association of zinc with new acquisition. “Persistently colonised” group was defined as participants who remained colonised throughout all study visits. “Colonisation clearance” group was defined as the participants in whom GBS colonisation was cleared by

last visit (Visit-4) and was used as a comparator group against persistently colonised group for estimating association of zinc with colonisation clearance.

The visit-1 and visit-4 zinc concentrations were compared using Wilcoxon matched-pairs signed-rank test. Groups were compared using Mann-Whitney test for skewed data sets or Student unpaired t- test for data sets in Gaussian distribution. The association between zinc and GBS acquisition or colonisation clearance was evaluated using a logistic regression model reporting the odds ratio. For all analyses, p values, geometric means, and 95% confidence interval (95% Cl) were reported. For categorical variables, groups were compared using Chi-square test reporting the odds ratio (OR). Reverse cumulative plots were constructed and threshold concentrations were determined. Data were analyzed using GraphPad Prism version 7.04 software (GraphPad Software, San Diego, California) and Stata version 13 software (StataCorp, College Station, Texas). For all analyses, a p value <0.05 was considered statistically significant.

2.9 Ethics

The primary study was approved by the Human Research Ethics Committee (HREC) of the University of the Witwatersrand (Wits); IRB/Protocol-M090937 and informed consent was taken from all the participants of this study. The current study was approved by Wits-HREC (Certificate# M220562); Appendix 2. All records were identified only by a coded number to maintain participant confidentiality and were kept in a secured area.

3. RESULTS

Of the 507 women who completed all four visits in the primary study, GBS colonisation and zinc status was available for 466 (92%); figure 3.1. At visit-1, 69.3% (323/466) women were not colonised with GBS, of whom 25% (81/323) had GBS new acquisition in one of the subsequent visits. The rate of recto-vaginal GBS new acquisition was 11.6%, 5.6%, and 8% between visits-1 and -2, visits-2 and -3, visits-3 and -4, respectively (table 3.1). Among 143 women detected with GBS colonisation at visit-1, 53.1% (76/143) cleared GBS colonisation by visit-4. The rate of colonisation clearance was 20.3% and 32.9% between visit-1 and -2, and visit-2 and -3, respectively (table 3.1).

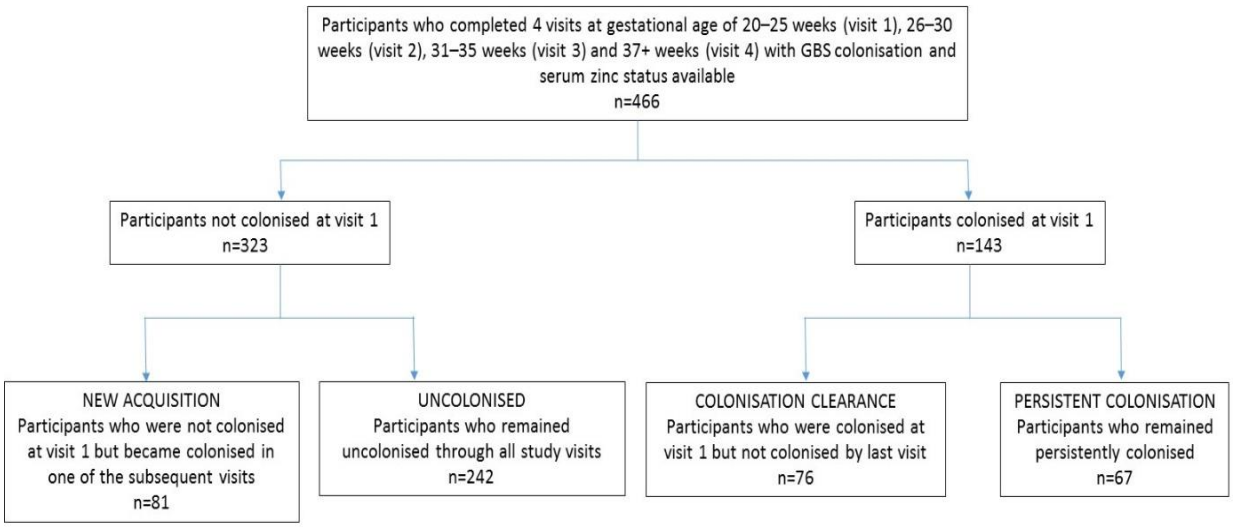

Figure 3.1: Eligible participants for the study

Table 3.1: Rate of GBS new acquisition and colonisation clearance

Cases	**Visit-2**	**Visit-3**	**Visit-4**
New Acquisition (n=81)	11.6% (37/323)	5.6% (18/323)	8% (26/323)
Colonisation clearance (n=76)	20.3% (29/143)	32.9% (47/143)	

3.1 Zinc nutrition in pregnant women

All study participants (n=466) were analysed for serum zinc concentration. The zinc concentration <7.6 µmol/L, indicative of zinc deficiency, was present in 0.42% (2/466) and 1.7% (8/464) of women at Visit-1 and Vist-4, respectively. There was no difference in zinc geometric mean concentration (GMC) at visit-1 (18.32 µmol/L; 95%CI 16.98-19.77) and visit-4 (20.63 µmol/L; 95%CI 18.86-22.57, p=0.30), figure 3.2.

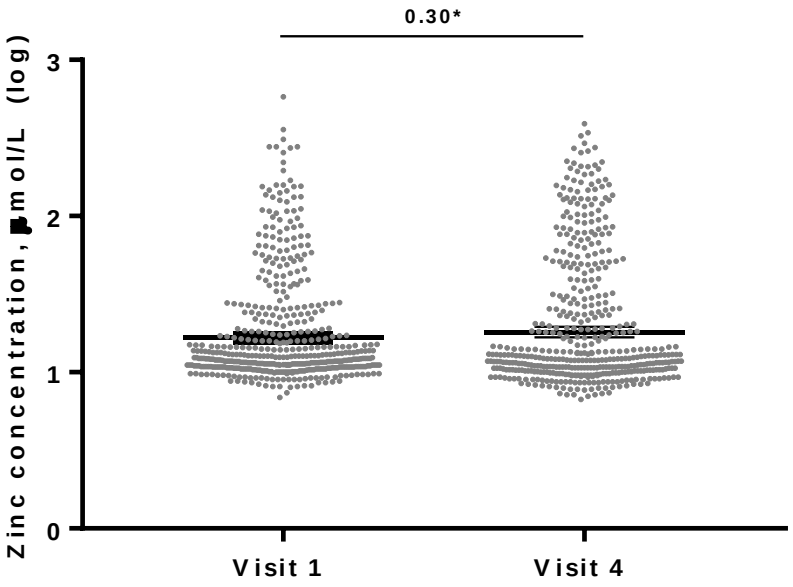

Figure 3.2: Zinc concentrations of women at visit-1 and visit-4

Represented are visits on X axis and zinc concentrations (black dots) in µmol/L (in log base 10) on Y axis, GMC (thick solid line), 95%CI (thin solid line), *p value. Visit-1 zinc GMC (18.32 µmol/L, 95% CI 16.98-19.77); Visit-4 zinc GMC (20.63 µmol/L, 95% CI 18.86-22.57)

3.2 Association of zinc nutrition with GBS new acquisition in pregnant women

There were no differences in the demographic characteristics between women who had GBS new acquisition compared with persistently un-colonised women (table 3.2). Zinc GMC at visit-1 was higher among the persistently un-colonised group (20.18 µmol/L; 95%CI 17.99-22.64) compared with the new-acquisition group (13.68 µmol/L; 95%CI 12.59-14.87; p=0.0255); table 3.3. Consequently, in the logistic regression analysis, zinc concentration was associated with lower odds of GBS colonisation acquisition (Odds ratio (OR), 0.15, 95%CI 0.05-0.44; p= 0.001). The strength of association between zinc concentration and reduced odds of new acquisitions trended to be stronger at higher zinc titers (table 3). The lowest zinc concentration threshold associated with 45% reduced odds of GBS acquisition was ≥15 µmol/L (27.2% in persistently un-colonised vs 40.5% in new-acquisition group, OR: 0.55, 95%CI 0.31- 0.96; p=0.03); table 3.3; figure 3.3.

3.3 Association of zinc nutrition with GBS colonisation clearance in pregnant women

There were no differences in demographic characteristics between the GBS colonisation clearance and the persistently colonised group; table 2. Zinc GMC was higher at visit-1 (20.03 µmol/L; 95%CI 16.54-24.27) among the colonisation-clearance compared with the persistently colonised group (16.45 µmol/L; 95%CI 13.32-20.31); p=0.04 (table 3.4). In logistic regression analysis, higher odds of GBS clearance was observed with high zinc concentration, albeit, not significant (OR, 1.92 (0.75- 4.86); p=0.17). Analysis at increasing zinc concentrations, indicated a trend of higher odds of GBS colonisation clearance with zinc concentration between 15 and 25 µmol/L (table 3.4). The lowest zinc threshold significantly associated with GBS colonisation clearance was ≥17 µmol/L (38.2% in colonisation clearance vs 20.9% persistently colonised group; OR: 2.34; p=0.02); table 3.4. Reverse

cumulative distribution plots with zinc concentrations of colonisation clearance and persistently colonised groups are shown in figure 3.4.

Table 3.*2*: Baseline demographic and clinical characteristics of women

Variable	**New Acquisition (n=81)**	**Persistently un-colonised (n=242)**	**P value**
Mean age; years ± SD	25.81 ± 5.11	25.96 ± 5.74	0.98[#]
Mean gestational age at visit-1; weeks ± SD[§]	22.86 ± 2.32	22.71 ± 2.11	0.65[#]
Gravidity; median [IQR]	2; [1, 2]	2; [1, 2]	0.25[##]
Parity; median [IQR]	0; [0, 1]	0; [0, 1]	0.87[##]
	Colonisation clearance (n=76)	**Persistently colonised (n=67)**	
Mean age; years ± SD	26.05 ± 5.15	26.85 ± 6.38	0.52[###]
Mean gestational age at visit-1; weeks ± SD[§]	23.03 ± 2.32	22.83 ± 2.22	0.54[#]
Gravidity; median [IQR]	2; [1, 3]	2; [1, 3]	0.96[##]
Parity; median [IQR]	1; [0, 1]	1; [0, 1]	0.90[##]

Abbreviations: IQR: interquartile range; SD: standard deviation

[#] P value derived using Mann Whitney test, [##] P value derived using logistic regression, [###] P value derived using student unpaired t-test, §Based on 80 new acquisition cases and 66 persistently colonised cases for which gestational age was available

Table 3.3: Association between zinc concentration and GBS new acquisition

Participants (n=323)	**New Acquisition** (n=81)	**Persistently un-colonised** (n=242)	**P value***	**OR (95% CI); P value****
Zinc GMC at visit-1, umol/L (95% CI)	13.68 (12.59-14.87)	20.18 (17.99-22.64)	0.03	0.15 (0.05-0.44); 0.001
Zinc concentration at visit-1, umol/L	New Acquisition, Proportion (%)	Persistently un-colonised, Proportion (%)	OR (95% CI); P value[#]	
>=7.6	80/81 (98.8%)	241/242 (99.6%)	0.33 (0.017-6.38); 0.41	
>=10	71/81 (87.7%)	215/242 (88.8%)	0.89 (0.41- 1.85); 0.77	
>=15	22/81 (27.2%)	98/242 (40.50%)	0.55 (0.31- 0.96); 0.03	
>=17	16/81 (15.1%)	90/242 (84.9%)	0.42 (0.23 - 0.76); 0.004	
>=20	11/81 (13.6%)	80/242 (33.1%)	0.32 (0.16- 0.63); 0.0007	
>=25	8/81 (9.9%)	66/242 (27.3%)	0.29 (0.14-0.63); 0.001	
>=50	1/81 (1.2%)	45/242 (18.6%)	0.05 (0.005-0.32); 0.0001	

Abbreviations: GMC: geometric mean concentration; 95% CI: 95% confidence interval; OR: Odds ratio

* P value derived using Mann-Whitney test,** P value derived using logistic regression analysis,[#] P value derived using Chi-square test

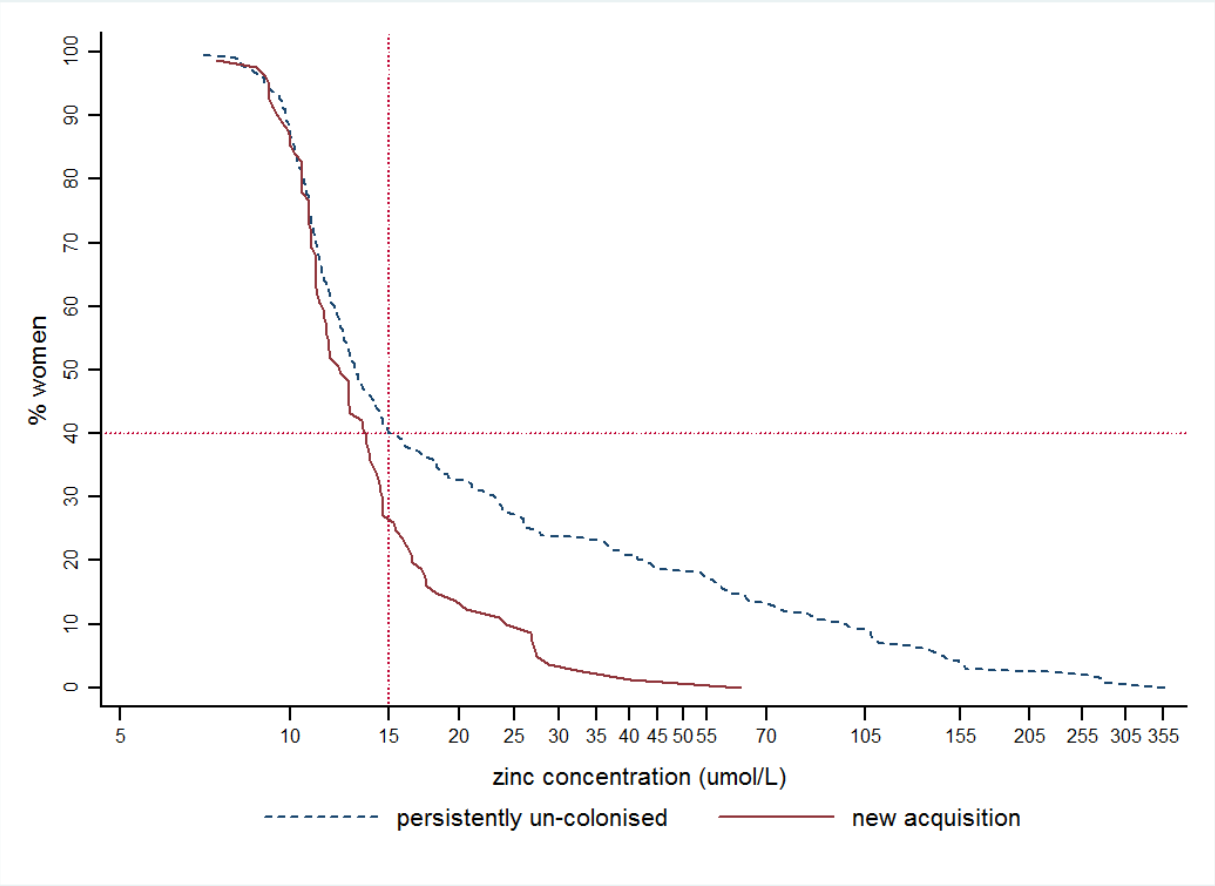

Figure 3.3: Reverse cumulative plots of zinc concentrations of women who had GBS new acquisition and those who remained persistently un-colonised with GBS.

Vertical and horizontal line on X axis and Y axis represent zinc concentration of 15 µmol/L at which 40% women are likely to remain GBS un-colonised.

Table 3.4: Association between zinc concentration and GBS colonisation clearance

Participants (n=143)	**Colonisation clearance** (n=76)	**Persistently colonised** (n=67)	**P value ***	**OR (95% CI); p- value ****
Zinc GMC at visit-1, umol/L (95% CI)	20.03 (16.54-24.27)	16.45 (13.32-20.31)	0.04	1.92 (0.75- 4.86); 0.17
Zinc concentration at visit-1, umol/L	Colonisation clearance, Proportion (%)	Persistently colonised, Proportion (%)	OR (95% CI); P value[#]	
>=7.6	76/76 (100%)	67/67 (100%)	Odds Ratio >0.9999	
>=10	68/76 (89.5%)	54/67 (80.6%)	2.0 (0.83 to 5.24); 0.13	
>=15	32/76 (42.1%)	18/67 (26.9%)	1.98 (0.99- 3.91); 0.06	
>=17	29/76 (38.2%)	14/67 (20.9%)	2.34 (1.09- 4.74); 0.02	
>=20	26/76 (34.2%)	13/67 (19.4%)	2.16 (1.03- 4.60); 0.047	
>=25	24/76 (31.6%)	12/67 (17.9%)	2.12 (0.98 to 4.78); 0.06	
>=50	14/76 (18.4%)	10/67 (14.9%)	1.29 (0.52- 3.19); 0.58	

Abbreviations: GMC: geometric mean concentration; 95% CI: 95% confidence interval; OR: Odds ratio

*P value derived using Mann-Whitney test,** P value derived using logistic regression analysis,[#] P value derived using Chi-square test

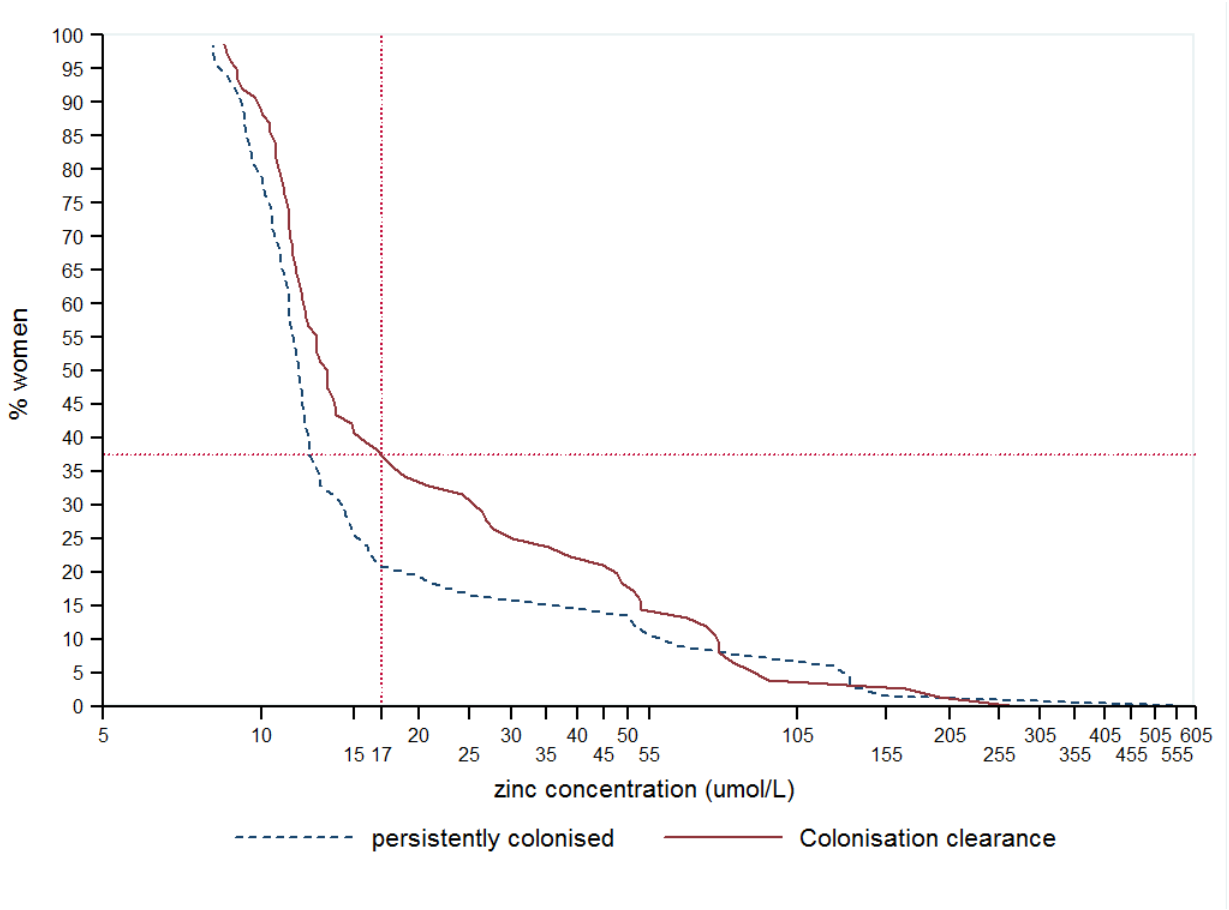

Figure 3.4: Reverse cumulative plots of zinc concentrations of women who cleared GBS colonisation and those who remained persistently colonised with GBS.

Vertical and horizontal line on X axis and Y axis represent zinc concentration of 17 µmol/L at which 38% women are likely to clear GBS colonisation.

4. DISCUSSION

This study demonstrated an association between serum zinc concentration and reduced risk of GBS new acquisition in pregnant women not colonised at visit-1. A threshold of 15 µmol/L of zinc was significantly associated with reduced odds of GBS acquisition. These data suggest that adequate zinc nutrition could reduce risks of GBS new acquisition in pregnant women. Further, a trend of higher odds for GBS clearance with increase in zinc concentration was noted, indicating that adequate zinc nutrition could also have a therapeutic role in GBS colonisation clearance. Currently, there is no data from human studies, which have evaluated association of serum zinc concentration with GBS colonisation. In a recent study by Burcham *et al* in murine model, a higher burden of GBS vaginal colonisation was observed in mice fed with zinc-deficient diet than those fed with diet containing zinc, after 7 days post challenge of both groups with GBS (67), which supports findings of our study.

The globally dominant GBS strains (including serotypes Ia, III and V and hypervirulent ST-17), form strong biofilms under acidic conditions, enhancing their ability to colonise and persist on vaginal epithelium (25, 68, 69). Zinc is known to exhibit anti-biofilm properties; and the inhibitory effects of zinc on biofilm formation is observed for several bacterial pathogens including diverse *Streptococcus* species (11-13). Due to the anti-biofilm properties of zinc, it could be mitigating GBS colonisation or persistence thereof on vaginal epithelial surface. In a study by Francis et al, a panel of colonising or invasive GBS strains' cultures that varied by capsular serotype, ST, isolation source, and clinical presentation were exposed to physiologically plausible zinc concentrations (70). A significant inhibition in culture growth of GBS strains was noted with increase in zinc concentrations (70).

Several studies have reported beneficial role of zinc nutrition in reduced bacterial colonisation burden on respiratory or enteric mucosa, attenuation of bacterial virulence and lowered risks of infection (71-77). In studies by Eijkelkamp *et al*, and Strand *et al*, mice fed on standard chow diet (with restricted zinc), showed extensive pneumococcal colonisation in the nasal mucosa and reduced survival time after 36 hours post challenge with *Streptococcus pneumoniae* compared with mice fed on zinc supplemented diet (71, 72). In another study, guinea pigs fed on zinc restricted diet showed a higher bacterial load of *Salmonella dublin* in liver and spleen tissues than those fed on diet with adequate zinc, at 15days post challenge with the bacteria (73). Low serum zinc concentration in elderly individuals has been observed to be associated with an increased risk of pneumonia caused by *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Legionella pneumophila*, and *Haemophilus influenzae* (74). Furthermore, zinc supplementation also reduces risk of oropharyngeal infections by Candida and *Staphylococci* species in immunocompromised patients (75). In addition, reduced risks of acute lower respiratory infection and pneumonia associated with nasopharyngeal *Streptococcus pneumoniae* colonisation has been reported for children supplemented with zinc (76, 77).

During pregnancy, the average physiological requirement of zinc increases up to 38% in third trimester compared with pre-pregnancy (78). Consequently, serum zinc concentrations decrease during the course of pregnancy, predisposing pregnant women to be at risk of zinc deficiency (65, 79). Although, majority of pregnant women had serum zinc concentration within the normal range for pregnancy (≥7.6 µmol/L) in this study, the level of zinc concentration was an important determinant associated with reduced odds of GBS new acquisition and high odds of GBS clearance. A serum zinc concentration of >=15 µmol/L reduced the risk of GBS new acquisition by 45%. Furthermore, a trend of higher odds of GBS

colonisation clearance with increasing serum zinc concentration signifies a therapeutic role of zinc in colonisation clearance. A serum zinc concentration of 17 µmol/L increased the odds of GBS clearance by 2.34 fold. Zinc nutrition for reducing GBS colonisation is unlikely to be achieved by dietary intake alone as previous studies have reported a high percentage of women with inadequate dietary zinc intake (80-85). In South Africa and worldwide, 84% and 82% of pregnant women, respectively, reportedly have inadequate zinc intake (80, 81). Changing food environments, food insecurity or food choices have contributed to energy-dense diet replacing the nutrient-rich diet globally (78), predisposing several pregnant women to inadequate serum zinc levels or zinc deficiency (82, 83). National surveys have found that more than 20% of women worldwide (84) and around 46-76% of pregnant women in sub-Saharan Africa are zinc deficient (80, 85), indicating zinc nutrition is critically low in some countries. High rates of HIV infection particularly in South Africa further compromise maternal micronutrient status (85), increasing risks of zinc inadequacy in pregnant women living with HIV.

Zinc supplementation could be a potential strategy for addressing inadequate zinc nutrition in pregnant women. The potential role of maternal zinc supplementation is also evident in the immunity and reducing morbidity of infants and children (86, 87), including supplementation being associated with 18%, 41% and 50% reduction in incidence of diarrhoea, pneumonia, malaria and child mortality, respectively (88). Zinc therapy is also recommended by the World Health Organisation to reduce the severity and duration of acute diarrhoea (89, 90). Also, zinc supplementation reportedly reduces the severity or duration of pneumonia in children and elderly (74, 77). Post zinc supplementation, the incidence of pneumonia was 48% lower (RR, 0.52; 95% CI, 0.36, 0.76) in elderly with serum zinc concentration ≥70 µg/dL (equivalent to 10.7 µmol/L) compared with those with <70 µg/dL (74).

To address micronutrients deficiencies or inadequate intake, the National Department of Health (NDoH) of South Africa implemented the national food fortification programme in 2003, which provided for the mandatory fortification of maize meal and wheat flour, the two most commonly eaten food items (91). However, the fortification strategy has not been successful in meeting the micronutrient dietary needs (92, 93). Challenges include poor affordability of fortified products for low-income families, poor regulation in ensuring fortification compliance, leaching of fortified nutrients during processing, and non-fortification of sorghum (the second most consumed sorghum meal after maize) (92, 93). The NDoH also implemented antenatal micronutrients supplements, but, currently iron, calcium and folic acid are the only recommended supplements in pregnant women (94). The findings from this study warrants evaluation of zinc supplements on GBS colonisation outcomes in pregnant or non-pregnant women that would justify the need of antenatal zinc supplements implementation in South Africa or elsewhere.

Limitations: The sensitivity of selective media for detection of GBS is estimated at 85%, which as a result could lead to an overestimation of new acquisitions or an underestimation of persistent colonisation (95). The serum zinc concentrations as a biomarker for determining zinc status has a few limitations. Some factors that may reduce serum zinc concentrations are consumption of supplemental iron, acute and chronic infection and systemic inflammation (96, 97). In addition, serum zinc concentrations are reported to have diurnal variation, may have low sensitivity for marginal zinc status, and fluctuate throughout the day in response to meals and time of day (96, 97).

5. CONCLUSION

Adequate level of zinc nutrition through zinc supplementation in early pregnancy could reduce GBS colonisation risk and/or persistence in mothers; thereby could possibly reduce risks of invasive GBS disease in their newborns.

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7. APPENDICES

Appendix 1: Ethics clearance certificate

Appendix 1: Ethics Clearance Certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R49 Dr N Dhar

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M220562

NAME: **(Principal Investigator)**	Dr N Dhar
DEPARTMENT:	School of Pathology Wits VIDA - Vaccines and Infectious Diseases Analytics Research Unit Chris Hani Baragwanath Academic Hospital
PROJECT TITLE:	*Association between serum zinc level and dynamics of Group B Streptococcus colonisation amongst pregnant women in South Africa*
DATE CONSIDERED:	Ad hoc
DECISION:	Approved unconditionally
CONDITIONS:	Sub-study under M18/11/137
NOTE:	If contact information regarding student study participants is required, please contact the Registrar's office - <Nicoleen.Potgieter@wits.ac.za>
SUPERVISOR:	Professor SA Madhi
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2022/06/17

This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office secretariat on the 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to submit details to the Committee. I agree to submit a yearly progress report. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **May** and therefore reports and re-certification will be due in the month of **May** each year. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).

Signature of Principal Investigator

24/06/2022
Date