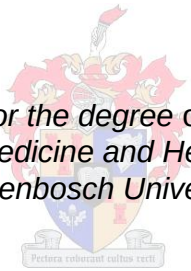
The use of probiotics in the management of Necrotizing Enterocolitis in HIV-exposed premature very-low birth weight infants.

by
Evette van Niekerk



Dissertation presented for the degree of *Doctor of Philosophy in the Faculty of Medicine and Health Sciences at Stellenbosch University*

Supervisor: Prof. Gert Kirsten
Co-supervisor: Prof. Reneé Blaauw

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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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ABSTRACT

Introduction: An association between maternal human immunodeficiency virus (HIV) infection and Necrotizing Enterocolitis (NEC) in preterm infants has been reported. The impact of probiotics in an HIV-exposed very low birth weight (VLBW) infant on the occurrence of NEC is uncertain at present; however it is known that probiotics have protective effects against inflammation and prevent NEC. Postnatal growth restriction is a major issue in preterm, especially extremely-low-birth-weight (ELBW) infants and probiotics have been found to improve feeding tolerance in preterm infants. Human milk oligosaccharides (HMO) also known as the prebiotics of human milk, are known to have bifidogenic and anti-adhesive effects. Infants that receive human milk show a reduced incidence of NEC compared to those who receive infant formula. Very little is known about the composition of breast milk in the HIV-infected mother.

Objective: The primary objective of the study was to assess the effect of probiotics on the incidence and severity of NEC in high-risk infants born to HIV-positive and HIV-negative women. The secondary objectives were to assess the effect of probiotic administration on feeding tolerance and growth outcomes of HIV-exposed but uninfected preterm infants, to describe the HMO composition of HIV-infected mothers breast milk and lastly to determine if HMO composition affects the incidence of NEC in HIV-exposed preterm very low birth weight infants.

Patients and Methods: A randomized, double blind, placebo controlled trial was conducted for the period July 2011 to August 2012. HIV-exposed and HIV-unexposed premature (<34 weeks gestation) infants with a birth weight of ≥500g and ≤1250g were

randomized to receive either a probiotic or a placebo. The probiotic consisted of $1x10^9$ CFU, *L. rhamnosus GG* and *B. infantis* per day and was administered for 28 days. NEC was graded according to Bell's criteria. Anthropometrical parameters and daily intakes were monitored. Breats milk samples were analysed for oligosaccharide content.

Results: 74 HIV-exposed and 110 HIV-unexposed infants were enrolled and randomized (mean birth-weight, 987g; mean gestational 28.7 weeks). The incidence of death and NEC did not differ significantly between the HIV-exposed and unexposed groups but a significantly higher NEC incidence was found in the control group. There was no difference in the average daily weight gain for treatment groups or HIV exposure. The HIV-exposed group achieved significantly higher z-scores for length and head circumference at day 28 than the unexposed group ($p<0.01$ and $p=0.03$, respectively). There were no differences in the incidence of any signs of feeding intolerance and abdominal distension between the groups. Our results show significantly higher absolute concentrations of 2'-fucosyllactose, laco-N-tetraose and lacto-N-fucopentaose 1 and higher relative abundance of 3'-sialyllactose, difucosyl-lacto-N-tetraose and fucosyl-disialyllacto-N-hexaose in HIV-infected compared to -uninfected Secretor women. DSLNT concentrations were significantly lower in the breast milk of mothers whose infants developed NEC compared to infants without NEC.

Conclusion: Probiotic supplementation reduced the incidence of NEC in the premature infants; however results failed to show a lower incidence of NEC in HIV-exposed premature infants. Probiotic supplementation did not affect growth outcomes or the incidence of any signs of feeding intolerance in HIV-exposure. The data confirms previous reports that HIV-infected mothers have higher 3'sialyllactose milk

concentrations. Most intriguing though, the data also indicates that low levels of DSLNT in the mother's milk increase the infant's risk for NEC, which is in accordance with results from previously published animal studies and warrants further investigation.

OPSOMMING

Inleiding: ’n Verwantskap tussen moederlike menslike immuniteitsgebreksvirus (MIV) en nekrotiserende enterokolitis (NEK) in premature babas is aangemeld. Die impak van probiotika in ’n MIV-blootgestelde baie lae geboortemassa (BLGM) baba op die voorkoms van NEK is tans nog onseker, maar dit is wel bekend dat probiotika ’n beskermende effek het teen inflammasie en die voorkoms van NEK. Nageboortelike groei beperkings is ’n groot probleem in premature, veral ekstreme lae geboortemassa (ELGM) babas. Daar is gevind dat probiotika voeding toleransie in premature babas kan verbeter. Menslike melk oligosakkariede (MMO), ook bekend as die prebiotika van menslike melk, is bekend om bifidogeniese en anti-kleef effekte te hê. Babas wat moedersmelk ontvang toon ’n verlaagde voorkoms van NEK in vergelyking met diegene wat baba formule melk ontvang. Baie min inligting is bekend oor die samestelling van borsmelk in die MIV-positiewe moeder.

Doel: Die primêre doel van die studie was om die effek van probiotika op die voorkoms en die graad van NEK in hoë risiko babas van MIV-positiewe en MIV-negatiewe vroue te bepaal. Die sekondêre doelwitte was om die effek van probiotika op voeding verdraagsaamheid en groei uitkomste van MIV-blootgestelde, maar nie- geinfekteerde premature babas te evalueer sowel as die MMO samestelling van MIV-positiewe moeders se borsmelk te beskryf en laastens om die invloed van die MMO samestelling op die voorkoms van NEK in baie lae geboortegewig MIV-blootgestelde premature babas te beskryf.

Pasiënte en Metodes: ’n Gerandomiseerde, dubbelblinde, plasebo-beheerde studie is vir die tydperk Julie 2011 tot Augustus 2012 onderneem. MIV-blootgestelde en nie-blootgestelde premature (<34 weke) babas met 'n geboorte gewig van ≥500g en ≤1250g was ewekansig verdeel om probiotika of plasebo te ontvang. Die probiotika het bestaan uit 1x10^9 kolonie vormende eenhede, *L. rhamnosus GG* en *B. infantis* per dag en is toegedien vir 28 dae. NEK is gegradeer volgens Bell se kriteria. Antropometriese parameters en daaglikse inname is gemonitor. Borsmelk monsters is geanaliseer vir oligosakkaried inhoud.

Resultate: 74 MIV-blootgestelde en 110 MIV-nie-blootgestelde babas is ingesluit en ewekansig ingedeel (gemiddelde geboorte gewig, 987g, gemiddelde gestasie 28,7 weke). Die voorkoms van die sterftes en NEK het nie beduidend verskil tussen die MIV-blootgestelde en nie-blootgestelde groepe nie, maar 'n beduidende verskil is gevind vir NEK voorkoms tussen die studie en die kontrole groep. Daar was geen verskil in die gemiddelde daaglikse gewigstoename tussen die behandelings groepe of MIV-blootstelling nie. Die MIV-blootgestelde groep het beduidend hoër z-tellings vir lengte en kopomtrek op dag 28 getoon teenoor die nie-blootgestelde groep ($p < 0.01$ en $p = 0,03$, onderskeidelik). Daar was geen verskille in die voorkoms van voeding onverdraagsaamheid en abdominale distensie tussen die twee groepe nie. Ons resultate dui op aansienlik hoër absolute konsentrasies van 2'-fucosyllactose, laco-N-tetraose en lakto-N-fucopentaose 1 en hoër relatiewe voorkoms van 3'-sialyllactose, difucosyl-lakto-N-tetraose en fucosyl-disialyllacto-N-hexaose in MIV-positiewe vroue in vergelyking met-negatiewe Sekretor vroue. DSLNT konsentrasies was aansienlik laer in

die melk van moeders wie se babas NEK ontwikkel het in vergelyking met babas sonder NEK.

Gevolgtrekking: Probiotika aanvullings verminder die voorkoms van NEK in premature babas, maar die resultate kon nie ’n laer voorkoms van NEK in MIV-blootgestelde premature babas bewys nie. Probiotiese aanvulling het geen invloed op groei uitkomste of die voorkoms van voeding onverdraagsaamheid in MIV-blootstelling getoon nie. Die data bevestig vorige verslae wat aandui dat MIV-besmette moeders hoër 3'sialyllactose borsmelk konsentrasies het. ’n Interessante aspek is dat lae vlakke van DSLNT in die moeder se melk beduidend is van ’n verhoogde risiko vir NEK, wat in ooreenstemming is met die resultate uit voorheen gepubliseerde dier studies en regverdig verdere ondersoeke.

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TABLE OF CONTENTS

LIST OF ABREVIATIONS

AGA: appropriate for gestational age

AIUGR: Asymmetrical intrauterine growth restriction

CFU: colony-forming units

CMV: cytomegalovirus

DFLNH: difucosyllacto-N-hexaose

DFLNT: difucosyl lacto-N-tetraose

DSLNT: disialyllacto-N-tetraose

DSLNH: disialyllacto-N-hexaose

ELBW: extremely low birth weight

FDSLNH: fucosyl disialyllacto-N-hexaose

2'FL: 2-fucosyllactose

3'FL: 3'fucosyllactose

FLNH: fuco lacto-N-hexaose

HC: Head circumference

HMO: human milk oligosaccaride

HIV: human immunodeficiency virus

HPLC: High-performance liquid chromatography

IgA: immunoglobulin A

KMC: Kangaroo Mother Care

KKMC: Khayelisha Kangaroo Mother Care

LGA: large for gestational age

LBW: low birth weight

LNFP: lacto-N-fucopentaose

LNT: lacto-Ntetraose

LNnT, lacto-N-neotetraose

LST c: sialyllacto-N-tetraose c

MCT: medium chain triglyceride

NCPAP: nasal continuous positive airway pressure

NEC: necrotizing enterocolitis

NICU: neonatal intensive care units

NPO: nil per os

PCR: polymerase chain reaction

PMTCT: prevention of mother to child transmission

PROM: premature rupture of membranes

SGA: small for gestational age

SIUGR: Symmetrical intrauterine growth restriction

3'SL: 3'-sialyllactose

TAH: Tygerberg Academic Hospital

TBCH: Tygerberg Children's Hospital

LST b: sialyllacto-N-tetraose b

VLBW: very low birth weight

MOTIVATION FOR THE STUDY

Necrotizing enterocolitis (NEC) is a condition predominantly seen in premature infants and is the leading cause of death due to gastrointestinal disease in neonatal intensive care units.[1] An association between maternal human immunodeficiency virus (HIV) infection and NEC in preterm infants has been reported.[2] Protocols for NEC treatment include nondrug treatments such as infants being nil per os, drug treatments and surgical interventions. Each of these has its own detrimental effects on patient outcome such as poor growth and neurodevelopmental delays.[3] Furthermore, HIV infection with acquired immunodeficiency syndrome (AIDS) is well known to be associated with a worse outcome in surgical patients.[4]

Evidence suggests that intestinal microbiota play an important role in general health and more importantly in the prevention of NEC in premature infants. Infants cared for in neonatal intensive care units develop very different intestinal microbiota with bifidobacteria being significantly less common.[5] Preterm very low birth weight (VLBW) and extremely low birth weight (ELBW) infants' initial exposure to environmental microorganisms is most often from equipment, air and other neonates, with the nursing personnel acting as carriers.[6] Furthermore, the effect of antibiotic treatment and the type of feed (breast milk vs formula milk) administered further determine the abundance of beneficial intestinal bacteria.[5]

The primary objective of the study was to evaluate the efficacy of probiotics in reducing the incidence and severity of NEC in premature, HIV-exposed, VLBW infants. Prophylactic probiotic treatment could be an effective, affordable and appropriate way of reducing the incidence of NEC in this highly vulnerable population. A reduction in the incidence of NEC would decrease mortality, morbidity, medical costs and length of hospital stay and improve the long-term neurodevelopmental outcomes of VLBW infants.

Secondly, this study aimed to determine the prebiotic quality (human milk oligosaccharides) of the breast milk of HIV-infected and HIV-uninfected mothers and to

determine whether there was an association between the prebiotic quality (human milk oligosaccharides) and NEC. If the results of this study could prove that the prebiotic quality of breast milk (HIV exposed/unexposed) reduced the incidence of NEC and thereby mortality and morbidity of preterm, VLBW babies, it could strengthen the views and perspectives of mothers and medical personnel on the importance of breastfeeding. Furthermore, breastfeeding may decrease the household financial burden and improve hygiene and safety measures. Prebiotics are available in breast milk, making the study highly sustainable as breast milk is free.

A systematic review of 16 randomised control trails indicated a significant reduction in the risk of NEC and death with the supplementation of probiotics. It is necessary to note that the majority of these trials included the use of both formula milk and breast milk. No research was found on the influence of HIV status on the use of probiotics and the effect that it had on the incidence of NEC in premature, VLBW infants. Limited research has been done on the prebiotic quality of breast milk in HIV-positive women, more specifically those who have given birth to a preterm infant. Very limited research also exists on the use of probiotics in a developing country such as South Africa.

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PhD CANDIDATE CONTRIBUTION TO THE RESEARCH

The candidate made the following contributions to this PhD thesis:

The study consceptualistion and design.

Applied and received funding to conduct the research.

Together with research assistants the candidate collected the data.

The candidate was responsible for the analysis and interpretation of the data together with the assistance of a statistician.

The candidate wrote all included papers, critically appraised all of the content and approved the final manuscript.

BRIEF OUTLINE OF THE THESIS

This thesis is devided into four chapters. A brief overview of each chapter wil now be given.

Chapter 1 covers the literature background and consist of the following three review articles:

Probiotics in premature infants: a focus on Necrotizing enterocolitis, focusses on the pathogenisis and diagnosis of necrotizing interocolitis (NEC) and provides broad guidelines on the use of probiotics in premature low-birth weight infants.

The use of probiotic bacteria in HIV-exposed premature infants: A focus on NEC, provides a in depth discussion on the role that HIV has in the development of NEC and the potential benefits that probiotics hold in affected infants.

Human milk oligosaccharides: Possible links to necrotizing enterocolitis and HIV-exposure. This article aims to describe the prebiotic and anti-adhesive effects of human milk oligosaccharides (HMO's) and how they contribute to the development of NEC in the presence of HIV infection.

Chapter 2 consist of the methodology of this randomized controlled trail.

Chapter 3 covers the results of the study and consists of three articles.

Probiotics and necrotizing enterocolitis in HIV-exposed premature infants. This article adresses the primary and a secondary outcome of the study. The primary objective of the study was to evaluate the efficacy of probiotics in reducing the incidence and severity of all stages of NEC in premature very-low birth weight infants that are exposed to HIV. The secondary objective that this article aimed to adress, assessed the incidence of NEC in very-low birth weight and extremely low birth weight infants born to HIV-infected and HIV-uninfected women.

Probiotics, feeding tolerance and growth: a comparison between HIV-exposed and unexposed very low birth weight infants. This article aimed to elucidate the role

of probiotics on feeding tolerance and subsequent growth in HIV exposed versus unexposed premature VLBW infants in a resource limited setting where practice involves early initiation of enteral feeds with expressed breast milk.

Human milk oligosaccharides differ between HIV-infected and -uninfected mothers and are related to necrotizing enterocolitis incidence in their preterm very low birth weight infants. This article adresses the final objectives of this study, namely to determine the oligosaccharides quality of own mother's breast milk of HIV-infected and HIV-uninfected mothers and wheather there is an association between the oligosaccharide quality of breast milk and the incidence of NEC in premature very-low birth weight infants.

Chapter 4 concludes the study objectives. This chapter briefly accepts or rejects the set null hypothosis of this clinical trail and provides recommendations.

Chapter 1

LITERATURE OVERVIEW

REVIEW 1

PROBIOTICS IN PREMATURE INFANTS: A FOCUS ON NECROTIZING ENTEROCOLITIS

Van Niekerk E. Probiotics in premature infants: focus on necrotising Enterocolitis. S Afr J Clin Nutr 2011;24(3): S35-S37.

Probiotics in premature infants: focus on necrotising enterocolitis

Van Niekerk, BScDietetics, MDietetics
Stellenbosch University
Correspondence to: Evette van Niekerk, evettev@sun.ac.za

Abstract

The premature infant may be exposed to an environment that is associated with the development of gastrointestinal complications. In preventing the latter, it is crucial that, if probiotics is chosen as a preventive measure, the selection of a safe product with documented probiotic properties together with close monitoring of patients is mandatory before offering this therapy for routine use in this high-risk deserving population. It is important to note that the effect of a probiotic bacterium is strain-specific. When considering the evidence from randomized control trials, researchers believe that probiotics should be offered as routine therapy for preterm infants, and that additional placebo-controlled trials are not necessary. The available guidelines may be a helpful tool in optimizing the use of probiotics in research settings.

 S Afr J Clin Nutr 2011;24(3): S35-S37

Introduction

Necrotising enterocolitis (NEC) is predominantly seen in premature infants and is the leading cause of mortality and morbidity in neonatal intensive care units (NICU).[1-3] NEC is rare in term infants, whereas in the preterm infant it begins at 10-15 days after birth.[1] It is characterized by bowel wall necrosis of various length and depth. NEC has an overall incidence of 2-5% in all premature infants and up to >10% in babies weighing less than 1 500 g at birth.[4,5]

Pathogenesis of necrotising enterocolitis

Although research has been conducted, the pathogenesis of NEC has not yet been fully elucidated but it is considered to be a multi-factorial disease.[6,7] The most common known risk factors are prematurity, enteral feeding, ischaemia, infective agents and bacterial colonization.[4,5,7,8] Epidemiological studies have reported a strong association between prematurity and NEC because of the structural and functional gastrointestinal incompetence of prematurity. Premature babies also present with lower gastric acid and pepsin production and lower levels of protective mucus.[4,9]

The fetal gut is exposed to amniotic fluid containing hormones and peptides that may have a role in intestinal maturation and preparation for postnatal feeding. Preterm infants may not have completed this maturation process when they are initially fed. Preterm infants are unable to digest carbohydrates and proteins completely, leading to the production of organic acids, which may be harmful to the developing gut.[9] Several studies have shown that formula-fed infants have a higher incidence of NEC compared to breast fed infants. Breast milk contains multiple factors that improve intestinal maturation. In addition, human milk provides passive immunity factors such as polymeric immunoglobulin A (IgA) and macrophages that have immune protective properties.[4,9]

The premature infant may also be exposed to antibiotic treatment during this early life stage, which may alter the intestinal micro flora, facilitate colonisation of the gut by more pathogenic organisms, and activate the inflammatory cascade, leading to high expressions of pro-inflammatory mediators. The combination of these events is currently thought to lead to the manifestation of NEC.[5]

Diagnostic criteria of NEC

NEC is diagnosed on clinical grounds and roentgenographic findings. The initial symptoms may be subtle and non-specific and include apnoea, irregular temperature and lethargy. The most common sign of NEC is abdominal distension, which may be accompanied by bilious vomiting and feeding intolerance with high gastric aspirates. Gross blood appears in the stool in 25-63% of cases whereas occult blood is present in 22-59%.[1,4,6] Severe NEC presents with respiratory failure, rapid cardiovascular and haemodynamic collapse, and shock.[4] An abdominal X-ray is the current investigation of choice to confirm the clinical diagnosis of NEC. The radiological signs in early NEC include dilated and tubular in appearance bowel loops. The pattern of pneumatosis intestinalis and portal venous gas is diagnostic of NEC.[10] Bell et al described three Stages of NEC, with Stage 1 being suggestive, Stage 2 being definitive, and Stage 3 being severe. Stage 1 is extremely non-specific and may reflect feeding intolerance, sepsis or gastrointestinal haemorrhage. These

signs may also simply be manifestations of severe prematurity. Stage 1 should not be considered as definitive NEC but is useful, primarily to alert the clinician to early signs that may predict the development of NEC. Stage 2 represents early definitive NEC, is usually diagnosed radiologically by the presence of pneumatosis intestinalis and/or portal venous gas. Stage 3 is indicative of more advanced disease and it is usually associated with major systemic signs such as shock, and bowel perforation. Stage 3 usually requires surgical intervention.[1,3,11]

The role of probiotics in premature infant nutrition

The intestinal microbial community is obtained from the birth canal and from close parental contact after birth.[12] In contrast, the preterm infants acquire colonizing bacteria from the intensive care environment rather than their mother's vaginal canal and skin surface.[13] These infants often also receive antibiotic treatment perinatally to prevent acute sepsis which may further alter the composition of intestinal bacteria. Moreover, preterm infants have delayed colonization with healthy bacteria, such as *Lactobacillus* and *Bifidobacterium* species, which may lead to decreased function of the gut microbial community and immune functions.[13-19]

The administration of probiotic to this vulnerable population may, at least on theoretical grounds, be an effective way to change the gut colonization with the so called healthy bacteria. It has been suggested that introducing probiotics to preterm infants might be beneficial to avoid overgrowth of pathogenic organisms. Probiotic supplementation has also been proposed to increase feeding tolerance, decrease the amount of days until full feeds are reached and prevent nosocomial infections in preterm infants. Probiotics administration potentially competes with other organisms for binding sites and substrate in the bowel, which increases the production of anti-inflammatory cytokines, decreases the production of pro-inflammatory cytokines, reduces intestinal permeability, and enhances enteral nutrition.[20]

A recent meta-analysis of 11 randomized clinical trials that involved 2 176 premature infants treated with oral probiotics concluded that there are significant benefits with regards to the use of probiotic supplements in reducing all-cause mortality and NEC in preterm neonates. Overall evidence indicates that additional placebo controlled trials are unnecessary, if a suitable probiotic product is available.[21] The data from a recently updated systematic review was used to develop basic guidelines on the use of probiotics in preterm infants. These guidelines give some clarity on specific strain selection, probiotic dose and the duration of supplementation.[22]

Strain selection

Bifidobacteria and lactobacilli have been found to be the most promising probiotic cultures in preterm neonates.[12,23,24] It is important to bear in mind that there are different mechanisms underlying the clinical benefits of probiotics and that such benefits are also strain-specific. Bifidobacteria are the dominant strains in infancy, and the combination of lactobacilli and bifidobacteria is known to enhance the bifidogenic effect.[12,25,26] *Lactobacillus* and *Bifidobacterium* produce acidic end products during their metabolism and in so doing they lower the pH of the intestinal environment and create a locally unfavourable setting for pathogens. The literature indicates that *Lactobacillus* binds to mucins and intestinal epithelial cells, and may be able to reverse the permeability of the immature gut.[27]

It would also appear that the functionality of a multistrain or multispecies probiotic preparation could be more effective and more reliable than that of a monostrain probiotic.[28-30] A high number of different probiotic strains is not, in itself, indicative of greater efficacy when compared to that containing a lower number of strains.[31] The guidelines indicate that it would be sensible to use probiotic products that have been shown to be effective in randomized clinical trials.

Recommended dose

An optimal dosage is vital for any probiotic strain to survive and optimally colonize the intestinal tract. The concept of viability refers to the ability of the probiotic strain to survive and proliferate in 'adequate' numbers to benefit the host. Evidence indicates that to be functional, probiotics have to be viable and in adequate dosage levels, typically 10^6 to 10^7 colony-forming units (CFU)/g of product.[28,32,33]

Based on the median dose used in clinical trials in preterm neonates, it is suggested that a daily dose of 3×10^9 CFU/day may be appropriate for neonates with a gestational age of less than 32 weeks. Currently, there are no data available regarding a dose beyond which the risk of probiotic complications will be high in extremely low birth weight (ELBW) neonates. Until such research is available it is recommended that the starting dose should be 1.5×10^9 CFU/day for ELBW neonates until they reach enteral feeds of 50-60 ml/kg/day. Because neonates are often intolerant to large enteral volumes, the probiotic dose may be decreased by half. [34] The reduced dose is still expected to be beneficial.[22]

When to start administration

The importance of early bacterial colonization in preterm neonates has been well established.[21] With this in mind, it is recommended that probiotic supplementation should be started as early as possible before pathogens colonize the gut or antibiotics destroy the existing beneficial organisms.[12,23,35] The majority of the clinical trials started probiotic supplementation when enteral feeds were initiated. The earliest reported age at which supplementation was initiated was four hours of life.[22] It is desirable that premature neonates should be clinically stable (no signs of sepsis or ileus) to ensure that gut function is optimal with minimal risk of intolerance or translocation. The optimal protocol for probiotic administration in ELBW neonates with intrauterine growth restriction needs to be confirmed.[36]

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REVIEW 2

THE USE OF PROBIOTIC BACTERIA IN HIV-EXPOSED PREMATURE INFANTS: A FOCUS ON NEC

THE USE OF PROBIOTIC BACTERIA IN HIV-EXPOSED PREMATURE INFANTS: A FOCUS ON NECROTIZING ENTEROCOLITIS

Evette Van Niekerk, Author[1], BSc, M Diet. Gert F Kirsten, Co-Author[2], Mb.,ChB, MMed (Pead), DCH (SA), FCP(Pead)(SA), MD. Renée Blaauw, Co-Author[1], PhD Nutrition.

Affiliations: [1]Division Human Nutrition. Faculty of Medicine and Health Sciences. Stellenbosch University; [2]Department of Pediatrics and Child Health, Division of Neonatology. Faculty of Medicine and Health Sciences, Stellenbosch University.

Address correspondence to: Evette van Niekerk. Division Human Nutrition; Faculty of Medicine and Health Sciences; Stellenbosch University. PO Box 19063; Francie van Zijl Drive. TYGERBERG 7505. South Africa. Telephone: +27 21 938 9474. Fax:+27 21 933-299 (evettev@sun.ac.za)

ABSTRACT

Necrotizing enterocolitis is a condition predominantly seen in premature infants and is the leading cause of mortality and morbidity in neonatal intensive care units. Recently an association between maternal human immunodeficiency virus (HIV) infection and necrotizing enterocolitis (NEC) in preterm infants was reported. The administration of probiotics to this vulnerable population may be an effective way to change the colonization pattern. However the effect of probiotics on the incidence of NEC in premature and low birth weight infants exposed to HIV is unknown. Published research indicates that probiotics reduce the risk of NEC and death in HIV-unexposed infants. A recent clinical trial by Van Niekerk et al found a reduction in the severity of disease per Bells criteria in HIV-exposed preterm infants. This article aims to provide an overview the general factors associated with NEC in premature low birth weight infants, to discuss factors that contribute to the incidence of NEC specific to HIV-exposed infants and to speculate on the role that probiotics may play in reducing the occurrence of NEC in HIV-exposed infants.

Key words: Human Immunodeficiency Virus, Necrotizing Enterocolitis, Premature infant, Probiotic, Very Low Birth Weight

INTRODUCTION

Necrotizing enterocolitis (NEC) is a condition predominantly seen in premature infants and is the leading cause of gastro-intestinal associated mortality and morbidity in neonatal intensive care units (NICU).[1] This acute gastrointestinal disease presents an overall incidence of 2-5% in all premature infants and 5 -15% in babies weighing less than 1500gr at birth.[2, 3] Recently an association between maternal human immunodeficiency virus (HIV) infection and NEC in preterm infants was reported.[4] Two mechanisms are proposed to explain the increased risk of NEC in premature infants exposed to HIV. Firstly fetal HIV exposure and secondly the use of antiretroviral medication during pregnancy.[4] Probiotics are live microbial food ingredients which have been shown to have health promoting effects.[5] *Lactobacillus Rhamnosus GG* and *Bifidobacterium Infantis* have been used as probiotics to reduce the incidence of NEC.[6] A recent meta-analysis of sixteen randomized, controlled trials indicated significant reduction in the risk of NEC and death in HIV unexposed infants following the use of probiotics.[7] The purpose of this review is to provide an overview of the factors that contribute to the incidence of NEC in HIV-exposed infants and the role that probiotics may play in reducing the occurrence of NEC.

INCIDENCE OF PREMATURE AND LOW BIRTH WEIGHT INFANTS BORN TO MOTHERS THAT ARE HIV INFECTED

Fetal complications such as preterm birth and intrauterine growth restriction are more common in untreated HIV-infected women than in women without HIV infection and

their occurrence correlates with the severity of HIV disease.[8] An association has also been found between the presence of maternal HIV infection and the incidence of small for gestational age (SGA) premature births.[3, 9, 10]. A recent cohort study found that the prevalence of low birth weight (LBW) (<2500g) deliveries in HIV-affected pregnancies was 11.5%.[11] Also in a randomized trial, Kawai et al reported prematurity rates were as high as 19% amongst HIV-infected mothers with a median CD4 cell count of 500 cells/ml.[11, 12] Stratification by stage of disease shows that symptomatic HIV-positive women tend to have a higher risk of LBW than do asymptomatic women.[13]

FACTORS CONTRIBUTING TO THE DEVELOPMENT OF NECROTIZING ENTEROCOLITIS

Gastrointestinal immunity of the preterm infant and NEC

Although the pathogenesis of NEC has been researched it has not yet been fully elucidated but it is considered multifactorial.[1, 14] The most common risk factors are prematurity, timing, volume and choice of enteral feeding, intestinal ischaemia and bacterial colonization.[14] The majority of premature infants may also be exposed to antibiotic treatment during hospitalization, which alters intestinal micro-flora to facilitate colonization by more pathogenic organisms.[15] Aberrant colonization[16] of the intestinal tract may activate the inflammatory cascade, leading to high expressions of pro-inflammatory mediators.[15, 16] The combination of these events leads to the manifestation of NEC.[15]

A strong association exists between prematurity and NEC because of structural and functional gastrointestinal incompetence. The intestinal epithelium's primary function is to provide a physical barrier between the inside of the body and the luminal environment of the gastrointestinal tract. The barrier function would however be incomplete in the absence of immunologic components of the intestinal defense system.[17]

The immunity of the newborn bowel is dependent upon the innate rather than the adaptive immune system. During this developmental phase, Toll-like (TLR) receptors survey the lumen for macromolecules with pathogenic characteristics. If they are activated, they induce apoptosis to entrap the pathogen. A bacterial receptor such as Toll-like receptor 4 (TLR4) is vital, as it senses lipopolysaccharides (LPS).[18] LPS is an endotoxin and a major component of the outer membrane of Gram-negative bacteria. Elevated circulating levels of LPS have been identified in patients with NEC. [19]

TLR4 expression correlates well with increasing gestational age, until term birth, where after it decreases rapidly. Furthermore TLR4 expression also increases in response to LPS.[18] Recent published research indicates that TLR4 expression is up regulated in infants that developed NEC. [18, 20] The more TLR4 is expressed, the more apoptosis will occur, therefore increasing the severity of NEC.[18] Chan et al suggest that bowel distension in overfed premature neonates potentiates LPS action on innate TLR4 immunity. It is hypothesized that bowel distension injures the intestinal mucosa, and exposes the enterocytes to bacterial LPS.[20]

Upon exposure to bacterial products such as LPS, fetal intestinal epithelial cells produce more interleukin (IL)-8 and may also produce tumor necrosis factor (TNF), IL-1, IL-6, and platelet-activating factor (PAF).[19] Concentrations of IL8 and IL10 have been found to be significantly higher in infants with stage 3 NEC than in infants with less severe stages of NEC.[21]

This inflammatory hyper-responsiveness of neonatal gut correlates with increased nuclear factor kappa B (NF-κB)-mediated gene transcription which, in turn, is related to a developmental deficiency of the inhibitor of kappa B (IκB). NF-κB signals undergo gradual down regulation in fetal intestinal epithelial cells following birth at term gestation.[18] The NF-κB pathway is important in microbial recognition and subsequent inflammatory response in the mature gut.[22]

The role of T cells in the pathogenesis of NEC has been understudied. [18] T cells are traditionally not considered in the pathogenesis of NEC. Studies on the genetic risk factors for NEC suggest that a Th1-mediated immune response is associated with more severe disease. [23] Weitkamp et al[24] investigated the effector T cell and T-regulated cell (Treg) proportions. They found a robust presence of CD4 and CD8 effector T cells in human NEC tissue. The proportion of lamina propria Treg is significantly reduced in the ileum of premature infants with NEC.[24]

Factors that may contribute to the incidence of NEC in HIV-exposed infants

The pathogenesis of NEC in neonates born to HIV-infected mothers has not been clearly elucidated, a few contributing factors will now be discussed.

Antiretroviral treatment schedules

Although there is a scarcity of data on the outcomes of premature infants born to HIV-infected mothers, maternal HIV infection and its treatment have been identified as independent risk factors for neonatal NEC. [4, 10] Furthermore a high incidence of preterm labour and premature birth has been found as treatment has evolved into regimens of highly active antiretroviral therapy (HAART).[25]

Normally pregnancy is characterized by an increase in Th2 cytokines and suppression of Th1 cytokine production, this is said to protect the pregnancy.[26, 27] The progression of HIV disease is also characterized by a shift from Th1 to Th2 cytokine production.[28] HAART counteracts this cytokine shift.[28] It has been hypothesized that the increased risk for preterm delivery observed in HIV-infected, HAART-exposed women is mediated through changes in the cytokine environment in pregnancy.[29] Fiore et al showed that HAART use in pregnancy was associated with increased IL-2 and decreased IL-10.[29] An inverse relationship exists between gestational ages (prematurity) and the incidence of NEC.[30] This could be a possible reason for the increased risk of NEC in infants born to HIV-infected mothers.

Thus although antiretroviral prophylaxis has decreased perinatal transmission of HIV and the benefit of antiretroviral prophylaxis in preventing HIV transmission outweighs the risks of side effects, the burden of NEC in the HIV-exposed newborn population is significant.[8, 31] A case report by De Carolis et al speculates that, rather than the maternal therapy, it is the neonatal therapy that plays a main role in increasing the risk of NEC. [32]

Anaemia of prematurity and transfusion of packed red blood cells (PRBCs) are common in very low birth weight infants.[33] A strong association has been shown between red blood cell transfusions and the incidence of NEC in premature infants.[33-35] Possible reasons why PRBC transfusions may lead to NEC include a decrease in nitric oxide in stored red blood cells[36] and an exaggerated intestinal immune response to PRBC transfusion.[33, 34] Locke et al could not show a systemic cytokine response after PRBC transfusion.[37] However, Paul et al points out that because intestinal ischaemia is part of the pathophysiology of NEC, any inflammatory response after transfusion may be initially limited to the intestine. [33] A recent meta-analysis concludes that transfusion-associated NEC has a higher risk of mortality than NEC which was not preceded by transfusion.[35] Feiterna-Sperling et al found a high proportion of ARV-exposed infants with anaemia, and the risk for anaemia was generally enhanced by the intensity of maternal ART.[38] Josephson et al also reported increased odds of NEC after transfusion in infants older than 4 weeks.[34]

Immunological abnormalities:

The pathogenesis of NEC in neonates born from HIV-positive mothers is still unclear. [32] Possible reasons for an increased incidence of NEC in neonates of HIV-infected mothers is the use of HAART,[25] an increased incidence of preterm births amongst HIV infected woman, [8] early-onset sepsis and lastly that HIV-infected mothers with advanced HIV disease may have lower antibody titers.[39, 40] The intricate immunological changes, involving cytokine production and T cell function, observed in HIV-exposed infants also support the hypothesis of a possible link between HIV-vertical-exposure and NEC.[41, 42] It

has been thought that exposure to HIV determines an alteration of interleukin (IL)-12 expression in the gut mucosa and decreased T-cell function.[32] IL-12 has been shown to induce and enhance cell-mediated immune responses. Thus, abnormalities in IL-12 regulation could contribute to the decreased cellular responses observed in infants who are vertically infected with HIV. Chougnet et al found that IL- 12 production was undetectable in infants born to HIV-positive mothers.[43]

Uninfected infants born to HIV-infected mothers also demonstrate immune deficiencies which could influence outcome, particularly in advanced NEC.[31] The absence of any HIV infection at follow-up of the infants does not rule out this hypothesis, as significant haematological and immunological abnormalities have been reported in HIV-uninfected infants of HIV-positive mothers. These deficiencies may make these infants more prone to infections, and possibly to developing NEC.[4, 41] Karpelowsky et al were the first to document an increased mortality among HIV-uninfected neonates with NEC born to HIV-infected mothers.[40] A recent retrospective study provided conflicting results. It reviewed cases of stage III NEC; HIV-exposed infants were compared with HIV-unexposed infants and the findings indicate that HIV-exposed infants do not have more severe disease or more adverse outcomes in stage III NEC than unexposed infants.[31] It is suggested by Desfrere et al that premature newborn infants of HIV-positive mothers should be monitored very carefully for a possible increased risk of NEC.[4]

Cytomegalovirus:

Similar to HIV, cytomegalovirus (CMV) transmission occurs during birth as the infant is exposed to the mother's infected cervical and vaginal secretions or post-natally through

breast milk. Approximately 50%–60% of infants fed breast milk containing CMV become post-natally infected with CMV, with a higher risk of symptomatic CMV among preterm infants and this may lead to a sepsis-like illness. [44] Identified risk factors for postnatal CMV transmission include a high viral load, early shedding of CMV in milk, duration of breast-feeding, and lower infant gestational age and birth weight.[45] HIV-1 viral load and CMV co-infection synergistically increased the frequency of activated CD4 T cells, suggesting that CMV co-infection may play an important role in CD4 T cell depletion during acute infant HIV-1 infection.[46] HIV-infected infants who acquire CMV infection have a significantly higher rate of disease progression than those infected with HIV-1 alone.[46, 47]

Premature infants receive significant nutritional and immunologic benefits from breast milk, because breast milk feeding is associated with lower incidence of NEC and late-onset sepsis.[48] Pasteurisation is highly effective method of removing viable CMV and HIV, but will also damage lymphocytes and immunoglobulins in the milk.[45] Postnatal CMV infection in preterm infants may present clinically as enterocolitis. Since CMV transmission via breast milk can occur early during postnatal life, CMV may be an infectious cause of NEC.[49] Clinical signs may range from minor to severe. It is suggested that clinical features such as diarrhoea, abdominal distension and abdominal tenderness in the absence of radiological features of NEC should increase concerns of acquired CMV infection in the preterm baby.[50] Despite the fact that CMV infection is well known to be an opportunistic infection in patients with HIV infections, contradictory Chokoe et al indicated in a retrospective study that CMV is not a risk factor for NEC in immune-compromised neonates.[51]

Maternal sepsis:

As intrauterine infection is a major cause of premature labor and delivery a high sepsis related mortality and morbidity rate is found in premature infants.[52] Chorioamnionitis is a common cause of pre-term labor and it is inversely correlated with gestational age,[52] furthermore an association exists between HIV transmission and the histologic findings of chorioamnionitis.[41]

Increased risk for preeclampsia:

With the routine use of HAART, the reported incidence of preeclampsia in HIV-infected pregnant women has increased.[8] Furthermore an association exists between preeclampsia and the development of NEC in the infant. This correlates with the pathological basis of NEC as it is suggested that NEC is due to antenatal ischemia secondary to redistribution of blood flow away from the bowel leading to ischemia.[53]

Pasteurization of breast milk:

Breast milk is important in NEC prophylaxis[54] and contains up to 106 microbes/mL in healthy mothers.[55, 56] In fact, breast milk has been shown to be an uninterrupted source of probiotic bacteria to the infant gut, including Lactobacillus sp. and Bifidobacteria.[55-58] Pasteurization of breast milk potentially destroys these beneficial bacteria. Go´mez de Segura et al emphasized that pasteurization at 62.58ºC for 30 minutes leads to the loss of beneficial micro-biota present in breast milk.[57] The World Health Organization (WHO)

recommends exclusive breastfeeding. Modifications to breast-feeding, such as breast milk expression and pasteurization is recommended by the WHO to reduce the risk of HIV transmission while providing breast milk's immune properties.[59] This same pasteurization conditions that was indicated by de Segura et al is used to pasteurize breast milk of HIV infected mothers and breast milk for donor purposes.[57, 59] The effect of this two edged intervention is firstly that by pasteurizing breast milk of HIV infected mothers it reduces the risk for mother to child HIV transmission although destroying the potential health promoting bacteria available in breast milk and thereby placing the infant at risk for the development of NEC.

THE ROLE OF PROBIOTICS IN THE MANAGEMENT OF NEC

Until recently the intestinal tract has been considered sterile at birth. However, some studies suggest that the meconium from healthy hosts is not sterile and that gut colonization may start before birth.[60, 61] The intestinal microbial community of full term infants is furthermore derived from the birth canal and close parental contact after birth.[62] In contrast, preterm infant's acquire colonizing bacteria from the intensive care environment, rather than their mother's vaginal canal and skin surface.[63] Postponed colonization with healthy bacteria, such as Lactobacillus and Bifidobacterium species, are seen in preterm infants. This delay may lead to an inappropriate gut microbial community and immune functions. Antibiotic treatment peri-natally to prevent acute sepsis also further alters the composition of intestinal bacteria.[63, 64]

The administration of probiotics to this vulnerable population may be an effective way to change the colonization pattern. It is suggested that introducing probiotics to preterm infants might be beneficial by preventing overgrowth of pathogenic organisms; probiotic supplementation may increase feeding tolerance, decrease the number of days until full feeds are reached and prevent nosocomial infections; probiotics potentially compete with other organisms for binding sites and substrate in the bowel.[7] The focal health-promoting effect of probiotics is their enhancement of mucosal immune response through increasing macrophage activity, elevating numbers of killer cells, T cells and interferon, the suppression of NF-κB signaling[40] and their action against pathogenic microbial colonization and translocation.[44]

Bengmark et al proposed that probiotic administration protects the gut surface and could delay progression of HIV.[65] The loss of intestinal CD4+ Th17 cells, which differentiate in response to normal micro-flora, occurs early in HIV disease. The depletion of Th17 cells from the gut in HIV infection is associated with microbial translocation, chronic immune activation, and disease progression.[66, 67] Disturbance of the micro-biota early in HIV infection leads to increases pathogen dominance, reduced levels of Bifidobacteria and Lactobacillus species and increased mucosal inflammation. Furthermore it has recently been hypothesized that probiotic bacteria may stabilize CD4+ T cell numbers in HIV-infected children and are likely to have protective effects against probable gastrointestinal inflammation.[67]

Based on the observation that NEC is known to develop after the intestine has been colonized with gram-negative bacteria, several research groups have sought to establish whether TLR4 may play a role in the pathogenesis of NEC. There are ten

known individual TLRs (TLR1 - TLR10). As previously mentioned TLR4 is known to be the receptor for the outer membrane component of gram-negative bacteria, namely LPS. The reciprocal expression of TLR9 and TLR4 was found to influence the extent of TLR4 signaling, and the development of NEC was accompanied by a relative increase in TLR4 with a related reduction in the protective TLR9. These findings support the relative protective value of probiotic administration to infants with NEC, as these probiotic preparations are rich in bacterial DNA, through which activation of TLR9, which is essential to mediate the anti-inflammatory effect of probiotics on the host, would be expected to limit TLR4 signaling and reduce NEC severity.[68, 69]

The latest updated Cochrane review summarizes the evidence of probiotics efficacy from sixteen randomized trials and more than 2700 preterm infants. Results show that probiotics reduced the incidence of severe NEC, mortality, and NEC related mortality.[70] Bifidobacteria and Lactobacilli has been found to be the most promising probiotic cultures, given the development of the intestinal flora in preterm neonates.[71, 72] It is important to bear in mind that there are different mechanisms producing the clinical benefits of probiotics and there are also strain-specific effects.[71] Bifidobacteria are the dominant strains in infancy, and the combination of Lactobacilli and Bifidobacteria is known to enhance the bifidogenic effect.[62] Lactobacillus and Bifidobacteria produce acidic end products during their metabolism; these lower the pH of the intestinal environment and create a locally unfavourable setting for pathogens. [73] Literature indicates that Lactobacillus binds to mucins and intestinal epithelial cells, and may be able to reverse the permeability of the immature gut.[73]

A recent randomized control trail evaluated the efficacy of probiotics in reducing the incidence and severity of all stages of NEC in premature very-low birth weight infants that are exposed to HIV. The study group received breast milk plus a daily probiotic supplement of *L. rhamnosus GG* (0.35×10^9 colony-forming units [CFU]) and *B. infantis* (0.35×10^9 CFU). The control group received breast milk plus a placebo consisting of medium chain triglyceride (MCT) oil.[74] Furthermore this trail found the use of probiotics to be safe in HIV-exposed infants with no difference found in the incidence of positive blood cultures.[74] Results from this randomized control trail (RCT) indicate that the overall incidence of NEC was 5% which is within the lower range of other reported studies.[75, 76] Among the nine cases of NEC that occurred, four episodes (2%) were severe NEC Bell's stage III cases. Furthermore the study found a reduced incidence of NEC (Bell's II and III) in the study group when compared to the control group (3% vs. 6% NEC incidence respectively), not specific to the HIV status. This study failed to show that probiotics lowered the incidence of NEC in HIV-exposed premature infants however it appears to reduce the severity of disease per Bells criteria.

CONCERNS REGARDING PROBIOTIC TREATMENT

Probiotics are often regulated as dietary supplements rather than as pharmaceuticals or biological products. Dietary supplements usually do not demonstrate requirements of safety, purity, or potency before marketing. [77] Probiotics are different from conventional drugs; there are many types, strains, and optimum production, transport, storage, dosage, and contraindications are unclear.[78]

Because the pathogenesis of NEC involves various complex pathways, different probiotic strains may create benefit by different pathways. Evidence suggests that the use of a multi-strain or multi-species probiotic should be more effective and more consistent than that of a mono-strain probiotic.[71] Randomized controlled trials that used a multi-strain probiotic reported a significant decline in NEC, whereas those reporting a lesser decline used a single organism, such as *Lactobacillus rhamnosus GG*.[7, 14, 71] Luoto et al. suggests that it may be prudent to avoid the use of this single strain alone, pending further evidence. [71, 79] However Deshpande et al also emphasized that using more than two or three strains (each with an optimal mass) may increase the risk of translocation because of the substantial increase in the total dose. Furthermore it is better to avoid untested combinations, because strain combinations can be antagonistic, compatible or synergistic.[71]

The most important area of concern with probiotic use is the risk of sepsis[77] AlFaleh et al reported that there was no significant difference among the probiotic and placebo group in the rate of culture proven sepsis from thirteen eligible trials.[70] This updated review of available evidence supports a change in practice.[70]

CONCLUSION

After considering the evidence provided from randomized control trails, researchers conclude that probiotics should be offered as a routine therapy for the prevention of NEC in preterm infants. With an awareness of the association between perinatal HIV exposure and the increased risk of NEC in premature infants, probiotic bacteria might

help to improve the management of these infants by reducing inflammation and improving immune function. Furthermore HIV-exposed infants that receive pasteurized breast milk from their HIV-infected mothers receive less health promoting bacteria available in breast milk compared to their HIV-unexposed counterparts, again increasing their risk for NEC. It should however be noted that live probiotic bacteria have the potential to become pathogenic when host defenses are compromised. At this time little evidence exists on the use of probiotics in HIV-uninfected neonates born to HIV-infected mothers. A recent clinical trial by Van Niekerk et al found a reduction in the severity of disease per Bells criteria in HIV-exposed preterm infants. We therefore support the use of probiotics is an effective method in reducing the risk for NEC in VLBW and ELBW birth weight infants. Furthermore studies are encouraged to establish the efficacy of probiotics in reducing the incidence of NEC in HIV-exposed infants.

List of abbreviations: CMV: cytomegalovirus; HAART: highly active antiretroviral therapy; HIV: Human Immunodeficiency Virus IgA: immunoglobulin A; IκB: inhibitor of kappa B; LPS: lipopolysaccharide; NEC: necrotizing enterocolitis; NF-κB: nuclear factor kappa B; PAF: platelet-activating factor; SGA: small for gestational age; TLR4: Toll-like receptor 4; TNF: necrosis factor; VLBW: very low birth weight.

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Authors' contibutions

Mrs. Evette van Niekerk: Mrs. van Niekerk wrote the paper and had primary responsibility for final content.

Prof Gert Kirsten: Prof Kirsten reviewed and revised the manuscript, and approved the final manuscript as submitted.

Prof Reneé Blaauw: Prof Blaauw critically reviewed the manuscript, and approved the final manuscript as submitted.

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REVIEW 3

HUMAN MILK OLIGOSACCHARIDES: POSSIBLE LINKS TO NECROTIZING ENTEROCOLITIS AND HIV-EXPOSURE.

Van Niekerk E, Kirsten GF, Nel DG, Blaauw R. Human milk oligosaccharides: possible links to necrotizing enterocolitis and HIV-exposure. S Afr J Clin Nutr 2014;27(2):51-55.

The role of human milk oligosaccharides in preventing necrotising enterocolitis and human immunodeficiency virus transmission

Van Niekerk E, BSc(Dietetics), M(Dietetics), Lecturer, Stellenbosch University; **Blaauw R,** PhD(Nutrition), Associate Professor
Division Human Nutrition, Faculty of Medicine and Health Sciences, Stellenbosch University
Kirsten GF, MBChB, MMed(Paed), DCH(SA), FCP(Paed)(SA), MD, Professor
Department of Paediatrics and Child Health, Division of Neonatology, Faculty of Medicine and Health Sciences, Stellenbosch University
Correspondence to: Evette van Niekerk, e-mail: evettev@sun.ac.za
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Abstract

The heavy burden of maternal human immunodeficiency virus (HIV) infection has resulted in a high prevalence of premature births, with an associated increase in the incidence of necrotising enterocolitis (NEC). NEC is a life-threatening inflammatory disease of the gastrointestinal tract that predominantly occurs in preterm infants. Human milk oligosaccharides (HMOs) are carbohydrate compounds which have been shown to have health-promoting effects through their bifidogenic and antiadhesive properties. There is a reduced incidence of NEC in infants who receive human milk, compared to those receiving infant formula. It is suggested that the oligosaccharides found within human milk may act as specific substrates in assisting the growth of selected beneficial bacteria, called probiotics. Probiotics are live microbial food ingredients which have been shown to have health-promoting effects. *Lactobacillus rhamnosus GG* and *Bifidobacterium infantis* have been used as probiotics to reduce the incidence of NEC. Furthermore, HMOs have been associated with protection against postnatal HIV transmission. HMOs may hold key responsibility for the prevention of NEC, and possibly the transmission of HIV, to infants in resource-limited settings and in a developing country, such as South Africa, where HIV plays a major role in the outcomes of preterm neonates.

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Introduction

Research, started over a century ago, on human milk oligosaccharides (HMOs) has focused predominantly on their prebiotic effects. Today it is known that the bifidogenic effects of HMOs are one of the many beneficial effects now being discovered.[1] In addition to providing a substrate for the growth of beneficial bacteria, HMOs also directly inhibit pathogens from adhering to the intestinal epithelia through their antiadhesive properties.[1] Recent data suggest that firstly, a relationship exists between the HMOs and the incidence of necrotising enterocolitis (NEC),[2,3] and secondly, they may have an influence on human immunodeficiency virus (HIV) transmission.[4] This article aims to describe the prebiotic and antiadhesive effects of HMOs, and how they could possibly prevent the development of NEC in the presence of HIV infection.

Human milk oligosaccharides

HMOs[5,6] are complex carbohydrates that are highly abundant in breast milk, but not in infant formula.[7] Oligosaccharides are carbohydrates made up of 3-9 monosaccharide units.[5,6] The oligosaccharide concentration of human milk varies with gestational age, lactation duration, and from one mother to another.[6,8] HMOs reach the highest concentration in colostrum (20-25 g/l), and then decrease after roughly two weeks to approximately 5-20 g/l in mature breast milk.[1,6,8-11]

HMOs consists of five different monosaccharide units:

- Glucose.
- Galactose.
- The sialic acid component, known as N-acetylglucosamine.
- Fucose.
- N-acteylneuaminic acid.[1,12]

With a few exceptions, all HMOs contain lactose at their reducing end. They may be elongated (an enzymatic attachment) by the addition of β1-3- or β1-6-linked lacto-N-biose or N-acetyllacetosamine. The latter can be further extended by one or two disaccharides. Chain branching may occur at the β1-6 linkage. Branched lactose molecules may form both linear (para-HMOs) and branched structures (iso-HMOs). Furthermore, oligosaccharide chains can be fucosylated (also known as neutral oligosaccharides) in the α1-2, α1-3 or α1-4 linkage, or sialylated (also known as acidic oligosaccharides) in the α2-3 or α2-6 linkage. The ABH secretor (*Se*) status and Lewis (*Le*) blood group determines the HMO composition of a mother's breast milk.[1] The presence or absence of blood group substances (A, B and H) in the saliva and other body secretions is controlled by a gene on chromosome 19, the fucosyltransferase

locus 2 and the fucosyltransferase locus 3.[13] Both genes are expressed in glandular epithelia and have dominant allele (*Le* and *Se*, respectively) coding for enzymes with fucosyltransferase activity and recessive alleles (*le* and *se*, respectively) that are not functional. Briefly, the fucosyltransferase encoded by the *Se* gene regulates the synthesis of HMOs containing fucose with an α-1,2 linkage. The *Le* gene promotes the synthesis of α-1,3 and α-1,4 linkages.[1] There are up to 200 identified HMOs and their effects appear to be strongly structure dependent.[8,11]

An atypical characteristic of HMOs is that the monosaccharides, of which they are comprised, are bound by specific bonds resistant to the digestive enzymes present in the small intestine.[8,14] Although intact HMOs may be absorbed, the majority of oligosaccharides ingested through breast milk pass through the small intestine undigested, and reach the colon where they act as a fuel source for bifidus-predominant bacteria, thereby gaining the status of prebiotics.[1,15,16] A small percentage of absorbed HMOs are excreted in the urine,[8,11] and may protect the infant from urinary tract infections.[17] High proportions of bifidobacteria are represented in the intestinal tract of breastfed infants, and through the generation of short-chain fatty acids, they protect the enterocyte from pathogens, and may contribute to mucosal integrity.[8,11,17]

HMO functionality is not limited to their prebiotic effects only. The possibility was raised more than a decade ago that HMOs may act as an anti-infective agent by blocking pathogen adhesion to the intestinal epithelial cell walls, thereby possibly preventing enteric infections, such as rotavirus diarrhoea and systemic infections, such as HIV/acquired immune deficiency syndrome.[17] The anti-infective effect that they hold has generated considerable interest. The bifidogenic and anti-adhesive properties of HMOs, as well as their role in NEC and the possible linkage to HIV, are now be described in further detail.

The bifidogenic (prebiotic) effect of human milk oligosaccharides

Factors that influence gastrointestinal tract colonisation can be grouped into two categories: extrinsic and intrinsic. Intrinsic factors include underlying neonatal health, immunological status, gastrointestinal transit time and pH. These factors affect the process of colonisation and the types of organisms established in the neonatal intestinal tract. Extrinsic factors include the geographical area, maternal and surrounding environment bacteria, mode of delivery, feeding practices and drug therapies.[18]

It is well established that the type of delivery has a significant influence on the development of intestinal microflora.[19,20] Infants born by vaginal delivery are more exposed to their mother's microbiota than those born by Caesarean section.[19] Initial exposure to microflora by those born by Caesarean delivery is most likely to be from environmental micro-organisms on the equipment, in the air and on other neonates, with the nursing personnel acting as carriers.[20]

Furthermore, the preterm infant is exposed to bacteria in the neonatal intensive care unit, where the frequent use of broad-spectrum antibiotics affects the colonisation of health-promoting bacteria.[21] The choice of feed plays an important role in the establishment of intestinal flora. *Bifidobacterium* is a predominant organism found in the intestinal tract of breastfed infants. *Lactobacillus* and *Streptococcus* are found in lesser quantities. By contrast, similar amounts of bacteroides and bifidobacterium are found in formula-fed infants, as well as components of the more pathogenic species, such as *Staphylococcus*, *Escherichia coli* and *Clostridia*.[21] An extraordinary characteristic of the intestinal environment of breastfed infants is the selective nourishment and protective support provided by the intestinal microbiotia.[22]

The term "prebiotic" was introduced and defined by Gibson and Roberfroid as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon".[23,24] Although HMOs fully conform to this definition, it is important to bear in mind that HMOs promote the growth of certain, but not all, health-promoting bacteria.[1] Glycoprofiling results by LoCascio et al[22] provide molecular and mechanistic evidence that specific strains of bifidobacteria have an affinity for selected classes of HMO.[8,11] This suggests that the diversity of specific oligosaccharide structures found within human milk do not provide a direct source of nutrition to the infant, but rather act as specific substrates to assist the growth of selected beneficial bacteria, that in their own right support the development and protection of the neonate.[22] N-acetylglucosamine oligosaccharides have been implicated as important cell wall components for bifidobacteria.[17] In the case of *B. longum by infantis*, this strain has the ability to preferentially consume the most abundant HMOs.[11,22]

Antiadhesive effect of human milk oligosaccharides

There is increasing evidence that oligosaccharides in human milk have a direct inhibitory effect on certain virulence capabilities of pathogenic microorganisms.[11] The destructiveness of most pathogenic microorganisms, e.g. *Campylobacter jejuni*, *E. coli* and *Salmonella* strains, often depends on their aptitude to adhere to epithelial surfaces.[25] In general, bacterial adhesion is a ligand-receptor interaction between structures on the bacterial surface and complementary structures on the mucosal surface of the host.[11]

HMOs structurally imitate epithelial cell surface carbohydrates, and therefore act as decoys to which infectious agents can bind, instead of to the host, thereby preventing possible infection.[26,27] These molecules can withstand the low pH of the stomach, and resist degradation through enzymes from the pancreas and brush border membrane.[28] With every feed, the gut of breastfed infants is flushed with various oligosaccharides that can effectively reach and block binding sites, thereby reducing the chances of probable pathogen adhesion to these locations.[26]

Human immunodeficiency virus and human milk oligosaccharides

Breastfeeding is the predominant postnatal transmission route for HIV-1 infection in children.[29] However, the majority of breastfed infants do not become HIV-infected, despite continuous exposure to the virus through their mothers' milk over many months. This raises the question: Does human milk have properties that protect the infant against HIV transmission?

Dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN)-bound pathogens are usually transferred into dendritic cell (DC) lysosomes, where they are processed before stimulating T cells to initiate an immune response.[25] However, HIV-1 pathogens are kept hidden within the DC for a number of days without prompting an immune response before they are transferred to CD4+ T lymphocytes, where they multiply and cause disease at a later stage.[11,30] DC-SIGN is a carbohydrate-binding protein. It recognises mannose-containing glycoconjugates, such as HIV-1-gp120, with an even higher binding affinity for *Le* blood group antigens.[25,29,31] Although monomeric *Le* epitopes bind to DC-SIGN, the presence of multivalent *Le* epitopes is required to compete with HIV-1-gp120 for DC-SIGN-binding. The occurrence of multiple *Le* blood group determinants as part of HMOs led scientists to postulate that HMOs compete with HIV-1-gp120 for binding to DC-SIGN, and therefore play a protective role against HIV-1 mother-to-child transmission during breastfeeding.[1,25] Since some HMOs structurally resemble the *Le* blood group antigens that show high-binding affinity for DC-SIGN, and some HMOs carry one or multiple *Le* epitopes, HMOs may compete with gp120 for binding to DC-SIGN and reduce HIV-1 mother-to-child transmission.[31,32]

However, the results by Bode et al suggest otherwise.[4] They found that the concentration of HMOs with secretor or *Le*-active epitopes did not correlate with HIV transmission risk. These results propose that the protection against postnatal HIV transmission is probably independent of the mother's secretor or *Le* blood group status, and that HMOs offer other protective properties, i.e. concentrations of lacto-N-neotetraose.[4] As described, more than 200 different HMOs have been characterised, and investigators have speculated whether or not the amount will rise to 1 000. Hong et al described the blocking of the DC-SIGN as a two-edged sword, indicating that although it may reduce the entrance of certain viruses, such as HIV-1, at the same time it may also reduce the ability of the infant's immune system to detect and fight other pathogens, leading to a potentially increased risk of bacterial or viral gastroenteritis.[29] Hong et al have shown that physiological concentrations of HMOs significantly reduce HIV-1gp120-binding to DC-SIGN.[25,29] Therefore, the question is: Which HMOs are the most potent inhibitors of HIV-1-gp120–DC-SIGN binding?[29] Once individual HMOs have been identified that block HIV-1-gp120 binding to DC-SIGN, it will be important to assess whether or not these HMOs trigger adverse effects.[25]

Necrotising enterocolitis

NEC is the most common serious acquired condition of the gastrointestinal tract in preterm infants.[33] NEC occurs in 4-11% of all premature infants born with a very low birthweight, and an inverse relationship exists between birthweight, gestational age and the incidence of NEC.[33,34] Generally, prematurity, enteral feeding, ischaemia and bacterial colonisation are the most common risk factors for the development of NEC.[35-38] The pathogenesis of NEC in neonates born to HIV-infected mothers has not been clearly elucidated. Some described contributing factors include maternal antiretroviral treatment, maternal sepsis and immunological abnormalities. Although antiretroviral prophylaxis has decreased the perinatal transmission of HIV, the burden of NEC in the HIV-exposed newborn population is significant.[39,40] Infants who receive human milk show a reduced incidence of NEC, compared to those receiving infant formula.[41] Numerous advantages of breast milk have been found over infant formula. These include the earlier achievement of full enteral feeding, the presence of active enzymes which enhance the maturation of the immature intestinal tract, and anti-infective agents which protect the neonate from infections.[42] The effect of prebiotics on NEC has not been studied partly because of the very large sample sizes that are required, and secondly because of ethical concerns with such trials.

Jantscher-Krenn et al found that disialyllacto-N-tetraose (DSLNT) has a protective effect against the development of NEC in neonatal rats.[2] Results by Van Niekerk et al[3] indicate that DSLNT may indeed protect against NEC, not only in neonatal rats as indicated by Jantscher-Krenn et al, but also in human preterm infants. These results suggest that infants diagnosed with NEC received breast milk that contained lower concentrations of DSLNT than infants who did not develop NEC. Specifically designed and well powered cohort studies are required to verify the link between DSLNT and NEC. If confirmed, DSLNT concentrations in mothers' milk could serve as a noninvasive marker to determine whether or not a breastfed infant is at risk of developing NEC.[3]

The role of probiotics in the management of necrotising enterocolitis

When focusing on bacterial colonisation, which plays a pivotal role in the development of NEC,[43,44] it is important to note that no single bacterial species has thus far been associated with NEC. Although *Enterobacteriaceae* spp. is the most frequently pronounced bacteria to be found in association with NEC, *Clostridia* spp. and *Staphylococcus* spp. have also been isolated.[45] Probiotics might be beneficial in preventing the overgrowth of pathogenic organisms, increased feeding tolerance,[46,47] decreasing the number of days until full feeds[48] are reached and preventing nosocomial infections. Furthermore, probiotics potentially compete with other organisms for binding sites and substrate in the bowel.[49,50] Microbiota disturbances early in HIV infection lead to greater dominance of potential pathogens, reduced levels of *Bifidobacteria* and *Lactobacillus* spp. and increasing mucosal inflammation.[51] As HMOs support the growth of these beneficial bacteria, it is therefore proposed that in conjunction with the use of probiotics, HMOs could potentially reduce the risk of NEC. A randomised clinical trial by van Niekerk et al was the first to examine the use of probiotics in the management of NEC in HIV-exposed versus non-exposed, very low-birthweight

infants.[52] The results from this trial concluded that the incidence of death and NEC did not differ significantly between the HIV-exposed and unexposed groups. However, a reduced severity of disease per Bell's staging criteria was found. A significantly lower incidence of NEC was found in the probiotic group than in the control group.[52]

Because the pathogenesis of NEC involves various complex pathways, different probiotic strains may create benefit through different pathways. Evidence suggests that the use of a multistrain or multispecies probiotic should be more effective and more consistent than that of a monostrain probiotic.[53] Randomised controlled trials that used a multistrain probiotic reported a significant decline in NEC, whereas those reporting a lesser decline used a single organism, such as *L. rhamnosus GG*.[49,53,54]

Conclusion

HMOs not only act as a substrate for beneficial bacteria, but also provide antiadhesive effects, thereby reducing the risk of infection.[1] Many unanswered questions remain that surround the mother-to-child transmission of HIV and the role of HMOs. Furthermore, the incidence of NEC in premature infants has increased as the burden of HIV has heightened.[55] Therefore, the question remains: Could HMOs serve as a prophylactic measure against this life-threatening gastrointestinal condition? Further studies are needed to investigate the influence of HMOs on the attachment of pathogens to the intestinal epithelial cell walls. To fully understand the immunological needs of the preterm infant, HMO concentrations in preterm breast milk need to be clearly defined. Future prospects for HMO research are expected to be very useful with regard to the development of management options that are effective in protecting babies from harmful infections.

Breast milk is widely recognised as the optimal feed of choice for both term and preterm infants, as it upholds metabolic, immunological and neurodevelopmental advantages.[56] The World Health Organization[57] recommends exclusive breastfeeding for all infants, and recommends modifications to breastfeeding, such as breast milk expression and pasteurisation, to reduce the risk of HIV transmission, while providing the advantages of breast milk's immune-stimulating properties.[58] The use of breast milk should be strongly advocated in neonatal units. Postnatal breastfeeding practices in resource-limited settings and developing countries such as South Africa, can be supported by practising kangaroo mother care in neonatal units. A 2012 Cochrane review concluded that kangaroo mother care improves anthropometric parameters, breastfeeding and mother satisfaction.[59] Mothers of preterm infants should be encouraged to express their breast milk and pasteurise it, if applicable. Breastfeeding must also be enforced when it is physiologically possible for preterm infants.

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Chapter 2

RESEARCH METHODOLOGY

2. RESEARCH DESIGN AND METHODOLOGY

2.1 RESEARCH QUESTION

Does the administration of probiotics reduce the incidence and severity of necrotizing enterocolitis (NEC) in premature, very low birth weight (VLBW) infants exposed to HIV?

2.2 AIM OF THE INVESTIGATION

The aim of this study was to determine whether the administration of probiotics reduced the incidence and severity of NEC in premature, VLBW infants exposed to HIV.

2.3 OBJECTIVES

2.3.1 Primary objective

The primary objective of the study was to evaluate the efficacy of probiotics in reducing the incidence and severity of NEC, as per Bell's criteria,[1] in premature, VLBW infants exposed to HIV.

2.3.2 Secondary objectives

The secondary objectives included the following:

2.3.2.1. To assess the incidence of all stages of NEC in VLBW infants born to HIV-infected and HIV-uninfected women.

2.3.2.2. To determine the prebiotic quality (human milk oligosaccharides) of own mother's breast milk of HIV-infected and HIV-uninfected mothers;

2.3.2.3. To determine whether there was an association between the prebiotic quality (human milk oligosaccharides) of breast milk and the incidence of NEC in premature, VLBW infants; and

2.3.2.4. To evaluate weight gain for both probiotic-exposed and -unexposed premature, VLBW infants.

2.4 NULL HYPOTHESES

The following null hypotheses guided the current study:

2.4.1 H_0: The administration of probiotics does not reduce the incidence and severity of NEC in premature, VLBW infants exposed to HIV.

2.4.2 $H_{0:}$ There will be no difference in the incidence of NEC stages in VLBW and ELBW infants born to HIV-infected and -uninfected women;

2.4.3 $H_{0:}$ There will be no difference between the prebiotic quality (human milk oligosaccharides) of the breast milk of HIV-infected and -uninfected mothers;

2.4.4 $H_{0:}$ There will be no association between the prebiotic quality (human milk oligosaccharides) of breast milk and the incidence of NEC in premature, VLBW infants;

2.4.5 $H_{0:}$ There will be no difference in weight gain for probiotic-exposed and -unexposed premature, VLBW infants.

2.5 CONCEPTUALISATION

The conceptual framework of the study is outlined in Figure 1 on the following page. As shown in this diagram, the aim of the study was to determine whether the administration of probiotics reduced the incidence of NEC in premature, VLBW infants exposed to HIV. The prebiotic quality of each breast milk sample was measured for oligosaccharide composition. Paediatric anthropometric measurements include the head circumference, length and weight.

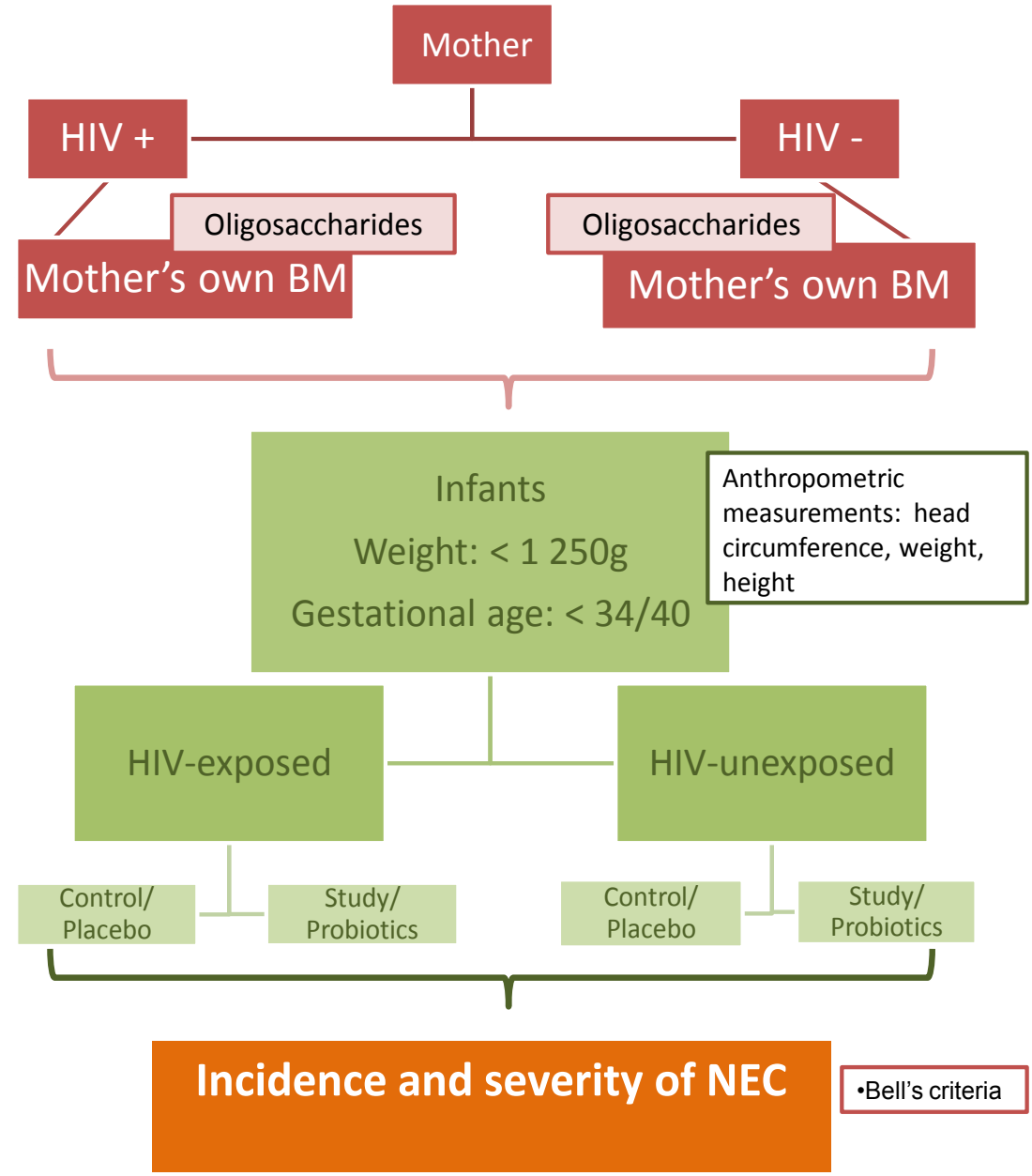

Figure 1: Conceptual framework of the study

The key words and phrases used in Figure 1 were defined for the purposes of this study as follows:

Premature infant: This refers to any infant born before 37 completed weeks' gestation, regardless of birth weight.[2]

Very low birth weight (VLBW) infant: This refers to any infant weighing less than 1 500 g at birth.[3]

Breast milk oligosaccharides: These are the third most abundant solid component in breast milk after lactose and lipids. The most abundant neutral oligosaccharides found in human milk are fucosylated tri- to hexasaccharides and the precursors to these, namely lacto-N-tetraose (LNT) and lacto-N-neotetraose (LNnT).[4]

Necrotizing enterocolitis (NEC): This is an acute inflammatory bowel disorder predominantly seen in premature infants. It is the leading cause of mortality and morbidity in neonatal intensive care units.[5-7]

Probiotics: Probiotics are live microbial food supplements or components of bacteria that have been shown to have beneficial effects on human health.[8]

Anthropometric measurements: This refers to the measurement of body size, proportion and weight. These measurements include length, weight and head circumference. Anthropometric measurements can be used to evaluate the nutritional status of an individual.[9]

Human immunodeficiency virus (HIV): An initial HIV infection causes an acute phase of HIV-specific humoral and cell-mediated immune responses. HIV primarily infects cells expressing the CD4 receptor molecule. The resulting T-cell depletion leads to decreased immune surveillance and thus receptiveness to opportunistic infections and progression to acquired immunodeficiency syndrome (AIDS).[10]

2.6 SETTING

Wards G1, G2, J3 and G8 of Tygerberg Children's Hospital (TBCH), situated in Tygerberg Academic Hospital (TAH), were selected for data collection. Infants who had been transferred from TBCH to the Khayelitsha Kangaroo Mother Care (KKMC) unit continued to form part of the study population. The KKMC unit is situated in TAH. The standard study procedures applied at the KKMC unit.

2.7 STUDY DESIGN

A double-blind, placebo-controlled, randomised clinical trial was conducted.

2.8 STUDY POPULATION

The study population consisted of male and female premature, VLBW infants who were HIV-exposed and HIV-unexposed. These patients had been born at TBCH and admitted or transferred to wards G1, G2, J3 and G8. Infants who had been transferred from TBCH to the KKMC unit continued to form part of the study population.

2.8.1 Sample size

According to the 2009 live birth statistics of TBCH, 1 883 infants were born with a birth weight of < 2 500 g and 498 infants were born with a birth weight of < 1 500 g. According to the 2009 statistics from the Prevention of Mother-to-Child Transmission (PMTCT) clinic at TAH, 541 HIV-infected mothers gave birth to 449 live low birth weight infants (< 2 500 g) or premature infants (< 37 weeks' gestation), of which 97 (19%) were VLBW babies (< 1 500 g).

Infants with a birth weight of < 1 250 g were included in this study due to the increased length of stay of these infants in hospital and to ensure that the majority of the study population was not exited prematurely from the study. The sample size was estimated according to the statistics mentioned above, the inclusion and exclusion criteria (refer to 2.8.2.1 and 2.8.2.2) and the recommendations of a statistician. The total study population consisted of 184 infant participants, of which 74 were HIV-exposed and 110

were not. A power analysis for two-way analysis of variance (ANOVA) with two levels for each factor with a power of 90% to detect interaction effect sizes of δ = 0.55 yielded samples of size n = 36 in each of the four groups (probiotic or placebo group for each of the HIV-exposed or HIV-unexposed groups). The sample sizes of n = 37, n = 37 (in the HIV-exposed group) and n = 54, n = 56 (in the HIV-unexposed group) exceeded this power specification. An effect size of δ = 0.55 is regarded as a smaller-than-medium effect.

Concerning the secondary objectives of the study that entailed the oligosaccharide analysis, the study population that consisted of HIV-infected and HIV-uninfected mothers also amounted to 184 participants. A sample size of 184 participants was calculated by the statistician to give a power of 80% to detect a minimum difference in core oligosaccharides of 7% between the HIV-infected and HIV-uninfected groups. It also gave an 80% power to detect a minimum difference of 5% at each of the measured time points. The infant mortality due to NEC was estimated at 30 patients within the last two years at TBCH. According to research, the incidence of NEC is approximately 5–6% in VLBW infants, with a significant morality rate of 20–25%.[11]

2.8.2 Sample selection

Preterm infants (< 34 weeks' gestation) with a birth weight of < 1 250 g who had been admitted or transferred to wards G1, G2, J3 and G8 at TBCH and the KKMC unit were randomly assigned, by a random number sequence, to either the placebo administration group (control group) or the probiotic administration group (study group). Randomisation was done by the assigned statistician. The inclusion and exclusion criteria that were used are outlined below.

2.8.2.1 Inclusion criteria

The following inclusion criteria were used for mothers:

a. HIV-infected and HIV-uninfected women who had given birth to a premature, VLBW infant at TBCH and who had consented to participate in the study were included and

b. Only mothers who had decided to breastfeed after counselling, regardless of their HIV status, were included. All HIV-infected mothers are counselled routinely at TBCH about the risks and benefits of breastfeeding and bottle-feeding in accordance with the PMTCT scheme.

c. HIV-infected mothers on the PMTCT treatment regimen were included. Mothers who received antiretroviral (ARV) medication (nevirapine, zidovudine [AZT] and/or highly active antiretroviral therapy [HAART]) were included in the study.

The following inclusion criteria were used for premature, VLBW infants:

a. Infants who had been admitted or transferred to wards G1, G2, J3 and G8 at TBCH were included; or

b. Infants who had been transferred to the KKMC unit in TBH were also included; and

c. Birth weight had to be below 1 250 g; and

d. Premature infants, born with < 34 weeks' gestation, were included; and

e. Male and female subjects formed part of this study; and

f. Infants born via either normal vaginal delivery or a caesarean section were included; and

g. Infants who received antibiotic treatment and those who did not were included in the study. The majority of infants in the wards received an initial dose of antibiotic treatment with penicillin G; and

h. Initially all infants were fed via an orogastric tube. Once infants had reached full enteral feeds, the orogastric tube was removed and infants received feeds via cup feeding; and

i. Patients had to receive their own mothers' breast milk; and

j. All infants had to receive their own mothers' breast milk with the use of donor breast milk in exceptional cases. Patients received donor breast milk when mothers own breast milk was not available. Donor breast milk was included in the following circumstances:

- As a supplemental feed due to insufficient breast milk supply from the mother.
- When the mother was unavailable, for example on home visits over weekends or for continuation of work.
- If the mother was ill and unable to breastfeed the infant, for example if the former had been admitted to an intensive care unit.

Due to the high costs involved and sparse availability of donor breast milk, it is only provided to infants when there is no alternative or in scenarios as sketched above. Donor breast milk is usually not provided for extended periods of time.

k. Premature VLBW infants who were HIV-exposed or HIV-unexposed were included in the study.

l. HIV-exposed, premature, VLBW infants who received ARV medication also formed part of the study population. Nevirapine was prescribed by the attending neonatologist. The dose administered did not affect infant inclusion in the study. Nevirapine doses were noted on the data collection forms.

2.8.2.2 Exclusion criteria

The following exclusion criteria were used for mothers:

a. Mothers who received medication that was contraindicated for breastfeeding were not included in this study. These medications include the following drug classes: anticoagulants (e.g. wafarin), cytotoxics and psychoactive drugs (e.g. tricyclic antidepressants and antipsychotics such as chlorpromazine) and individual drugs detectable in breast milk that pose theoretical risk. Examples of these drugs are listed in Table 1[12] ; or

b. Mothers with active *Mycobacterium tuberculosis* infection who were still potentially infectious and who should not have been in direct contact with their infants were excluded from this study [13]; or

c. HIV-infected mothers who presented clinically sick with a CD4 cell count < 200 cells/µl were also excluded.

The following exclusion criteria were used for premature, VLBW infants:

a. Infants with birth weight < 500 g were excluded; or

b. Infants who received total parenteral nutrition and were nil per os (NPO) were excluded; or

c. Premature infants with a birth age of < 25 weeks' gestation were excluded; or

d. Infants with a pre- or postnatal diagnosis of gastroschisis, large omphalocele or congenital diaphragmatic hernia did not form part of the study population; or

e. Infants with congenital intestinal obstruction or perforation were excluded; or

f. Infants with major congenital malformations and/or developmental disabilities pertaining to the gastrointestinal tract were excluded; or

g. Infants with a diagnosis of a complex heart disease were excluded; or

h. Infants with perinatal asphyxia were excluded. Perinatal asphyxia was defined as an Apgar score < 3 at five minutes and the need for resuscitation after birth.

Table 1: Some individual drugs contraindicated for breastfeeding mothers[12]

Medication	General concern and specific effects
Chloramphenicol	Can cause possible idiosyncratic bone marrow suppression.
Sulfapyridine, sulfisoxazole	Use with caution in infant with jaundice or G6PD deficiency and ill, stressed or premature infant.
Iodides, iodine	Can cause goitre.
Clofazimine	Potential for transfer of a high percentage of maternal dose; possible increase in skin pigmentation.
Corticosteroids	When given to the mother in large doses for weeks or months, high concentrations can be achieved in breast milk. This may suppress growth and interfere with endogenous corticosteroid production in the infant.
Lamotrigine	Potentially therapeutic serum concentrations can be reached in the infant.
Aspirin (salicylates)	Metabolic acidosis may occur in the infant. With large maternal doses and sustained use, breastfed infants < 1 month may achieve plasma concentrations that increase the risk of hyperbilirubinemia.
Clemastine	Can result in drowsiness, irritability, refusal to feed, a high-pitched cry and neck stiffness.
Primidone	Can cause sedation and feeding problems.

2.8.3 Exit criteria and adverse events

Infants who conformed to the exit criteria of the study were followed-up. Treatment (probiotic/placebo) was withdrawn and daily monitoring was continued. All exit criteria were discussed with the attending neonatologist. Participants were exited from the study in the following circumstances:

a. Infants who were NPO remained in the study until enteral feeds had been recommenced. These infants did not receive probiotic/placebo supplementation until feeds had been restarted;

b. Infants who had been NPO for more than three days and required the use of total parenteral nutrition were exited from the study, and probiotic/placebo supplementation was not reinstated. These infants remained part of the study population and continued to be assessed on a daily basis without probiotic/placebo supplementation;

c. Infants who developed any stage of NEC according to Bell's criteria were exited. Although Bell's Stage I is not recognised as definite NEC, it still has the potential to evolve into Stage II or Stage III NEC if managed inappropriately. For the purpose of this trial, infants with Stage I NEC were included in the analysis as no information existed on the effect of probiotics on HIV-exposed VLBW infants;

d. Infants who developed diarrhoea that lasted for more than three days were removed from the study. The enteral feeds of these infants were stopped and, for this reason, these infants no longer conformed to the inclusion criteria of the study (see 2.8.2.1);

e. HIV-exposed infants with a positive polymerase chain reaction (PCR) result after Day 14 of life did not remain in the study. The aim of this study was to determine the outcome of HIV-exposed infants;

f. Infants who developed an adverse event attributed to the use of probiotics, such as septicaemia with positive blood cultures containing probiotic strains,[14] were

also exited from the study. The following major risk factors for probiotic sepsis were proposed:

- An immune-compromised state, including a debilitating state, such as advanced HIV;
- Prematurity.[15]

g. All infants were exited at the end of the neonatal period, in other words Day 28 of life.

2.9 SUBJECT RECRUITMENT

2.9.1 Establishment of the HIV-exposed and HIV-unexposed groups

All admitted preterm VLBW infants in Ward G2 in TBCH who conformed to the inclusion and exclusion criteria with informed parental consent formed part of the total study population. Assessment continued for those patients who had been transferred to wards G1, G8 and J3 and the KKMC unit during the study period.

The HIV-exposed group consisted of all consenting HIV-infected mothers who had a confirmed HIV-infected rapid-test result, as obtained from the PMTCT programme during the prenatal period. The HIV-unexposed group consisted of all consenting HIV-uninfected mothers.

A total of 184 infant participants were included in the study. The HIV-unexposed group consisted of 110 participants. It was expected that the recruitment of the HIV-unexposed group would be faster than that of the exposed group. The HIV-exposed group consisted of approximately 20% of infants born, according to statistics (see 2.8.1). Due to limited funding and resources, data collection for the HIV-exposed group was halted at 74 participants, with the agreement of the statistician.

2.9.2 Establishment of the control and study groups

Study infants were allocated to the HIV-exposed or HIV-unexposed groups according to the mother's HIV status. All consenting HIV-infected mothers who had a confirmed HIV

rapid-test result, as obtained from the PMTCT programme during the prenatal period, were included in the study.

Randomisation was performed by HIV status; therefore, the exposed and unexposed infants were randomised separately. Participants from the two groups (HIV-exposed and HIV-unexposed) received a sequential number as they were entered into the study (hereafter described as the participant's number). Participants were randomised into the study or control groups (probiotic vs. placebo supplementation) with a random number sequentially allocated to each participant. All randomisation procedures were completed by the study statistician before commencement of the study. The attending physician and nurses caring for the infants were blinded to the group assignment. The investigator, research assistants and study participants were also blinded to the group assignment. Participants were exited from the study on Day 28 after birth (end of the neonatal period) or when infants were discharged from the hospital, if this was earlier.

2.10 METHODOLOGY: MEASUREMENTS, METHODS AND INSTRUMENTS

The methodology of this study consisted of different components. Firstly, infants were supplemented with either the probiotic or the placebo, breast milk samples were collected from the mother three times during the 28 days of the study and maternal saliva samples were collected as soon as possible after participant enrolment. Secondly, infants were weighed on a daily basis, and length and head circumference were measured on five occasions during the 28-day follow-up period. Lastly, infants were assessed on a daily basis, and clinical, medical and nutritional data were collected. It was also during these assessments that the presence of NEC was evaluated. The specific measurements, methods and instruments used to collect the data will be discussed in further detail below.

2.10.1 Probiotic and placebo supplementation

2.10.1.1 Blinding and storage of probiotic and placebo

The probiotic used in this trial was Pro-B2, produced and manufactured by C Pharm. Pro-B2 is an oil suspension that is stable at room temperature (see Addendum A for stability reports). The product was stored at cool room temperature in sealed containers. Blinding of the product was done by C Pharm, a pharmaceutical company based in Cape Town, South Africa. Medium-chain triglyceride (MCT) oil was chosen as the most suitable control agent with aesthetical properties similar to those of Pro-B2. No differences in the colour and appearance of the probiotic and placebo were noted. The probiotic and placebo were blinded with the use of a colour-coded label (orange or purple). After an infant had been included in the study and informed consent had been granted, the infant received a participant number. Each participant number had an allocated colour code (orange or purple). Each infant had his/her own container of either probiotic or placebo (labelled as orange or purple) for the 28 days that he/she was enrolled in the study. The participant number was noted on the container to ensure anonymity. Colour-coded (orange or purple) containers were kept in separate plastic holders. The investigator and assistants were blinded throughout the study. Blinding was terminated once the data collection had been completed.

2.10.1.2 Supplementation with the probiotic or placebo

Lactobacillus rhamnosus GG and *Bifidobacterium infantis* have been used as probiotics to reduce the incidence of NEC.[11] The product used in the current study was Pro-B2, which contains *L. rhamnosus GG* and *B. infantis*. The probiotic and placebo were supplied by C Pharm. The study group received *L. rhamnosus GG* and *B. infantis* for four weeks (28 days). This provided *L. rhamnosus GG* (0.35×10^9 colony-forming units [CFU]) and *B. infantis* (0.35×10^9 CFU) daily. The control group received MCT oil. A daily dose of five drops of the probiotic/placebo was administered to the infants. Table 2 indicates the daily supplement dose for the study and control groups.

Table 2: The daily supplement dose Pro-B2 that the study group received

Probiotic strain	**Five drops Pro-B2**
Lactobacillus rhamnosus GG	500 million cells
Bifidobacterium infantis	500 million cells
Total probiotics (CFU)	**1 billion cells (1 X 10^9 CFU)**

The probiotic/placebo was administered by the research assistants or investigator who followed the group randomisation assignment. Supplementation was monitored daily by the investigator or the research assistants. The date, time and signature of the research assistants or investigator were recorded each time that administration of the probiotic/placebo took place.

Hygiene practices were strongly adhered to during this study. Before supplementation with the probiotic/placebo, the investigator or research assistants washed their hands with an antiseptic soap and thereafter D-germ (a sterilising spray) was applied. Only after this had been done the infants received their supplementation.

The standard feeding protocol of the ward applied during the study. The breast milk of HIV-infected mothers was pasteurised using the flash method. Pasteurisation is standard protocol in the ward and was therefore not part of the methodology of this study. Flash heating will, however, be briefly described. Four hundred and fifty millilitres of water was brought to a boil in an aluminium pot and then removed from the heat. Breast milk in a glass peanut butter jar with a lid was placed in the water bath for 20 minutes; it was then removed from the water bath, uncovered and allowed to cool to 37 ºC.[16] Pasteurisation decreases HIV activity in human milk and has been found to be an effective way of preventing mother-to-child transmission of the virus.[17]

2.10.1.3 Events that may have affected the administration of the probiotic/placebo

The following probiotic/placebo supplementation guidelines were followed when the following events occurred:

- When newborn infants were admitted to Ward G2 in TBCH and were NPO, the probiotic/placebo was not administered until enteral feeds had commenced.
- Infants with reoccurring apnoea who were NPO did not receive the probiotic/placebo until the infant had been started on enteral feeds again.
- Infants with reoccurring apnoea who were not NPO received their probiotic/placebo supplementation through the enteral feeding tube and not orally. The probiotic was added to the mothers' breast milk and inserted into the feeding tube with a sterile syringe.
- When suspected NEC was noted in the patient folder, the infant continued with treatment until an abdominal X-ray had confirmed a diagnosis of NEC Stage I. If the infant remained a suspected NEC case and was NPO, the infant did not receive a probiotic/placebo until the enteral feeds had recommenced.

2.10.2 Breast milk samples

A full sample of expressed breast milk was used for the determination of prebiotic quality. Samples of HIV-infected and HIV-uninfected breast milk were tested. All breast milk samples were raw untreated specimens; therefore, milk samples did not undergo any pasteurisation procedures. Breast milk was collected in sterilised containers with lids. Expression of breast milk was by hand. Each sample contained a volume of 5-10 ml of breast milk for analysis.

It has been found that the highest oligosaccharide concentration is present on Day 4 of lactation and that it decreases by about 10% by Day 10 and by another 10% by Day 30.[18] The frequency at which the milk samples were collected was determined according to these results. Three milk samples were collected from each mother on the following days:

Milk sample 1: The first milk sample was collected on Day 4 of lactation.

Milk sample 2: The second milk sample was collected on the tenth day of lactation, namely six days after the first sample had been collected.

Milk sample 3: The third sample was collected on the final day of the study, Day 28 after birth and the end of the neonatal period.

If a mother was unavailable during the breast milk sample collection, the breast milk samples were collected at the first available opportunity. All milk samples were collected by the investigator or research assistants in a manner standardised for all protocol procedures. Milk samples were stored at -25 ºC prior to oligosaccharide extraction.

2.10.2.1 Subject selection for human milk oligosaccharide analysis

Out of the 184 mother-infant pairs enrolled in the original clinical trial, all infants who developed NEC and whose mothers had sufficient milk samples available for analysis were included in the substudy. Thereafter, using a random number sequence table, the study statistician equally included mother-infant pairs stratified according to the mother's HIV status (infected/uninfected) and the mother's secretor status (secretor/nonsecretor). When the included mother-infant pair did not have sufficient milk samples available, the next random participant sample was selected. In total, 82 mother-infant pairs (41 with HIV-infected and 41 with HIV-uninfected mothers) were included in the substudy, and the mothers' breast milk was analysed for oligosaccharide composition

The breast milk samples were analysed for oligosaccharide content at the University of California, San Diego, by Dr. Bode. The appropriate routes were followed to ensure export of the samples. The South African Department of Health (DOH) and Stellenbosch University had approved the export. The material transfer agreement and DOH permit are included in Addendum B. The samples were stored in sealed ziplock plastic packets and placed in a large waterproof container. Each container had dry ice inserted to ensure that the samples were kept frozen during transport. After the samples had been delivered at the University of California, the investigator was notified that the samples were intact and frozen.

2.10.2.2 Laboratory techniques used to analyse breast milk samples

Raffinose was added to 10 µL milk to serve as an internal standard. Samples were applied to HyperSEP HyperCarb columns, followed by HyperSEP C18 columns to remove lipids, proteins and salt. Human milk oligosaccharide (HMO) eluates were

lyophilised and labelled with 2-aminobenzamide (2AB) for two hours at 65 ºC. Samples were applied to HyperSEP Silica columns to remove unlabelled 2AB. HMO-containing eluates were incubated with beta-galactosidase for four hours at 37 ºC to remove lactose. 2AB-labelled HMO was analysed by HPLC on an amide-80 column (4.6 mm ID x 25 cm, 5 µm, Tosoh Bioscience, Tokyo) with a 50-mm ammonium formate/acetonitrile buffer system. Separation was performed at 25 ºC and monitored with a fluorescence detector at 360 nm excitation and 425 nm emissions. Peak annotation was based on standard retention times and mass spectrometric analysis on a Thermo LCQ Duo Ion trap mass spectrometer equipped with a Nano-ESI-source. HMO concentrations in nmol/ml were calculated based on the area under the curve for each HMO relative to the area under the curve for the internal standard raffinose. HMO concentrations in mg/ml were calculated based on the molecular weight of the respective oligosaccharide.[19]

2.10.3 Saliva samples

A saliva sample was collected from all consenting mothers to determine the ABH secretor status. This refers to the secretion of ABO blood group antigens. Saliva samples were collected in sterilised containers with lids. A saliva sample of 1–2 ml (excluding foam) was sufficient for analysis. All saliva samples were collected by the investigator or research assistants in a manner standardised for all protocol procedures. Saliva samples were taken by the investigator to the National Health Laboratory Service, situated in TAH, directly after collection for analysis. The collection of saliva samples was carried out as follows:

The participants were instructed to allow saliva to pool in the mouth. With the head tilted forward, the saliva sample was collected in the test tube. The test tubes were twice the capacity of the desired sample volume that was used as the saliva was expected to foam.

2.10.3.1 Laboratory techniques to determine salivary secretor status

Samples were analysed by Mrs. Rosemary de Beer, a medical technologist at TAH's Blood Grouping Department. The following laboratory techniques were used to analyse the saliva samples that had been collected as described above[20, 21]:

- One to two millilitres of saliva was collected and placed in the autoclave at 120 ºC for 10 minutes and then spun down at 3 200 rpm for 10 minutes.
- The clear supernatant was transferred to a clean tube and frozen at -20 ºC until tested.
- After thawing, saliva was again spun at 3 200 rpm for 10 minutes.
- The reagent used was Anti-H (*Ulex europaeus*), manufactured by Immucor Gamma.
- In a test tube, two drops Anti-H were added to two drops of saliva and mixed.
- This was incubated at room temperature for 10 minutes (neutralisation stage).
- One drop of 3–4% Group O red blood cells in saline was added and mixed.
- This was incubated for five minutes at room temperature.
- Tubes were then spun for 20 seconds at 900 rcf, gently resuspended and read macroscopically for agglutination.
- Positive and negative controls were included.

If the red blood cells were agglutinated, the Anti-H reagent was not neutralised by H substance and the saliva was from a nonsecretor; if the red blood cells were not agglutinated, the saliva contained H substance, which neutralised the reagent, so no agglutination was observed.

2.10.4 Clinical, medical and nutritional data

The clinical, medical and nutritional data were collected on four data collection forms (please refer to Addendum C). These data collection forms consisted of the following forms: the patient medical background, daily monitoring, infection screening and NEC

screening forms. Some of the sections in these data collection forms were repeated in the various forms, such as the blood sample collection section, birth anthropometrical measurements, infant medication received and phototherapy treatment, which merely served as a cross-check for quality control purposes. The data collection forms had been precoded. The coding list was supplemented as new possibilities/options occurred.

2.10.4.1 Patient medical background data collection form

This form was used to collect medical background information of the infants as well as the mothers. This information was documented by the investigator or research assistants upon the infants' entry into the study. The maternal information was divided into three sections and included general information, medical information and medication received. The infant information also consisted of these three sections with the addition of a section on blood sample collection. The blood samples collected in this study were all routine blood samples taken in the ward, and they were not specifically collected for this study.

The mode of delivery, namely either normal delivery or caesarean section, was noted for all mothers participating in the study. The premature rupture of membranes was noted. This information was documented by the investigator or research assistants upon the infant's entry into the study.

Table 3: Medical background information

Maternal information	
General information	• Age • Race • Gravida/parity • Mode of delivery • Whether prolonged rupture of membranes had occurred • Maternal HIV status • Maternal CD4 cell count • Whether the mother was enrolled on the PMTCT scheme and the duration of enrolment • Maternal tuberculosis history and treatment • Maternal syphilis (Venereal Disease Research Labarotory [VDRL] test) history and treatment
Medical information	Whether the following conditions were applicable to the mother: • Hypertension/preeclampsia • Diabetes • Antepartum haemorrhage • Abruptio placentae
Maternal medication	Maternal antibiotics and steroids given prior to/during labour were noted.

Table 3 continued: Medical background information

Infant information	
General information	• Gender • Date of birth • Apgar counts • Birth weight, length and head circumference • Gestational ages – dates • Ballard score • Infant syphilis (VDRL test) status and whether being treated
Medical information	• Respiratory complications Whether the infant had undergone any of the following: • Cardiac echo • Cranial ultrasound • Chest X-ray • Phototherapy
Infant medication	The following medications were noted: • Nevirapine • AZT • Surfactant • Antibiotics • Other

Table 3 continued: Medical background information

Infant information	
Blood sample collections	Whether the following blood samples had been collected: • CRP • Full blood count at birth • Blood culture • Blood gas at birth • HIV PCR after Day 14 of life

2.10.4.2 Daily monitoring form

Table 4 indicates the routine examinations and the complications related to the dysfunction of an immature organ system that were documented. The daily clinical assessments of the study babies by the attending doctors were documented by the investigator or research assistants.

Table 4: Complications related to the dysfunction of an immature organ system that were documented[22]

Organ/area that complication affected		Data that were documented
Lungs	Respiratory distress syndrome (RDS)	Medical notes and diagnosis of RDS were documented.
Central nervous system	Poor sucking and swallowing reflexes before 34 weeks' gestation Apnoea due to hypopharyngeal obstruction or immaturity of the respiratory system in the brain	Medical notes were documented.
Infections	Sepsis	Haematology results: White blood cell count, platelet count and bacterial cultures were documented.
Temperature regulations	Hypothermia	Medical notes were documented.
Metabolic problems	Hypoglycaemia and hyperglycaemia	Haematology results and medical notes were documented.

All medications, intravenous fluids and blood transfusions administered to the study infants were documented on the medical form by the investigator or research assistants. ARV treatment was documented for the HIV-exposed infants.

2.10.4.2.1 Daily feeding volumes

The time of initial feeding was at the discretion of the attending physician. The daily volume of milk feeds was determined according to the feeding protocol of the institution. According to ward protocol, feeds are increased in daily increments of 25 ml/kg/d. Furthermore, per ward protocol, breast milk fortifier is introduced when infants reach daily feeds of above 100 ml/kg. Fortification of feeds is, however, dependent on feeding

tolerance and may differ from one individual to another. The volume of feeds was advanced slowly, if tolerated by the infant, and feeding was stopped if there were any signs of feeding intolerance such as vomiting or abdominal distension. The dietary intake of all study infants was documented on a feed monitoring form. Any feeding intolerance was documented.

The following data were captured on the daily monitoring form:

- The daily administration of a probiotic or placebo and the amount (number) of drops given.
- The feeding route of the infant.
- The infant's weight, head circumference and length.
- The volume/amount of breast milk, Neonatelyte (glucose-electrolyte-containing solution) and saline provided to the infant.
- Supplementation with FM85 (a breast milk fortifier) and MCT oil, multivitamin and iron supplementation.
- The urinary output.
- The stool frequency and colour/consistency.
- Phototherapy and blood products received.
- Medication administered. All the medications that the infants received were noted.

The methods and techniques pertaining to the anthropometrical measurements will now be discussed. No anthropometric measurements were taken for the time period that infants received nasal continuous positive airway pressure (NCPAP). The following anthropometric measurements were taken:

2.10.4.2.2 Weight

All study infants, irrespective of whether or not these infants were incubated, were weighed daily by the nursing staff. Daily weights were recorded from each participant's medical file by the investigator or research assistants. Any significant weight changes were queried, and the measurement was repeated under the supervision of the investigator or research assistants.

The weight of each infant was determined as follows[23]:

- An electric scale was used.
- Electronic scales were calibrated once a week by the nursing staff.
- All instruments were zero calibrated before beginning the measurements.
- Infants were weighed without any clothing or a nappy. If a nappy was worn, the weight was corrected by subtracting the weight of the nappy.
- The infant was placed in the middle of the scale and had to lie still before the reading was taken.
- The measurements were read to the nearest 0.001 kg.
- The average of three measurements was taken.

2.10.4.2.3 Length

Infants were measured on Day 1 of the study; thereafter, the length was measured weekly. Upon exiting the study (Day 28), the length was measured again; therefore, five measurements, on days 1, 7, 14, 21 and 28, were taken. Length measurements were performed by the investigator or research assistants, with the help of the infant's mother, if needed.

The length of each infant was measured according to the following criteria:

- A standard nonstretchable measuring tape with 0.5-cm and 1-cm dimensions was used.

- All head coverings and socks were removed.[23]
- The infant was placed supine on the tape measure with the head and shoulders held by an assistant.
- The observer then extended the right leg and held the ankle at right angles to the surface. The length from the infant's crown to heel was then measured.
- Measurements were read to the nearest 0.5 cm.[23]
- The average of three measurements was taken.

2.10.4.2.4 Head circumference

Each infant's head circumference was measured on Day 1 of the study and subsequently measured on a weekly basis. Upon exiting the study (Day 28), the head circumference was measured again. Five measurements, on days 1, 7, 14, 21 and 28, were therefor recorded. Measurements were performed by the investigator or research assistants, with the help of the infant's mother, if needed.

The head circumference of each infant was measured according to the following criteria[23]:

- A standard nonstretchable measuring tape with 0.5-cm and 1-cm dimensions was used.
- All head coverings were removed.
- The measuring tape was placed on the supraorbital ridge and on the occiput.
- The tape was on the same plane on both sides of the head.
- Measurements were read to the nearest millimetre.
- The average of three measurements was used.

All infants' weight, height and head circumference measurements at birth and Day 28 of life were plotted on the Fenton-Babson growth chart (Addendum D).[24]

2.10.4.3 Blood cultures, routine bloods, blood gas and infection screening form

As infection may be associated with the use of probiotics in premature infants, all episodes of infection were monitored. A routine HIV DNA PCR test was done on every HIV-exposed baby after Day 14 postpartum. These results were documented by the investigator or research assistants as soon as the results became available. The results of laboratory investigations requested by the attending doctors, namely full blood counts and CRP assays, were documented.

The following data were captured on this form:

- CRP results.
- Culture results (blood cultures, stool cultures, swab cultures and lumbar puncture cultures).
- Full blood count results.
- HIV PCR results after Day 14 of life.
- Blood gas results.
- Possible sepsis diagnosis by the attending neonatologist.

2.10.4.4 Necrotizing enterocolitis screening form

Infants were evaluated daily for the development of NEC by the attending neonatologists. Whenever a study infant was suspected to have NEC, the infant was also evaluated by the attending neonatologist. If the diagnosis of NEC was made, it was staged according to Bell's classification, as outlined below. The following diagnostic criteria of NEC, according to Bell's staging, were used.[1] This NEC staging system is based upon historical, clinical and radiographic data.

Stage I (suspect)

Any one or more of the following historical factors producing perinatal stress:

- Systemic manifestations, such as temperature instability, lethargy, apnoea or bradycardia, are present.

- Gastrointestinal manifestations, for example poor feeding, increasing pregavage residuals, emesis (may be bilious or test positive for occult blood), mild abdominal distension or the presence of occult blood in the stool, are present.
- Abdominal radiographs may show distension with mild ileus.

Stage II (definite)

Any one or more of the following historical factors:

- The above signs and symptoms are present, along with persistent occult or gross gastrointestinal bleeding and marked abdominal distension.
- Abdominal radiographs show significant intestinal distension with ileus, small bowel separation (oedema in bowel wall or peritoneal fluid), unchanging or persistent 'rigid' bowel loops, pneumatosis intestinalis or portal vein gas.

Stage III (advanced)

Any one or more of the following historical factors:

- The above signs and symptoms are present, and there is deterioration of vital signs, evidence of septic shock or marked gastrointestinal haemorrhage.
- Abdominal radiographs may show pneumoperitoneum, in addition to the other anomalies listed in Stage II.

The basic feeding patterns (with any evidence of feeding intolerance), clinical staging, an abdominal X-ray and growth data for all infants were recorded during the 28 days of the study. The following data were captured on the NEC screening form:

- Feeding intolerances: For the purpose of this study, a feeding intolerance was identified when vomiting and aspirates occurred. The colour of vomits and aspirates were noted. Furthermore, abdominal distension and stool volume and consistency were noted on the daily monitoring form.
- The presence of abnormal clinical signs. The following were noted as abnormal signs: Breathing > 65/min, apnoea, bradycardia (< 100 beats/min),

tachycardia (> 180 beats/min), abdominal distension, convulsions, lethargy and jaundice.

- The presence of bowl sounds.
- Possible NEC complications (bowl perforations, shock).
- The presence of an abdominal drain.
- Bell's staging grade.
- Whether an abdominal X-ray had been performed.

2.11 DATA COLLECTION

The data collection took place at TBCH and KKMC. Patients who had been admitted or transferred to wards G1, G2, J3 or G8 were recruited for the study upon conforming to the inclusion and exclusion criteria (see 2.8.2.1 and 2.8.2.2). Infants who had been transferred to the KKMC unit continued to be included in the study. Written informed consent was obtained from the mother of each study infant after the full procedure had been explained to her, and she had the opportunity to ask any questions or clarify any uncertainty. The data were collected by the investigator and two research assistants. The investigator as well as the research assistants had received good clinical practice (GCP) training. The research assistants were a qualified nurse and a dietician. The assistants had received a month's training to conform to the data collection procedures and to ensure standardisation. Data collection took place from 4 July 2011 to 31 August 2012.

2.12 DATA CAPTURING

Data capturing was done using Microsoft Excel 2007®.

2.13 STATISTICAL ANALYSIS

Microsoft Excel® was used to capture the data, and STATISTICA version 11 (StatSoft Inc. [2013] STATISTICA [data analysis software system], www.statsoft.com.) was used to analyse the data. Data were analysed by the investigator, with the help of a statistician.

Summary statistics were used to describe variables such as the breast milk intake data of infants, routine examinations, medication, intravenous fluids, blood transfusions and mode of delivery. Distributions of variables were presented with histograms and/or frequency tables. Medians or means were used as the measures of central location for ordinal and continuous responses and standard deviations and quartiles as indicators of spread. Descriptive statistics were used to describe the incidence of NEC among the study infants. ANOVA was used to detect significant associations between the breast milk samples of HIV-infected and HIV-uninfected mothers.

The relationships between continuous response variables and nominal input variables, for example different diets, were analysed using appropriate ANOVA or pooled or Welch t-tests if only two groups were involved. Appropriate repeated measures ANOVA were used when responses were measured at specific time intervals. When only two times were compared, this was done with paired t-tests.

When ordinal response variables versus a nominal input variable were compared, nonparametric ANOVA methods were used. For completely randomised designs, the Mann-Whitney test (for two groups) was used, and for repeated measures designs at two times, the Wilcoxon test was used.

Two factors influenced the variables, namely treatment (probiotic or placebo) and HIV exposure (HIV exposed or HIV unexposed). Two-way ANOVA was done to compare the treatment means per HIV exposure (in other words, to check the interaction effects), and no differences were observed. The residuals were checked for normality, and if they were nonnormal, bootstrap multiple comparisons were done to accommodate for the nonnormality. This analysis confirmed the nonsignificant interactions.

Furthermore, these variables were also compared with Mann-Whitney nonparametric tests for the treatments per HIV-exposure group, which confirmed the nonsignificance detected with the bootstrap multiple comparisons. The Mann-Whitney tests were reported, and the median (lower quartile, upper quartile) was included in the reported data (tables and text).

The relation between nominal variables was investigated with contingency tables and appropriate chi-square tests, such as the likelihood ratio chi-square test. A p-value of $p < 0.05$ represents statistical significance in hypothesis testing, and 95% confidence intervals were used to describe the estimation of unknown parameters.

2.13.1 Anthropometric measurements of infants

All infants' weight, length and head circumference measurements from birth to Day 28 of life were plotted on the Fenton-Babson growth chart (Addendum D).[24] Birth weight was classified as small for gestational age (SGA), appropriate for gestational age (AGA) or large for gestational age (LGA). Infants were subclassified as being VLBW and ELBW. Growth restriction (symmetrical and asymmetrical) was determined with the use of the weight, length and head circumference. A birth weight below the 10th percentile was used to identify infants who were SGA. Z-scores were used in the interpretation of anthropometrical data as these are superior to percentiles for infants whose size is outside the normal range of a growth chart, that is, beyond the 3rd and 97th percentiles.[25] Z-scores were calculated with the use of Microsoft Excel® spreadsheets provided by Professor Fenton (see URL link ucalgary.ca/fenton).[26] These calculated spreadsheets were developed according to the Fenton-Babson growth chart.[24]

2.14 PILOT STUDY

A pilot study was performed to streamline all data collection procedures. All samples (breast milk and saliva) were collected during the pilot study. Ten pilot study participants were recruited for a period of one week. Since the protocol was not affected or changed during the pilot study period, it was decided that the participants in the pilot study could

be included in the main study. The use of these data was approved by the Human Research Ethics Committee of the Faculty of Health Sciences, Stellenbosch University.

2.15 FINANCIAL DISCLOSURE

Funding for this study was received from various sources, the main sources being the National Research Foundation, Nestlé Nutrition Institute Africa, the Medical Research Council and the Faculty of Medicine and Health Sciences, Stellenbosch University.

2.16 ETHICS APPROVAL

The study protocol was submitted to the Human Research Ethics Committee of the Faculty of Health Sciences, Stellenbosch University and TAH for approval (M10/09/035). The researcher herewith discloses that the study protocol was also submitted and approved by Karl Bremer Hospital for the continuation of data collection at the facility in the event that infants were transferred from TAH. Karl Bremer Hospital was, however, never used as a study site during the study period, so the details for this are not included in the study setting description (see 2.6). The South African clinical trial registration number was DOH-27-0413-4277. The trial was, furthermore, registered at the United States National Institutes of Health registry (www.clinicaltrials.gov), with reference number NCT01868737. Insurance for this study was provided by Stellenbosch University. The intellectual property of the study protocol will remain that of Stellenbosch University.

2.17 GOOD CLINICAL PRACTICE

2.17.1 Informed consent and voluntary participation

The investigator and all others involved in the study adhered to GCP guidelines during the study period. All participating mothers were informed about the study in their first language (Afrikaans, English and Xhosa) (see Addendum E). In some instances,

participants received 24 hours to consider partaking in the study before the written consent form was signed. Each participant received a copy of the consent form. Participating in the study was voluntary. Participants could have left the study at any time and were not penalised or prejudiced in any way for doing so.

2.17.2 Confidentiality and quality of care

Confidentiality and anonymity were maintained during this study. All mothers received an allocated participant number that was used for the identification of milk samples as well as allocation to the control or study group. The attending physician and nurses caring for the infants were blinded to the group assignment, as were the researcher and research assistants. The HIV status of the mother and her infant was treated with confidentiality and was not revealed to any persons other than the nursing staff, the patient's doctor, the research assistant and the investigator.

The milk samples of the control and study groups were collected and analysed in the same manner, thereby maintaining patient confidentiality. No feeds were withheld from any study infant due to milk sample collection. If an infant needed to be fed at the time of milk sample collection, the infant was first either breastfed or fed expressed breast milk that had been collected previously. Infants of HIV-infected mothers received pasteurised expressed breast milk. Infants of HIV-uninfected mothers received unpasteurised breast milk (expressed or breastfed).

Participation in this study did not affect the quality of care that infants and mothers received during or after the study. Appropriate medical care was provided to HIV-infected mothers as determined by the provincial protocol for the treatment of HIV/AIDS.

2.17.3 Probiotic product (Pro-B2)

The product (Pro-B2) was supplied by C Pharm. It is registered with the Medicines Control Council (no 133279). It is classified as a complementary medicine. Stability reports (see Addendum A) were provided by C Pharm twice during the study duration, in other words every six months. The product proved to be stable during the study period. The results were analysed and repeated according to ethical standards.

2.17.4 Potential risk factors attributed to the use of probiotics.

A potential risk factor attributed to the use of probiotics is septicaemia. Any infant with a positive blood culture containing a probiotic strain was exited from the study. Furthermore, an immune-compromised state such as advanced HIV and prematurity are major risk factors for septicaemia.

2.18 POTENTIAL INDIVIDUAL AND COMMUNITY BENEFITS

The primary objective of the study was to evaluate the efficacy of probiotics in reducing the incidence and severity of NEC in premature, VLBW infants exposed to HIV. Prophylactic probiotic treatment could be an effective and appropriate way of reducing the incidence of NEC in this highly vulnerable population. A reduction in the incidence of NEC will decrease medical expense, decrease length of hospital stay and improve overall patient outcomes. Secondly, this study aimed to determine the prebiotic quality (human milk oligosaccharides) of the breast milk of HIV-infected and HIV-uninfected mothers and to determine whether there was an association between the prebiotic quality (human milk oligosaccharides) and NEC. The results of this study could strengthen the views and perspectives of mothers and medical personal on the importance of breastfeeding. HIV-infected mothers might become more favourable to breast milk, thereby decreasing the household financial burden and improving hygiene and safety measures. Prebiotics are available in breast milk, making the study highly sustainable as breast milk is free.

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Chapter 3

RESULTS

ARTICLE 1

PROBIOTICS AND NECROTIZING ENTEROCOLITIS IN HIV-EXPOSED PREMATURE INFANTS.

Probiotics and necrotizing enterocolitis in HIV-exposed premature infants: a randomized controlled trial

Evette Van Niekerk, Author[1], BSc, M Diet. Gert F Kirsten, Co-Author[2], MbChB, MMed (Pead), DCH (SA), FCP(Pead)(SA), MD. Reneé Blaauw[3], Co-Author[1], PhD Nutrition. Daniel G Nel, Co-Author[4], PhD.

Affiliations:

[1] Division Human Nutrition; Faculty of Medicine and Health Sciences; Stellenbosch University. PO Box 19063; Francie van Zijl Drive. Tygerberg 7505. South Africa. Email: evettev@sun.ac.za

[2]Department of Pediatrics and Child Health, Division of Neonatology. Faculty of Medicine and Health Sciences, Stellenbosch University. PO Box 19063; Francie van Zijl Drive. Tygerberg 7505. South Africa. Email: gfk@sun.ac.za

[3] Division Human Nutrition; Faculty of Medicine and Health Sciences; Stellenbosch University. PO Box 19063; Francie van Zijl Drive. Tygerberg 7505. South Africa. Email: rb@sun.ac.za

[4]Department of Statistics and Actuarial Science, Stellenbosch University. Email: dgnel@sun.ac.za

Address correspondence to: Evette van Niekerk. Division Human Nutrition; Faculty of Medicine and Health Sciences; Stellenbosch University. PO Box 19063; Francie van Zijl Drive. TYGERBERG 7505. South Africa. Telephone: +27 21 938 9474. Fax:+27 21 933-299 (evettev@sun.ac.za)

Abstract

Background: An association between maternal human immunodeficiency virus (HIV) infection and Necrotizing Enterocolitis (NEC) in preterm infants has been reported. The impact of probiotics in an HIV-exposed very low birth weight infant on the occurrence of NEC is uncertain at present; however it is known that probiotics have protective effects against inflammation and prevent NEC.

Objective: To assess the effect of probiotics on the incidence of NEC in high risk infants born to HIV-positive and HIV-negative women.

Patients and Methods: HIV-exposed and HIV-unexposed premature (<34 weeks gestation) infants with a birth weight of ≥500g and ≤1250g were randomized to either the probiotic or placebo group. The probiotic consisted of $1x10^9$ CFU, *L. rhamnosus GG* and *B. infantis* per day and was administered for approximately 4 weeks. NEC was graded according to Bell's criteria.

Results: 74 HIV-exposed and 110 HIV-unexposed infants were enrolled and randomized (mean birth-weight, 987g; mean gestational 28.7 weeks). The incidence of death and NEC did not differ significantly between the HIV-exposed and unexposed groups but a significant difference (p=0.029) was found for total NEC incidence between the study and control groups.

Conclusion: Probiotic supplementation reduced the incidence of NEC in the premature very low birth weight infants; however results failed to show a lower incidence of NEC in

HIV-exposed premature infants. A reduction in the severity of disease was found in the HIV-exposed group.

Clinical trial registration number: NCT01868737

The South African clinical trial registration number: DOH-27-0413-4277.

Key words: Human Immunodeficiency Virus, Necrotizing Enterocolitis, Premature infant, Probiotic, Very Low Birth Weight.

Introduction:

Necrotizing enterocolitis (NEC) is the most common gastrointestinal insult in preterm infants and major cause of infant mortality in neonatal intensive care units. [1] NEC occurs in 4–11% of all premature infants born with a very low birth weight (VLBW) and an inverse relationship exists between birth weight, gestational age and the incidence of NEC.[1, 2] The precise pathogenesis of NEC is still unclear but is widely considered to be multifactorial [3] The most common risk factors are prematurity, enteral feeding, gut ischemia, patent ductus arteriosus (PDA), packed red blood cell (PRBC) transfusion and bacterial colonization. [4-6]

In recent literature an association between maternal human immunodeficiency virus (HIV) infection and NEC in preterm infants has been reported.[7] Furthermore, the prevalence of low birth weight (LBW) and preterm birth among infants who are born to HIV infected women has increased as treatment has evolved into regimens of highly active antiretroviral therapy (HAART).[8] The exposure to HIV during fetal life and maternal treatment with antiretroviral drugs are two probable mechanisms proposed to explain the increased incidence of NEC in this population.[9] Changes in the cytokine environment in pregnancy due to maternal HAART use has been hypothesized to increase the risk for preterm delivery observed in HIV-infected woman.[10] An inverse relationship exists between gestational age and the incidence of NEC.[11] It is therefore hypothesized that the increase in lower gestational ages is associated with the increased risk of NEC in infants born to HIV-infected mothers.

Probiotic bacteria are live microorganisms that colonize the gastrointestinal tract and potentially provide benefit to the host.[12, 13] There is increasing interest in the potential health benefits of proactive colonization of the gastrointestinal tract of preterm infants.[14] Probiotics may prevent NEC by promoting colonization of the gut with beneficial organisms, preventing colonization by pathogens, improving the maturity and function of the gut mucosal barrier, and modulating the immune system.[15] The most frequently used probiotics are *Lactobacillus* and *Bifidobacterium.*[15, 16] The potential impact of probiotics in an HIV-exposed VLBW preterm infant on the occurrence of NEC is at present uncertain. In view of this uncertainty we have studied the potential effect of probiotics in VLBW HIV-exposed infants. The null-hypothesis for this study was that the administration of probiotics does not reduce the incidence and severity of NEC in premature very-low birth weight infants that are exposed to HIV. A secondary aim of this study was to assess the incidence of NEC in very-low birth weight and extremely low birth weight infants born to HIV-infected and HIV-uninfected women.

Patients and Methods:

A randomized, double blind, placebo controlled clinical trial was conducted in the neonatal high care unit of Tygerberg Children's Hospital (TBCH) Cape Town, South Africa for the period July 2011 to August 2012. All mothers and infants pairs that conformed to the inclusion criteria and provided written informed consent were included into the study. The infants were randomized into the study or control groups by a random-number table sequence assigned by a statistician. Randomization was

performed by HIV-status; therefore the exposed and unexposed infants were randomized separately. All randomization procedures were done before the study commencement by the study statistician and an independent entity for both the probiotic and placebo. Sample size was determined by a statistician according to the live birth statistics for infants born to HIV-positive mothers at the institution. Only infants with a birth weight of <1250g were included in this study due to the increased length of stay of these infants in hospital and to ensure that the majority of the study population was not exited prematurely from the study. Participants were enrolled and assigned to the respective groups by the researcher and two research assistants. Inclusion criteria for the mothers included: (1) HIV-positive or-negative mothers who gave birth to a premature and very-low birth weight baby at TBCH and consented to participate in the study; (2) Only breastfeeding mothers, regardless of their HIV status and (3) HIV-positive mothers that were on the prevention of mother to child transmission (PMTCT) treatment schedule.

Babies were included if they (1) had a birth weight of of ≥500g and ≤1250g; (2) were HIV exposed or unexposed; (3) received breast milk (either from their mothers or donor breast milk). Breast milk of HIV-positive mothers was pasteurized (according to ward protocol) before it was administered to the infants. Infants were excluded if they had major abnormalities such as gastroschisis, a large omphalocele or congenital diaphragmatic hernia

Pro-B2® (C Pharm, Cape Town, South Africa) is an oil suspension and may be stored at room temperature. Stability reports were provided by C-Pharm twice during the study duration. The product proved to be stable during the study period. Throughout the

study period, the standard of care protocol consisted of one dose (5 drops) probiotic/placebo daily for 4 weeks (28 days). This provided the study group with *L. rhamnosus GG* (0.35×10^9 colony-forming units [CFU]) and *B. infantis* (0.35×10^9 CFU) daily. The control group received placebo consisting of medium chain triglyceride (MCT) oil. MCT oil was chosen as the most suitable control agent with esthetic properties similar to that of Pro-B2. Supplementation of the probiotic/placebo was initiated when enteral feeds started. Probiotic/ placebo supplementation was delayed/ halted in the event of: the infants being nill per os (NPO); when a query NEC was suspected the infant continued with treatment until a confirmed a positive diagnosis of NEC I was made through abdominal X-ray; if the infant remained a query NEC and was NPO the infant did not receive probiotics/ placebo until the enteral feeds were commenced again. Supplementation was discontinued when HIV-exposed infants had a positive polymerase chain reaction (PCR) result on day 14 of life due to the uncertainty of probiotic associated septicemia in immune compromised HIV-positive VLBW infants on HAART

All study participants received human breast milk. Both the probiotics and placebo were mixed with the mothers own breast milk or donor breast milk before administration via the orogastric tube or orally. The probiotic/ placebo was added to the breast milk by the researcher and two research assistants. The attending physician and nurses caring for the infants, as well as the researcher, research assistant and study participants were blinded to the group assignment. Participants exited the study on day 28 after birth or upon discharge from the hospital.

Data on birth weight, estimated gestational age, gender, type of delivery, and Apgar scores were collected. Gestational age was determined by the best estimate of the neonatal and obstetrical care providers based upon physical examination of the infants. Anthropometrical measurements (weight, length and head circumference), intake and output and daily clinical progress notes were reviewed. Infants were evaluated daily for the development of NEC by the attending neonatologists. Whenever a study infant was suspected to have NEC the infant was evaluated by the attending neonatologists in conjunction with the pediatric radiologist and categorized by modified Bell's classification. Infants who developed any stage of NEC according to Bells Criteria were exited. Although Bells stage I is not recognized as definite NEC, it still has the potential to evolve to stage II or III NEC if managed inappropriately. For the purpose of this trial the infants with stage I NEC were included in the analysis as no information exist on the effect of probiotics on HIV exposed VLBW infants. Infants with stage II and III NEC will however be described separately. Ethical approval was granted by the Human Research Ethics Committee of the Faculty of Health Sciences, Stellenbosch University and Tygerberg Academic Hospital. The clinical trial registration number: NCT01868737. A power analysis for two way-ANOVA with 2 levels for each factor with a power of 90% to detect interaction effect sizes of $\delta=0.55$ yield samples of size n=36 in each of the four groups. Our sample sizes of n=37, n=37 (in the HIV exposed group) and n=54, n=56 (in the HIV-unexposed group) exceeds this power specification. Data analyses were performed with Statistica Software (version 11). Frequencies between groups were compared using the likelihood ratio chi-square test and means between groups using t-tests. Statistical significance was defined as a p-value less than 0.05.

Results:

A total of 219 infants were screened for the study. Ten infants were not included in the trial due to declined parental consent and twenty five infants did not conform to the inclusion criteria, thus leaving 184 infants enrolled in the clinical trial (Figure 1). Of these, 74 (40%) infants were HIV-exposed and 110 (60%) were HIV-unexposed infants. Within the HIV-exposed group 37 (50%) infants were randomized into the study and control group respectively. The HIV-unexposed group was randomized into 54 (49%) study infants and 56 (51%) control infants. Of the 184 enrolled in the study, 156 completed the full 28 day study follow-up period (Figure 1). Infant outcomes were as follows: 3 infants were withdrawn from the study, 7 discharges, 11 deaths occurred (5 deaths were NEC associated), 4 NEC survivals and 3 positive PCR results at day 14 of life.

Demographics and major characteristics are depicted in Table 1. The mean birth weight was 987g ±160.24 (range: 560g-1244g) and the mean gestational age 28.7 ±2.95 (range: 24-36) weeks. Apgar scores at 5 minutes differed significantly between probiotic and placebo groups among HIV-exposed infants (p= 0.042).

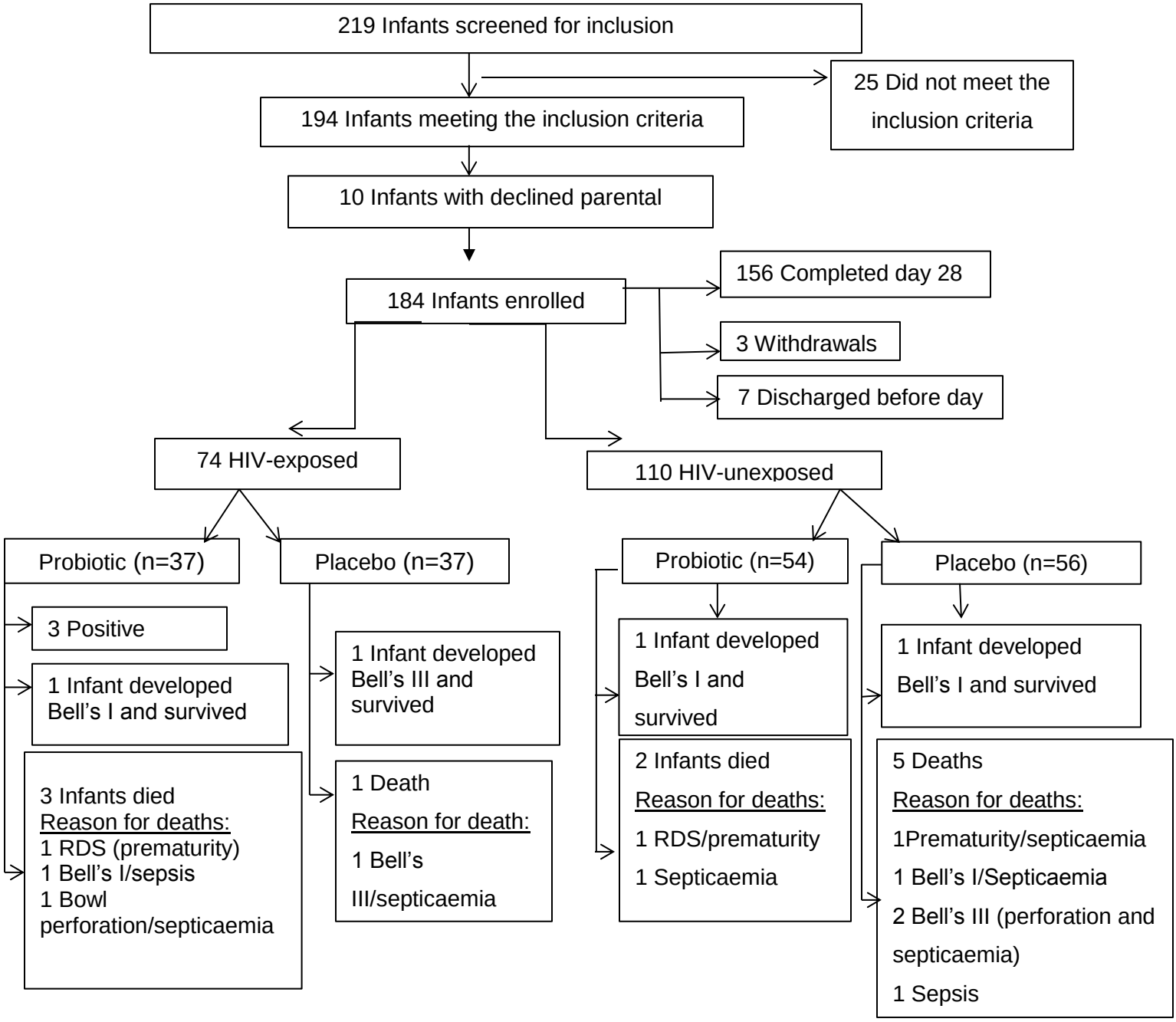

Figure 1: Flow diagram of infants included in the clinical trial on the use of probiotics in HIV-exposed and unexposed premature infants.

HIV: human immunodeficiency virus; NEC: necrotizing enterocolitis; PCR: polymerase chain reaction; RDS: respiratory distress syndrome.

Table1: The demographic and clinical characteristics of all infants enrolled in study (n=184).

	HIV-exposed			**HIV-unexposed**		
	Probiotic (n=37)	**Placebo (n=37)**	**Significance**	**Probiotic (n=54)**	**Placebo (n=56)**	**Significance**
Gender	**n=37**	**n=37**	**p = 0.24**	**n=54**	**n=56**	**p = 0.26**
Male	14 (38%)	19 (51%)		29 (54%)	24 (43%)	
Female	23 (62%)	18 (49%)		25 (46%)	32 (57%)	
Race	**n=37**	**n=37**	**p = 0.23**	**n=54**	**n=56**	**p = 0.22**
Black	32 (87%)	35 (95%)		20 (37%)	29 (53%)	
Mixed Race	5 (13%)	2 (5%)		33 (61%)	24 (42%)	
White				1 (2%)	2 (3%)	
Other					1 (2%)	
Birth weight (g)	**n=37**	**n=37**	**p = 0.68**	**n=54**	**n=56**	**p = 0.31**
500-750	4 (11%)	2 (5%)		4 (7%)	8 (14%)	
751-1000	14 (38%)	14 (38%)		21 (40%)	25 (45%)	
1001-1250	19 (51%)	21 (57%)		29 (53%)	23 (41%)	
Gestational age (wk.)	**n=34**	**n=35**	**p = 0.82**	**n=53**	**n=55**	**p = 0.39**
24-28	18 (53%)	16 (46%)		23 (43%)	31 (56%)	

29-32	15 (44%)	18 (51%)		28 (53%)	22 (40%)	
33-36	1 (3%)	1 (3%)		2 (4%)	2 (4%)	
Apgar score (at 5 min)						
<4	1 (3%)		0.042*	2 (4%)	1 (2%)	0.66
4-7	6 (16%)	15 (41%)		7 (13%)	10(18%)	
>7	29 (81%)	22 (60%)		45 (83%)	45 (80%)	

*Statistical significance p<0.05

Table 2: Clinical characteristics of HIV infected and uninfected mothers

	Total Mothers (n=184)	**HIV-Infected Mothers (n=74)**	**HIV-uninfected Mothers (n=110)**	**Significance**
CD4 cell (mean, SD)		401 ±230.6		
ARV treatment duration 4 weeks or more		54 (75%)		
Delivery information				
PROM	17 (9%)	6 (9%)	11 (12%)	0.51
Mode of delivery	**n=183**	**n=74**	**n=109**	**0.53**
C-section	139 (76%)	58 (78%)	81 (74%)	
Vaginal delivery	44 (24%)	16 (22%)	28 (26%)	
Maternal medical treatment				
Maternal antibiotics	46 (25%)	21 (30%)	25 (24%)	0.42
Steroids received	126 (68%)	54 (75%)	72 (67%)	0.23

PROM: Prolonged rupture of membranes

Table 2 depicts the maternal clinical characteristics. The maternal CD4 cell counts ranged from 29 cells/dl - 1091 cells/dl, with a mean CD4 of 401.73 cells/dl. Seventy five percent (n=54) of the HIV infected mothers received ARV treatment for four weeks or more prior to labor and 15 (20%) mothers received insufficient treatment (<4weeks). Three (4%) of the HIV-infected mothers did not receive any treatment and no treatment information was available for two (3%) of the mothers. All the infants that were HIV-

exposed received nevirapine. Three of the 74 infants had a confirmed HIV-positive PCR result and subsequently started AZT treatment (Figure 1). Interestingly, all infants with a positive PCR were within the study group and did not develop NEC.

A total of 4 227 *L. rhamnosus GG* and *B. infantis* doses were administered (mean doses 22.9 per infant). Nine cases of all stages of NEC occurred (incidence rate 5%); while four infants had developed Bells stage III (incidence rate 2.2%) (Table 3). None of the infants in the study developed NEC stage II. The incidence of all stages of NEC in the HIV exposed group differed significantly (p=0.045) for Bells staging criteria. Five percent (n=2) of the HIV-exposed study infants developed Bell's I and two (5%) infants in the control group developed Bell's III. There was no difference in the incidence of stage III NEC in the HIV-unexposed group compared to the HIV-exposed group [n=2 (4%) vs. 2 (5%); p=1.0, respectively]. Only one of the infants in the HIV-exposed group with stage III NEC survived (Figure 1). The incidence of death [4 (5.4%) vs. 7 (6%); p=0.79] or all stages of NEC [4(5%) vs. 5(5%); p=0.76] did not differ significantly between the HIV-exposed and unexposed groups (Figure 1). There was no difference in the incidence of positive blood cultures between the HIV-exposed group and the HIV-unexposed group [8 (11%) vs. 17 (16%); p<0.68 respectively] as well as between the study group and the control group [15 (16%) vs. 10 (11%); p<0.32 respectively]. None of the positive blood cultures grew *Lactobacillus* or *Bifidobacterium* species. The mean antibiotic treatment days did not differ significantly between the HIV-exposed an unexposed groups (p=0.3). Enteral feeds were started at 3.13 (±1.21) mean days of life, with full feeds achieved at 10.21(±3.57) mean days of life.

Table 3: Clinical outcomes of HIV-exposed and unexposed infants

	HIV-exposed			**HIV-unexposed**		
	Probiotic (n=37)	**Placebo (n=37)**	**Significance**	**Probiotic (n=54)**	**Placebo (n=56)**	**Significance**
NEC	**n=2**	**n=2**		**n=1**	**n=4**	
NEC Bell's I	2 (5%)	None	0.045*	1 (2%)	2 (4%)	0.36
NEC Bell's III	None	2 (5%)		None	2 (4%)	
Positive Cultures						
†Positive Blood cultures	5 (14%)	3 (8%)	1.00	10 (19%)	7 (12%)	0.13
†Positive LP culture				1 (2%)	1 (2%)	
Days to achievement of full feeding (DOL, mean ±SE)	10.19 ±0.62	9.68 ±0.62	0.56	9.63 ±0.47	11.14 ±0.46	0.022*
Use of antibiotics (mean days,±SD)	3.99 ±1.85	3.60 ±1.44	0.35	3.39 ±1.60	4.00 ±2.47	0.14

LP: lumbar puncture; DOL: day of life; *: Statistical significance: $p<0.05$; † Gram– bacteria: Acinetobacter baumannii, Enterococcus Faecium, Esterichia coli, Klebsiella Pneumonia, Proteus Mirabilis. Gram+bacteria: Corynebacterium, MRSA, Streptococcus, Staphylococcus. Viral: Herpes. Fungal: Candida.

Table 4: Characteristics of infants with Bell's I and Bell's III.

	Bell's I n=5	Bell's III n=4	Significance
Probiotic	3 (60%)	None	0.029*
Placebo	2 (40%)	4(100%)	
HIV-exposure			
HIV-exposed	2 (40%)	2 (50%)	0.76
HIV-unexposed	3 (60%)	2 (50%)	
AGA	1 (20%)	2 (50%)	0.64
SGA	4 (80%)	2 (50%)	
Weight (gr)			
500-750	1 (20%)	1(25%)	0.53
751-1000	3 (60%)	3(75%)	
1001-1250	1(20%)	None	
Maternal CD4	376.5 ±122.3	609 ±242.5	0.69
Antenatal Steroids	4 (80%)	3 (75%)	0.86
RBC transfusion	2 (4%)	1(25%)	0.71
Antibiotic treatment	4.5 (±2.5)	4.7 (±2.4)	0.89
Surfactant	2 (40%)	1 (25%)	0.63
Blood cultures	2 (40%)	None	
Total days CPAP (mean, ±SD)	7.4 ±9.42	12 ±6.06	0.22

AGA: Appropriate for gestational age; SGA: Small for gestational age; RBC: Red blood cell; *: Statistical significance: $p<0.05$

Apgar scores at 5 minutes were the only factor that affected the overall NEC incidence (p=0.014) (results not shown). As depicted in Table 4, the incidence of all stages of NEC was significantly lower in the study versus the control group [3(3%) vs. 6 (6%); p=0.029]. Four infants (4%) in the control group developed Bells Stage III. None of the infants that developed NEC had a clinical classification of PDA. Three of the infants that developed NEC had red blood cell (RBC) transfusions during clinical manifestations of NEC. Within 24 hours NEC was confirmed and both cases resulted in death due to NEC. The mean maternal CD4 cell count for HIV infected mothers whose infants developed NEC was 493 cells/µl (min: 290 cells/µl; max: 781cells/µl). All of the HIV-positive mothers (n=4) whose infants developed NEC received sufficient antiretroviral medication during pregnancy.

Discussion:

Supplementing probiotics to premature infants has emerged in recent years as a promising strategy to prevent NEC. The multifactorial pathogenesis of NEC is well discussed in literature and although many variables are associated with the development of NEC, prematurity and low birth weight remain a major risk factor.

The heavy burden of maternal HIV infection in developing countries such as South Africa has resulted in a high prevalence of premature birth and NEC.[17] There is a scarcity of data on the outcomes of premature infants born to HIV-infected mothers. A case control study in 2005 suggests an association between maternal HIV status and an increased risk of NEC in premature infants.[7] A cohort analysis by Karpelowsky in 2010 suggested an increase in mortality rates, but no increase in the severity of NEC.[17,

[18] The reported incidence of NEC was 5 - 15% between 2003 and 2007. [19, 20] In our study the overall incidence of all stages of NEC was 5% and 2.2% for severe stages of NEC which is within the lower range of other reported studies.[20, 21] Furthermore the study found a reduced incidence of NEC (Bell's I and III) in the study group when compared to the control group (3% vs. 6% NEC incidence respectively), not specific to the HIV status. Recent evidence shows that very-low–birth weight premature infants are deficient in anaerobic bacterial colonization of the intestinal tract and that lack of these organisms may allow for overgrowth of more pathogenic bacteria, leading to initiation of NEC.[22] Evidence from our study supports the hypothesis of the immune stimulating properties that probiotics may hold in this at risk population. The focal health-promoting effect of probiotics is their enhancement of mucosal immune defense; through enhancing macrophage activity, increasing numbers of killer cells, T cells and interferon; suppression of NF-κB signaling[23] and action against pathogenic microbial colonization and translocation.[24]

The primary outcomes of this study were to determine the incidence and severity of NEC in HIV-exposed infants when treated with probiotic cultures. This has not previously been reported. The intricate immunological changes, involving cytokine production and T cell function, observed in HIV-exposed infants also supports the hypothesis of a possible link between HIV vertical exposure and NEC.[25, 26] HIV-exposed uninfected infants may also demonstrate immune deficiencies,[26-29] which could influence their outcome, particularly in advanced NEC.[17] The incidence of all stages of NEC within the HIV group differed significantly (p=0.045). Five percent (n=2) of the HIV-

exposed trial infants receiving probiotics developed Bell's I and two (5%) infants in the control group developed Bell's III. Although there were cases of NEC reported within the study group, it is noteworthy that it was less severe cases of NEC (Bell's I). Infants with stage I NEC were included in the analysis as there is no literature available on the implications of probiotic use as well as the role of oral anti-retroviral drugs in VLBW HIV-exposed infants. A retrospective, double-blind study showed no difference between clinical presentation and histopathological features in HIV-exposed and HIV-unexposed infants.[22]

The higher incidence of severe stages of NEC in the HIV-exposed and unexposed control group compared to the study group could be related to the administration of probiotics. The similar incidence of positive blood cultures between the HIV-exposed and unexposed infants are probably related to exclusive breast milk feeding. All infants that were enrolled in this trial received mothers own breast milk or donor breast milk. The breast milk of HIV-positive mothers was pasteurized before administration to infants. The use of breast milk is associated with a reduced incidence of NEC. Human milk may however also be a vehicle for microorganisms derived from the mother or the environment during collection. It has been found that coagulase-negative staphylococci, alpha hemolytic streptococci and pathogens such as Escherichia coli and others are present in 60-80% of all donor breast milk samples collected.[30] Pasteurization is known to decrease the number of pathogens present in breast milk. Therefore it is possible that the pasteurization of breast milk could have served as a protective mechanism for the HIV-exposed infants. Limitations of the study: Although statistics are significant, a small

group of NEC cases limits an over enthusiastic interpretation of the results. All infants in this study were exclusively breast milk fed which is associated with a very low incidence of NEC.

Conclusion:

This study is the first to document the use of Probiotics *L. rhamnosus GG* and *B. infantis* in premature VLBW infants born to HIV-infected mothers. This study failed to show that probiotics lowered the incidence of NEC in HIV-exposed premature infants however it appears to reduce the severity of disease. Furthermore we support the use of probiotics as an effective method in reducing the risk for NEC in low and very low birth weight infants. These results support the need for further RCT's in HIV-exposed preterm infants and the use of probiotics.

Abbreviations: HIV: human immunodeficiency virus; MCT: medium chain triglyceride; NEC: necrotizing enterocolitis; TBCH: Tygerberg Children's Hospital; VLBW: very low birth weight.

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Contributor's Statement:

Mrs. Evette van Niekerk: Mrs. van Niekerk conceptualized and designed the study, coordinated and supervised data collection and analysis, drafted the manuscript and revised all the manuscripts.

Prof Gert Kirsten: Prof Kirsten reviewed and revised the manuscript, and approved the final manuscript as submitted.

Prof Reneé Blaauw: Prof Blaauw critically reviewed the manuscript, and approved the final manuscript as submitted.

Prof Daniel Nel: Prof Nel took responsibility for the statistical analysis of data.

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ARTICLE 2

PROBIOTICS, FEEDING TOLERANCE AND GROWTH: A COMPARISON BETWEEN HIV-EXPOSED AND UNEXPOSED VERY LOW BIRTH WEIGHT INFANTS

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Probiotics, feeding tolerance, and growth: A comparison between HIV-exposed and unexposed very low birth weight infants

Evette Van Niekerk BS.c., M. Diet. [a,*],
Gert F. Kirsten Mb., Ch.B., M.Med. (Pead), D.C.H. (SA), F.C.P. (Pead)(SA), M.D. [b],
Daniel G. Nel Ph.D. [c], Reneé Blaauw Ph.D. [a]

[a] Division Human Nutrition, Faculty of Medicine and Health Sciences, Stellenbosch University, Tygerberg, South Africa
[b] Department of Pediatrics and Child Health, Division of Neonatology, Faculty of Medicine and Health Sciences, Stellenbosch University, Tygerberg, South Africa
[c] Department of Statistics and Actuarial Science, Stellenbosch University, Tygerberg, South Africa

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ABSTRACT

Objective: The aim of this study was to compare the effect of administration of probiotics on feeding tolerance and growth outcomes of HIV-exposed (but uninfected) versus HIV non-exposed preterm infants. The null hypothesis of this study states that there will be no difference in the feeding tolerance and growth outcomes for both probiotic-exposed and unexposed premature very low birth weight infants.

Methods: A randomized, double-blind, placebo-controlled trial was conducted during the period from July 2011 to August 2012. HIV-exposed and non-exposed premature (<34 wk gestation) infants with a birth weight of ≥500 g and ≤1250 g were randomized to receive either a probiotic mixture or placebo. The multispecies probiotic mixture consisted of 1×10^9 CFU, *Lactobacillus rhamnosus GG* and *Bifidobacterium infantis* per day and was administered for 28 d. Anthropometrical parameters, daily intakes, and feeding tolerance were monitored.

Results: Seventy-four HIV-exposed and 110 unexposed infants were enrolled and randomized (mean birth weight 987 g ± 160 g, range, 560–1244 g; mean gestational age 28.7 wk). In all 4227 probiotic doses were administered (mean 22.9/infant). There was no difference in the average daily weight gain for treatment groups or HIV exposure. The HIV-exposed group achieved significantly higher *z* scores for length and head circumference at day 28 than the unexposed group ($P < 0.01$ and $P = 0.03$, respectively). There were no differences in the incidence of any signs of feeding intolerance and abdominal distension between the groups.

Conclusion: Probiotic supplementation did not affect growth outcomes or the incidence of any signs of feeding intolerance in HIV exposure.

Introduction

Postnatal growth restriction and failure have been recently identified as a major issue in preterm, especially extremely low birth weight (ELBW) neonates [1,2]. It has been demonstrated that anthropometrical parameters, such as weight, length, and head circumference, in neonates of HIV-positive mothers are significantly lower due to lower baseline values compared with neonates of HIV-negative mothers. Furthermore the postnatal growth of uninfected exposed infants is significantly affected by the maternal HIV status [3]. Poorer neurodevelopmental outcomes in very low birth weight (VLBW) infants are associated with delayed achievement of full enteral feeds [4]. The fear of necrotizing enterocolitis (NEC) and the unclear definition of feeding intolerance (intolerance due to prematurity versus early NEC) further complicate the urgency of early enteral feeding. Several factors have been associated with feeding tolerance such

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* Corresponding author. Tel.: +27 21 938 9474; fax: +27 21 933 2991.
E-mail address: Evettev@sun.ac.za (E. Van Niekerk).

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as antenatal corticosteroid use, patent ductus arteriosus, sepsis, phototherapy, formula versus preterm human milk, continuous nasogastric versus intermittent bolus milk feeding, and volume and rate of advancement of feeds [1]. Probiotics have been found to improve feeding tolerance [5] and total weight gain in preterm infants [6,7]. No studies were found in the literature that examined the effect of probiotics on malnutrition/growth in low birth weight infants born to HIV-positive mothers. Therefore, the aim of this study was to elucidate the role of probiotics on feeding tolerance and subsequent growth in HIV-exposed versus unexposed premature VLBW infants in a resource-limited setting where practice involves early initiation of enteral feeds with expressed breast milk where feasible, with progressive advancement of these feeds and concomitant use of IV dextrose.

Participants and methods

This randomized, double-blind, placebo-controlled clinical trial was conducted in the neonatal high-care unit of Tygerberg Children's Hospital (TBCH) Cape Town, South Africa. Between July 2011 and August 2012, mother and infant pairs that conformed to the inclusion criteria and provided written informed consent were included into the study. Premature (<34 wk gestation) and VLBW (<1250 g) HIV-exposed and unexposed infants were randomized into the study or control groups by a random-number table sequence assigned by a statistician. All randomization procedures were done before the study commencement by the study statistician and an independent entity for both the probiotic and placebo. Sample size was determined by a statistician according to the life birth statistics for infants born to HIV-positive mothers at the institution. A sample size of 184 participants was calculated to give a power of 90%. Inclusion criteria were consecutive HIV-positive or HIV-negative mothers who gave birth to a premature baby with a birth weight ≥500 g and ≤1250 g at TBCH and consented to participate in the study. Only mothers who decided to breastfeed after counseling, regardless of their HIV status were included. HIV-positive mothers who were on the prevention of mother-to-child transmission treatment schedule received nevirapine and zidovudine as well as those who received highly active antiretroviral therapy (HAART) were enrolled in the study, as well as HIV-exposed infants that received antiretroviral (ARV) medication. Infants with major abnormalities such as gastroschisis, a large omphalocele or congenital diaphragmatic hernia were excluded.

The study group received breast milk plus a daily probiotic supplement of *Lactobacillus rhamnosus GG* (0.35×10^9 colony-forming units [CFU]) and *Bifidobacterium infantis* (0.35×10^9 CFU). The control group received breast milk plus a placebo consisting of medium-chain triacylglycerol (MCT) oil. Pro-B2® (C Pharm, Cape Town, South Africa) is an oil suspension and may be stored at room temperature. Stability reports were provided by C-Pharm twice during the study duration. The product proved to be stable during the study period. MCT oil was chosen as the most suitable control agent with esthetic properties similar to that of Pro-B2. Infants received mother's own breast milk. The HIV-exposed infants received pasteurized mother's breast milk as per ward protocol. Throughout the study period, the standard-of-care protocol consisted of a dose of five drops probiotic per placebo daily. Both the probiotics and placebo were either mixed with the mother's own breast milk or donor breast milk before feeds or were administered via the orogastric tube after feeds. Infants who had reached full feeds and were fed orally received probiotic or placebo supplementation orally. Supplementation of the probiotic or placebo was initiated when enteral feeds started. Probiotic or placebo supplementation was delayed or halted (1) when infants were NPO with admittance or after; (2) when a query NEC was noted in the patient folder and the infant continued with treatment until the abdominal x-ray had confirmed a positive diagnosis of NEC I; or (3) when a positive polymerase chain reaction (PCR) result was obtained from HIV-exposed infants on day 14 of life. Daily supplementation continued until 28 d postconceptual age. HIV-exposed infants received pasteurized breast milk. The probiotic or placebo was added to breast milk by the researcher and two research assistants who were blinded and not involved in the care of the infant and who followed randomization procedures. According to ward protocol, donor breast milk was provided to infants when they required a supplemental feed, when there was insufficient breast milk supply from the mother, or when the mother was unavailable or ill and unable to breastfeed the infant.

Infants were evaluated on a daily basis, at which time feeding and growth data were recorded. Data on birth weight, estimated gestational age, sex, type of delivery, and Apgar scores were collected. Infants were evaluated daily for the development of NEC by the attending neonatologists. All study infants were weighed daily by nursing staff. Daily weights were recorded from each participant's medical file by the investigator or research assistant. Any drastic weight changes were queried and the measurement was repeated under supervision of the investigator or research assistants. An electric scale was used, with an accuracy of 0.001 kg. A small for gestational age (SGA) birth was defined as a newborn weighing10th percentile of gestational age-specific birth weight distribution [8]. Length and head circumference were measured by the investigator or research assistants on days 1, 7, 14, 21, and 28 of the study. A standard, non-stretchable measuring tape with 0.5- and 1-cm dimensions was used [9]. Daily weight was measured to the nearest gram and weekly head circumference and recumbent length to the nearest 0.5 cm. *z* Scores were used in the interpretation of anthropometrical data because they are superior to percentiles for infants whose size is outside of the normal range of a growth chart, that is, beyond the third and 97th percentiles [10].

Ethical approval was granted by the Human Research Ethics Committee of the Faculty of Health Sciences, University of Stellenbosch and Tygerberg Academic Hospital. Data analyses were performed with Statistica Software (version 11), Johannesburg, South Africa. Frequencies between groups were compared using the likelihood ratio χ^2 test and means between groups using pooled *t* tests. Statistical significance was defined as observing *P*-value <0.05.

Results

Of the 194 infants meeting inclusion criteria, 184 were enrolled after parental consent was obtained. Seventy-four (40%) infants were exposed to HIV and 110 (60%) were not. The HIV-exposed group was randomized into 37 (50%) infants in the study and control group, respectively. The HIV-unexposed group was randomized into 54 (49%) study infants and 56 (51%) control infants. The 28-d study follow-up period was completed by 156 (85%) of infants (Fig. 1). Three infants withdrew from the study, 7 were discharged, 11 died (5 deaths were NEC-associated), 4 were NEC survivors, and 3 had positive PCR results at day 14 of life.

The mean birth weight in infants born to HIV-positive women was 1009 g (± 153 g) and 972 g (± 164 g) in infants born to HIV-negative women ($P = 0.12$). There was no difference in the incidence of positive blood cultures between exposed and unexposed groups (8 [11%] versus 17 [16%]; $P = 0.68$, respectively) as well as between study and control groups (15 [16%] versus 10 [11%]; $P = 0.32$, respectively; Table 1). None of the positive blood cultures grew *Lactobacillus* or *Bifidobacterium* species.

Table 2 depicts the maternal clinical characteristics and medical treatment. The maternal CD4 cell counts ranged from 29/dL to 1091/dL, with a median CD4 cell count of 399/dL. Maternal CD4 cell counts were not related to infant birth weight ($P = 0.93$). A high incidence of cesarean deliveries was found in the study population (139 [76%]). Maternal steroid administration was higher in HIV-positive mothers than HIV-negative mothers (54 [75%] versus 72 [67%]). The majority (75%) of

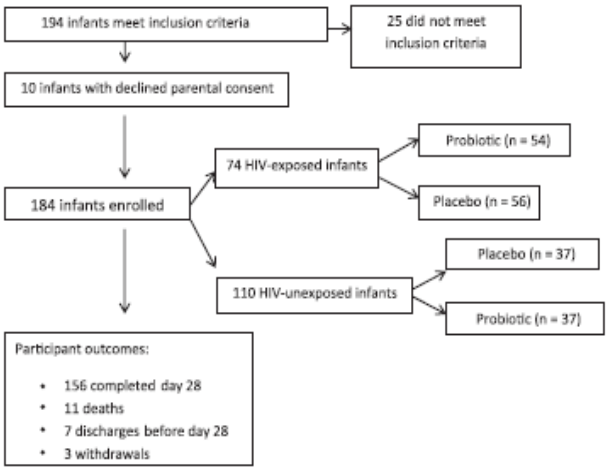

Fig. 1. Flow diagram of infants included in the clinical trial on the use of probiotics in HIV-exposed and unexposed premature infants.

Table 1
Demographic and clinical characteristics of study infants

	HIV-exposed			HIV-unexposed		
	Probiotic (n = 37)	Placebo (n = 37)	Significance	Probiotic (n = 54)	Placebo (n = 56)	Significance
Sex						
Male (n, %)	14 (38)	19 (51)		29 (54)	24 (43)	
Female (n, %)	23 (62)	18 (49)		25 (46)	32 (57)	
Race					5	
Black (n, %)	32 (87)	35 (95)		20 (37)	29 (53)	
Mixed race (n, %)	5 (13)	2 (5)		33 (61)	24 (42)	
White (n, %)	0	0		1 (2)	2 (3)	
Other (n, %)	0	0		0	1 (2)	
Gestational age (n)	34	35	0.82	53	56	0.39
24–28 wk (n, %)	18 (53)	16 (46)		23 (43)	31 (56)	
29–32 wk (n, %)	15 (44)	18 (51)		28 (53)	22 (40)	
33–36 wk(n, %)	1 (3)	1 (3)		2 (4)	2 (4)	
Apgar (5 min)						
< 4 (n, %)	1 (3%)	0	0.042*	2 (4%)	1 (2%)	0.66
4–7 (n, %)	6 (16%)	15 (41%)		7 (13%)	10 (18%)	
>7 (n, %)	29 (81%)	22 (60%)		45 (83%)	45 (80%)	
CPAP						
CPAP days (median; quartiles range)	4.00 (2.00–7.00)	4.5 (3.00–7.00)	0.58	5.00 (2.0–9.0)	5.00 (2.00–9.00)	0.78
Positive cultures						
Positive blood cultures† (n,%)	5 (14%)	3 (8%)	1.00	10 (19%)	7 (12%)	0.13
Positive LP culture† (n,%)	0	0		1 (2%)	1 (2%)	

CPAP, continuous positive airway pressure; LP, lumbar puncture; MRSA, methicillin-resistant *Staphylococcus aureus*
* χ^2 test statistical significance $P < 0.05$.
† Gram – bacteria: *Acinetobacter baumannii*, *Enterococcus faecium*, *Esterichia coli*, *Klebsiella pneumonia*, *Proteus mirabilis*. Gram + bacteria: *Corynebacterium*, MRSA, *Streptococcus*, *Staphylococcus*. Viral: Herpes. Fungal: Candida.

HIV-positive mothers received sufficient ARV treatment > 4 wk. Three (4%) of the HIV-infected mothers did not receive any treatment and no treatment information was available for two (3%) of the mothers. The prevalence of hypertension was significantly higher in HIV-negative rather than HIV-positive mothers (35 [47%] versus 64 [58%]; $P = 0.002$, respectively).

In all, 4227 *L. rhamnosus GG* and *B. infantis* doses were administered (mean doses 22.9/infant). Table 3 depicts the general feeding characteristics. Study infants in the HIV-exposed group were NPO for fewer days than those in the control group (2.8 [± 0.9] versus 3.1 [± 2.2]; $P = 0.43$, respectively). The initiation of enteral feeds was on similar days for both study and control groups (HIV-exposed infants: 4.00 ± 0.99 versus 3.00 ± 0.98 compared with HIV-unexposed infants: 3.00 ± 1.66 versus 3.00 ± 0.82, respectively). Full enteral feeds are defined for the purpose of this review as when infants no longer required the use of IV fluids. Inverse results were found for the achievement of full feeds between the HIV-exposed and unexposed groups. No difference was found for HIV exposure or per supplementation group for the achievement of full feeds. The HIV-exposed infants reached full feeds later than the control group (10.19 ± 4.055 versus 9.68 ± 3.46; $P = 0.56$), whereas the HIV-unexposed group reached full feeds in significantly fewer days (9.63 ± 2.42 versus 11.14 ± 4.15; $P = 0.022$). Feeding volumes on day 7 of life were significantly lower for HIV-exposed infants who received probiotic supplementation than those who did not (62.04 ± 35.42 versus 79.47 ± 28.09; $P = 0.036$). It is, however, noteworthy that the quartile range in feeding volumes for the control group on day 7 ranged from 51.68 to 105.55 mL, putting caution to the interpretation of these results. There were no differences in the incidence of any signs of feeding intolerance and abdominal distension between the groups.

Table 2
Maternal clinical characteristics and medical treatment

	Mothers (N = 184)	HIV-infected mothers (n = 74)	HIV-uninfected mothers (n = 110)	Significance
CD4 cell (median, quartiles range; min/max)		399 (169–556; 29–1091)		
Mode of delivery (n)		74	109	0.53
Cesarean delivery (n, %)	139 (76)	58 (78)	81 (74)	
Vaginal delivery (n, %)	44 (24)	16 (22)	28 (26)	
Duration of ARV treatment (n)		72		
≥4 wk (n, %)		54 (75)		
3 wk		8 (11)		
2 wk		1 (1.4)		
1 wk/during labor		6 (8)		
No treatment		3 (4)		
Maternal medical treatments				
Maternal antibiotics (n, %)	46 (25)	21 (30)	25 (24)	0.42
Maternal steroids (n, %)	126 (68)	54 (75)	72 (67)	0.23
Maternal medical conditions				
Hypertension/preeclampsia (n, %)	99 (54)	35 (47)	64 (58)	0.00257*
Diabetes (n, %)	6 (3)	2 (3)	4 (4)	0.22
PROM (n, %)	17 (9)	6 (9)	11 (12)	0.51

ARV, antiretroviral; PROM, prolonged rupture of membranes
* χ^2 test statistical significance $P < 0.05$.

Table 3
Feeding characteristics of HIV-exposed and unexposed infants

	HIV-exposed			HIV-unexposed		
	Probiotic (n = 37)	Placebo (n = 37)	Significance	Probiotic (n = 54)	Placebo (n = 56)	Significance
Participant intake (median; ± SD [quartile range])						
Total days NPO (mean, ± SD)	2.8; ±0.9	3.1; ±2.2	0.43	2.3; ±1.3	2.3; ±1.6	0.827
Time to initiation of enteral feeding	4.00; ±0.99 (3.00–4.00)	3.00; ±0.98 (3.00–4.00)	0.48	3.00; ±1.66 (2.00–3.00)	3.00; ±0.82 (2.00–3.00)	0.17
Time to achievement of full feeding	9.00 ±4.055 (8.00–11.00)	9.00 ±3.46 (8.00–11.00)	0.66	9.5; ±2.42 (8.00–11.00)	10.00; ±4.15 (9.00–11.5)	0.15
Feeding amount day 7 (mL/kg)	62.04; ±35.42 (38.20–79.87)	79.47; ±28.09 (51.68–105.55)	0.035*	76.00; ±33.28 (52.32–98.44)	72.28; ±34.50 (54.28–100.52)	0.70
Feeding amount day 14 (mL/kg)	159.43; ±36.94 (142.91–174.87)	159.07; ±38.48 (140.90–172.69)	0.84	164.65; ±40.96 (151.02–170.42)	163.32; ±49.08 (143.98–175.67)	0.82
Feeding amount day 21 (mL/kg)	170.71; ±31.69 (163.61–173.49)	168.57; ±44.34 (147.36–175.82)	0.23	167.63; ±27.55 (151.02–170.42)	169.23; ±43.40 (153.61–176.79)	0.71
Feeding amount day 28 (mL/kg)	173.31; ±27.82 (164.81–177.61)	173.03; ±28.95 (162.60–177.61)	0.76	171.57; ±32.40 (153.31–178.84)	170.38; ±34.71 (157.40–176.68)	0.61
Breast milk supplementation (median; ±SD [quartile range])						
Days of FM85	15.00; ±6.46 (10.00–18.00)	16.00; ±6.21 (12.00–18.00)	0.49	15.50; ±4.96 (10.00–17.00)	15.00; ±6.59 (8.00–18.00)	0.21
Days of MCT oil	11.00; ±3.37 (9.00–12.00)	6.50; ±3.67 (6.00–8.00)	0.15	7.5 ±3.29 (5.00–11.00)	8.00; ±4.14 (5.00–12.00)	0.62
Feeding tolerance (median; ±SD [quartile range])						
Days that feeding intolerance occurred	2.00; ±1.32 (1.5–3.00)	2.00; ±2.16 (1.00–3.00)	0.82	3.00; ±1.80 (1.00–4.00)	3.00; ±1.99 (1.5–4.00)	0.39
Abdominal distention	0.00; ±1.99 (0.0–2.00)	0.00; ±2.79 (0.00–2.00)	0.50	0.5; ±1.92 (0.00–2.00)	1.00; ±3.07 (0.00–4.00)	0.07

FM85, breast milk supplement; MCT, medium chain triacylglycerol
* χ^2 test statistical significance $P < 0.05$.

As evident from Table 4, a significant difference could not be found for birth weights between the HIV-exposed and unexposed groups. A higher but non-significant incidence of selective intrauterine growth restriction (SIUGR) was found in the HIV-exposed group than in the unexposed group (19 [26%] versus 20 [18%]; $P = 0.23$, respectively). A significant difference in the average daily weight increase for either treatment groups or HIV exposure (Fig. 2 and 3) could not be found. The HIV-exposed group did, however, show better daily weight gain. When comparing the study with the control group, a difference could not be found in the *z* scores for any of the anthropometrical parameters within the 28 d. However the HIV-exposed group showed significantly higher *z* scores for length and head circumference at day 28 than the unexposed group ($P = 0.003$ and $P = 0.03$, respectively; Figs. 4–6). Although it is evident from Table 4 and Figure 4 that the HIV-exposed infants showed more days of weight gain in the 28-d period and that the *z* scores were noticeably higher by day 28, *z* scores still remained suboptimal.

Discussion

Our results indicate that SIUGR was more prevalent in neonates of HIV-positive mothers than their unexposed counterparts. These results are supported by other studies that found a significant effect of maternal HIV status on the neonatal anthropometric parameters [3]. One study found that infants born to mothers with a CD4 cell count >200/μL were 70% less likely to be SGA compared with infants born to mothers with CD4 cell count ≤200/μL [11]. Our study found no relation for CD4 cell count and infant birth weight.

The results of this study demonstrate was no difference in feeding volumes between HIV-exposed and unexposed groups or between study and control groups with exception of day 7. In contrast with other studies, this study could not show a difference for feeding intolerance and abdominal distension between study and control groups [12,13].

Although there were no significant differences in growth (head circumference, length, and weight) between study and control groups, concurring with other studies [12,14–16], it is worthwhile to recognize that when only taking HIV exposure into account the HIV-exposed group showed significantly higher *z* scores for length and head circumference at day 28. Although these *z* scores were significantly higher, they continually show inappropriate growth for both groups, where infants fall below their initial *z* scores at birth. Postnatal growth failure is extremely common in the VLBW and ELBW infant. Data from the National Institute of Child and Human Development Neonatal Research Network indicates that not only are 16% of ELBW infants SGA at birth, but by 36 wk corrected age, 89% show growth failure [17]. The American Academy of Pediatrics (AAP) recommended a target daily growth rate of 15 g/kg for ELBW preterm infants [18]. The HIV-exposed group had more days of growth velocity and a daily weight gain more in accordance with the AAP recommendation than unexposed infants. No association between growth velocity and HIV-exposure status was found in another study [19]. The present study shows weight gain patterns similar to those reported previously [20–22] It is noteworthy that, after postnatal weight loss, the weight of these infants does not achieve the birth *z* score (which is slightly below the 25th centile), but remains at approximately the third centile up to the 28th d postmenstrual age [22]. It has been reported that at hospital discharge, most infants born between 24 and 29 wk of gestation had not achieved median birth weight [21].

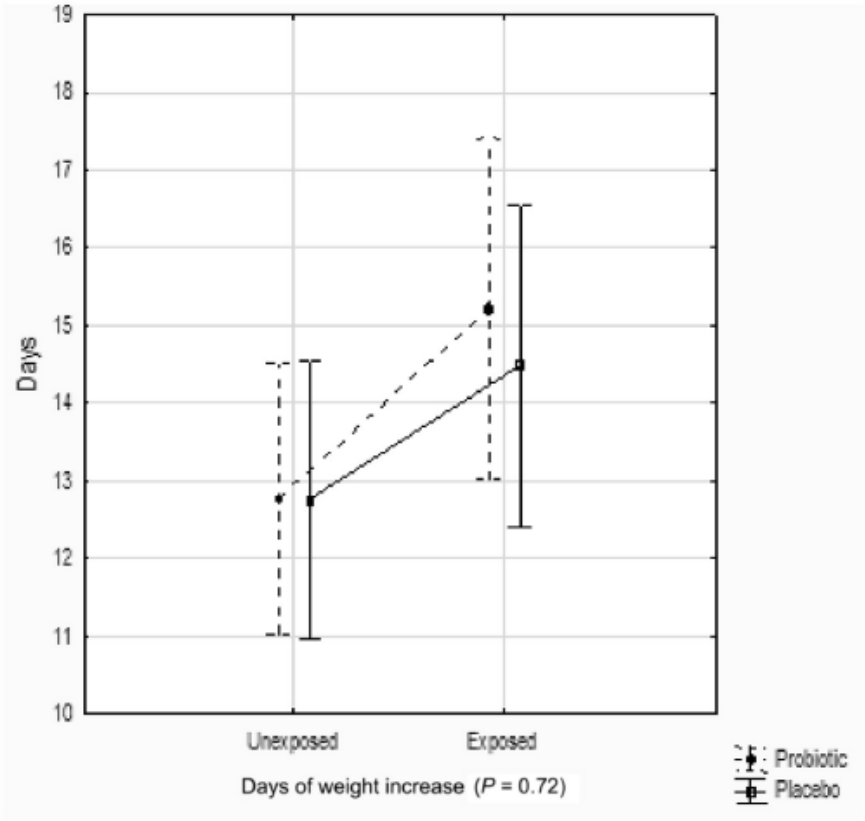

Fig. 2. Days that HIV-exposed and unexposed infants showed weight increase during the study period.

Although the growth is suboptimal, the significant difference in head circumference and length in the HIV-exposed group remains an area for further investigation. Results indicated that SIUGR did not contribute to difference in growth between the HIV-exposed and unexposed groups. Prenatal maternal dietary and lifestyle habits, maternal and infant ARV treatment and an in-depth study of infant's nutritional intake may be areas of interest to clarify this unexpected phenomenon. It is evident from our results that the HIV-exposed group showed a lower incidence, however, non-significant difference, of positive blood cultures than the HIV-unexposed group. A large cohort study found that neonatal infections among ELBW infants are associated with poor growth outcomes, specifically head circumference [23]. The further implication of these results are that this faltering in growth and head circumference is associated with poor neurodevelopmental outcomes and cerebral palsy [23].

The similar incidence of positive blood cultures between HIV-exposed and unexposed infants are probably related to exclusive breast milk feeding. All infants who were enrolled in this trial received mother's own breast milk or donor breast milk. The breast milk of HIV-positive mothers was pasteurized before administration to infants. Pasteurization is known to decrease the number of pathogens present in breast milk. Therefore, it is possible that the pasteurization of breast milk could have served as a protective mechanism for HIV-exposed infants.

Conclusion

The use of probiotic supplementation did not affect growth outcomes or feeding tolerance in HIV-exposed and non-exposed VLBW infants. Therefore, we accept the null hypothesis that there will be no difference in the weight gain for both probiotic-exposed and unexposed premature VLBW infants. The significant difference in head circumference and length in the HIV-exposed group remains an area for further investigation. The focal health-promoting effect of probiotics is their enhancement of mucosal immune response and their action against pathogenic microbial colonization and translocation [24]. The latest updated Cochrane review results show that probiotics reduced the incidence of severe NEC, mortality, and NEC-related mortality [25]. Although results from the present study could not determine a difference in weight gain, the use of probiotics in premature VLBW infants is supported due to the beneficial properties of probiotics.

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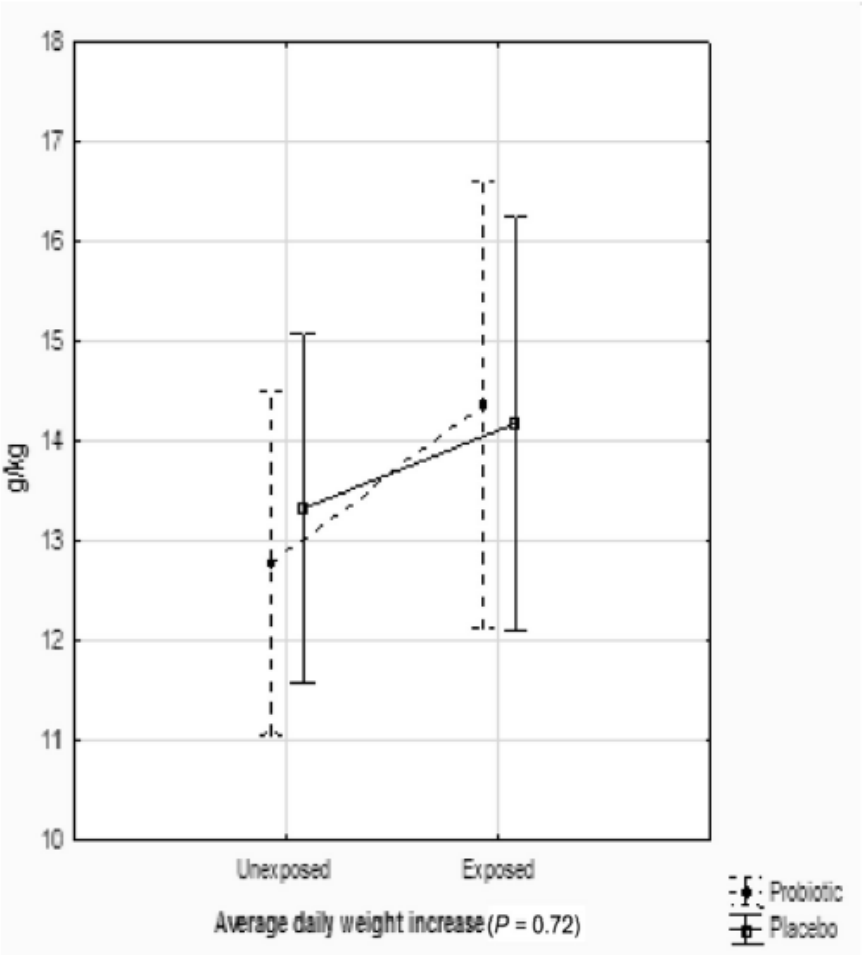

Fig. 3. Average daily weight increase for the HIV-exposed and unexposed infants.

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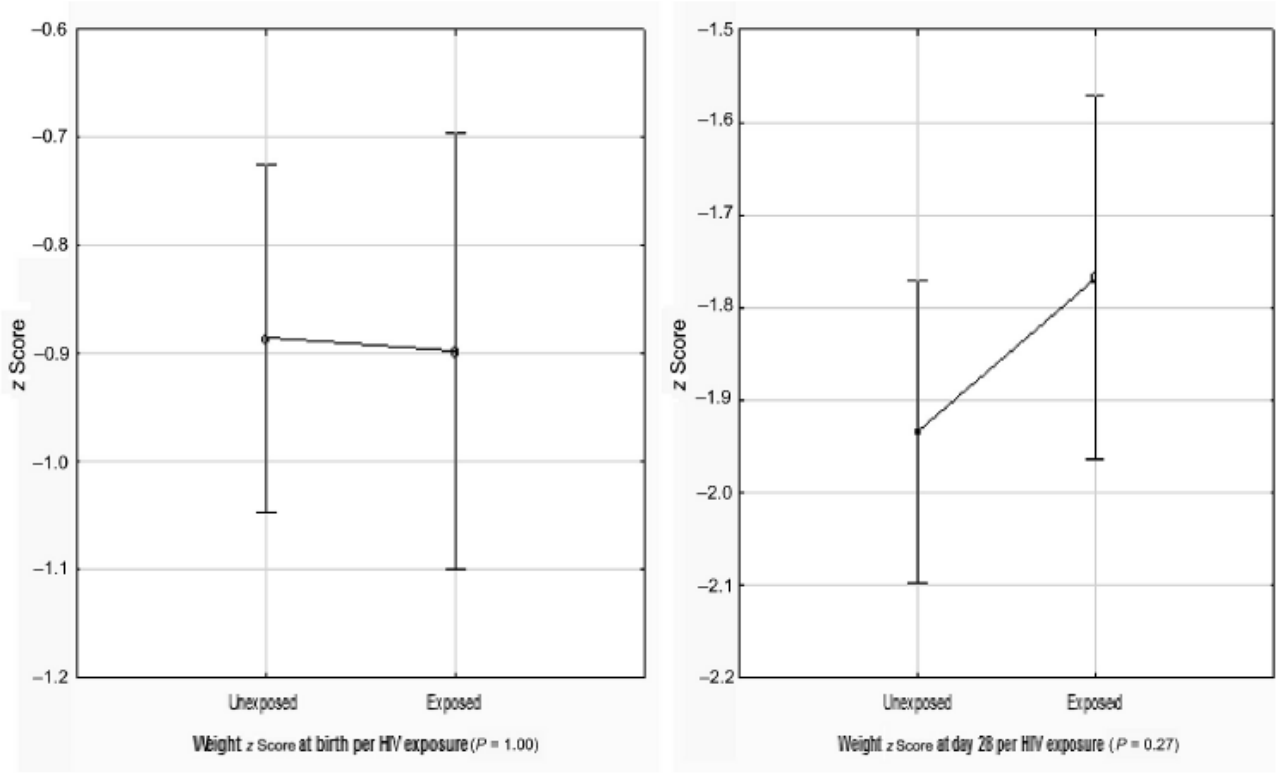

Fig. 4. Weight *z* scores at birth and day 28 per HIV exposure.

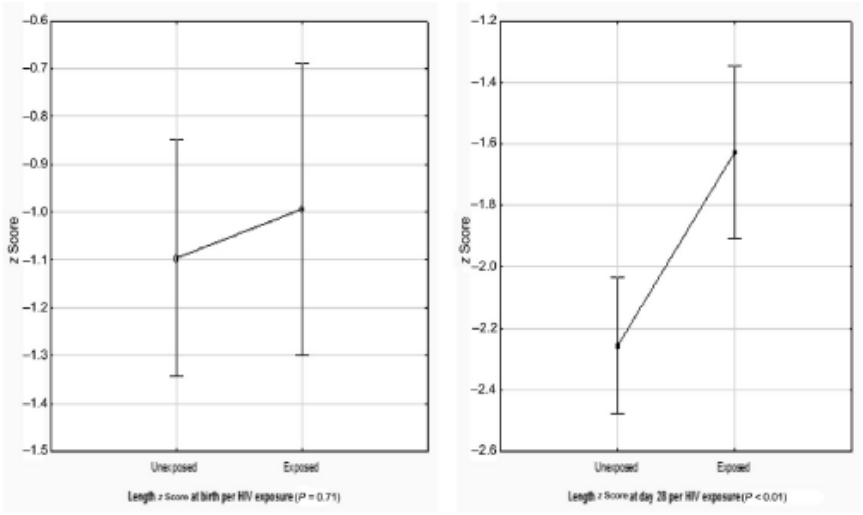

Fig. 5. Length *z* scores at birth and day 28 per HIV exposure.

ARTICLE 3

HUMAN MILK OLIGOSACCHARIDES DIFFER BETWEEN HIV-INFECTED AND -UNINFECTED MOTHERS AND ARE RELATED TO NECROTIZING ENTEROCOLITIS INCIDENCE IN THEIR PRETERM VERY LOW BIRTH WEIGHT INFANTS

Van Niekerk E, Kirsten GF, Nel DG, Blaauw R. Human milk oligosaccharides differ between HIV-infected and -uninfected mothers and are related to necrotizing enterocolitis incidence in their preterm very low birth weight infants. Journal of Nutrition.2014; 144:1227-1233.

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Human Milk Oligosaccharides Differ between HIV-Infected and HIV-Uninfected Mothers and Are Related to Necrotizing Enterocolitis Incidence in Their Preterm Very-Low-Birth-Weight Infants[1–3]

Evette Van Niekerk,[4]* Chloe A. Autran,[7] Daniel G. Nel,[6] Gert F. Kirsten,[5] Reneé Blaauw,[4] and Lars Bode[7]

[4]Division of Human Nutrition and [5]Department of Pediatrics and Child Health, Division of Neonatology, Faculty of Medicine and Health Sciences, and [6]Department of Statistics and Actuarial Science, Stellenbosch University, Stellenbosch, South Africa; and [7]Divisions of Neonatology and Gastroenterology, Hepatology, and Nutrition, Department of Pediatrics, University of California, San Diego, San Diego, CA

Abstract

The heavy burden of maternal HIV infection has resulted in a high prevalence of premature birth and associated necrotizing enterocolitis (NEC). Human milk oligosaccharides (HMOs) were recently associated with HIV infection and transmission through breastfeeding and were also shown to reduce NEC in an animal model, particularly the HMO disialyllacto-N-tetraose (DSLNT). The primary aim of this study was to verify differences in HMO composition between HIV-infected and HIV-uninfected women. The secondary aim was to assess whether the HMO composition in the milk of mothers whose infants were diagnosed with NEC differs from that of mothers whose infants did not develop NEC. This study forms part of a larger clinical trial conducted at the Tygerberg Children's Hospital, Cape Town, South Africa, which recruited HIV-infected and HIV-uninfected mothers and their preterm infants (<34 wk gestation; $500 and #1250 g). Eighty-two mother-infant pairs were selected for the substudy. Mother-infant pairs were stratified according to the mother's HIV (infected/uninfected) and secretor status (secretor/nonsecretor). HMOs in 4- and 28-d postpartum milk samples were analyzed by HPLC and compared between groups. Our results confirm previous reports that HIV-infected mothers have higher relative abundances of 3#-sialyllactose in their milk compared with HIV-uninfected mothers (10.7% vs. 6.8%; $P < 0.01$). Most intriguingly, the data also indicated that low concentrations of DSLNT in the 4-d milk samples in the mother's milk increased the infant's risk of NEC (200 ± 126 vs. 345 ± 186 mg/mL; $P < 0.05$), which is in accordance with results from previously published animal studies and warrants further investigation. This trial was registered at clinicaltrials.gov as NCT01868737. J. Nutr. 144: 1227–1233, 2014.

Introduction

The heavy burden of maternal HIV infection has contributed to the prevalence of premature birth and necrotizing enterocolitis (NEC)[8] (1,2), one of the most common intestinal disorders in preterm infants (3). Human milk oligosaccharides (HMOs) (4,5), complex carbohydrates that are highly abundant in breast milk but not in infant formula (6), were recently associated with HIV infection and transmission through breastfeeding (7) and were also shown to reduce NEC in an animal model (8). More than 100 structurally distinct HMOs have been identified (9). Concentrations of individual oligosaccharides vary with gestation (term vs. preterm), over the course of lactation, and from one mother to another (5). Interpersonal HMO variations are most pronounced for α1-2-fucosylated HMO. α1-2-Fucosylation of milk oligosaccharides is catalyzed by the enzyme fucosyltransferase 2 (FUT2), encoded by the secretor (Se), which also affects a person's Se blood group status (10,11). Women with an active Se locus (secretors) express a functional FUT2 enzyme, and their milk contains high concentrations of α1-2-fucosylated HMOs [e.g., 2#-fucosyllactose or lacto-N-fucopentaose (LNFP) 1]. Nonsecretors lack a functional FUT2 enzyme, and their milk does not contain α1-2-fucosylated HMOs. Given the accumulating evidence in support

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[3] Supplemental Figure 1 is available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
[8] Abbreviations used: DFLNH, difucosyllacto-N-hexaose; DSLNT, disialyllacto-N-tetraose; FUT2, fucosyltransferase 2; HMO, human milk oligosaccharide; LNFP, lacto-N-fucopentaose; NEC, necrotizing enterocolitis; Se, secretor; TBCH, Tygerberg Children's Hospital; 3#SL, 3#-sialyllactose.
* To whom correspondence should be addressed. E-mail: evettev@sun.ac.za.

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of the beneficial effects of individual HMOs (12), mother-to-mother variations in HMO composition may provide an explanation for why some breast-fed infants are at lower risk of certain diseases than others and in the context of HIV infection (7) and NEC (12,13).

A recent study in a cohort of mother-infant pairs in Lusaka, Zambia, showed that HIV-infected women with total HMOs above the median were less likely to transmit HIV via breast-feeding (7). However, the relative abundance of 1 particular HMO, 3#-sialyllactose (3#SL), was significantly higher among transmitting versus nontransmitting women. Moreover, 3#SL concentrations and relative abundance were significantly higher in HIV-infected compared with HIV-uninfected women. The primary aim of this study was to verify differences in HMO composition between HIV-infected and HIV-uninfected women in an independent cohort of mother-infant pairs who were selected from a larger clinical trial originally conducted in the neonatal high care unit of Tygerberg Children's Hospital (TBCH), Cape Town, South Africa (clinical trial registration at https://clinicaltrials.gov/: NCT01868737).

In addition, the burden of HIV infection increases the prevalence of preterm delivery (1,2) and associated complications, including NEC. NEC risk is 6- to 10-times lower in breast-fed compared with formula-fed infants. The results of a recent study in a neonatal rat model of NEC suggest that the presence of HMOs in breast milk and their absence in formula could be one of the reasons for the protective effects of breastfeeding against NEC (8). HMOs significantly improved survival and reduced NEC pathology scores in neonatal rats. One specific HMO, disialyllacto-N-tetraose (DSLNT), was shown to be responsible for the beneficial effects. It remains unknown, however, whether these results translate from the rat model to human preterm infants. The secondary aim of this study was to assess whether HMO composition in the milk of mothers whose infants were diagnosed with NEC differs from that of mothers whose infants did not develop NEC.

Participants and Methods

Original clinical trial. This study is part of a larger randomized, double blind, placebo-controlled clinical trial that was conducted in the neonatal high care unit of TBCH. Between 4 July 2011 and 22 August 2012, 184 mother and infant pairs that met the inclusion criteria and whose mothers gave written informed consent were enrolled in the clinical trial. Included were HIV-infected or HIV-uninfected mothers who consecutively gave birth to a premature infant with a birth weight of $500 and #1250 g at TBCH and consented to participate in the trial. Only mothers who decided to breastfeed after counseling, regardless of their HIV status, were included. HIV-infected mothers who were on the prevention of mother to child transmission treatment schedule receiving nevirapine and zidovudine, mothers administered highly active antiretroviral medication, and HIV-exposed infants who were administered antiretroviral medication were enrolled in the trial. Infants with major abnormalities such as gastroschisis, a large omphalocele, or congenital diaphragmatic hernia were excluded.

As part of the original clinical trial, infants were randomly assigned to a study or a control group by a random-number table sequence assigned by a statistician. The study group consumed breast milk plus a daily probiotic supplement of *Lactobacillus rhamnosus* GG (0.35×10^9 CFUs) and *Bifidobacterium infantis* (0.35×10^9 CFUs). The control group consumed breast milk plus a placebo consisting of medium-chain TG (MCT) oil. Van Niekerk et al. (14) gave a detailed description of the methodology and the results of the probiotics administration.

For the purpose of this substudy, the data collected on use of probiotics are not discussed. The primary aim of this substudy was to determine whether HMO composition varies between HIV-infected and HIV-uninfected mothers. The secondary aim of this study was to determine whether HMO composition in the mother's milk can be linked to the incidence of NEC in her infant. Whenever an infant was suspected to have NEC, he or she was evaluated by the attending neonatologists in conjunction with the pediatric radiologist. If the diagnosis of NEC was made, it was staged according to Bell's classification, ranging from Bell stage I (suspect) to Bell stage III (advanced) (15).

Participant selection for the substudy. Of the 184 mother-infant pairs enrolled in the original clinical trial, all infants who developed NEC and whose mothers had sufficient milk samples available for analysis were included in the substudy. Thereafter, using a random-number table sequence, the study statistician equally included mother-infant pairs stratified according to the mother's HIV status (infected/uninfected) and the mother's Se status (secretor/nonsecretor). Fourteen of the included mother-infant pairs did not have sufficient milk samples available, in which case the next random participant sample was selected. In total, 82 mother-infant pairs (41 with HIV-infected and 41 with HIV-uninfected mothers) were included in the substudy and their milk analyzed for HMO composition (Fig. 1).

Milk sample collection. A sample of hand-expressed breast milk was collected from each mother at 4 and 28 d postpartum. Untreated, unpasteurized milk samples were immediately frozen at 225°C, and aliquots were shipped on dry ice to the University of California, San Diego, for HMO analysis. HMO analysis was performed at the University of California, San Diego, as previously described (7).

Se status analysis. To determine the mother's Se status (secretor/nonsecretor), 1–2 mL of the mother's saliva was collected in sterile containers when maternal consent for the study was granted or at the earliest time thereafter. Samples were immediately taken to the National Health Laboratory Service at the TBCH for analysis (16,17).

Trial registration and statistical analysis. Ethical approval was granted by the Human Research Ethics Committee of the Faculty of Health Sciences, University of Stellenbosch, and Tygerberg Academic Hospital (clinical trial registration number: NCT01868737). The study was conducted in accordance with the Good Clinical Practice guidelines.

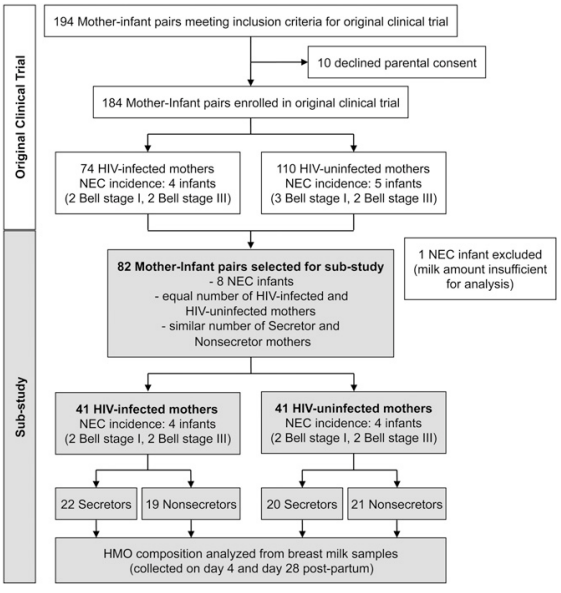

FIGURE 1 Flow diagram of mother-infant pairs recruited in the original clinical trial and selected for the substudy. HMO, human milk oligosaccharide; NEC, necrotizing enterocolitis.

The South African Department of Health and Stellenbosch University approved milk sample export to the United States. Data analyses were performed with Statistica software (version 11; StatSoft). A power analysis to determine sample sizes needed for this study was conducted in which 2 levels for each factor and a power of 90% to detect interaction effect sizes of d = 0.55 were used. The 2 factors mainly considered could be factors such as HIV status and NEC incidence. When comparing continuous variables between 2 groups (e.g., oligosaccharide concentrations for HIV groups), 1-factor ANOVA was used; if residuals were not normally distributed, a nonparametric test, the Mann-Whitney test, was used to confirm the conclusions from the ANOVA. Where necessary, heteroscedasticity was addressed with Welch-Satterthwait ANOVA. Nominal variables (e.g., race, Se status) were compared between groups with contingency tables and the maximum likelihood chi-square test. When group means were compared, they were reported as means ± SDs, where the SD refers to the SD of the group. Significance was achieved in all hypothesis tests if $P < 0.05$.

Results

Table 1 shows the clinical characteristics and medical treatments for all 82 mothers included in this substudy, separated by HIV-infected (n = 41) and HIV-uninfected (n = 41) women. Most important for this study, there was no difference in Se status between HIV-infected and HIV-uninfected mothers. However, there was a significant difference in the racial distribution between the 2 groups, with black women making up 93% of the HIV-infected group but only 51% of the HIV-uninfected group. There was no significant difference between HIV-infected and HIV-uninfected mothers with respect to age, gravida and parity, mode of delivery, use of antibiotics or steroids, and incidence of hypertension/pre-eclampsia or prolonged rupture of membranes. CD4 cell counts in the HIV-infected group ranged from 29 to 862 cells/100 mL, with a mean CD4 of 373 cells/100 mL (normal CD4 cell count range: 500–1000 cells/100 mL) (18). Thirty-four of the HIV-infected mothers (83%) were administered antiretroviral treatment for ≥4 wk prior to labor and 7 mothers (17%) received insufficient treatment (<4 wk).

Table 2 shows the demographic and clinical characteristics of all 82 infants included in this substudy, separated by HIV-exposed and HIV-unexposed infants. There was no significant difference between HIV-exposed and HIV-unexposed infants with respect to gender distribution, birth weight, gestational age, or 5-min Apgar scores. All HIV-exposed infants were administered nevirapine.

Because Se status dramatically affects HMO composition in the mother's milk, results for secretor and nonsecretor women were analyzed separately and are shown in Figs. 2 and 3, respectively. Results are shown for the 2 different sample collection times (4 and 28 d postpartum) and given as absolute oligosaccharide concentrations in micrograms per milliliter as well as relative abundancies in percentages of all analyzed oligosaccharides.

Among secretor women, those who were HIV infected had significantly lower concentrations of the oligosaccharides

TABLE 1 Maternal clinical characteristics and medical treatment[1]

	Total mothers (n = 82)	HIV-infected mothers (n = 41)	HIV-uninfected mothers (n = 41)	P[2]
Race, n (%)				<0.0001
Black	59 (72)	38 (93)	21 (51)	
Mixed	22 (27)	3 (7)	19 (46)	
White	1 (1)	0 (0)	1 (2)	
Age, y	27.7 ± 5.4	28.7 ± 5.1	26.7 ± 5.5	0.08
CD4 cells, cells/100 mL	—	373 ± 207.6	—	
Se status,[3] n	82	41	41	0.66
Secretor, n (%)	42 (51)	22 (54)	20 (49)	
Nonsecretor, n (%)	40 (49)	19 (46)	21 (51)	
Gravida/parity,[3] n	82	41	41	
Gravida	2.5 ± 1.3	2.3 ± 1.2	2.6 ± 1.3	0.22
Parity	2.1 ± 0.9	2.1 ± 1.0	2.2 ± 0.9	0.91
Mode of delivery,[3] n	81	41	40	0.0259
Cesarean section, n (%)	63 (78)	36 (88)	27 (68)	
Vaginal delivery, n (%)	18 (22)	5 (12)	13 (32)	
Duration of antiretroviral treatment,[3] n	—	41	—	
≥4 wk, n (%)	—	34 (83)	—	
<4 wk, n (%)	—	7 (17)	—	
Maternal medical treatments				
Maternal antibiotics,[3] n	67	39	37	
Prevalence, n (%)	19 (28)	11 (28)	8 (21)	0.51
Maternal steroids,[3] n	81	40	41	
Prevalence, n (%)	58 (72)	32 (80)	26 (63)	0.09
Maternal medical conditions				
Hypertension/pre-eclampsia,[3] n	82	41	41	
Prevalence, n (%)	44 (54)	21 (51)	23 (56)	0.66
Prolonged rupture of membranes,[3] n	67	36	31	
Prevalence, n (%)	9 (13)	4 (11)	5 (16)	0.55

[1] Values are means ± SDs unless otherwise indicated. Se, secretor.
[2] Determined by using chi-square test or 1-factor ANOVA.
[3] n indicates population with available data.

TABLE 2 Demographic and clinical characteristics of infants[1]

	HIV-exposed (n = 41)	HIV-unexposed (n = 41)	P[2]
Gender, n (%)			0.45
Male	15 (37)	18 (44)	
Female	26 (63)	23 (56)	
Birth weight, g	988.9 ± 157.9	974.5 ± 151.8	0.67
Gestational age, wk	28.5 ± 1.9	28.8 ± 1.7	0.39
Apgar score at 5 min, n (%)			0.28
4–7	11 (27)	7 (17)	
≥7	30 (73)	34 (83)	

[1] Values are means ± SDs unless otherwise indicated.
[2] Determined by using chi-square test or 1-factor ANOVA, $P \leq 0.05$.

2#-fucosyllactose (697 vs. 1227 mg/mL; $P < 0.05$), lacto-N-tetraose (534 vs. 665 mg/mL; $P < 0.05$) and LNFP 1 (538 vs. 791 mg/mL; $P < 0.05$) compared with HIV-uninfected secretor women (Fig. 2A) in the 4-d samples. In addition to these differences in absolute concentrations, the relative abundance of 3#SL (10.7% vs. 6.8%; $P < 0.01$), difucosyllacto-N-tetraose (14.1% vs. 10.2%; $P < 0.05$), and fucosyl-disialyllacto-N-hexaose (2.0% vs. 1.4%; $P < 0.05$) was higher in HIV-infected secretor women than in HIV-uninfected secretor women (Fig. 2B). There were no significant differences in HMO composition between HIV-infected and HIV-uninfected secretor women for the 28-d milk sample (Fig. 2C, D).

In nonsecretors, there was no significant difference in absolute HMO concentrations between HIV-infected and HIV-uninfected women in the 4-d milk samples (Fig. 3A). However, the relative abundance of difucosyllacto-N-hexaose (DFLNH) was higher in HIV-infected compared with HIV-uninfected nonsecretors (4.4% vs. 2.8%; $P < 0.05$) (Fig. 3B).

In the 28-d milk samples, HIV-infected nonsecretors had significantly lower concentrations of 3-fucosyllactose (40 vs. 65 mg/mL; $P < 0.01$), LNFP 3 (35 vs. 64 mg/mL; $P < 0.01$), and fucosyllacto-N-hexaose (31 vs. 74 mg/mL; $P < 0.01$) compared with milk of uninfected nonsecretors (Fig. 3C). Similar to the 4-d samples, the 28-d sample in HIV-infected nonsecretors also had a higher relative abundance of DFLNH compared with the uninfected group (5.1% vs. 3.3%; $P < 0.01$) (Fig. 3D). In addition, HIV-infected nonsecretors had a higher relative abundance of lacto-N-neotetraose (9.4% vs. 4.0%; $P < 0.05$) and a lower relative abundance of 3-fucosyllactose (2.4% vs. 1.6%; $P < 0.05$) compared with uninfected nonsecretors.

Nine NEC cases occurred within the larger clinical trial cohort (5% incidence). Five of the infants were diagnosed with Bell stage I and four with Bell stage III (2.2% incidence). One infant with NEC (Bell stage I) was excluded because the mother's milk was not sufficient for HMO analysis. The remaining 8 NEC cases were included in this substudy. The HIV-exposed and HIV-unexposed infant groups each had 2 NEC stage I and 2 NEC stage III cases.

Comparing infants who developed NEC with infants who did not develop NEC (irrespective of HIV or Se status of the mother), the mother's milk showed no difference in the total concentration of all analyzed HMOs in the 4-d (Supplemental Fig. 1A) and the 28-d (Supplemental Fig. 1B) samples. There were no significant differences in the concentrations of any of the individual HMOs, except for 1 HMO. Concentrations of DSLNT were significantly lower in the 4-d milk samples of mothers whose infants developed NEC compared with all other infants (200 ± 126 vs. 345 ± 186 mg/mL; $P < 0.05$) (Supplemental Fig. 1C). There was no difference in DSLNT concentrations whether infants were diagnosed with NEC Bell stage I or stage III disease severity (each $n = 4$). DSLNT concentrations in the 28-d milk samples of the remaining 5 NEC cases were lower than in all infants without NEC (Supplemental Fig. 1D). One reason for this lack of significance could be the lower number of 28-d milk samples available for analysis. Three of the infants diagnosed with NEC (1 with Bell stage I and 2 with Bell stage III) did not survive until the 28-d milk sample was collected. Thus, only the 5 remaining 28-d milk samples were available for HMO analysis. After review of the 4-d milk samples of infants with NEC, DSLNT concentrations were indeed lower in infants who did not survive compared with infants who survived past day 28. However, the difference was not significant ($P = 0.657$), likely due to the very small number of samples in both groups.

Discussion

Our results confirm data from a previous study conducted in Zambia that reported differences in HMO composition between HIV-infected and HIV-uninfected women (7). The primary goal of the previous study was to determine whether HMO composition is linked to a higher transmission risk through breastfeeding, and milk from uninfected mothers was only included to serve as a control. Surprisingly, the milk of HIV-infected women had significantly higher concentrations of the sialylated HMO 3#SL, which is confirmed by the present study.

A woman's Se status strongly determines what oligosaccharides are present in her milk. Therefore, the HMO composition in the milk of secretor and nonsecretors is very different, especially with respect to fucosylated HMOs. When selecting samples for this substudy, we purposely separated the cohort into secretor and nonsecretors and within each group compared HMO profiles between HIV-infected and HIV-uninfected women. With this approach we were able to identify additional, mostly fucosylated HMOs whose concentrations significantly differed between HIV-infected and HIV-uninfected women. The biologic or pathophysiologic relevance of these findings remains elusive, however. At least 2 scenarios are possible: 1) HIV infection changes the glycosylation machinery in the mammary gland epithelial cell, which changes the composition of oligosaccharides that are secreted with the milk; 2) individuals who express more or less of certain glycosylation-related genes are more or less likely to acquire an HIV infection. In parallel, these glycosylation-related genes also determine what oligosaccharides are synthesized and secreted with the milk and at what concentration. A combination of in vitro and in vivo studies will be required to approach and answer this difficult question.

Although the primary aim of this study was to determine whether HMO composition varies between HIV-infected and HIV-uninfected mothers, secondary data analysis revealed intriguing results that link HMOs to NEC. Breast-fed infants are known to have a lower risk of developing NEC (19), but the components in human milk that are responsible for the protective effects of breastfeeding remain to be elucidated. A recent study suggested that the HMO DSLNT might be involved because it improved survival and reduced ileum pathology scores in neonatal rats (8). The effects were highly structure-specific because the removal of just 1 sialic acid led to a complete loss of protection. So far, there has been no evidence that these observations translate from the rat model to human preterm infants and that HMOs in general or DSLNT in particular helps

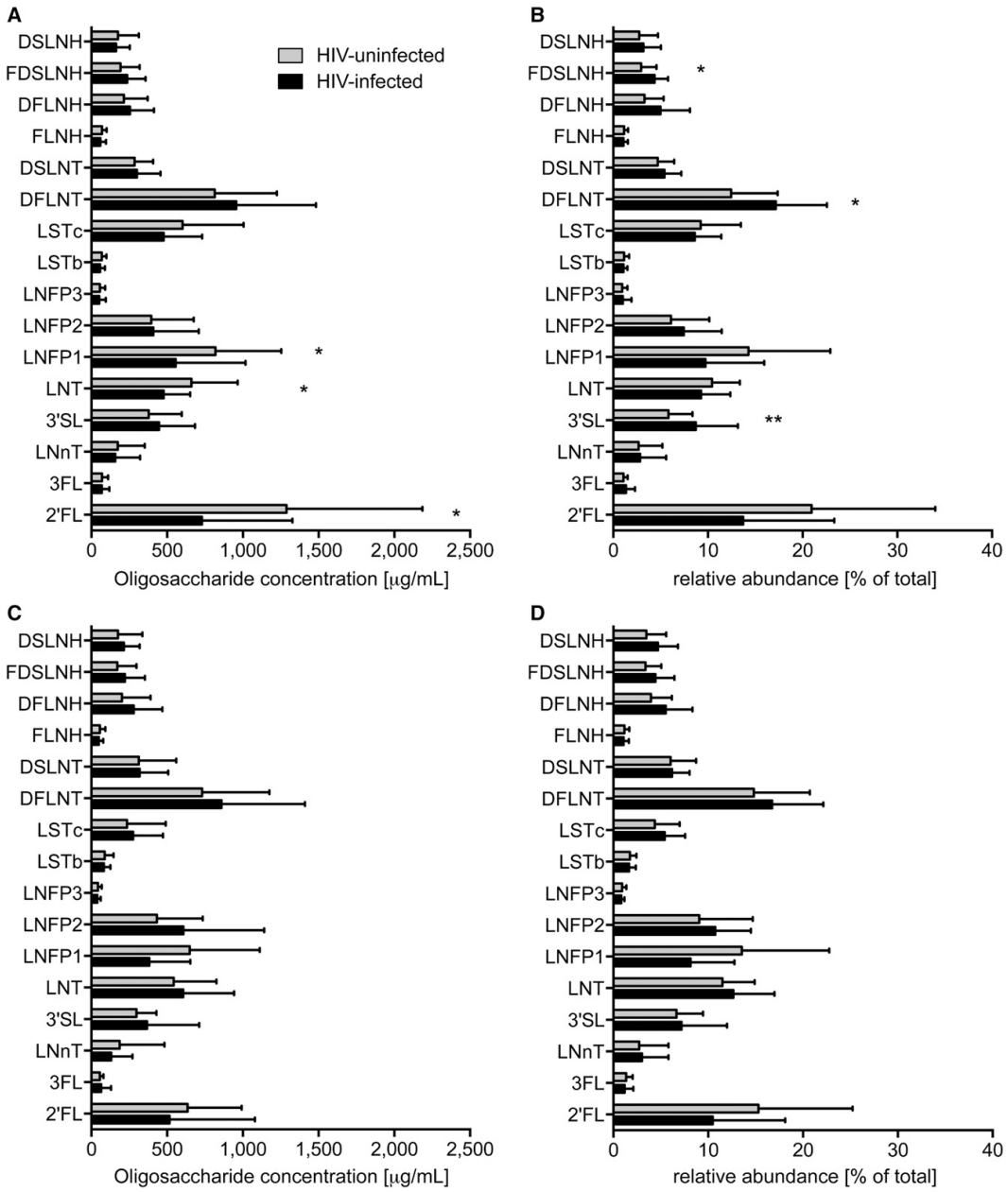

FIGURE 2 HMO composition in the milk of HIV-infected (n = 22) and HIV-uninfected (n = 20) secretor women at 2 different stages of lactation. Values are means ± SDs or percentages. Absolute HMO concentrations in 4-d milk samples (A); relative HMO abundance in 4-d milk samples (B); absolute HMO concentrations in 28-d milk samples (C); and relative HMO abundance in 28-d milk samples (D). *P , 0.05, **P , 0.01. DFLNH, difucosyllacto-N-hexaose; DFLNT, difucosyllacto-N-tetraose; DSLNH, disialyllacto-N-hexaose; DSLNT, disialyllacto-N-tetraose; FDSLNH, fucosyl-disialyllacto-N-hexaose; FLNH, fucosyllacto-N-hexaose; HMO, human milk oligosaccharide; LNFP, lacto-N-fucopentaose; LNnT, lacto-N-neotetraose; LNT, lacto-N-tetraose; LST, sialyllacto-N-tetraose; 2#FL, 2#-fucosyllactose; 3FL, 3-fucosyllactose; 3#SL, 3#-sialyllactose.

to prevent NEC. Our new results now suggest that infants diagnosed with NEC indeed received milk that contained lower concentrations of DSLNT compared with infants who did not develop NEC. The effects seem to be structure-specific because no other individual HMO was associated with NEC and the total sum of all analyzed HMOs was not different whether or not the infants was diagnosed with NEC. It is important to note that only 4 of the 8 NEC cases were categorized as confirmed

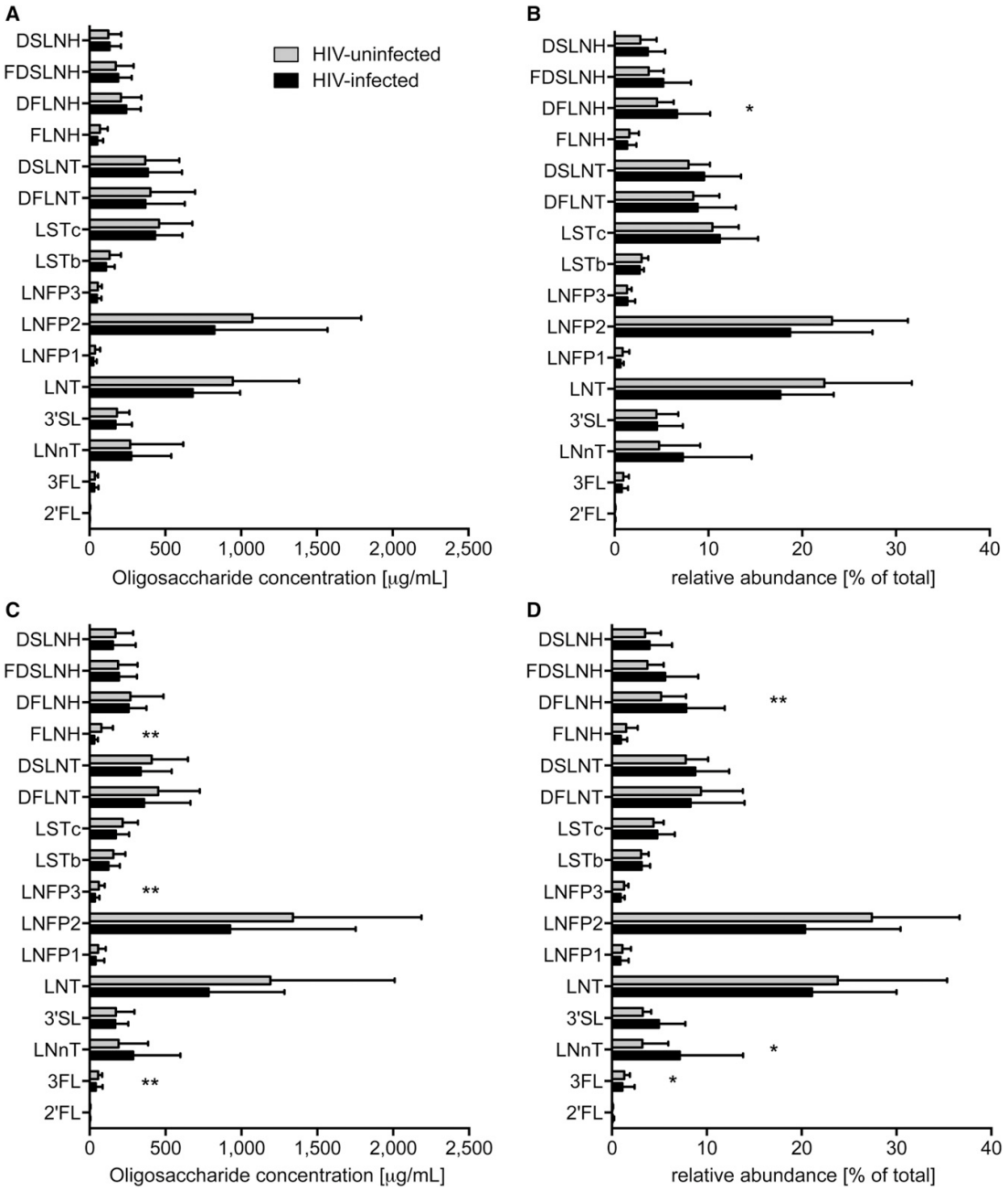

FIGURE 3 HMO composition in the milk of HIV-infected (n = 19) and HIV-uninfected (n = 21) nonsecretor women at 2 different stages of lactation. Values are means ± SDs or percentages. Absolute HMO concentrations in 4-d milk samples (A); relative HMO abundance in 4-d milk samples (B); absolute HMO concentrations in 28-d milk samples (C); and relative HMO abundance in 28-d milk samples (D). *P < 0.05, **P < 0.01. DFLNH, difucosyllacto-N-hexaose; DFLNT, difucosyllacto-N-tetraose; DSLNH, disialyllacto-N-hexaose; DSLNT, disialyllacto-N-tetraose; FDSLNH, fucosyl-disialyllacto-N-hexaose; FLNH, fucosyllacto-N-hexaose; HMO, human milk oligosaccharide; LNFP, lacto-N-fucopentaose; LNnT, lacto-N-neotetraose; LNT, lacto-N-tetraose; LST, sialyllacto-N-tetraose; 2#FL, 2#-fucosyllactose; 3FL, 3-fucosyllactose; 3#SL, 3#-sialyllactose.

survivors and nonsurvivors was not significant ($P = 0.657$), likely due to the small number of cases.

Although the original clinical trial was not designed to determine whether individual HMOs in the mother's milk are linked to infant NEC risk, our results indicate that DSLNT may indeed protect from NEC, not only in neonatal rats but also in human preterm infants. Specifically designed and well-powered cohort studies will be required to verify the link between DSLNT and NEC. If confirmed, DSLNT concentrations in mother's milk could serve as a noninva- sive marker to determine whether a breast-fed infant is at risk of developing NEC. In addition, DSLNT or DSLNT-like compounds could be developed as novel and desperately needed supplements or drugs for the treatment or prevention of NEC.

Although differences were significant, the small group of NEC cases limits the interpretation of the results. All of the infants in this study were exclusively breast-milk fed, which is associated with a very low incidence of NEC.

Acknowledgments

E.V.N., G.F.K., R.B., and L.B. designed the research; E.V.N., C.A.A., and L.B. conducted the research; E.V.N., C.A.A., D.G.N., and L.B. analyzed the data; E.V.N. and L.B. wrote the manuscript; R.B. and G.F.K. critically reviewed the manuscript; and L.B. had primary responsibility for final content. All authors read and approved the final manuscript.

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Chapter 4

CONCLUSIONS AND RECOMMENDATIONS

4.1 SUMMARY OF STUDY OBJECTIVES AND DESIGN

During the 20th century, Nobel Prize winner Elie Metchinkoff proposed the idea that nonpathogenic bacteria were associated with a prolonged lifespan. The term 'probiotic' was first introduced in 1965, and the definition has since undergone many variations.[1] The Food and Agriculture Organization of the United Nations and the World Health Organization defined probiotics in 2001 as "live microorganisms which when administered in adequate amounts confer a health benefit on the host".[2]

In 1999, Hoyos et al. were the first to report a significant reduction in the incidence of necrotizing enterocolitis (NEC) following probiotic supplementation. *Lactobacillus acidophilus* and *Bifidobacterium infantis* were given to preterm neonates. A significant decrease in NEC (85 cases *vs.* 34 cases) was noted in the probiotics group.[3, 4] Numerous clinical trials have since been conducted to elucidate the role of probiotics in this fatal neonatal condition. Clinical trials that have used *Lactobacillus rhamnosus* or *Lactobacillus infantis* found a significant reduction in the incidence of severe Stage II–III NEC. These trials were not specific to HIV status.[5-9] It is also noteworthy to mention that many of the clinical trials conducted on the use of probiotics and the incidence of NEC included formula milk and were not specific to only breast milk as the choice of feed.[5] The literature indicates that the use of formula milk in preterm neonates increases the risk of developing NEC.[10]

The primary objective of the current study was to evaluate the efficacy of probiotics in reducing the incidence and severity of all stages of NEC in premature, very low birth weight (VLBW) infants exposed to HIV. The secondary research objectives were as follows:

i. To assess the incidence of all stages of NEC in VLBW and extremely low birth weight (ELBW) infants born to HIV-infected and HIV-uninfected women; and
ii. To evaluate weight gain for both probiotic-exposed and probiotic-unexposed, premature, VLBW infants; and
iii. To determine the prebiotic quality of the breast milk of HIV-infected and HIV-uninfected mothers, in terms of oligosaccharide content; and
iv. To determine whether there was an association between the prebiotic quality of breast milk in terms of oligosaccharide content and the incidence of NEC in premature VLBW infants.

A randomised, double-blind, placebo-controlled clinical trial was conducted in the neonatal high care unit of Tygerberg Children's Hospital (TBCH), Cape Town, South Africa, during the period July 2011 to August 2012. Babies were included if the following criteria were met: (i) birth weight was in the range of 500–1 250 g and (ii) the baby received breast milk, either from the mother or a donor. Both HIV-exposed and -unexposed babies were included in this study. Throughout the study period, the standard of care protocol consisted of one dose (five drops) of either a probiotic or a placebo daily for four weeks (28 days). This provided the study group with *L. rhamnosus GG* (0.35×10^9 colony-forming units [CFU]) and *B. infantis* (0.35×10^9 CFU) daily. The product used was Pro-B2®. Pro-B2® (C Pharm, Cape Town, South Africa) is an oil suspension and may be stored at room temperature. The control group received a placebo consisting of medium-chain triglyceride (MCT) oil.

Supplementation with the probiotic or placebo was initiated when enteral feeds started. Probiotic or placebo supplementation was delayed or halted in the following circumstances: (i) when an infant was nill per os (NPO) , the infant did not receive a probiotics or placebo until the enteral feeds had commenced again; (ii) when NEC was suspected, the infant continued with treatment until a positive diagnosis of NEC Stage I had been made through an abdominal X-ray; (iii) if the infant remained a possible NEC case and was NPO, the infant did not receive a probiotic or placebo until the enteral feeds had commenced again; (iv) supplementation was discontinued if HIV-exposed infants had a positive polymerase chain reaction (PCR) result on Day 14 of life, due to the uncertainty of probiotic-associated septicaemia in immunocompromised, HIV-positive, VLBW infants on highly active antiretroviral medication (HAART).

All study participants received human breast milk. Both the probiotic and placebo were mixed with the mothers' own breast milk or donor breast milk before administration via an orogastric tube or orally. The probiotic or placebo was added to the breast milk by the researcher and two research assistants. The attending physician and nurses caring for the infants as well as the researcher, research assistants and study participants were blinded to the group assignment. Participants exited the study on Day 28 after birth or upon discharge from the hospital.

Medical and clinical data were collected. These included data on birth weight, estimated gestational age, gender, type of delivery, Apgar scores and daily intake and output. Daily clinical progress notes were also reviewed. Each infant's weight was recorded from his/her

medical file on a daily basis, whilst the length and head circumference was measured by the investigator or research assistants on days 1, 7, 14, 21 and 28 of the study. Z-scores were used in the interpretation of anthropometrical data. Infants were evaluated daily for the development of NEC by the attending neonatologists. Infants who developed any stage of NEC, according to Bell's criteria, were exited from the study. When an infant was exited from the study, the supplementation was halted and daily follow-up continued. NEC Stage I has the potential to develop into more severe stages of NEC. Therefore, the standard of care practice in the specific neonatal unit entails that those infants who develop NEC Stage I are treated as a confirmed NEC case. For the purpose of this trial, infants with Stage I NEC were included in the analysis as there was no information on the effect of probiotics on HIV-exposed, VLBW infants.

A full sample of expressed breast milk was used for determination of the human milk oligosaccharide (HMO) content of HIV-infected and HIV-uninfected mothers. All breast milk samples were raw, untreated specimens; therefore, milk samples did not undergo any pasteurisation procedures. An aliquot of 5–10 ml of breast milk, expressed by hand, was collected in sterilised containers with lids. Three breast milk samples were collected from each mother on Day 4 and Day 10 of lactation, and the final sample was collected 28 days postpartum. Only the first and final breast milk samples were analysed. A saliva sample (1–2 ml) was collected from the mother when maternal consent for the study was granted or at the earliest opportunity thereafter in order to determine the maternal ABH secretor status.

A total of 219 infants were screened for the study. Ten infants were not included in the trial due to parents not providing consent, while 25 infants did not conform to the inclusion criteria. This left a final sample size of 184 infants in the clinical trial. Of these, 74 infants (40%) were HIV-exposed and 110 (60%) were not. Within the HIV-exposed group, 37 infants (50%) were randomised into the study and control groups, respectively. In the HIV-unexposed group, 54 infants (49%) were randomised into the study group and 56 infants (51%) into the control group.

Of the 184 infants enrolled in the study, 156 completed the full 28-day study period. Infant outcomes were as follows: three infants were withdrawn from the study; seven were discharged; 11 deaths occurred, five of which were NEC associated; there were four NEC survivals; and there were three positive PCR results at Day 14 of life.

4.2 NECROTIZING ENTEROCOLITIS IN HIV-EXPOSED VERY LOW BIRTH WEIGHT AND EXTREMELY LOW BIRTH WEIGHT INFANTS: INCIDENCE AND SEVERITY

NEC is the most common serious acquired disease of the gastrointestinal tract in preterm infants.[11, 12] Infants with NEC have a higher incidence of nosocomial infections, supoptimal nutrient intake and inappropriate growth, and also have longer durations of intensive care and hospital stay.[13] Possible risk factors for NEC are prematurity,[14] enteral feeding,[15] growth restrictions,[16] preeclampsia and maternal hypertensive disease,[17] low Apgar scores[14] and red blood cell transfusions.[18] Furthermore, the prevalence of low birth weight and preterm birth among infants who are born to HIV-infected women has increased as treatment has evolved into regimens of HAART.[19] Exposure to HIV during foetal life and maternal treatment with antiretroviral (ARV) drugs are two probable mechanisms proposed to explain the increased incidence of NEC in this population.[20]

The results of the current randomised control trail indicate that the overall incidence of NEC was 5%, which is within the lower range of other reported studies.[6, 21] Among the nine cases of NEC that occurred, four episodes (2%) were classified as severe NEC, namely Bell's Stage III. Furthermore, the study found a reduced incidence of NEC (Bell's stages II and III) in the study group when compared to the control group (3% *vs* 6% NEC incidence, respectively), not specific to HIV status.

This study failed to show that probiotics lowered the incidence of NEC in HIV-exposed premature infants; however, probiotic supplementation appeared to reduce the severity of disease, assessed using Bell's criteria. The severity of NEC within the HIV-exposed group differed significantly ($p = 0.045$), with 5% ($n = 2$) of the infants in the study group developing Bell's Stage I NEC and 5% ($n = 2$) of the infants in the control group developing Bell's Stage III NEC (Refer to article 1: Probiotics and necrotizing enterocolitis in HIV-exposed premature infants: a randomised controlled trial – Table 3: Clinical outcomes of HIV-exposed and -unexposed infants). The latest updated Cochrane review summarises the evidence of probiotic efficacy from 16 randomised trials and more than 2 700 preterm infants. The results show that probiotics reduced the incidence of severe NEC, mortality and NEC-related mortality.[5] The incidence of death for all stages of NEC did not differ significantly between the HIV-exposed and -unexposed groups in this study.

The researcher therefore partially accepts the null hypothesis (see 2.4) that the administration of probiotics does not reduce the incidence of NEC in premature, VLBW infants exposed to HIV. We do, however, reject the null hypothesis that the use of probiotics does not reduce the severity of NEC in these infants. A secondary objective of this study (see 2.3.2) was to assess the incidence of NEC in VLBW and ELBW infants born to HIV-infected and HIV-uninfected women. The results did not show a significant difference in the incidence of NEC in the case of HIV exposure. We therefore accept the null hypothesis (see 2.4) that there is no difference in the incidence of NEC in VLBW and ELBW infants born to HIV-infected and HIV-uninfected women.

4.3 FEEDING TOLERANCE

The administration of probiotic organisms has been shown in previous studies to shorten the time to full feeds.[5] This is supported by the results of the current study, in which it was found that infants in the HIV-unexposed study group reached full feeds in significantly fewer days than infants in the HIV-unexposed control group (9.63 ± 2.42 days *vs* 11.14 ± 4.15 days; p = 0.022). A different scenario was found in the HIV-exposed study group, in which infants reached full feeds later than in the HIV-exposed control group (10.19 ± 4.055 days *vs.* 9.68 ± 3.46 days p = 0.56). The initiation of enteral feeds was on similar days for both the study and control groups.

Feeding volumes on Day 7 of life were significantly lower for the HIV-exposed infants who received probiotic supplementation than for those who did not (63.83 ± 35.42 ml/kg *vs.* 78.45 ± 28.09 ml/kg; p = 0.036). The quartile range in feeding volumes for the control group on Day 7 ranged from 51.68 ml to 105.55 ml. This increased feeding volume was a data outlier, and conclusions should not be drawn from it. Except for the abovementioned feeding volumes on Day 7, the results show that, overall, there was no difference in feeding volumes between the HIV-exposed and -unexposed groups or between the study and control groups. In contrast to other studies,[22, 23] the current study did not show a difference in feeding intolerance and abdominal distension between the study and control groups.

4.4 POSTNATAL GROWTH

This study evaluated the weight gain of premature, VLBW infants receiving probiotic supplementation compared to similar infants who did not receive such supplementation. The results indicate that the use of probiotic cultures did not affect growth outcomes for head

circumference, length and weight in VLBW infants. These results are supported by other clinical trials.[22, 24-26]

In contrast to what was expected, the HIV-exposed group showed significantly higher z-scores for length and head circumference at Day 28. After ruling out all other factors that could have affected growth, such as gestational age, the use of mechanical ventilation and NEC, the question remains why this group reached better z-scores for head circumference and length than the HIV-unexposed infants.

The study found no difference in the incidence of positive blood cultures; however, the HIV-exposed group showed a lower but nonsignificant trend of positive blood cultures than the HIV-unexposed group. A large cohort study found that neonatal infections amongst ELBW infants were associated with poor growth outcomes, specifically head circumference.[27] The current study found no relationship between maternal CD4 cell count and infant birth weight. The majority (75%) of HIV-positive mothers had been receiving ARV treatment for more than four weeks. Three of the 74 infants (incidence rate of 4%) had a confirmed HIV-positive PCR result and were subsequently started on HAART. The results indicate that symmetrical intrauterine growth restrictions (SIUGR) were more prevalent in the neonates of HIV-positive mothers than in their HIV-unexposed counterparts. These results are supported by other studies that have found that maternal HIV status has a significant effect on neonatal anthropometric parameters.[28] Although the z-scores for length and head circumference were significantly higher in the HIV-exposed group, growth was nevertheless inappropriate for both the HIV-exposed and HIV-unexposed groups, in which infants fell below their initial z-scores at birth.

We could not find any evidence of the effects of ARV drug use and growth in premature infants. The idea was postulated that the use of these medications had an immune-supportive effect and gave an HIV-exposed premature infant an advantage above an HIV-unexposed premature infant who was immune suppressed. This could, however, not be supported by the literature.

There was no difference in feeding volumes between the HIV-exposed and -unexposed groups or between the study and control groups. Feeding volumes and the timing of initiation of enteral feeds had no effect on growth outcomes. The HIV-exposed group showed more days of growth velocity and a daily weight gain more in accordance with the American

Academy of Pediatrics recommendation[29] than the HIV-unexposed infants. Lango et al. could not show an association between growth velocity and HIV exposure.[30] The overall study population of the current study showed weight gain patterns similar to those reported in the literature.[31-33] We therefore accept the null hypothesis (see 2.4) that there is no difference in weight gain between probiotic-supplemented and -nonsupplemented premature, VLBW infants.

4.5 POSSIBLE RISK FACTORS ASSOCIATED WITH NECROTIZING ENTEROCOLITIS

The literature indicates that possible risk factors are associated with the development of NEC. These include prematurity,[14] enteral feeding,[15] growth restrictions,[16] preeclampsia and maternal hypertensive disease,[17] low Apgar scores[14] and red blood cell transfusions.[18] The study observed some of these risk factors.

i. The results of this study indicate that the prevalence of hypertension was significantly higher in HIV-negative mothers compared to HIV-positive mothers (64 [58%] *vs.* 35 [47%]; p = 0.002).
ii. Three of the infants who developed NEC had red blood cell transfusions during clinical manifestations of NEC. Within 24 hours, NEC was confirmed and both cases resulted in death due to NEC.
iii. Apgar scores at five minutes differed significantly between the probiotic and placebo groups among HIV-exposed infants (p = 0.042). This was, however, not relevant to the interpretation of the results as it was purely due to chance and the process of randomisation.
iv. Although the study found no difference in the incidence of positive blood cultures, the HIV-exposed group showed a lower but nonsignificant trend of positive blood cultures compared to the HIV-unexposed group. Furthermore, the severity of NEC in the HIV-exposed study group was less compared to the HIV-exposed control group.

It is suggested that introducing probiotics to preterm infants might be beneficial by preventing overgrowth of pathogenic organisms and potentially competing with other organisms for binding sites and substrate in the bowel.[24] The focal health-promoting effect of probiotics is their enhancement of mucosal immune response through increasing macrophage activity, elevating numbers of killer cells, T-cells and interferon, the suppression of NF-κB signalling[34] and their action against pathogenic microbial colonisation and translocation.[35] Bengmark et

al. have proposed that probiotic administration protects the gut surface and could delay the progression of HIV.[36] These results support the findings of Bengmark et al. and should be further investigated.

4.6 SAFETY OF PROBIOTICS

A systematic review found a benefit in a trend towards the reduction of sepsis in preterm infants.[5] The current study showed a 5% decrease in the incidence of positive blood cultures between the HIV-exposed and HIV-unexposed groups, but this was not statistically significant (8 [11%] *vs.* 17 [16%]; $p < 0.68$). There was also a 5% decrease between the study group and the control group, which was not statistically significant (15 [16%] *vs.* 10 [11%]; $p < 0.32$). These results support Deshpande et al's finding that there is no evidence of a reduced risk of late-onset sepsis in premature infants.[24] None of the positive blood cultures in the current study grew *Lactobacillus* or *Bifidobacterium* species.

The similar incidence of positive blood cultures in the HIV-exposed and -unexposed infants could be the result of the feeding protocol used in the specific unit, according to which each infant received the mother's own breast milk or donor breast milk. The breast milk of HIV-positive mothers was pasteurised according to ward protocol before administration to infants. Breast milk is known to prevent NEC. Human milk may, however, also be a vehicle for transmission of microorganisms derived from the mother or the environment during collection. It has been found that coagulase-negative staphylococci, alpha hemolytic streptococci and pathogens such as *Escherichia coli* and others are present in 60–80% of all donor breast milk samples collected.[37] Pasteurisation is known to decrease the number of pathogens present in breast milk. Therefore, it is possible that the pasteurisation of breast milk could have served as a protective mechanism for the HIV-exposed infants. The use of probiotics in VLBW and ELBW infants exposed to HIV was thus found to be safe in this clinical trial.

4.7 OLIGOSACCHARIDE QUALITY OF HIV-INFECTED MOTHERS' BREAST MILK

Distinct differences in oligosaccharides were found between the breast milk of HIV-infected and HIV-uninfected mothers. A significantly higher relative abundance of 3-fucosyllactose (3'SL), difucosyl lacto-N-tetraose (DFLNT) and fucosyl disialyllacto-N-hexaose (FDSLNH) was found in the HIV-infected secretor group. Higher sialyted HMO, 3'SL in the breast milk of

HIV-infected mothers have previously been postulated to have pro-inflammatory effects. The relative abundance of the neutral oligosaccharide difucosyllacto-N-hexaose (DFLNH) was significantly higher in the HIV-infected nonsecretors in the first and final samples, and there were higher concentrations of lacto-N-neotetraose (LNnT) in the second sample. Bode et al. found a nonsignificant trend towards higher concentrations of LNnT (mg/L) and postpartum HIV transmission.[38]

The HIV-uninfected secretors showed significantly higher concentrations for the neutral oligosaccharides 2'-fucosyllactose (2'FL), lacto-N-tetraose (LNT) and lacto-N-fucopentaose (LNFP I) in the first milk sample than the HIV-infected secretors. The high content concentrations of oligosaccharides such as LNT, LNFP I and LNDFH I in early stage milk/colostrum could affect the formation of bifidus flora in the infant colon significantly.[39] We therefore reject the null hypothesis (see 2.4) as distinct differences were found in the HMO content of HIV-infected and HIV-uninfected mothers. Furthermore, given previously mentioned supporting evidence, it seems that specific oligosaccharides found in higher quantities in HIV-infected mothers' breast milk may have pro-inflammatory effects that could potentially affect disease transmission and progression.[38]

Jantscher-Krenn et al. found that DSLNT had a protective effect against the development of NEC in neonatal rats.[40] The results of the current study indicate that DSLNT levels were significantly higher in the group that did not develop NEC, supporting the possibility that DSLNT also protects against NEC in humans. Greenhill et al. concur that DSLNT could be used as a biomarker to determine the preterm infant's risk of developing NEC. We therefore reject the final null hypothesis (see 2.4) that an association does not exist between the prebiotic quality of breast milk and the incidence of NEC in premature, VLBW infants. The results of this study are the first to support evidence from animal studies that certain oligosaccharides may be markers for the occurrence of NEC.

The findings support the relative protective value of probiotic administration to infants with NEC. Microbiota disturbances early in HIV infection lead to greater dominance of potential pathogens, reduced levels of *Bifidobacteria* and *Lactobacillus* species and increased mucosal inflammation.[41] HMO supports the growth of these beneficial bacteria, and it is therefore proposed that in conjunction with the use of probiotics, HMO potentially reduces the risk of NEC.

4.8 LIMITATIONS OF THE STUDY

There were numerous limitations to this randomised control trail. The following limitations occurred and could have impacted the quality of the results:

i. Due to slow enrolment of the HIV-exposed group, the sample sizes were recalculated by the study statistician. A power analysis for two-way ANOVA with a power of 90% to detect interaction effect sizes of $\delta = 0.55$ was calculated. Only nine infants in this study developed NEC. Although the statistics are significant, a small sample size of NEC cases precludes an overenthusiastic interpretation of the research results. Of the nine NEC cases that occurred within the clinical trial, only eight were included for HMO analysis.

ii. Of the eight cases included in the HMO analysis three of the infants that developed NEC Stage I were in the probiotic group. With the randomisation patients were included that received probiotics, however with the very small numbers of patients that did develop NEC, the role of probiotics would not be valid in testing.

iii. All infants in this study received breast milk. Although we are delighted with the very low incidence of NEC, the use of breast milk alone may have affected the incidence of NEC. An ethical dilemma exists when conducting clinical trials in which NEC is the measured endpoint. It is known that the use of formula milk increases the incidence of NEC in premature infants.

iv. The probiotic product used in this study underwent stability tests twice during the study period. This was done by C Pharm, and reports were not supported by an independent entity.

v. The infants' length was measured with a nonstretchable measuring tape and not with a length board. This technique was used to ensure that infants were handled to the minimum and for infection control purposes. However, using this technique could have affected the accuracy of length data.

vi. Only a subsample of breast milk samples was analysed due to financial constraints. Analysis of the total sample of approximately 500 breast milk samples would have strengthened the data. Further funding is sought to analyse these samples.

vii. The proportionate prevalence of HIV-exposed versus HIV-unexposed infants led to lower patient numbers and a strenuous data collection period with financial constraints.

viii. The 28-day data collection period did lead to seven infants being discharged to other institutions before the end of the 28-day follow-up period.

ix. This study had a 5% incidence of declined parental consent. HIV status remains a sensitive matter in South Africa. Obtaining parental consent was a time-consuming procedure that had to be handled with respect and sensitivity.

x. Before the supplementation of probiotics/ placebo the investigator/ assistant washed her hands with an antiseptic soap and thereafter D-germ (a sterilizing spray) was applied. Thereafter infants received their supplementation. Medical gloves are not routinely used in the unit where the study was performed. However, gloves were used during all patient-care activities that involved exposure to blood and all other body fluid. Although hygienic practices were strongly adhered to during this study, stricter guidelines should have been in place to ensure minimal contamination risks.

Furthermore, interesting results could have been obtained by means of collection of the following data:

i. A secondary PCR result at a later point in time could have been used to determine further HIV transmission risks and links to HMO content.

ii. Faecal sample collection and analysis of stool cultures could have provided insight into bacterial colonisation in HIV-exposed infants.

iii. Cytokine profiles in an HIV-exposed infant would have been novel and could have presented interesting results for future studies.

4.9 RECOMMENDATIONS

Practical recommendations to address the specific research problem are as follows:

i. This study found a reduced incidence of NEC (Bell's stages II and III) in the study group when compared to the control group (not specific to HIV status). This study supports the notion that a change in practice is needed and that the use of probiotics is an effective method to reduce the risk of NEC in VLBW and ELBW infants in general and infants who are immune compromised. Many important issues need to be addressed, however, before accepting probiotics as a routine therapy in preterm neonates.

ii. The optimal strains, dose and duration of probiotic organisms are not clear in published research. It is recommended that a probiotic organism and dose *that have previously been evaluated in a well-designed clinical trial* be used.

iii. Maturity of the host is also an important factor in colonisation by probiotic organisms. Very little published literature is available on the use of probiotics in infants < 1 000 g. In the current study, probiotics proved to be safe in this vulnerable group but due to a lack of data, *caution should be used in these infants*.

iv. Furthermore, probiotics have been found to be safe in VLBW and ELBW infants exposed to HIV. This study did not show a significant difference in the incidence of positive blood cultures, but it is evident from the results that the HIV-exposed group showed a lower trend of positive blood cultures than the HIV-unexposed group. The safety of probiotic supplements is an important issue in preterm neonates. It is reassuring to know that sepsis caused by probiotic organisms has not been reported in any of the trials included in the systematic reviews.[24] However, *caution is necessary before adopting probiotics for prophylaxis in immunocompromised hosts* such as preterm and HIV-exposed neonates given that the possibility of probiotic sepsis can never be ruled out.

v. Other practical issues include difficulties in accessing a suitable, stable and safe product with recognised probiotic properties. Should the use of probiotics become routine practice, *it is essential that probiotic products be approved by the regulatory agencies to ensure the safety, viability and quality of probiotic organisms available.*

vi. A large cohort study found that neonatal infections amongst ELBW infants were associated with poor growth outcomes, specifically head circumference.[27] It is recommended that poor growth patterns (as seen in this study) in VLBW and ELBW infants be supported by *early and optimal initiation of enteral feeds, the use of probiotics to encourage achievement of full feeds, exclusive use of breast milk, appropriate supplementation of breast milk and optimal infection control in neonatal units.*

vii. Three of the 74 infants (incidence rate of 4%) had a confirmed HIV-positive PCR result. Low HIV transmission rates such as these should be upheld by strict adherence to prevention of mother-to-child transmission (PMTCT) protocols. It is therefore recommended that PMTCT guidelines be upheld postnatally with feeding protocols that adhere to baby-friendly guidelines. Furthermore, where possible,

exclusive breastfeeding should be encouraged; the breast milk of HIV-infected mothers should be pasteurised, and ARV treatment regimens should be properly implemented.

The following are recommendations for future research:

i. Postnatal growth failure is common in the VLBW and ELBW infant. Although growth was suboptimal, the significant difference in head circumference and length between the HIV-exposed and -unexposed groups in this study remains an area for further investigation. The results indicate that SIUGR did not contribute to the difference in growth between the HIV-exposed and -unexposed groups. Prenatal maternal dietary and lifestyle habits, maternal and infant ARV treatment and the infant's nutritional intake may be areas of study to clarify this unexpected phenomenon. *Longitudinal studies are also recommended to assess the progress, growth and outcomes, including neurodevelopmental outcomes, of premature, VLBW and ELBW infants infected with HIV.*

ii. The literature indicates that HMO may potentially protect against postnatal HIV transmission.[38] The results of the current study indicate that low levels of DSLNT in the mother's milk increase the infant's risk for NEC, which is in accordance with the results of previously published animal studies and warrants further investigation. Future studies should also assess *HIV-transmission risk and oligosaccharide quality of HIV-infected women*. *Cytokine profiles of HIV-exposed infants compared to their maternal oligosaccharide breast milk content might provide interesting results.*

iii. Large clinical trials are recommended to assess the *use of probiotics in HIV-exposed VLBW infants.* Additional outcomes of these trials should include *stool analysis and cytokine profiles of HIV-infected infants.*

iv. It is suggested that oligosaccharides found within human milk may act as specific substrates for assisting the growth of selected beneficial bacteria. An important research question that remains is the *possible relationship that exists between probiotic supplementation and maternal HMO profiles.* Future studies should assess the *use of different probiotic cultures and maternal HMO profiles on gut colonisation.*

v. Finally, several infant formula-producing companies searched for inexpensive alternatives to HMO and developed mixtures of galactooligosaccharides and fructooligosaccharides or inulin that mimicked the prebiotic effects of HMO. Extensive research is needed *to clarify the specific effects of these 'artificial' glycans and, more importantly, to understand the mechanisms by which HMO benefits the breastfed infant.*

vi. From the study results, it is clear that it would be worthwhile to further investigate the *synbiotic effect of probiotic supplementation in infants at risk of NEC.*

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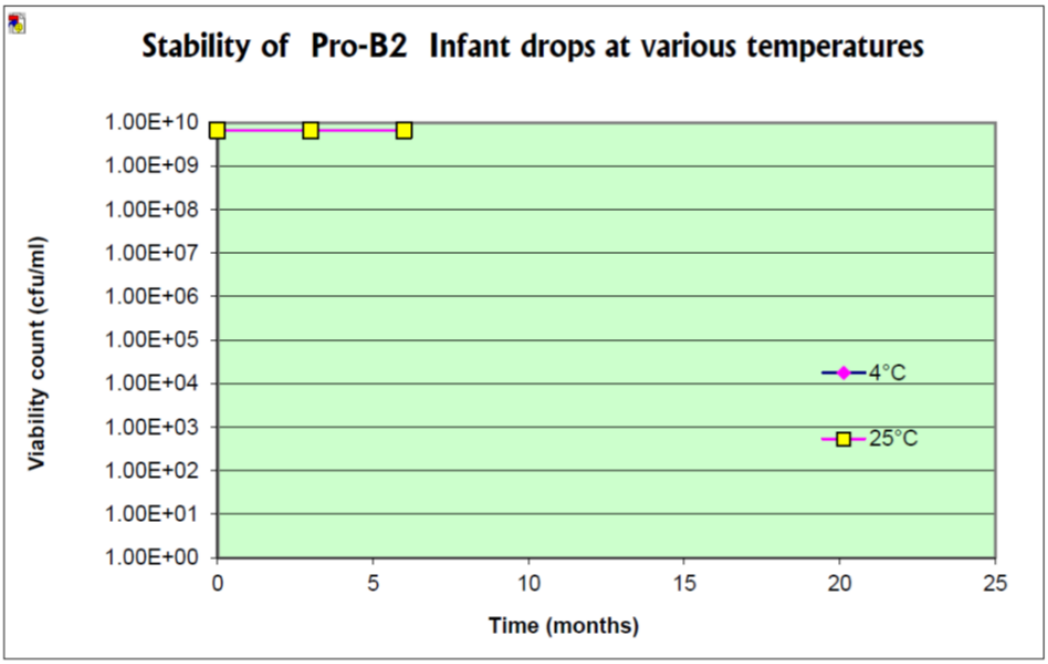
ADDENDA

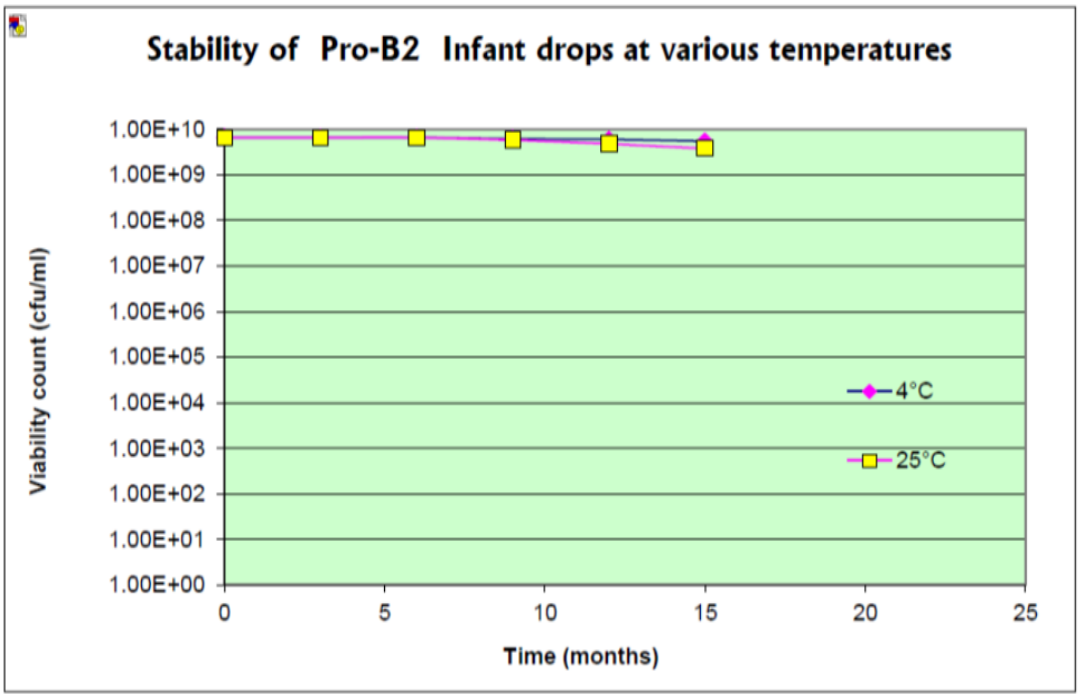
ADDENDUM A: Stability reports for Pro-B2

Stability report 1:



Viability count is indicated as CFU/ml. this starts at increments of 1 x 10^0 and raises in increments of power 1. Therefore the claim made by C-Pharm is is 1 x 10^8. The product remained stable during months 0-6 of the study at 25ºC.

Stability report 2



Viability count is indicated as CFU/ml. this starts at increments of 1 x 100 and raises in increments of power 1. Therefore the claim made by C-Pharm is is 1 x 10^8. The product remained stable during months 6-15 of the study at 25ºC.

ADDENDUM B: Export permit, material transfer agreement (MTA)

health

Department:
Health
REPUBLIC OF SOUTH AFRICA

Private Bag X828, PRETORIA, 0001
Civitas Building Corner Andries and Struben Street, PRETORIA, 0001
Tel (012) 395 0922 • Fax (012) _____

Tel (012) 395-9197/8366 Ms L Motopi

Fax (086) 632 2606/6815 J1/2/4/2 No 1/12

Evette van Niekerk
Lecturer Therapeutic Nutrition
Stellenbosch of University
Division of Human Nutrition
P O Box 19063
TYGERBERG
7505

Dear Ms van Niekerk

EXPORT PERMIT

Attached please receive one export permit as requested by your fax dated 18 June 2012.

Please note that the Department is committed to processing all requests for permits as soon as possible. However, the Department cannot guarantee the issuing of a permit within a specified time. You are therefore advised that applications for permits reach the Department well in advance of shipping dates and/or requirements.

The Department will not be responsible for any losses due to applications that are not received timeously.

Kind regards

DIRECTOR-GENERAL: HEALTH
Date: 21/06/2012
Ms P Netshidzivhani

health

Department:
Health
REPUBLIC OF SOUTH AFRICA

Private Bag X828, PRETORIA, 0001
Civitas Building Corner Andries and Struben Street, PRETORIA, 0001
Tel (012) 395 0922 - Fax (012) _____

Reference : J1/2/4/2 No 1/12
Enquiry : Ms L Motopi
Tel : (012) 395 8366/9197
Fax : (086) 632 6815/2606

EXPORT PERMIT

In terms of Section 68 of the National Health Act 2003 (Act No. 61 of 2003) –

Evette van Niekerk
Lecturer Therapeutic Nutrition
Stellenbosch University
Division of Human Nutrition
P O Box 19063
TYGERBERG
7505 RSA
Tel. No.: (021) 938 9474 Fax. No.: (021) 933 2991

Is hereby authorised to export from the Republic of South Africa –

170 Human breast milk

to –

Dr Lars Bode
University of California
San Diego
212 West Dickinson Street
Lab CTE B111 and B114
San Diego, CA 92103
Tel. No: 619 534 7545 Fax. No: 619 534 7537

For – Breast milk samples will be analyzed for oligosaccharides.

This export permit is subject to the following conditions:

1. The substance shall be imported into the country specified above, within the legal requirements of that country.
2. The substance shall be exported from South Africa and handled in accordance with the provisions of the National Health Act 2003 (Act No. 61 of 2003), and the regulations made in terms of the Act.
3. The export permit shall not be used for any trade or advertising purposes.
4. **This export permit shall expire on 30 September 2013.**

DIRECTOR-GENERAL: HEALTH
Date: 21/09/2012
Ms P Netshidzivhani

MATERIAL TRANSFER AGREEMENT

Between

STELLENBOSCH UNIVERSITY ("SU")

Physical Address	Admin. Building B 3223, Victoria Street, Stellenbosch, 7600, South Africa
Postal Address	Private Bag X1, Matieland, Stellenbosch, 7602, South Africa
Telefax Number	+27 (0)21 808 4537
Telephone Number	+27 (0)21 808 3546
Contact Person: **Contract** related matters	Manager Research Contracts: Mrs Cornelia Malherbe cmalherbe@sun.ac.za
Contact Person: **Project** related matters Email Address	Evette van Niekerk evettev@sun.ac.za
Signature who warrants that s/he is duly authorised to sign	
Name	Evette van Niekerk
Position	Lecturer Therapeutic Nutrition
Date	12/06/2012

and

[ ] ("**Recipient**")

Physical Address	UCSD Dept. Of Pediatrics 9500 Gilman Drive, MC 8450 La Jolla, CA 92093 USA
Postal Address	UCSD Health Sciences Asst. Vice Chancellor 9500 Gilman Dr. MC 0602 La Jolla, CA 92093 USA
Telefax Number	858.822.6691
Telephone Number	858.822.3474
Contact Person	Robin Samit -contract matters Lars Bode –project matters
Email Address	rsamit@ucsd.edu lbode@ucsd.edu
Signature who warrants that s/he is duly authorised to sign	
Name	Lars Bode
Position	
Date	

Recipient Scientist

Facility/Laboratory Address	UCSD Dept. Of Pediatrics 9500 Gilman Drive, MC 8450 La Jolla, CA 92093 USA
Telephone Number	858.822.3474
Email Address	lbode@ucsd.edu

Signature	
Name	Lars Bode
Position	
Date	

Research Project title: The use of probiotics in the management of Necrotising Enterocolitis in HIV exposed premature and very-low birth weight infants	**Contract number: S002863**
Research Period: Sample analysis: Sept 2012 – February 2013	**SU Ethical Clearance number: M10/09/035**

Material type:

Human tissue or blood samples [x]; Cell components [ ]; Plants or organisms [ ]; Animals [ ]; Genetically Modified Organisms [ ]

Bioprospecting: [YES / NO]

Use of indigenous biological resources: [YES / NO]

1 The Recipient acknowledges that the Material is confidential and proprietary to SU. The transferred Materials shall be used solely for non-commercial research purposes to carry out the Research Project (clause 15) only at the Recipient's facility/laboratory under the direction of the Recipient's Scientist.

2 Legal title to the Material will remain with SU. Nothing in this Agreement: (i) grants the Recipient any rights over the Material (other than as specifically granted by this Agreement) or under any patent, plant breeders' right or other intellectual property right, nor any right to use, or (ii) permits the use of any products or processes containing, using, or directly derived from the Material, for profit-making or commercial purposes.

3 The Recipient will not make use of, or permit anyone else to make use of, the Material or a product directly derived from the Material for commercial purposes or for any other purposes other than for the Research Project without SU's prior written consent. In such event, it agrees to negotiate in good faith with SU for the grant of an appropriate licence or the conclusion of a revenue sharing agreement, if justified. SU will have no obligation to grant a licence.

4 It is expressly acknowledged and agreed by the Recipient that the Materials may not be used for work on humans, in clinical trials or for diagnostic purposes involving human subjects and that the Materials must at all times be used in accordance with all applicable laws and regulations.

5 The Recipient agrees not to transfer, transmit or in any other way provide access to the Materials, to any third party without the written consent of SU. Such consent will not be unreasonably withheld if the third party is an academic research institution as long as such third party signs an equivalent Material Transfer Agreement with SU. Upon request by SU, the Recipient will return such of the Material as may be required to the SU or dispose of such Material as directed by SU and Recipient shall certify such disposal upon request.

6 Upon request the Recipient will notify SU of the results of the Research Project and provide SU with samples of such results developed through the use of the Material. Neither party may register or apply for the registration of any intellectual property right with respect to the results of the Research Project (including without limitation under patent or plant breeders' right) without the prior written consent of the other party. The Recipient grants SU a fully paid-up, non-exclusive right to use any results developed through the use of the Material transferred under this Agreement for its own internal, non-profit academic research and teaching purposes.

7 Material delivered pursuant to this agreement by SU to the Recipient is understood to be experimental in nature and may have hazardous properties. SU makes no representations and provides no warranties of any kind, either expressed or implied by law, with respect to the Material or any information associated to it. In particular, SU hereby excludes and disclaims any express or implied warranty of reasonable quality, merchantability or fitness for a particular purpose, that the

Material or any information associated with it, is free from defects (latent or otherwise), or that use of the Material will not infringe any intellectual property right or other third party rights.

8 In no event will SU or its personnel be liable to the Recipient for any direct or indirect losses or damages whatsoever, arising in connection with this agreement, save to the extent that the limitation of liability contained herein is not permitted by applicable law.

9 Risk of loss or damage to the Material will pass to the Recipient upon delivery to the transport carrier. The use of the Material will be at the sole and exclusive risk of the Recipient. The Recipient hereby indemnifies and agrees to hold SU harmless against any and all losses that may arise in connection with the Materials, including any loss or damage to the Material in transit.

10 The Recipient agrees to treat all information pertaining to the Material as confidential and proprietary to SU, including the properties, characteristics, content and composition thereof and the potential uses and methods of use thereof and will not disclose any information pertaining to the Material to any third party without the written consent of the SU including pursuant to clause 11. The Recipient will also ensure that the Recipient's Scientist and all other persons allowed to access the Material comply with this clause.

11 SU recognises the desire of the Recipient to publish details of academic research in scientific journals or theses and SU agrees that the Recipient will be free to publish results of the Research Project, providing that SU is provided with a copy of any such manuscript or abstract at least thirty (30) days prior to submitting such publication to the scientific journal or to exmaminers, to give SU the opportunity of requesting the removal of any proprietary confidential information pertaining to the Material. Recipient must comply with SU's removal requirement to the reasonable satisfaction of SU prior to submitting such publication. Manuscripts should be sent to SU's technology transfer company, InnovUS Pty Ltd, or its Research Contracts Office, Division for Research Development, for review of such proprietary information content. SU agrees to maintain such results, copy of any such manuscript or abstract in confidence and not to engage in any written dissemination or in any dissemination by other methods of results obtained by Recipient from use of the Materials for the purpose described in Clause 6.

However, this clause 11 shall not preclude either party's attribution of authorship in, and distribution of academic literature reporting the results of research conducted with the Materials, where applicable.

12 The Recipient agrees to provide appropriate acknowledgement of the source of the Materials in all publications. Each party agrees not to use or refer to this Agreement in any promotional activity, or use the names or marks of the other without express written permission.

13 General

13.1 This Agreement shall come into force on the date on which it is signed by both parties and shall remain in force for the duration of the Research Period, or as long as the Recipient has possession of the Materials if longer.

13.2 Either the Recipient or SU may terminate this Agreement forthwith by thirty (30) days prior notice of termination in writing:

13.2.1 If either party commits a material breach of this Agreement, which in the case of a breach capable of remedy is not remedied within thirty (30) days of the receipt by the party in default of notice identifying the breach and requiring its remedy, or.

13.2.2 Termination without cause

Upon termination of this Agreement, Recipient's rights to use the Material will cease and Recipient will discontinue all use of the Material, but all other terms hereunder will continue unaffected.

13.3 Neither party shall assign or transfer any interest in this Agreement without prior written approval of the other party.

13.4 No amendment, consent or waiver of terms of this Agreement shall bind either party unless in writing and signed by all parties. Any such amendment, consent, or waiver shall be effective only in the specific instance and for the specific purpose given.

13.5 This Agreement embodies the entire agreement between the parties hereto and no provision of this Agreement may be changed except by the mutual written consent of the parties hereto.

13.6 This Agreement shall be governed by the South African Law and the South African Courts shall have exclusive jurisdiction to deal with any dispute which may arise out of or in connection with this Agreement.

13.7 The use of South African biological resources is governed by the National Environmental Management Biodiversity Act, Act 10 of 2004 (NEMBA) and its associated subordinate legislation. To engage in any bioprospecting activity using biological material with South African origin a bioprospecting permit must be obtained from the Department of Environmental Affairs:

The Director General

Department of Environmental Affairs

Private Bag x447

PRETORIA

0001

Enquiries:

The Director: Resource Use

Mr. Muleso Kharika

Tel: +27 12 310 3578 / 3451

Fax: +27 12 320 4087 / 7026

Email: Jkharika@environment.gov.za
www.environment.gov.za

14 The Material

This agreement concerns the following types and quantities of material to be provided to the Recipient:

Type of Material	Quantity	Place of Origin	Already Identified Potential Uses
Human Breast Milk	180	Tygerberg Hospital. Cape Town South Africa	Analysis of Oligosaccharides for PhD Study

Material includes all progeny generated from the Material supplied and that part of all derivatives and the derivative's progeny which contains any of the Material supplied or its progeny.

15 The Research Project

The manner in which and the extent to which the Material may be used by the Recipient are as follows:

~ END ~

ADDENDUM C: Data collection forms

	PARTICIPANT NO AND CODE				DATE OF ENTRY INTO STUDY			
	ENVELOPE NO							
	Infant's sticker				Mother's sticker			
	MATERNAL INFORMATION		CONSENT FORM SIGNED: Y/N					
			SALIVA SAMPLE TAKEN: Y/N					
	HIV status:		Pos =1 Neg=2					
	CD4 cell count:							
	PMTCT:		HAART =1; TREATMENT IN LABOUR= 2; NO TREATMENT= 3					
	Duration PMTCT Med:							
	TB:		YES=1; NO=2					
	TB treatment:		YES=1; NO=2					
	VDRL + :		YES=1; NO=2					
	Syphilis treated:		YES=1; NO=2	Treatment completed:		YES=1; NO=2		
GENEREAL	Age:							
GENEREAL	Race:		BLACK=1; COLOURED=2; WHITE=3; OTHER=4		If other - Specify:			
GENEREAL	Gravida:							
GENEREAL	Para:							
GENEREAL	Mode of delivery:		NVD=1; BREECH=2; C-SECTION=3					
GENEREAL	PROM:		YES=1; NO=2					
MEDICAL	Hypertension/ pre-eclampsia:		YES=1; NO=2					
MEDICAL	Diabetes:		Type1= 1; Type 2= 2; Gestational=3; None=4					
MEDICAL	Antepartum haemorrhage:		YES=1; NO=2					
MEDICAL	Abruptio placenta:		YES=1; NO=2					
MEDICAL	Other (please state):							
MEDICATION	Maternal antibiotics:		YES=1; NO=2	Specify:				
MEDICATION	State antibiotics:							
MEDICATION	Maternal steroids:		YES=1; NO=2					
MEDICATION	Other medication- specify:							
	INFANT INFORMATION							
	GENDER:		Male= 1; Female=2					
	DOB:							
	APGAR:	/1min	/ 5min	/10min				
	Birth weight:				LENGTH:			
	GA- dates:				HEAD CIRCUMFERENCE:			
	GA- BALLARD:							
	IU GROWTH		SGA=1; AGA=2; LGA=3		COMPLETE FENTON GROWTH CHART			
	VDRL + :		YES=1; NO=2					
	Syphilis treatment:		YES=1; NO=2					
MEDICAL	Respiratory:		None:1; RDS/ HMD=2; Wet lung=3; Pneumonia=4; TTN=5; Other=6					
MEDICAL	Other - specify:							
MEDICAL	nCPAP:		YES=1; NO=2					
MEDICAL	Cardiac echo:		Normal=1; PDA=2; Other=3; Not done= 4		Other -Specify:			
MEDICAL	Cranial ultrasound:		YES=1; NO=2					
MEDICAL	Cranial ultrasound results:		Normal=1; IVH 1/2=2; IVH 2/3=3					
MEDICAL	Chest X-ray		YES=1; NO=2					
MEDICAL	Jaundice req Phototherapy:		YES=1; NO=2	Total days:				
MEDICATION	ARVs		YES=1; NO=2;NOT APPLICABLE=3					
MEDICATION	Nevirapine:		YES=1; NO=2	Dose:				
MEDICATION	AZT:		YES=1; NO=2	Dose:				
MEDICATION	ARV Treatment period:							
MEDICATION	Antibiotics from birth:		YES=1; NO=2 **COMPLETE DAILY MONITORING**					
MEDICATION	Bloods taken at birth:		YES=1; NO=2 **COMPLETE INFECTION SCREENING**					
MEDICATION	Surfactant		YES=1; NO=2					
MEDICATION	Antibiotics later:		YES=1; NO=2	Specify:				
BLOODS	CRP:		YES=1; NO=2					
BLOODS	FBC at birth:		YES=1; NO=2	FBC later:		YES=1; NO=2	COMPLETE INFEC SCREEN	
BLOODS	Blood culture		YES=1; NO=2	Blood culture later:		YES=1; NO=2	COMPLETE INFEC SCREEN	
BLOODS	Blood gas at birth:		YES=1; NO=2	Blood gas later:		YES=1; NO=2	COMPLETE INFEC SCREEN	
	PCR DAY 14 RESULTS		Pos =1 Neg=2 **COMPLETE INFECTION SCREENING**					

											INTAKE AND OUTPUT														RECEIVING PHOTO	BLOOD TRANSF	FEED INTOLERANCE	CLINICAL	NEC	INFECTION	MEDICATION			
											Fluid intake						Supplementation					Output												
Exit reason (codes)	BM Day 1 lactation and collection date	Study parameters (BM) COMPETED	Study parameters(Head circumference)	Study parameters (Length)	Day	Date (dd/mm/yy)	CPAP	Weight	Probiotic/ Placebo administered	Amount of drops administered	Feeding route ORAL=1; ENTERAL=2; NPO=3	Breast milk Amount	Neonatelyte	Saline	Other	TOTAL ml/kg	FM85	MCT oil	Vitamins	Fe	Other	Urine (nappies)	Stool Frequency	Stool Consistency: CODE	RECEIVED Phototherapy	RECEIVED Blood product type	COMPLETED Intolerance	COMPLETED Abnormal clinical signs	COMPLETED Suspected NEC/NEC	COMPLETED Infection screen	Medication type	Other medication and Medication reason	Written notes in patient file	ADVERSE EVENTS COMPLETED
	x	Y/N	cm	cm			Y/N	Gram	Y/N	5	1;2	ml	ml	ml			Gram	ml	Y/N	Y/N					Y/N	Code	Y/N	Y/N	Y/N	Y/N	Code	Code	Y/N	Y/N
					Birth																													
					2																													
					3																													
					4																													
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Participant no:

		INFECTION SCREENING																								
		CRP	CULTURE SCREEN															FBC						PCR	BLOOD GAS				SEPSIS	
			BLOOD		STOOL		SWAB			LP																			
Day	Date	CRP	Blood culture		Stool culture		SWAB	SWAB SITE		LP		Hct	Hb	WBC	Neutrophil	Lymph	Platelet count	PCR	PH	Bicarbonate	TOTAL CO2	Base deficit	Grade	Final Sepsis Diagnosis		
		Result	Pos/Neg	Organism	Pos/Neg	Organism	Pos/Neg	CODE	Organism	Pos/Neg	Organism	Result	Result	Result	Result	Result	Result	Pos/Neg	Result	Result	Result	Result	Code			
Birth																										
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Participant no:

	FEED		CLINICAL		NEC MARKERS					
Day	Vomiting&aspirates	Intolerance	Abnormal clinical signs	Bowl Sounds	NEC complications	Abdominal X- Ray	Bells NEC grade	Surgery required	Abdominal drain	NEC ADDITIONAL NOTES
	CODE	Y/N	CODE	Y/N	CODE	Y/N	i/ii/iii	Y/N	Y/N	
Birth										
2										
3										
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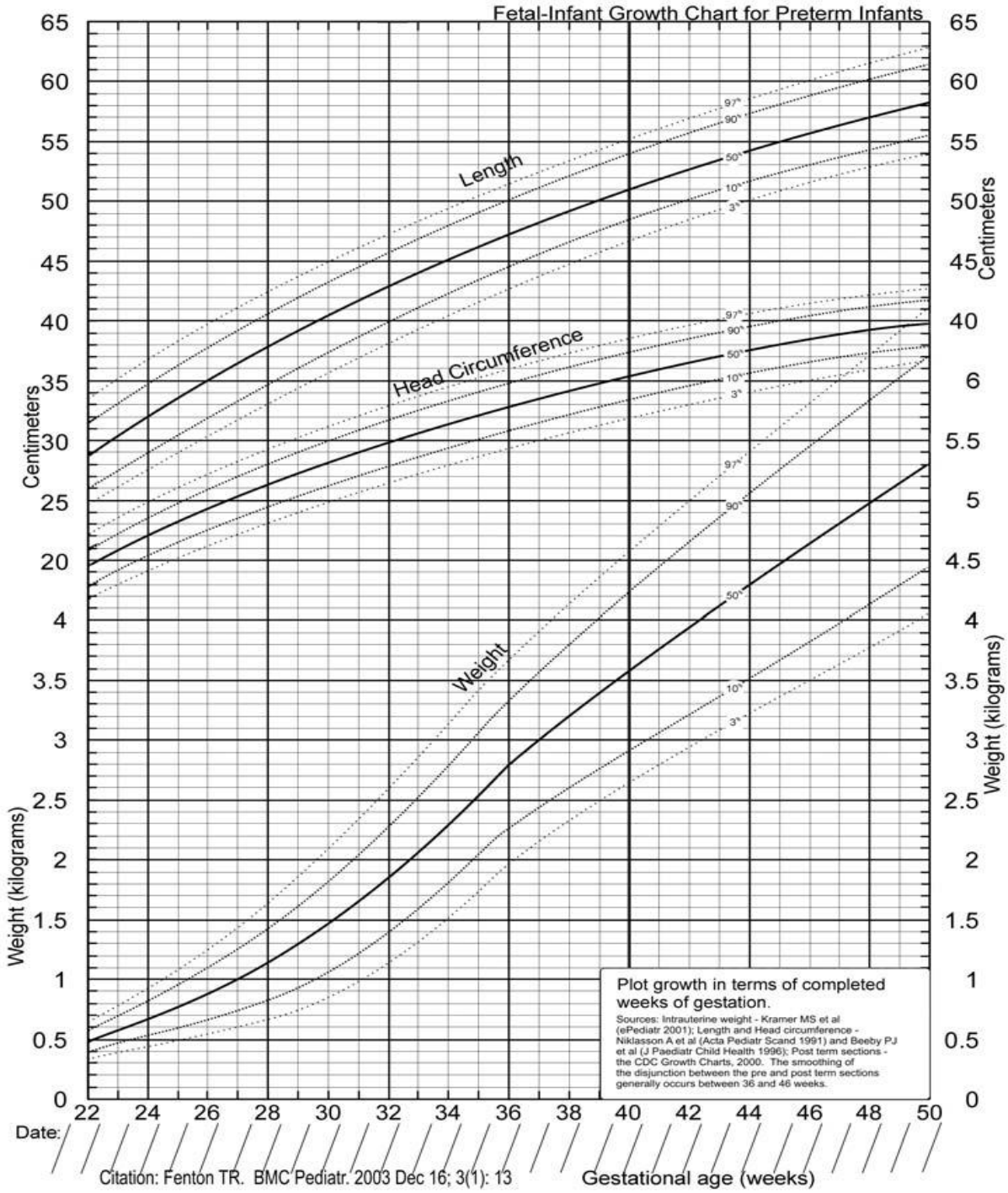
ADDENDUM D : Fenton-Babson growth chart [17]



ADDENDUM E: Informed consent forms (English, Afrikaans and Xhosa)

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITLE OF THE RESEARCH PROJECT:

THE USE OF PROBIOTICS IN THE MANAGEMENT OF NECROTISING ENTEROCOLITIS IN HIV EXPOSED PREMATURE AND VERY-LOW BIRTH WEIGHT INFANTS

REFERENCE NUMBER: M10/09/035

PRINCIPAL INVESTIGATOR: MISS EVETTE VENTER/ van Niekerk

ADDRESS:
DIVISION OF HUMAN NUTRITION
TYGERBERG CAPMUS
THIRD FLOOR
TYGERBERG
7505

CONTACT NUMBER:
WORK: 021 938 9474
CELL: 084 941 3832

1. **INVITATION**

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask

the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you or your baby in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the **Human Research Ethics Committee at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

WHAT IS THIS RESEARCH STUDY ALL ABOUT?

Five to ten percent of babies born before 8 months suddenly develop a condition called necrotising enterocolitis. It is a very serious condition where large parts of the bowel may die and for which the baby may require surgery to remove dead bowel. The exact cause of this condition in babies born too early is not clear but we do know that it is more common in premature babies who receive formula milk or where there are certain germs present in the baby's intestines. It is also more common in premature babies born to mothers who are HIV+. The antiretroviral medication that mothers use and the HIV infection itself might increase the risk. Breast milk stimulates the growth of "good" germs in the gut or intestines of babies. Studies have shown that the "good" germs in breast milk may protect the gut of a small premature baby against necrotising enterocolitis. These so-called "good" germs can be added to breast milk of a mother in order to grow in the babies bowel and to prevent "bad" germs causing damage to the gut. The live "good" germs that we want to add to your breast milk are called Probiotics. In this study we want to see if the "good" germs will help to protect your baby against developing necrotising enterocolitis.

Babies who are born very early are admitted to Ward G2 in Tygerberg Children's Hospital. Very small premature babies who weigh less than 1250g born to mothers who are HIV+ or HIV- will take part in the study. Probiotics ("good" germs) will be given to half of the babies in the study for the first 28days. The other half of the babies will receive a product that looks similar but with no added bacteria ("good" germs).
With this study we want to determine if Probiotics ("good" germs) will:

- help prevent a baby from getting necrotising enterocolitis
- result in shorter hospitalisation
- improve the daily weight gain

This study also wants to determine whether there are differences in the composition of breast milk of HIV+ and HIV- women

HOW WILL YOU AND YOUR BABY PARTICIPATE IN THE STUDY?

If your baby has a birth weight lower than 1250g your baby will be admitted to Ward G2 while you will be admitted to Ward J5 or J2. We will visit you in the ward after the birth of your baby to explain the study to you and to invite you and your baby to participate in the study. We will give you a letter which explains the study and if you agree to participate, you must consent. Within a few days after the birth of your baby, you will be transferred to stay with your baby in ward G2. Once the baby is bigger, you and your baby will be transferred to ward G8 in Tygerberg Children's Hospital where you will stay until your baby is ready to be transferred to another hospital where you will remain until your baby weighs more than 1800g and is ready to go home.

The babies will be divided into two Groups; for the one group Probiotics will be given and for the other group a placebo will be given. The placebo looks the same as the Probiotic but has none of the "good" germs inside.

Babies will be in the study for the first 28 days after birth.

WHAT WILL HAPPEN TO ME IF I DO TAKE PART?

Small premature babies are too weak to suckle on the breast. The mother has to express milk from her breast which is then given to the baby by means of a thin plastic tube that goes from the baby's mouth to the baby's stomach. The nurses in G2 baby ward will teach you how to express breast milk for your baby. As part of the study, we want to measure the quality of some of the components in your breast milk. The most important one is oligosaccharride which also helps to protect your baby against infection. We will therefore need a 5-10ml sample of your breast milk at specific times.

The first milk sample we will need from you is on day 4 of life, then on day 10 and again on day 28 of life. These 3 milk samples will be frozen before being tested in the laboratory. A saliva sample of 2ml will also be needed from you. The sample will be collected in a sterile container. After informed consent is given by you the saliva sample will be taken. Only one saliva sample will be needed. The composition of your saliva affects the oligosaccharide content of your breast milk. The saliva samples will be frozen before being tested in the laboratory.

Information from your hospital file will be used in the study such as how your baby was delivered, what medications you received during your pregnancy, etc. All pregnant women in the Western Cape are tested for HIV during pregnancy. As it is important for us to know your HIV status, we will obtain your HIV status from your hospital folder.

What will happen to the baby if I give consent?

Small babies are weighed every day to see if they are getting enough milk. Your baby will be weighed daily and his/her length and head size will be measured weekly.

The study group (the babies that will receive the Probiotics) will receive 5 Probiotic drops per mouth once daily, for 4 weeks (28 days). The control group (the babies that will receive the placebo without the "good" germs) will receive 5 placebo drops per

mouth once daily, for 4 weeks (28 days). Your baby's folder will be read by the researcher every day and the results of laboratory tests will be recorded.

WHY HAVE YOU BEEN INVITED TO PARTICIPATE?

You have been chosen because you gave birth to a very small baby that was born too early, and was admitted to ward G2 at Tygerberg Children's Hospital (TCH) and you are able to express a sample of breast milk.

WHAT WILL YOUR RESPONSIBILITIES BE?

You will be responsible for expressing milk every day for your baby. On days 4, 10 and 28. 5ml - 10ml of the breast milk expressed by you, will be frozen and taken to the laboratory. A saliva sample of 2ml will also be needed from you. The saliva samples will be frozen before being tested in the laboratory.

WILL YOU BENEFIT FROM TAKING PART IN THIS RESEARCH?

You will benefit from the knowledge that you were a valuable contributor in providing the medical team with important information that might help them in their search for the best way of feeding "early born" and very small babies and to help prevent necrotising enterocolitis.

ARE THERE ANY RISKS INVOLVED IN TAKING PART IN THIS RESEARCH?

Your baby will be treated exactly the same as any of the other small babies admitted to ward G2 at Tygerberg Children's Hospital. Some of the babies will have Probiotics and others will receive a placebo (without the "good" germs). All of the babies (those

receiving the Probiotics and those who receive placebo) will be monitored for signs of infection. Babies will be removed from the study should the following happen:

If the baby does not take any breast milk by mouth for more than 3 days and requires the use of special food

If the baby develops necrotising enterocolitis

If the baby develops diarrhoea (runny stomach)

If the baby develops a blood infection from the Probiotics. The risk of developing a blood infection from the ‘good” germs is very low.

IF YOU DO NOT AGREE TO TAKE PART, WHAT ALTERNATIVES DO YOU HAVE?

It is your decision whether you would like to take part or not. If you decide not to take part in the study it will not affect the care given to your baby. Furthermore you are free to withdraw at any point without explanation or any negative consequences. Your and your baby’s routine health care will not be negatively affected

WHO WILL HAVE ACCESS TO YOUR MEDICAL RECORDS?

Only the researcher, research assistant and persons directly associated with the study will have access to your medical records. All information will always be kept confidential.

The milk samples sent to the laboratory cannot be traced back to you, because your name will not be put on any of your milk samples. Only the researcher and research assistant will know which milk sample belongs to which mother. The researcher and research assistant will treat all information gathered as strictly confidential and no information that can indentify you or your baby will be released to any person who is not not directly associated with the study.

The information obtained/collected will be handled with complete confidentiality and anonymity, but will be published in scientific journals and presented at congresses. Under no circumstances will your or your baby's name be revealed.

WHAT WILL HAPPEN IN THE UNLIKELY EVENT OF SOME FORM INJURY OCCURRING AS A DIRECT RESULT OF YOUR TAKING PART IN THIS RESEARCH STUDY?

It is unlikely that something will go wrong. If you are not able to express milk at the planned collection time, you can try to express milk again later. The researcher will inform you of the next appropriate time to express the breast milk. Throughout the study standard procedures will be used, thus making the chances that something goes wrong, very slim.

WILL YOU BE PAID TO TAKE PART IN THIS STUDY AND ARE THERE ANY COSTS INVOLVED?

No you will not be paid to participate in the study. There will be no costs involved for you, if you do participate.

WHAT HAPPENS WHEN THE RESEARCH STUDY STOPS?

The information obtained from the study will be published in medical journals.

WHAT IF NEW INFORMATION BECOMES AVAILABLE?

Sometimes during the course of a research project, new information becomes available about the topic that is being studied. If this happens, your researcher will tell you about

it and discuss with you whether you want to continue the study. If you decide to withdraw, the standard of care that you and your baby receive will not be affected. If you decide to continue in the study you will be requested to sign an updated consent form. The relevant information that arises during the course of the study will be incorporated into documentation where appropriate.

IS THERE ANYTHING ELSE THAT YOU SHOULD KNOW OR DO?

You can contact Miss Evette Venter at tel 021 938 9474/ 084 941 3832 if you have any further queries or encounter any problems.

You can contact the Committee for Human Research at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by the researchers.

You will receive a copy of this information and consent form for your own records.

Declaration by participant

By signing below, I ……..…….....…. agree to take part in a research study entitled THE USE OF PROBIOTICS IN THE MANAGEMENT OF NECROTISING ENTEROCOLITIS IN HIV EXPOSED PREMATURE AND VERY-LOW BIRTH WEIGHT INFANTS

I also give consent that my baby …….....may take part in a research study entitled THE USE OF PROBIOTICS IN THE MANAGEMENT OF NECROTISING ENTEROCOLITIS IN HIV EXPOSED PREMATURE AND VERY-LOW BIRTH WEIGHT INFANTS

I declare that:

I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.

I have had a chance to ask questions and all my questions have been adequately answered.

I understand that taking part in this study is voluntary and I have not been pressurised to take part.

I may choose to leave the study at any time and will not be penalised or prejudiced in any way.

I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) .....…..…….. on (*date*) ……...…….. 2011.

....

Signature of participant **Signature of witness**

Declaration by researcher

I *(name)* ……..…… declare that:

- I explained the information in this document to ……..
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use a interpreter. (*If a interpreter is used then the interpreter must sign the declaration below.*

Signed at (*place*) .....…..…….. on (*date*) ……...…….. 2011

....

Signature of investigator **Signature of witness**

Declaration by interpreter

I *(name)* ……..…… declare that:

- I assisted the investigator (*name*) ……. to explain the information in this document to (*name of participant*) ……..…….. using the language medium of Afrikaans/Xhosa.

- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) .....…..…….. on (*date*) ……...……..

....

Signature of interpreter **Signature of witness**

DEELNEMERINLIGTINGSBLAD EN -TOESTEMMINGSVORM

TITEL VAN DIE NAVORSINGSPROJEK: DIE GEBRUIK VAN PROBIOTIKA IN DIE BEHANDERLING VAN NEKROTISERENDE ENTEROKOLITIS IN MIV BLOOTGESTELDE PREMATUUR EN BAIE-LAE GEBOORTE MASSA BABAS.

VERWYSINGSNOMMER: M10/09/035

HOOFNAVORSER: MEJ EVETTE VENTER/ van Niekerk

ADRES:

AFDELING MENSLIKE VOEDING

TYGERBERG KAMPUS

VLOER 3

TYGERBERG

7505

KONTAKNOMMER:

WERK: 021 938 9474

CELL: 084 941 3832

UITNODIGING

U word genooi om deel te neem aan ’n navorsingsprojek. Lees asseblief hierdie inligtingsblad op u tyd deur aangesien die detail van die navorsingsprojek daarin verduidelik word. Indien daar enige deel van die navorsingsprojek is wat u nie ten volle verstaan nie, is u welkom om die navorsingspersoneel of dokter daaroor uit te vra. Dit is baie belangrik dat u ten volle moet verstaan wat die navorsingsprojek behels en hoe u daarby betrokke kan wees. U deelname is ook volkome vrywillig en dit staan u vry om deelname te weier. U sal op geen wyse hoegenaamd negatief beïnvloed word indien u sou weier om deel te neem nie. U mag ook te eniger tyd aan die navorsingsprojek onttrek, selfs al het u ingestem om deel te neem.

Hierdie navorsingsprojek is deur die Menslike Navorsing Etiek Komitee van die Universiteit Stellenbosch goedgekeur en sal uitgevoer word volgens die etiese riglyne

en beginsels van die Internasionale Verklaring van Helsinki en die Etiese Riglyne vir Navorsing van die Mediese Navorsingsraad (MNR).

WAT BEHELS HIERDIE NAVORSINGSPROJEK?

Vyf tot tien persent van babas wat gebore is voor 8 maande ontwikkel skielik 'n toestand genaamd nekrotiserende enterokolitis. Dit is 'n baie ernstige toestand waar groot dele van die derm kan sterf en waarvoor chirurgie vereis mag word om die dooie derm te verwyder. Dit is nie duidelik wat die presiese oorsaak van hierdie toestand in die babas wat te vroeg gebore is nie, maar ons weet dat dit meer algemeen in premature babas is wat ŉ melkformule ontvang of waar daar sekere kieme teenwoordig is in die baba se ingewande. Dit is ook meer algemeen in premature babas van moeders wat MIV+ is. Die antiretrovirale medikasie wat moeders gebruik en die MIV-infeksie self kan die risiko verhoog. Borsmelk stimuleer die groei van "goeie" kieme in die derm of ingewande van babas. Studies het getoon dat die "goeie" kieme in borsmelk kan die derm van 'n klein premature baba beskerm teen nekrotiserende enterokolitis. Hierdie sogenaamde "goeie" kieme kan bygevoeg word in die bors melk van 'n moeder om in die baba se derm te groei en om die "slegte" kieme wat skade aan die ingewande veroorsaak te voorkom. Hierdie lewende "goeie" kieme wat ons vir die babas wil gee word Probiotika genoem. In hierdie studie wil ons sien of die "goeie" kieme sal help om jou baba te beskerm teen die ontwikkeling van nekrotiserende enterokolitis

Babas wat baie vroeg gebore word sal toegelaat word tot Saal G2 in Tygerberg Kinder Hospitaal. Baie klein premature babas wat minder as 1250g weeg met moeders wat MIV + of HIV- is sal deel neem in die studie. Probiotika ("goeie" kieme) sal gegee word aan die helfte van die babas in die studie vir die eerste 28 dae. Die ander helfte van die babas sal 'n produk ontvang wat soortgelyk lyk, maar dit het geen bygevoeg bakterieë ("goeie" kieme) nie.

Met hierdie studie wil ons bepaal of Probiotika ("goeie" kieme) sal:

• help verhoed dat 'n baba nekrotiserende enterokolitis kry

• lei tot ’n korter hospitalisasie tydperk

• lei tot die verbetering van die daaglikse gewigstoename

Hierdie studie wil ook bepaal of daar verskille in die samestelling van borsmelk van MIV + en MIV- vroue is.

Hoe sal jy en jou baba aan die studie deelneem?

As jou baba het 'n geboorte gewig laer as 1250g het sal jou baba toegelaat word tot Saal G2 terwyl jy sal toegelaat word tot Saal J5 of J2. Ons gaan julle in die saal besoek ná die geboorte van jou baba om die studie te verduidelik aan jou en vir jou en jou baba uit te nooi om deel te neem aan die studie. Ons sal vir jou ’n brief gee wat die studie verduidelik en as jy besluit om deel te neem, moet jy toestemming gee. Binne 'n paar dae na die geboorte van jou baba, sal jy oorgeplaas word om by jou baba te bly in Saal G2. Sodra die baba groter is, sal jy en jou baba oorgeplaas word na G8 in Tygerberg Kinder Hospitaal waar jy sal bly totdat jy jou baba gereed is om oorgeplaas te word na 'n ander hospitaal waar jy sal bly totdat jy jou baba meer as 1800g weeg en gereed is om huis toe gaan.

Die babas sal verdeel word in twee groepe; vir die een groep sal Probiotika gegee word en vir die ander groep sal 'n plasebo sal gegee word. Die plasebo lyk dieselfde as die Probiotika, maar bevat nie van die "goeie" kieme nie.

Babas sal in die studie wees vir die eerste 28 dae na geboorte.

Wat sal gebeur met my as ek deel te neem?

Klein premature babas is te swak om te drink aan die bors. Die ma moet haar bors uitmelk en die melk word dan aan die baba gegee deur middel van 'n dun plastiese pyp

wat strek vanaf die baba se mond na die baba se maag. Die verpleegsters in Saal G2 sal jou leer om jou borsmelk uit te melk vir jou baba. As deel van die studie, wil ons die gehalte van sommige van die komponente in jou borsmelk meet. Die belangrikste een is oligosakkariede wat ook help om jou baba te beskerm teen infeksies. Ons sal dus 'n 5-10ml monster van jou bors melk benodig op spesifieke tye.

Die eerste melk monster word van jou benodig op dag 4 van die laktasie, dan op dag 10 en weer op dag 28 van die baba se lewe. Hierdie 3 melkmonsters sal gevries word voordat dit getoets word in die laboratorium. 'n Speeksel monster van 2ml sal ook van jou benodig word. Die monster sal geneem word in 'n steriele houer. Nadat jy ingeligte toestemming gegee het, sal die speeksel monster geneem word. Slegs een speeksel monster sal nodig wees. Die samestelling van jou speeksel affekteer die oligosakkaried inhoud van jou borsmelk. Die speeksel monsters sal gevries word voordat dit getoets word in die laboratorium.

Inligting van jou hospitaal lêer sal gebruik word in die studie. Dit sluit in: inligting van die geboorte, medisyne wat jy ontvang het tydens jou swangerskap, ens. Alle swanger vroue in die Wes-Kaap word vir MIV getoets tydens swangerskap. Aangesien dit belangrik is vir ons om jou MIV status te weet, sal ons jou MIV status verkry van jou hospitaal lêer.

Wat sal gebeur met die baba, as ek toestemming gee?

Klein babas word elke dag geweeg om te sien of hulle genoeg melk kry. Jou baba sal daagliks geweeg word en sy/haar lengte en kop omtrek sal weekliks gemeet word.
Die studie groep (die babas wat die Probiotika ontvang) sal een keer per dag, vir 4 weke (28 dae), 5 druppels Probiotika per mond ontvang. Die kontrole groep (die babas wat die plasebo ontvang sonder die "goeie" kieme) sal een keer per dag, vir 4 weke (28

dae), 5 druppels plasebo per mond ontvang. Jou baba se lêer sal elke dag deur die navorser gelees word en die resultate van laboratoriumtoetse sal aangeteken word.

3. WAAROM IS U UITGENOOI OM DEEL TE NEEM?

Jy is gekies omdat jy geboorte geskenk het aan 'n baie klein baba wat te vroeg gebore is en opgeneem is in Saal G2 van Tygerberg Kinder Hospitaal. Jy is ook in staat om ’n monster van jou borsmelk uit te melk.

4. WAT SAL JOU VERANTWOORDELIKHEDE WEES?

Jy sal elke dag verantwoordelik wees vir die uitmelk van jou borsmelk vir jou baba. Op dae 4, 10 en 28 sal ’n monster van 5 – 10ml van die borsmelk uitgemelk word deur jou, dit sal gevries word en na die laboratorium geneem word. 'n Speeksel monster van 2ml sal ook van jou benodig word. Die speeksel monsters sal gevries word voordat dit getoets word in die laboratorium.

5. SAL U VOORDEEL TREK DEUR DEEL TE NEEM AAN HIERDIE NAVORSING?

Jy sal voordeel trek uit die wete dat jy 'n waardevolle bydrae gelewer het deur die verskaffing van belangrike inligting aan die mediese span wat hulle kan help in hul soeke na die beste manier om "vroeë gebore" en baie klein babas te voed en om nekrotiserende enterokolitis te voorkom.

6. IS DAAR ENIGE RISIKO'S BETROKKE DEUR DEEL TE NEEM AAN HIERDIE NAVORSING?

Jou baba sal presies dieselfde behandel word as enige van die ander klein babas wat toegelaat word in Saal G2 van Tygerberg Kinder Hospitaal. Sommige van die babas sal Probiotika kry en ander sal 'n plasebo (sonder die "goeie" kieme) ontvang. Al die babas (die wat Probiotika ontvang en diegene wat plasebo ontvang) sal gemonitor word vir

tekens van infeksie. Babas sal verwyder word van die studie indien die volgende gebeur:

As die baba nie borsmelk per mond vir meer as 3 dae kan inneem nie en die gebruik van spesiale kos nodig het

As die baba nekrotiserende enterokolitis ontwikkel

As die baba diarree (loopmaag) ontwikkel

As die baba 'n bloed-infeksie ontwikkel van die Probiotika. Die risiko van die ontwikkeling van 'n bloed-infeksie as gevolg van die "goeie" bakterieë is baie laag.

7. WATTER ALTERNATIEWE IS DAAR INDIEN U NIE INSTEM OM DEEL TE NEEM NIE?

Dit is jou besluit of jy wil deelneem of nie. As jy besluit om nie deel te neem aan die studie nie dit sal geen invloed op die versorging van jou baba hê nie. Verder is jy vry om te onttrek op enige stadium sonder enige verduideliking of enige negatiewe gevolge. Jou en jou baba se roetine gesondheidsorg sal nie negatief geaffekteer word nie.

8. WIE SAL TOEGANG HÊ TOT JOU MEDIESE REKORDS?

Slegs die navorser, navorsingsassistent en die persone wat direkte verband het met die studie sal toegang hê tot jou mediese rekords. Alle inligting sal altyd vertroulik gehou word. Die melk monsters wat na die laboratorium gestuur word kan nie terug gespoor word na jou toe nie want jou naam sal nie op enige van jou melk monsters gesit word nie. Slegs die navorser en navorsing assistent sal weet watter melk monster behoort aan wat moeder. Die navorser en navorsing assistent sal alle inligting wat versamel word as streng vertroulik hanteer en geen inligting wat jou of jou baba kan identifiseer sal vrygestel word aan enige persoon wat nie direkte verband het met die studie nie.

Die inligting wat verkry is/ingesamel word sal hanteer word met volle vertroulikheid en anonimiteit maar dit sal gepubliseer word in wetenskaplike tydskrifte en by kongresse voorgedra word. Jou of jou baba se naam sal onder geen omstandighede bekend gemaak word nie.

9. WAT SAL GEBEUR IN DIE ONWAARSKYNLIKE GEVAL VAN ’N BESERING WAT MAG VOORKOM AS GEVOLG VAN U DEELNAME AAN HIERDIE NAVORSINGSPROJEK?

Dit is onwaarskynlik dat daar iets verkeerds sal gaan. As jy nie in staat om uit te melk teen die beplande versameling tyd nie, kan jy later weer probeer om uit te melk te. Die navorser sal u inlig oor die volgende gepaste tyd om die borsmelk uit te melk. Standaard prosedures sal regdeur die studie gebruik word, wat die kanse dat iets verkeerd loop baie skraal maak.

10. SAL U BETAAL WORD VIR DEELNAME AAN DIE NAVORSINGSPROJEK EN IS DAAR ENIGE KOSTE VERBONDE AAN DEELNAME?

Nee, jy sal nie betaal word om deel te neem aan die studie nie. Daar sal geen kostes aan jou verbonde wees indien jy deel te neem nie.

11. WAT GEBEUR WANNEER DIE NAVORSING PROJEK EINDIG?

Die inligting verkry uit die studie sal gepubliseer word in mediese joernale.

12. WAT GEBEUR AS DAAR NUWE INLIGTING BESKIKBAAR RAAK?

Soms in die loop van 'n navorsingsprojek, kan nuwe inligting beskikbaar raak oor die

onderwerp wat bestudeer word. As dit gebeur, sal jou navorser vir jou vertel daaroor en met jou bespreek of jy wil voortgaan met die studie. As jy besluit om te onttrek, sal die standaard van die versorging wat jy en jou baba ontvang nie geaffekteer word nie. As jy besluit om voort te gaan in die studie sal jy versoek word om 'n opgedateerde toestemming vorm te onderteken. Die relevante inligting wat ontstaan in die loop van die studie sal opgeneem word in dokumentasie waar van toepassing

13. IS DAAR ENIGIETS ANDERS WAT U MOET WEET OF DOEN?

Jy kan gerus vir Mej Evette Venter bel by tel 021 938 9474 / 084 941 3832 indien u enige verdere navrae of enige probleme ondervind.

Jy kan die Menslike Navorsing Etiek Komitee by 021-938 9207 kontak indien u enige probleme of klagtes het wat nie voldoende aangespreek word deur die navorsers nie.

U sal ’n afskrif van hierdie inligting- en toestemmingsvorm ontvang vir u eie rekords.

Verklaring deur deelnemer

Met die ondertekening van hierdie dokument onderneem ek,
……..……...……..……., om deel te neem aan ’n navorsingsprojek getiteld DIE GEBRUIK VAN PROBIOTIKA IN DIE BEHANDERLING VAN NEKROTISERENDE ENTERO KOLITIS IN MIV BLOOTGESTELDE PREMATUUR EN BAIE-LAE GEBOORTE MASSA BABAS.

Ek gee ook toestemming dat my baba
…….....mag deelneem aan ’n navorsingsprojek getiteld DIE GEBRUIK VAN PROBIOTIKA IN DIE BEHANDERLING VAN NEKROTISERENDE ENTERO KOLITIS IN MIV BLOOTGESTELDE PREMATUUR EN BAIE-LAE GEBOORTE MASSA BABAS.

Ek verklaar dat:

Ek hierdie inligting- en toestemmingsvorm gelees het of aan my laat voorlees het en dat dit in ’n taal geskryf is waarin ek vaardig en gemaklik mee is.

Ek geleentheid gehad het om vrae te stel en dat al my vrae bevredigend beantwoord is.

Ek verstaan dat deelname aan hierdie navorsingsprojek vrywillig is en dat daar geen druk op my geplaas is om deel te neem nie.

Ek te eniger tyd aan die navorsingsprojek mag onttrek en dat ek nie op enige wyse daardeur benadeel sal word nie.

Ek gevra mag word om van die navorsingsprojek te onttrek voordat dit afgehandel is indien die studiedokter of navorser van oordeel is dat dit in my beste belang is, of indien ek nie die ooreengekome navorsingsplan volg nie.

Geteken te *(plek)* .....…….. op *(datum)* ……...…….. 2011.

Handtekening van deelnemer Handtekening van getuie

Verklaring deur navorser

Ek *(naam)* ……..…… verklaar dat:

Ek die inligting in hierdie dokument verduidelik het aan
…….....……..

Ek hom/haar aangemoedig het om vrae te vra en voldoende tyd gebruik het om dit te beantwoord.

Ek tevrede is dat hy/sy al die aspekte van die navorsingsprojek soos hierbo bespreek, voldoende verstaan.

Ek ’n tolk gebruik het/nie ’n tolk gebruik het nie. (*Indien ’n tolk gebruik is, moet die tolk die onderstaande verklaring teken.)*

Geteken te *(plek*) .....…….. op *(datum*) ……...…….. 2011.

Handtekening van navorder Handtekening van getuie

Verklaring deur tolk

Ek *(naam)* ……..…… verklaar dat:

Ek die navorser *(naam*) …….……. bygestaan het om die inligting in hierdie dokument in Afrikaans/Xhosa aan *(naam van deelnemer)* ……..... te verduidelik.

Ons hom/haar aangemoedig het om vrae te vra en voldoende tyd gebruik het om dit te beantwoord.

Ek ’n feitelik korrekte weergawe oorgedra het van wat aan my vertel is.

Ek tevrede is dat die deelnemer die inhoud van hierdie dokument ten volle verstaan en dat al sy/haar vrae bevredigend beantwoord is.

Geteken te *(plek*) .....……. op *(datum*) ……...…….. 2011.

Handtekening van tolk Handtekening van getuie

INKCUKACHA ELUNGISELWE UMTHATHI NXAXHEBA KUNYE NEMVUME EYAZISIWEYO

ISIHLOKO SOPHANDO:

UKUSETYENZISWA KWENTSHOLONGWANE EZILUNGILEYO EKUNYANGENI UKUFA KWETHUMBU KWINTSANA EZIZALWE KWIMEKO ZE HIV NEZIZELWE ZINCINCI KAKHULU

REFERENCE NUMBER: M10/09/035

PRINCIPAL INVESTIGATOR: MISS EVETTE VENTER/ van Niekerk

ADDRESS:

DIVISION OF HUMAN NUTRITION

TYGERBERG CAPMUS

THIRD FLOOR

TYGERBERG

7505

INOMBOLO YOQHAKAMSHELWANO:

EYOMSEBENZI: 021 938 9474

ISELI: 084 941 3832

ISIMEMO

Uyamenywa ukuba uthathe inxaxheba kwiprojekti yophando. Nceda uthathe ixeshana ufunde lenkcazelo ebhaliweyo apha, eyakuthi ikucacisele ngenkcukacha zale projekti. Nceda buza kumsebenzi wesisifundo okanye ugqirha nawuphina umbuzo nangayiphina indawo ongayiqondiyo. Kubalulekile ukuba uqonde ngokupheleleyo kwaye waneliseke ukuba oluphando lungantonina nendlela onokuthi ubandakanyeke ngayo wena. Kwakhona, ukuthatha inxaxheba **akusosinyanzeliso kwaphela** yaye uvumelekile ukuba ungavumi ukuthatha inxaxheba. Ukuba uthi hayi, lonto ayizokucaphazela wena nomntwana nangayiphina indlela. Uvumelekile ukuba urhoxe koluphando nangaliphina ixesha nokuba ubusele uvumile ukuthatha inxaxheba.

Oluphando luvunyiwe yiKomiti yezophando zoluntu nemimiselo (ethics) kwiYunivesithi yase Stellenbosch yaye luza kuqhutywa ngokwemigaqo yophando olwamkelekileyo kwisaziso sehlabathi saseHelsinki, nemigaqo yoMzantsi Afrika yophando olwamkelekileyo kwakunye nemigaqo yophando olwamkelekileyo lweMedical Research Council (MRC).

OLUPHANDO LUNGANTONI KANYE?

Isihlanu ukuya kwishumi lepesenti zabantwana abazalwe phambi kwnyanga ezisibhozo, ngesaquphe baye baphuhle imeko ekuthiwa kukufa kwamathumbu (necrotising enterocolitis). Le yimeko enxunguphalisayo kakhulu apho amalungu amakhulu emathunjini athi afe kunyanzeleke ukuba umntwana afumane uqhaqho ukususa elithumbu lifileyo. Eyonanto eyenziwa yilemeko ebantwaneni abazalwe phambi kwexesha ayicacanga ncam kodwa siyazi ukuba ixhaphakile kubantwana abazalwe phambi kwexesha abafumana ubisi lwabantwana olusezitotini okanye apho kukho intsholongwane zithile ezikhoyo emathumjini omntwana. Kwaye ikwaninzi kubantwana abazalwe phambi kwexesha bezalwa ngabazali aba HIV+. Amachiza okunyanga abathi

omama bawasebenzise kunye nokosuleleka yintsholongwane ka gawulayo ingawenyusa amathuba okuba semngciphekweni. Ubisi lwebele lukhuthaza ukukhula kwe ntsholongwane ezilungileyo emathunjini omntwana. Izifundo zibonakalisa ukuba intsholongwane ezilungileyo ebisini lwebele zingakhusela amathumbu omntwana omcinci ozalwe phambi kwexesha kwesi sifo. (necrotising enterocolitis). Ezintsholongwane zibizwa ngokuba zilungile zingafakwa kubisi lwebele lukamama ukwenzela zikhule ethunjini lomntwana kwaye zinqande intsholongwane ezingalunganga zingenzi umonakalo ethunjini. Ezintsholongwane zilungileyo zihleliyo esifuna ukuzinika iintsana zibizwa ngokuba zi (Probiotics). Koluphando sifuna ukubona ukuba ezintsholongwane zilungileyo zingamkhusela na umntwana angabi safunyanwa sesisifo sethumbu elifayo (necrotising enterocolitis).

Abantwana abazalwa phambi kwexesha balaliswa kwi Ward G2 e Tygerberg Children's Hospital. Abantwana abancinci kakhulu bezalwe phambi kwexesha abangumlinganiselo ongaphantsi kwe 1250g bezalwa ngomama abanentsholongwane kagawulayo (HIV+) okanye (HIV-) bayakuthatha inxaxheba koluphando. Probiotics (Intsholongwane ezilungileyo) ziyakufakwa kwisiqingatha sabantwana abakoluphando intsuku zokuqala ebomini eziyi 28. Esinye isiqingatha sabantwana sakufumana into ekhangeleka ifana nezi Probiotics kodwa yona ingenazo ezintsholongwane zilungileyo.

Ngoluphando sifuna ukubona ukuba ingaba iProbiotics (intsholongwane ezilungileyo) ziya::

Kunceda ekukhuseleni abantwana bangafunyanwa sisifo sokufa kwamathumbu (necrotising enterocolitis),

Baya kulaliswa ixesha elifutshane esibhedlele.

Ukuba ubunzima bomzimba bungcono mihla le.

Oluphando lufuna nokuqonda ukuba kukho umahluko na kwizinga lobisi lomama onentsholongwane yeHIV nongenayo.

Wena nomntwana wakho niyakuyithatha njani inxaxheba koluphando

Ukuba umntwana wakho unobunzima obungaphantsi kwe 1250g umntwana wakho uyakulaliswa kwa Ward G2 ngaxeshanye wena ulaliswe kwa Ward J5 okanye kwa J2. Siyakundwendwela ewadini emva kokuzalwa komntwana wakho sicacise oluphando kuwe yaye sikumeme wena nomntwana wakho nithathe inxaxheba koluphando. Siza kunika ileta eyakuthi icacise oluphando yaye ukuba uyavuma ukuthatha inxaxheba, kufuneka wenze isivumelwano. Ngentsuku ezimbalwa emva kokuzalwa komntwana wakho, uyakutshintshwa ukuba uyokuhlala nomntwana wakho kwaward G2. Nje ukuba umntwana akhule, wena nomntwana wakho niyakuthi nithunyelwe kwaward G8 e Tygerberg Children's Hospital apho niya kuhlala kude kube umntwana wakho ukulungele akuba angatshintshelwa kwesinye isibhedlele apho niya kuhlala khona ade umntwana wakho abe unomlinganiselo ongaphezu kwe 1800g yaye ukulungele ukuya ekhaya.

Abantwana bayokwehlulwa babe ngamaqela amabini; iqela lokuqala liyakunikwa iProbiotics, elinye iqela linikwe into efana nazo kodwa ingezizo, ebizwa ngokuba yi plasibo. Le plasibo ifana nqwa ne Probiotics kodwa ayinazo ezintsholongwane zilungileyo.

Iintsana ziyakuba koluphando intsuku eziyi28 zokuqala emva koba zizelwe.

Kuyakwenzeka ntoni kum xa ndithe ndathatha inxaxheba?

Abantwana abancinci abazalwe phambi kwexesha abanamandla okuncanca emabeleni. Umama kufuneka asenge ubisi emabeleni oluthi lunikwe umntwana ngethumbu elibhityileyo lweplastiki ehamba emlonyeni ukuyakutsho esuswini somntwana. Abongikazi abakwi wadi labantwana iG2 bayakuthi bakufundise ukuba lusengwa njani ubisi ukulungiselela umntwana. Ngokoluphando, sifuna umlinganiselo wokulunga nezinga lobisi lwebele lwakho. Eyona nto ibalulekileyo yi "oligosaccharide" ethi nayo

incede ukukhusela umntwana wakho ekusulelekeni. Siyakuthi kengoko sifune isampulu engange 5-10ml yobisi lwebele ngamaxesha athile.

Isampulu yobisi yokuqala esizakuyifuna kuwe ingosuku lwesine uncancisa, kwakhona ngosuku lweshumi nangosuku lwe28. Ezisampulu zintathu ziyakufakwa kumkhenkce phambi kokuba zihlolwe elebhu. Isampulu yamathe engange 2ml iyakuthi ifunwe kuwe. Isampulu zamathe ziyakuthi zifakwe kwisikhongozelo esingenazintsholongwane. Ekubeni unike imvume amathe akho aya kuthathwa. Kufunwa isampulu enye qha yamathe. Indlela amathe akho ayiyo iyayichaphazela imo ye “oligosaccharide” yobisi lwebele lakho. Isampulu zamathe ziyakufakwa emkhenkceni (frozen) phambi kokuba zihlolwe elebhu

Inkcazelo yexwebhu lwakho esuka kwisibhedlele sakho iyakusetyenziswa kuphando enje ngokuba umntwana wabelekwa njani, ngawaphi amayeza owawafumana ngokuya wawukhulelwe, njalo,njalo. Onke amakhosikazi akhulelweyo eNtshona Koloni ayahlolwa iHIV ngexesha bekhulelwe. Njengokuba kubalulekile ukuba siyazi i HIV status yakho, Siyakuyifumana i HIV status kwixwebhu lwenkcukacha zakho lwesibhedle.

Kuzakwenzeka ntoni kumntwana ukuba ndinike isivumelwano?

Abantwana abancinci bafakwa kwisikali yonke imihla ukubona ukuba balufumana ngokwaneleyo ubisi. Umntwana wakho uzakubekwa esikalini ukujongwa ubunzima yonke imihla, ubude kunye nomlinganiselo wentloko ziyakulinganiswa qho ngeveki.

Iqela lophando (abantwana abazakufumana iProbiotics) bazakufumana amathontsi ayi 5 eProbiotics emlonyeni kanye ngosuku, iveki ezine (intsuku eziyi 28). Iqela lesibini (abantwana abazakuthi bafumane iplasibo) bazakufumana amathontsi ayi 5 eplasibo engenazo intsholongwane ezilungileyo ngomlomo kanye ngemini iveki ezine (intsuku eziyi 28) . Uxwebhu lwenkcukacha zomntwana wakho ziyakuthi zifundwe ngumphandi ntsuku zonke yaye iziphumo zovavanyo lwaselebhu zakubhalwa phantsi.

KUTHENI UMENYIWE UKUBA UTHATHE INXAXHEBA?

Uchongiwe kuba ubeleke umntwana omncinci kakhulu ozelwe phambi kwexesha, kwaye walaliswa kwa ward G2 at Tygerberg Children's Hospital (TCH) yaye uyakwazi ukucudisa amabele unikele ngesampulu yobisi lwebele.

ZINTONI EZILINDELEKILEYO KUWE?

Uyakulindeleka ukuba ucudise amabele kubekho ubisi ntsuku zonke ukulungiselela umntwana wakho. Ngezintsuku 4, 10 and 28 isampulu eyi 5ml - 10ml yobisi lwebele olunikezelwe nguwe luya kufakwa emkhenkceni kwilebhu. Isampulu yamathe akho engange 2ml iyakufunwa kuwe. Lesampulu yamathe iya kufakwa emkhenkceni (frozen) phambi kokuba ihlolwe elebhu.

KUKHO INZUZO EKUTHATHENI INXAXHEBA KOLUPHANDO?

Uzakuzuza ngolwazi kuba wenze umnikelo obalulekileyo ngokunika igqiza lezempilo ulwazi olubalulekileyo oluya kuthi lubancede kuphando lwabo xa bekhangela eyona ndlela elungileyo yokutyisa abantwana abazalwe "phambi kwexesha" nabantwana abancinci kakhulu nokunceda ukukhusela isifo sokufa kwethumbu. (necrotising enterocolitis.)

INGABA KUKHO INGOZI EKUTHATHENI KWAKHO INXAXHEBA KOLUPHANDO?

Umntwana wakho uyakuthi anyangwe kanye kanye njengabanye abantwana abancinci abalaliswe kwa-ward G2 e Tygerberg Children's Hospital. Abanye kwababantwana

bakuthi banikwe i-Probiotics abanye bafumane iplasibo (engenazo intsholongwane ezilungileyo). Bonke abantwana (abo bafumana i- Probiotics nabo bafumana i- placebo) bayakubekwa esweni ukukhangela iimpawu zosuleleko. Abantwana bayakususwa koluphando ukuba kuthe kwenzeka oku kulandelayo:

Ukuba umntwana akancanci ubisi lwebele ngomlomo kangangentsuku ezingaphezu ko-3 yaye kufuneka kusetyenziswe ukutya okukhethekileyo

Ukuba umntwana uye wafa ithumbu (necrotising enterocolitis)

Ukuba umntwana uthe wahambisa (ukubaleka kwesisu)

Ukuba umntwana uthe wafumana usuleleko ngegazi (blood infection) kwi- Probiotics. Ingozi yosuleleko ngegazi kwi ntsholongwane ezilungileyo iphantsi kakhulu.

UKUBA AKUVUMI UKUTHATHA INXAXHEBA UNOKUTHINI?

Isigqibo sesakho ukuba ufuna ukuthatha inxaxheba okanye akufuni. Ukuba ugqiba ukuba ungathathi nxaxheba koluphando,lonto ayizokuchaphazela inkathalo eyakuthi inikwe umntwana wakho. Ngaphezukoko ukhululekile ukuba urhoxe nagaliphi ixesha ngaphandle kokunika inkcazelo kungekho nanto imbi eyakuthi ikwehlele. Impilo yakho neyosana lwakho ayisayi kuchaphazeleka kakubi.

NGUBANI OZAKUFIKELELA KWINKCUKACHA ZESIGULO SAKHO?

Ngumphandi kuphela, umphandi oncedisayo kunye nabantu ababandakanyekayo koluphando abayakuthi babenokufikelela kwinkcukacha zesigulo sakho. Yonke inkcazelo iyakusoloko igcinwe ngokhuseleko.

Isampulu zamabisi ezithunyelwe elebhu aziyi kwaziwa ukuba zisuka kuwe ngoba igama lakho alizokufakwa kwisampulu zobisi lwakho. Ngumphandi kunye nomncedisi mphandi abayakuthi bayazi ukuba ngeyiphi isampulu yobisi yomama ngamnye. Umphandi kunye nomncedisi mphandi bayakuyithatha yonke inkcazelo edityanisiweyo

ngokuyimfihlelo engqongqo kwaye akukho nkcazelo iyakuchaza ngawe kunye nomntwana wakho eyakuthi ikhululwe nakuwuphina umntu ongabandakanyekiyo koluphando.

Inkcukacha efunyenweyo/eqokelelweyo iyakuthi iphathwe ngokhuseleko olukhulu yaye ingachazwa, kodwa iyakuthi ipapashwe kwincwadi zogqira yaziswe nakwindibano zogqira ezinje nge congressi. Akukho ndlela konke konke apho igama lakho okanye lomntwana wakho liyakuthi livezwe khona.

KUYAKWENZEKA NTONI UKUBA KUNOKWENZEKA KUBEKHO INGOZI EYENZEKAYO NGENXA YOKUTHATHA KWAKHO INXAXHEBA KOLUPHANDO?

Akuqhelekanga ukuba kungakho into enokungahambi ngendlela. Ukuba awukwazi ukunikela ngobisi ngexesha elibekiweyo, ungazama ukunikela ngobisi ngelinye ixesha. Umphandi uyakuthi akwazise ngelinye ixesha elifanelekileyo lokunikela ngobisi.

Ngexesha kuqhubeka oluphando imiqathango eqhelekileyo iyakuthi isetyenziswe, lonto yenze ukuba amathuba okuba kubekho into engahambi kakuhle, abemancinci kakhulu.

UZAKUHLAWULWA NA NGOKUTHATHA INXAXHEBA KOLUPHANDO OKANYE KUKHO INDLEKO EZIBANDAKANYWAYO?

Hayi, awuzokuhlawulwa ngokuthatha inxaxheba koluphando. Akuyikubakho ndleko ezikubandakanyayo, ukuba uyavuma ukuthatha inxaxheba.

KWENZEKA NTONI XA OLUPHANDO LWESISIFUNDO LUYEKA?

Inkcukacha efunyenwe koluphando iyakuthi ipapashwe kwincwadi zogqira. (medical journals.)

KUYAKUTHINI XA KUFUMANEKA INKCUKACHA ENTSHA?

Ngamanye amaxesha ngexesha leprojekthi yophando, inkcukacha entsha iyafumaneka engesihloko eso sifundwayo. Ukuba lento iyenzeka, umphandi wakho uyakuthi akuxelele ngayo yaye niyixoxe ukuba ngaba ufuna ukuqhubeka ngoluphando. Ukuba ugqibe ekubeni urhoxe, indlela enikhathalelwa ngayo wena nomntwana ayiyi kuchaphazeleka. Ukuba ugqibe ekubeni uqhubekeke nophando uyakuthi ucelwe utyikitye ifomu ehlaziyiweyo yesivumelwano. Inkcukacha ebandakanyekayo ethi ivuke ngexesha kuqhubeka uphando Iya kufakelwa kweso sivumelwano sitsha.

INGABA IKHONA ENYE INTO OFUNA UKUYAZI OKANYE UKUYENZA?

Ungaqhakamshelana no Miss Evette Venter ngomnxeba 021 938 9474/ 084 941 3832 ukuba uneminye imibuzo okanye ufumana ezinye ingxaki.

Ungaqhakamshelana ne Komiti yezophando zoluntu (Committee for Human Research) kwa: 021-938 9207 ukuba unezikhalazo okanye izinto ezingakonwabisanga ezithe azadweliswa ngababaphandi.

Uyakufumana ikopi yalenkcukacha kunye nesivumelwano oyakuthi uzigcinele, ezizezakho.

Isifungo somthathi nxaxheba

Ngokutyikitya apha ngezantsi, mna……..…….....…. ndiyavuma ukuthatha inxaxheba koluphando olubizwa: **UKUSETYENZISWA KWENTSHOLONGWANE EZILUNGILEYO EKUNYANGENI UKUFA KWETHUMBU KWINTSANA EZIZALWE KWIMEKO ZE HIV NEZIZELWE ZINCINCI KAKHULU**

(THE USE OF PROBIOTICS IN THE MANAGEMENT OF NECROTISING ENTEROCOLITIS IN HIV EXPOSED PREMATURE AND VERY-LOW BIRTH WEIGHT INFANTS)

Ndikwanika isivumelwano ukuba umntwana wam…….....angathatha inxaxheba koluphando olubizwa: **UKUSETYENZISWA KWENTSHOLONGWANE EZILUNGILEYO EKUNYANGENI UKUFA KWETHUMBU KWINTSANA EZIZALWE KWIMEKO ZE HIV NEZIZELWE ZINCINCI KAKHULU**. (**THE USE OF PROBIOTICS IN THE MANAGEMENT OF NECROTISING ENTEROCOLITIS IN HIV EXPOSED PREMATURE AND VERY-LOW BIRTH WEIGHT INFANTS)**

Ndazisa ukuba:

Ndiyifundile okanye ndiyifundelwe le nkcukacha neliphepha lesivumelwano yaye ibhalwe ngolwimi endiluthethayo yaye endikholisekileyo lilo.

Ndilifumene ithuba lokubuza imibuzo yaye imibuzo yam iphenduleke ngokwanelisayo.

Ndiyayiqonda into yokuba ukuthatha inxaxheba koluphando **ayisosinyanzelo yaye andinyanzeliswanga ukuba ndithathe inxaxheba.**

Ndinako ukukhetha ukulushiya oluphando nageliphina ixesha yaye akukho sohlwayo ndiyakusifumana ngenxa yoko.

Ndinokucelwa ukuba ndilushiye oluphando phambi kwexesha ukuba ugqirha wophando ucinga ukuba oko kufanelekile okanye ukuba andithanga ndalandela imigaqo yophando njengoko besivumelene.

Ityikitywe e (indawo) .....…..…….. ngo (umhla) ……...……..
2011.

Umtyikityo womthathi nxaxheba **Umtyikityo wengqina**

Isifungo somphandi

mna *(igama)* ……..…… ndazisa ukuba:

Ndiyicacisile inkcukacha equlethwe loluxwebhu ku……..

Ndimkhuthazile ukuba abuze imibuzo ndathatha ixesha elaneleyo ukumphendula.

Ndanelisekile ukuba uziqondile ngokwanelisayo zonke izinto-yinto zoluphando njengoko zixoxiwe ngentla

Ndiyisebenzisile/andiyisebenzisanga itoliki. (ukuba itoliki isetyenzisiwe kufuneka ityikitye isifungo esisezantsi).

Ityikitywe e(indawo.....…..……ngo(umhla) ……...……..2011

Umtyikityo womphandi **Umtyikityo wengqina**