SUPPLEMENTATION OF MOTHER'S MILK FOR PRETERM INFANTS:
MINERAL BIOAVAILABILITY

By

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MINERAL BIOAVAILABILITY FROM DIETS FOR PRETERM INFANTS
TITLE: Supplementation of Mother's Milk for Preterm Infants: Mineral Bioavailability

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ABSTRACT

Use of multi-nutrient supplements for preterm infants fed mother's milk is common practice in neonatal intensive care units to provide sufficient amounts of minerals and other nutrients. The research delineated in this thesis investigated calcium, magnesium, zinc and iron bioavailability from supplemented mother's milk for preterm infants. Multi-nutrient supplementation to mother's milk had the benefit for preterm infants of achieving short-term growth rates parallel to the fetus of similar gestational age without reducing the dietary bioavailability of calcium, magnesium and zinc. In the infant-piglet model it was demonstrated that the addition of calcium and phosphorus to the diet in similar proportions as to preterm infants fed fortified mother's milk did not reduce iron bioavailability. Multi-nutrient supplementation to mother's milk did not result in better short- or long-term growth, greater whole body bone mineral content or better zinc status in comparison to supplementation with calcium and phosphorus alone. If it, however, desirable for the preterm infant to attain short-term growth rates similar to the intrauterine fetus of the same post-menstrual age, future investigations should address the impact of multi-nutrient supplementation on the bioavailability of other minerals important for growth and development.

Long term outcomes of whole body bone mineral, lean and fat mass and
zinc status appeared to be determined by post-hospital discharge nutrition rather than nutrition in early neonatal life. Growth and body composition of breast-fed premature infants in the first year of life followed a different pattern in comparison to preterm infants fed a standard term formula. Therefore, future investigations should establish appropriate references for growth and body composition derived from breast-fed term infants in order to evaluate these outcomes in preterm infants fed mother's milk.

The research described in this thesis has contributed to the concept that moderate nutrient and mineral intakes from mother's milk supplemented with a multi-nutrient fortifier or from mother's milk alone with only supplemental calcium and phosphorus may be appropriate for the healthy preterm infant even though current nutrient recommendations are not met. Based on the information provided in this thesis, functional responses to mineral nutrition should be considered in setting nutrient recommendations for preterm infants.
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Special thanks to my parents Mia and Guus for their love and for giving me the encouragement and opportunity to explore my academic horizons. Finally, I want to thank Walter, who has given my life a new direction; thank you for your support, friendship and love.
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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BBMV</td>
<td>brush border membrane vesicles</td>
</tr>
<tr>
<td>BMC</td>
<td>bone mineral content</td>
</tr>
<tr>
<td>CaGP</td>
<td>calcium glycerophosphate</td>
</tr>
<tr>
<td>DXA</td>
<td>dual energy x-ray absorptiometry</td>
</tr>
<tr>
<td>MM</td>
<td>mother’s milk</td>
</tr>
<tr>
<td>MNF</td>
<td>multi-nutrient fortifier</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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PREFACE

The research described in this thesis investigated mineral bioavailability from supplemented mother's milk for preterm infants. This thesis contains an assembly of data obtained from both infant and piglet studies addressing the research questions generated to investigate the hypotheses stated in this thesis. Chapter one provides the rationale for this research in a general introduction which: discusses the importance of the respective minerals calcium, magnesium, zinc and iron in the diet for the premature infant; describes the approaches and techniques used in the measurement of mineral bioavailability and; presents current knowledge regarding mineral bioavailability from preterm infants' diets for the minerals of interest. Chapters two through six contain data which have been prepared as manuscripts either submitted or to be submitted to scientific journals, as indicated at the beginning of each chapter. Each chapter describing experimental results has multiple authors, but each study was designed and performed by the principal author and author of this thesis under supervision of Dr. SA Atkinson, PhD who was involved in all aspects of the research described in this thesis. In chapters two, four, five and six, Dr Bosco Paes, MD and Dr Jay K Shah, MD assisted in identification and recruitment of the preterm infant population and further gave valuable suggestions during the preparation of the manuscripts. Vinay Bhide contributed to the experiments performed in the addendum to chapter two as part of his undergraduate thesis. Filomena Incitti assisted
in the method development for the in-situ and in-vitro iron uptake studies described in chapter three as part of her undergraduate thesis. Terri Grad contributed to data collection and organization of the study described in chapter four, as this was part of her MSc training. Dr. RS Gibson, PhD was involved in the design of the study described in chapter six and provided suggestions during the preparation of the manuscript. Chapter seven discusses the importance of the work described in this thesis in relation to current knowledge and nutritional management of preterm infants. The discussion also highlights future directions and research questions that should be addressed to advance nutritional management of preterm infants fed mother's milk. References for chapters one and seven are provided at the end of this thesis. Chapters two through six each contain a separate reference list. The appendices contain additional data obtained in the infant studies that were not described in the individual manuscripts but are useful as additional information.
Chapter One

INTRODUCTION
Chapter One

INTRODUCTION

1.1 Rationale and Hypotheses

Mother's milk feeding as compared to formula feeding for the preterm neonate has many advantages for their development. Clinical evidence has emerged that preterm infants fed human milk compared to formula have better neurocognitive development (Lucas et al. 1992a) and a lower incidence of necrotizing enterocolitis (Lucas & Cole 1990). The latter may be related to the presence of cellular elements that contribute to host defence (Heine 1992). In addition, the presence of hormones and growth factors in human milk may stimulate growth, motility and maturity of the gastrointestinal tract (Sheard & Walker 1988) possibly contributing to better enteral feed tolerance (Lucas & Cole 1990).

When the preterm infant is born, the continuous delivery of nutrients via the placenta which provides for rapid fetal growth and nutrient accretion especially during the third trimester of pregnancy is disrupted. In order for the preterm infant to resume growth at a similar rate as the intrauterine fetus of similar gestational age, nutrient requirements will be greater in comparison to the infant that is born at term (Lucas 1993). Preterm mother's milk, therefore, may result in inadequate nutrient intakes in preterm infants as a result of the compositional variability of preterm mother's milk (Anderson 1984), decline of nutrient content through lactation and inherently lower
concentrations than those needed by rapidly growing preterm infants (Heine 1992).

Current nutrient recommendations for premature infants are set to provide sufficient amounts to achieve nutrient accretion and growth rates similar to those of the fetus of similar gestational age (American Academy of Pediatric [AAP] 1985). Human milk contains inadequate amounts of calcium and phosphorus to allow for retention of calcium and phosphorus which parallel intrauterine accretion. Therefore, the preterm infant fed mother's milk is at risk of developing osteopenia (Steichen & Tsang 1992). As well, excessive mineral* losses in feces and urine for magnesium, zinc and copper have been observed in preterm infants fed human milk (Dauncy et al. 1976) resulting in negative mineral accretions or mineral accretions below the intrauterine accretion rates. This may in part contribute to the slower growth rates observed in preterm infants fed mother's milk compared to preterm infants receiving formulas that have a nutrient composition which more closely meets their estimated nutritional needs (Carey et al. 1987, Greer & McCormick 1988, Schanler et al. 1988).

*A mineral is defined as any inorganic material found in the earths' crust, such as metallic ore. This definition includes those elements that are present in very small quantities in the body, such as zinc and iron, which are referred to as trace elements in the discipline of nutrition. For continuity within this thesis the main elements of interest, calcium, magnesium, zinc and iron, will all be considered under the term mineral. Although phosphorus is not a true mineral it is categorized as a mineral in the text. Please bear in mind the licence being exercised in the use of the term "mineral".
In order to provide the preterm infant with the benefits of mother's milk together with meeting their estimated nutrient requirements, multi-nutrient supplements or fortifiers have been designed. These fortifiers are either in a powdered or liquid form or consist of donor human milk components. Such fortifiers contain calcium, phosphorus, protein, energy, minerals and sometimes vitamins.

The potential improvements in growth, mineral accretion, biochemical indices of mineral status and bone mineral mass in preterm infants by addition of multi-nutrient fortifiers to mother's milk have been investigated in several randomized controlled trials (Modanjou et al. 1986, Gross et al. 1987, Carey et al. 1987, Greer & McCormick 1988, Pettifor et al. 1987, Venkataraman & Blick 1988, Kashyap et al. 1990). Although these studies demonstrated better short-term weight growth with multi-nutrient supplementation to mother's milk in comparison to unsupplemented mother's milk, inconsistent results have been presented regarding the improvement of linear growth, mineral retention, mineral status and bone mineral mass. Several factors may have contributed to this, such as differences in the characteristics of the study populations, study design, and limitations of the measurement of mineral absorption and retention and bone mineral mass. Dissimilarities in the quantity and quality of multi-nutrient supplements added to mother's milk may also play an important role as is explained below.

Mineral salts incorporated into different multi-nutrient fortifiers will vary in their physicochemical characteristics and their uptake at absorption sites will be
dependent upon the conversion to an absorbable form during digestion. Further, minerals may biochemically interact with other minerals either in a synergistic or antagonistic manner (Solomons 1988). Thus, by changing the mineral:mineral ratios in mother's milk by addition of a multi-nutrient supplement, the bioavailability of certain minerals may be reduced as a result of mineral interactions. The issue of mineral bioavailability has been addressed in the literature in relation to different food matrices and different populations (Clydesdale 1988, Solomons 1988). Not much attention, however, has been paid to mineral bioavailability, specifically mineral interactions in the diet for preterm infants in relation to mother's milk supplementation.

While multi-nutrient supplementation of mother's milk for preterm infants in hospital is common practice, supplementation is usually discontinued after hospital discharge. To date no investigations have studied long-term outcomes of growth, bone mineral mass, fat and lean mass in relation to multi-nutrient fortification of mother's milk pre-hospital discharge. Further no information is available on these long-term outcomes in preterm infants fed their mother's milk alone after hospital discharge.

The overall objective of the research described in this thesis was to determine the effect of amount and type (eg. in the form of a multi-nutrient fortifier) of dietary calcium and phosphorus on calcium, magnesium, zinc and iron bioavailability in both the preterm infant and in the infant-piglet model. Further, a descriptive follow-up study was conducted to determine if supplementation of mother's milk with a multi-nutrient fortifier in hospital and/or mother's milk compared to
formula feeding post-hospital discharge influenced the long-term outcomes of growth, body composition and zinc status.

1.1.1 Global hypotheses

I) The provision of calcium, phosphorus, protein, lactose and additional minerals in the form of a new multi-nutrient fortifier (MNF) in comparison to provision of calcium and phosphorus alone for preterm infants fed their mother's milk will:

   a) not alter dietary calcium, magnesium and zinc bioavailability;

   b) improve short-term growth and bone mineral mass;

   c) improve long-term outcomes of growth, bone mineral mass and zinc status.

II) The addition of calcium and phosphorus to the diet in proportionally similar amounts as to preterm infant diets will not alter iron bioavailability in the infant-piglet model.

1.2 Measurements of Mineral Bioavailability

The bioavailability of a mineral is the fraction of an ingested mineral that is absorbed and ultimately presented to tissues in forms that can be used to meet functional demands (O'Dell 1989, Lonnerdal & Glazier 1989). The bioavailability of a mineral will be dependent on extrinsic or dietary variables such as physicochemical characteristics of a mineral within the food matrix and biochemical interactions between minerals or between nutrients and minerals in the gastrointestinal tract. The physicochemical characteristics of a mineral include solubility and molecular
dimensions of a mineral in a food source, digesta and lumen influencing mucosal uptake (Clydesdale 1988). The majority of mineral interactions exhibit antagonistic characteristics which will occur between minerals whose physical and chemical properties are similar. Most antagonistic mineral interactions involve competition at the brush border membrane in the gut lumen (Solomons 1988). Intrinsic variables influencing mineral bioavailability are physiological variables which in response to changing relationships between mineral supply and demand can influence mineral absorption and retention, storage or incorporation into functional sites (Southgate 1989).

Three basic approaches which can be taken to measure mineral bioavailability are: 1) direct measurements of mineral absorption and retention; 2) indirect measurements of the mineral present in the body by analysis of body fluids and tissues; 3) functional responses elicited by the mineral of interest. Each of these approaches has its advantages and limitations (Solomons 1988) as will be discussed in the following sections.

1.2.1 In-vitro and in-situ methods - mineral uptake at absorption sites

Mineral uptake at absorption sites can either be measured in-vitro, in-situ or in-vivo. In-vitro models such as simulated digestion (Lonnerdal & Glazier 1989), everted gut sacs (Fischer et al. 1981) or brush border membrane vesicles (Kessler et al. 1978, Muir et al. 1984) can provide very important information regarding the uptake step in intestinal absorption as well as to identify and characterize membrane
carriers that are responsible for the uptake step in absorption. The results of these methods should be interpreted with caution, however, because such experiments are not performed under physiological conditions. Further, more complex mineral binding and ligand exchange reactions that are present in the food matrix in-vivo but not in-vitro conditions may limit the extrapolation of results obtained using these models to humans or animals (Flanagan 1987). In-situ methods, such as ligated loops (Simpson et al. 1986) or intestinal perfusion (Flanagan et al. 1980), in which segments of the intestine are laparotomized in anaesthetized animals have the advantage that intestinal absorptive functions are measured under conditions resembling physiological conditions in-vivo. A kinetic disadvantage may exist as the test solution may become depleted as absorption proceeds in these in-situ methods. However, when careful attention to experimental parameters is implemented useful data can be obtained (Flanagan 1987).

1.2.2 In-vivo methods - mineral absorption and retention

The measurement of mineral absorption in-vivo provides information on the dietary bioavailability, but does not necessarily reflect the amount that is ultimately presented to the body because of urinary and endogenous losses. Therefore, mineral retention should always be considered in addition to mineral absorption when evaluating mineral bioavailability in-vivo (Lonnerdal & Glazier 1989).

Two methods can be applied to measure mineral absorption and retention in-vivo:
**Mass balance techniques** - Apparent absorption and retention can be calculated by the difference between oral intake of a mineral and its excretion in feces and urine. Fractional mineral absorption, the percent of intake, is valuable when a comparison is made between the dietary bioavailability of a mineral in different diets (Solomons 1988). The mass balance technique, however, cannot distinguish between dietary minerals and the endogenous mineral pool. (Jangorbani et al. 1985). Thus when endogenous losses of a mineral are substantial, the dietary bioavailability of the mineral may be underestimated.

**Isotope tracers** - With the use of isotope tracers a mineral from a specific source can be distinguished from other sources of the mineral in a complex environment. Thus the use of isotope tracers has the benefit of being able to distinguish dietary mineral from the endogenous mineral pool. Isotope tracers are usually applied as a single-meal absorption test, where systemic uptake into the circulation or faecal and/or urinary output is monitored (Janghorbani et al. 1985, Weaver 1988). For applications of these techniques in preterm infants, stable isotope tracers impose less health risk in comparison with radioisotopes. To further minimize invasive procedures, such as blood sampling, faecal and/or urinary monitoring is usually applied when measuring true mineral absorption and retention. When using a single stable isotope to measure mineral absorption and retention, the assumption has to be made that no re-excretion, or only negligible amounts, of the absorbed isotope occurs as endogenous losses (Weaver 1988). With dual isotope tracer methodology
endogenous losses can be determined (Abrams et al. 1991); this technique, however, requires intravenous administration of stable isotopes which may not always be feasible in infant populations. Single isotope tracers have been successfully used in preterm infants to measure mineral absorption; however, the possibility of re-excretion of the stable isotope as endogenous losses then has to be considered in the interpretation of the results.

**1.2.3 Biochemical indices of mineral status**

Beside physicochemical characteristics of minerals and biochemical interactions between minerals in multi-nutrient supplements for preterm infants fed mother's milk, physiological variables, such as development changes in absorptive processes, maturity of metabolic functions, disease state as well as rapid growth can further influence mineral bioavailability (Southgate 1989). Thus, once a mineral has become available from its food matrix and has been absorbed and retained by the body, it needs to be available in such form to fulfil its metabolic function. For these reasons, biochemical indices of mineral status and functional outcomes are important when investigating mineral bioavailability (O'Dell 1989, Lonnerdal & Glazier 1989).

The bioavailability of a mineral can be measured indirectly by dietary provoked changes in the redistribution of the circulating mineral pool or in storage sites. Depending on the mineral of interest and its homeostatic regulation, different indices can be used. For example, serum phosphorus concentrations can be used in preterm infants as an indicator of dietary sufficiency of phosphorus. Serum calcium
and magnesium, however, are maintained within a narrow range and thus are not sensitive as indicators of dietary intake (Halbert & Tsang 1992). Therefore, functional outcomes or biochemical indices which reflect calcium or magnesium homeostasis need to be assessed alternatively.

Serum or plasma zinc is not a specific measure of zinc status nor a sensitive indicator of dietary zinc intake (Prasad 1988). As no other direct and specific indices of zinc status are available, which reflect active or recent body zinc (King 1990), hair zinc concentrations can be used as an indicator of zinc status (Gibson 1980).

To determine iron status haemoglobin is routinely used. However, haemoglobin is not very sensitive nor specific. When haemoglobin concentrations fall, iron stores have already been depleted. Alternatively, serum iron, ferritin or transferrin receptors may be used in preterm infants since they reflect iron stores more precisely (Jacobs & Worwood 1981, Flowers et al. 1989).

Further in appropriate animal models, distribution of minerals in tissues can be used as an index of mineral status (Solomons 1988).

1.2.4 Functional responses

Specific physiological or pathophysiological responses to dietary delivery or sufficiency of a mineral can be used as a functional measure of mineral bioavailability. The limitations of using functional outcomes are that they may be interdependent on other nutrients as well as influenced by physiological processes.

**Bone Mineral Mass** - The measurement of bone mineral mass has been
used to determine adequacy and dietary needs of calcium and phosphorus. Bone mineral mass may also be dependent on other minerals such as magnesium, zinc, copper and manganese (Ernst & Neal 1992). Bone mineral content (BMC) in preterm infants has traditionally been measured with single photon absorptiometry (SPA) in the radius or humerus (Greer et al. 1983). Although SPA has been the most practical tool to measure BMC in preterm infants while hospitalized, this method has several limitations. The repositioning of the bone with multiple measurements and the variability of the thickness of surrounding tissue may result in errors of measurement. Further, BMC in a single bone may not be representative of BMC in the whole body. For these reasons, SPA is not the most sensitive measurement of metabolic changes induced by the diet (Salle & Glorieux 1993).

Dual Energy X-ray Absorptiometry (DXA) has the capacity to measure BMC and bone mineral density (BMD) in the whole body. BMD is an areal density measurement which is expressed as g/cm². The third dimension, "body thickness" is not evaluated and since this increases with growth, it will influence BMD. For this reason BMD should not be used in infants (Salle & Glorieux 1993) and a body size adjustment can be made by weight, length or lean mass. Braillon et al. (1992) demonstrated a 7 % overestimation of BMC by DXA when measuring very small amounts of hydroxyapatite from isolated bones of stillborn infants. They and others (Venkataraman & Ahluwalia 1992) demonstrated, however, acceptable coefficients of variation of repeated measurements of whole body BMC in newborns infants. Brunton
et al. (1993) validated measurements of BMC by DXA with carcass analysis of piglets of greater than 1.6 kg of body weight. Because lower precision and accuracy were observed in smaller subjects for the measurement of BMC, DXA is limited in preterm infants to measurements made after term age.

*Growth and quality of growth* - Growth is a traditional measure of overall nutritional status in early life, but is not a very specific indicator of mineral nutrition. Length growth has been associated with BMC (Lucas 1993) and may therefore reflect dietary calcium and phosphorus indirectly. Both weight and length growth have been associated with dietary zinc in preterm infants (Friel et al. 1985). Head circumference growth provides an indirect measurement of brain growth and is therefore an important part of nutritional assessment (Pereira & Georgieff 1992).

Since growth should be represented by a proportional increase in lean, fat and bone mass, it is important to assess the contributions of lean and fat mass to growth. Although, lean and fat mass are not specifically related to mineral nutrition, they provide information on the quality of growth. Body composition and composition of weight gain in preterm infants was traditionally measured by skinfold thickness from multiple sites (Pereira & Georgieff 1992) or derived from protein and energy balance techniques (Reichman et al. 1983). These methods, however, are likely not very accurate because skinfold measures assume that subcutaneous fat is representative of internal fatness and this technique does not allow estimation of fat free mass. The balance technique determines composition of weight gain indirectly and does not
represent whole body composition. Other methods which have been applied to
determine lean and fat mass in preterm infants are bioelectrical impedance and total
body electrical conductivity. Most of these methods, however, have limitations and do
not correlate with results of carcass analysis (Klisch 1989). DXA, however, can
measure lean mass accurately and precisely in subjects larger than 1.6 kg (Brunton et

1.3 Clinical Trials of Multi-Nutrient Supplementation for Preterm Infants fed Mother's
Milk: Mineral Bioavailability

In this section the present state of knowledge regarding mineral nutrition and
bioavailability in preterm infants' diets is described. This information was used to
formulate the rationale for the research described in this thesis. The specific
objectives that were generated from this literature review are highlighted.

1.3.1 Literature search strategy

This review was generated from reports published prior to (before or in the
year of 1993) the development of the hypotheses that are described in this thesis.
Published clinical trials were searched with MEDLINE (National Library of Medicine,
Bethesda, MD). Studies regarding mineral bioavailability from human milk for
preterm infants were mainly identified by key words and their combinations. The
literature search was also occasionally performed by author, for those known to be
involved in related research described in this thesis. The most commonly used key
words in the Medline search were "preterm (premature, very-low-birth-weight) infants, nutritional needs, mother's (human) milk, fortified mother's (human) milk, mineral (calcium, phosphorus, magnesium, zinc, iron) balance, mineral homeostasis (status), growth, bone mineralization (bone mineral content), body composition (lean mass, fat mass), mineral interactions".

1.3.2 Calcium, phosphorus and magnesium

Calcium, phosphorus and magnesium are fundamental for tissue structure and function. An overview of calcium and magnesium metabolism is demonstrated in Appendix Ia and Appendix Ib, respectively. Calcium is the most abundant mineral in the body and together with phosphorus, forms the major inorganic component of bone. During the third trimester of gestation, approximately 80% of the body's total calcium, magnesium and phosphorus will be accumulated by the fetus. Approximately 99% of total body calcium, 80% of total body phosphorus and 60% of the body's magnesium is in bone (Koo & Tsang 1993). For the healthy growing preterm infant, inadequate intakes of calcium, magnesium and phosphorus from the diet will result in failure to appropriately mineralize bone (Minton et al. 1979, Rowe et al. 1979, Sagy et al. 1980, Koo & Tsang 1991).

*Calcium, phosphorus and magnesium absorption and retention* - Preterm mother's milk does not provide sufficient dietary calcium and phosphorus to achieve intrauterine accretion rates (Atkinson et al. 1983, Kashyap et al. 1990). Current recommendations advise that preterm infants fed mother's milk should receive
supplemental calcium and phosphorus to prevent osteopenia (AAP 1985). Only a few randomized controlled trials have reported mineral balance in response to multi-nutrient fortification of mother's milk. For this reason, also descriptive studies were considered in Tables 1a, 1b and 1c.

Fortification of mother's milk with a multi-nutrient fortifier for preterm infants resulted in achievement of intrauterine rates of accretion for calcium and phosphorus in some studies but not all (summarized in Tables 1a and 1b). Magnesium retention similar to intrauterine accretion was observed in preterm infants fed mother's milk in one study (Kashyap et al. 1990) but not in another investigation (Atkinson et al. 1983). Addition of multi-nutrient fortifiers to mother's milk resulted in magnesium retention which was similar to or exceeded intrauterine references (summarized in Table 1c).

These inconsistent findings can in part be explained by dissimilarities of quantity and quality of these multi-nutrient supplements. Fractional absorption ranged from 35 to 90 % for calcium, from 63 to 95 % for phosphorus and from 47 to 86 % for magnesium (Tables 1a, 1b and 1c) with the lowest values being observed with the use of a liquid multi-nutrient fortifier. Further, the lack of precision and accuracy of the mass balance techniques used to determine fractional mineral absorption may account for the wide range observed in fractional mineral absorption among studies. As illustrated in Tables 1a and 1c, the greatest values for fractional mineral absorption were observed when stable isotope tracers were used. In addition to these
considerations, mineral interactions may occur when a multi-nutrient supplement is added to mother's milk. For instance, a high calcium and phosphorus intake from preterm formulas depressed fractional magnesium absorption in preterm infants (Giles et al. 1990, Rodder et al. 1992). Therefore, a high calcium and phosphorus content of a multi-nutrient fortifier may reduce dietary magnesium bioavailability. Kashyap et al. (1990) did not show a significant decrease in fractional magnesium absorption by addition of a multi-nutrient supplement to mother's milk; however, this study may lack precision as mass balance techniques were applied to measure mineral absorption.

Calcium bioavailability and calcium and/or phosphorus: mineral interactions will in part be dependent upon the quality of calcium and phosphorus salts used in a multi-nutrient fortifier. A relatively new calcium/phosphorus salt, calcium glycerophosphate (CaGP), was demonstrated to have greater solubility in-vitro compared to conventional calcium and phosphorus salts (Hanning et al. 1989). When CaGP is incorporated into a multi-nutrient fortifier, it may allow for lower calcium and phosphorus intakes than current recommendations which would simultaneously decrease the potential for calcium: mineral interactions. The absorption and retention of calcium and magnesium in preterm infants fed mother's milk supplemented with either a new multi-nutrient fortifier (MNF) (containing CaGP as its calcium and phosphorus source) or CaGP alone, as well as the antagonistic effect of calcium on fractional magnesium absorption are investigated using stable isotope tracers, by the research described in this thesis.
Table 1a. Calcium balance in preterm infants fed mother's milk supplemented with or without a multi-nutrient supplement

<table>
<thead>
<tr>
<th>Reference</th>
<th>Diets and subjects (n)</th>
<th>Ca intake (mmol/kg.d⁻¹)</th>
<th>Ca absorption (%)</th>
<th>Ca retention (mmol/kg.d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Randomized controlled trials</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ehrenkranz et al. 1985</td>
<td>MM (4)</td>
<td>1.10</td>
<td>90⁻</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>MM+PF (4)</td>
<td>2.40</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>Kashyap et al. 1990</td>
<td>MM (41)</td>
<td>1.07</td>
<td>71</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>MM+PF (13)</td>
<td>4.97</td>
<td>68</td>
<td>3.03</td>
</tr>
<tr>
<td></td>
<td>FHM+PF (15)</td>
<td>4.47</td>
<td>61</td>
<td>2.40</td>
</tr>
<tr>
<td><strong>Descriptive studies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Schanler et al. 1985</td>
<td>MM+DMC (14)</td>
<td>1.62</td>
<td>54</td>
<td>0.67</td>
</tr>
<tr>
<td>Schanler &amp; Garza 1988</td>
<td>HM+DMC (16)</td>
<td>3.27</td>
<td>71</td>
<td>2.14</td>
</tr>
<tr>
<td>Schanler et al. 1988</td>
<td>MM+LF (10)</td>
<td>3.42</td>
<td>35</td>
<td>1.07</td>
</tr>
<tr>
<td>Ehrenkranz et al. 1989</td>
<td>MM+PF (6)</td>
<td>3.06</td>
<td>76</td>
<td>2.05</td>
</tr>
<tr>
<td>Raschko et al. 1989</td>
<td>MM+LF (10)</td>
<td>4.16</td>
<td>59</td>
<td>2.12</td>
</tr>
<tr>
<td>Liu et al.1989</td>
<td>HM+PF (9)</td>
<td>3.57</td>
<td>82⁺</td>
<td>NA</td>
</tr>
</tbody>
</table>

MM= Mother's milk, HM= Human donor milk (term or preterm), PF= Powdered multi-nutrient fortifier, LF= Liquid multi-nutrient fortifier, DMC= Donor milk components, NA= Not available.

⁺ Percent absorption determined with stable isotope tracer

Recommended intake: 4.6-5.2 mmol/kg.d⁻¹ (AAP 1985).
Intrauterine accretion rate: 2.3-3.0 mmol/kg.d⁻¹ (Ziegler et al. 1976, Widdowson et al. 1988).
Table 1b. Phosphorus balance in preterm infants fed mother's milk supplemented with or without a multi-nutrient supplement

<table>
<thead>
<tr>
<th>Reference</th>
<th>Diets and subjects (n)</th>
<th>P intake (mmol/kg.d(^{-1}))</th>
<th>P absorption (%)</th>
<th>P retention (mmol/kg.d(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Randomized controlled trial</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kashyap et al. 1990</td>
<td>MM (41)</td>
<td>0.70</td>
<td>93</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>MM+PF (13)</td>
<td>2.97</td>
<td>76</td>
<td>2.23</td>
</tr>
<tr>
<td></td>
<td>HM+PF (15)</td>
<td>2.66</td>
<td>73</td>
<td>1.92</td>
</tr>
<tr>
<td>Descriptive studies</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Schanler et al. 1985</td>
<td>MM+DMC (14)</td>
<td>1.10</td>
<td>92</td>
<td>0.94</td>
</tr>
<tr>
<td>Schanler &amp; Garza 1988</td>
<td>MM+DMC (16)</td>
<td>2.35</td>
<td>95</td>
<td>1.81</td>
</tr>
<tr>
<td>Schanler et al. 1988</td>
<td>MM+LF (10)</td>
<td>2.29</td>
<td>63</td>
<td>1.26</td>
</tr>
<tr>
<td>Ehrenkranz et al. 1989</td>
<td>MM+PF (6)</td>
<td>2.31</td>
<td>82</td>
<td>1.89</td>
</tr>
<tr>
<td>Raschko et al. 1989</td>
<td>MM+LF (10)</td>
<td>2.71</td>
<td>68</td>
<td>1.81</td>
</tr>
</tbody>
</table>

MM= Mother's milk, HM= Human donor milk (term or preterm), PF= Powdered multi-nutrient fortifier, LF= Liquid multi-nutrient fortifier, DMC= Donor milk components.

= Significantly different from comparison group(s)
Recommended intake: 3.8-4.5 mmol/kg.d\(^{-1}\) (AAP 1985).
Intrauterine accretion rate: 1.9-2.4 mmol/kg.d\(^{-1}\) (Ziegler et al. 1976, Widdowson et al. 1988).
Table 1c. Magnesium balance in preterm infants fed mother’s milk with or without a multi-nutrient supplement

<table>
<thead>
<tr>
<th>Reference</th>
<th>Diets and subjects (n)</th>
<th>Mg intake (mmol/kg.d⁻¹)</th>
<th>Mg absorption (%)</th>
<th>Mg retention (mmol/kg.d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Randomized controlled trial</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kashyap et al. 1990</td>
<td>MM (41)</td>
<td>0.26</td>
<td>77</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>MM+PF (13)</td>
<td>0.37</td>
<td>70</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>HM+PF (15)</td>
<td>0.36</td>
<td>67</td>
<td>0.15</td>
</tr>
<tr>
<td><strong>Descriptive studies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Schanler 1985</td>
<td>MM+DMC (14)</td>
<td>0.34**</td>
<td>NA</td>
<td>0.20**</td>
</tr>
<tr>
<td>Schanler &amp; Garza 1988</td>
<td>MM+DMC (16)</td>
<td>NA</td>
<td>NA</td>
<td>0.17</td>
</tr>
<tr>
<td>Schanler et al. 1988</td>
<td>MM+LF (10)</td>
<td>0.45</td>
<td>47</td>
<td>0.16</td>
</tr>
<tr>
<td>Liu et al. 1989</td>
<td>HM+PF (9)</td>
<td>NA</td>
<td>86°</td>
<td>NA</td>
</tr>
</tbody>
</table>

MM= Mother’s milk, HM= Human donor milk (term or preterm), PF= Powdered multi-nutrient fortifier, LF= Liquid multi-nutrient fortifier, DMC= Donor milk components, NA= Not available.
° Percent absorption determined with stable isotope tracer
** Derived from figures in reference
°° Significantly different from comparison group(s)
Recommended intake: 0.35-0.41 mmol/kg.d⁻¹ (AAP 1985).
Intrauterine accretion rate: 0.10-0.14 mmol/kg.d⁻¹ (Ziegler et al. 1976, Widdowson et al. 1988).
Biochemical indices of calcium, phosphorus and magnesium status -

The classical symptoms of phosphorus depletion syndrome, which result in rickets of prematurity, are hypophosphatemia (< 1.3 mmol/L) and hypercalcemia (>2.6 mmol/L). A serum phosphorus concentration of less than 1.8 mmol/L may indicate dietary phosphorus deficiency which has been associated with poor bone mineralization. Low serum phosphorus concentrations have been observed more frequently in preterm infants fed human milk compared to preterm infants fed a preterm formula (Steichen & Tsang 1992). Dietary phosphorus intake will affect renal handling of phosphorus and subsequently serum phosphorus concentrations (Halbert & Tsang 1992). Normal values reported for serum phosphorus in preterm infants range from 1.8 to 3.0 mmol/L (Hammond 1993). No consistent significant increase in serum phosphorus concentrations was observed by addition of a multi-nutrient fortifier to mother's milk (summarized in Table 2). Phosphorus intake below current recommendations and low dietary bioavailability of phosphorus from different multi-nutrient fortifiers may have accounted for this. Dietary phosphorus bioavailability is dependent on calcium:phosphorus ratio, absolute quantity of minerals added in the multi-nutrient supplement (Koo & Tsang 1993) and the possibility of calcium and phosphorus forming insoluble compounds possibly with other minerals and nutrients (Brink et al. 1992, Flanagan et al. 1985). As shown in Table 1b, phosphorus absorption was reduced by addition of a powdered multi-nutrient fortifier from approximately 93 % to approximately 75 %. 
Serum alkaline phosphatase has frequently been used as an indicator of bone mineral homeostasis; a reduction in alkaline phosphatase activity is used as a therapeutic marker for rickets (Delmas 1993) and has been measured in response to dietary interventions. However, the usefulness of this marker is limited and depends on the ability to quantify the activity of the specific skeletal isoenzyme of alkaline phosphatase. A significant decrease in serum alkaline phosphatase activity in response to multi-nutrient supplementation of mother's milk was only observed in one study (Table 2). More sensitive and specific markers for bone turnover and bone formation can now be measured such as plasma or serum osteocalcin and type I procollagen peptides or urinary pyridinoline excretion (Delmas 1993), however, these have not been validated in preterm infants as indices of bone mineral homeostasis. Such markers may be useful to provide information on bone mineral homeostasis in addition to measurements of bone mineral mass. *In this thesis plasma osteocalcin concentrations and serum alkaline phosphatase activities as biochemical indices for bone turnover and formation are described in preterm infants fed mother's milk supplemented with either MNF or CaGP alone.*

Biochemical indicators for magnesium status have not been measured in preterm infants. Magnesium deficiency in healthy premature infants is not likely to occur, since even with unsupplemented mother's milk intrauterine accretion rates of magnesium can be obtained (Table 1c).
Table 2. Serum calcium, phosphorus and alkaline phosphatase in preterm infants fed mother's milk with or without a multi-nutrient supplement.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Diets and subjects (n)</th>
<th>Serum Ca (mmol/L)</th>
<th>Serum P (mmol/L)</th>
<th>Alkaline Phosphatase (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Randomized controlled trials</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Modanlou et al. 1986</td>
<td>MM (10)</td>
<td>2.37 ±</td>
<td>1.97</td>
<td>1075</td>
</tr>
<tr>
<td></td>
<td>MM+PF (8)</td>
<td>2.44</td>
<td>2.06</td>
<td>790</td>
</tr>
<tr>
<td>Carey et al. 1987</td>
<td>MM (9)</td>
<td>2.52</td>
<td>1.55</td>
<td>375</td>
</tr>
<tr>
<td></td>
<td>MM+PF (6)</td>
<td>2.47</td>
<td>1.74</td>
<td>356</td>
</tr>
<tr>
<td>Gross 1987</td>
<td>HM (10)</td>
<td>NA</td>
<td>1.60**</td>
<td>550**</td>
</tr>
<tr>
<td></td>
<td>HM+LF (9)</td>
<td></td>
<td>2.06</td>
<td>360</td>
</tr>
<tr>
<td></td>
<td>HM+PF (8)</td>
<td></td>
<td>1.87</td>
<td>500</td>
</tr>
<tr>
<td>Greer &amp; McCormick 1988</td>
<td>MM (10)</td>
<td>2.52</td>
<td>1.79</td>
<td>382</td>
</tr>
<tr>
<td></td>
<td>MM+PF (10)</td>
<td>2.49</td>
<td>1.78</td>
<td>446</td>
</tr>
<tr>
<td>Pettifor et al. 1987</td>
<td>MM (30)</td>
<td>2.42</td>
<td>1.99</td>
<td>678</td>
</tr>
<tr>
<td></td>
<td>MM+LF (29)</td>
<td>2.36</td>
<td>2.03</td>
<td>488</td>
</tr>
<tr>
<td>Moyer-Milleur et al. 1992</td>
<td>MM+PF (18)</td>
<td>2.52</td>
<td>2.19 ±</td>
<td>394</td>
</tr>
<tr>
<td></td>
<td>MM+LF (17)</td>
<td>2.44</td>
<td>1.61</td>
<td>451</td>
</tr>
</tbody>
</table>

MM= Mother's milk, HM= Human donor milk (term or preterm), PF= Powdered multi-nutrient fortifier, LF= Liquid multi-nutrient fortifier, NA= Not available.
** Derived from figures in reference
· Significantly different from comparison group
1.3.3 Zinc

For the preterm infant zinc is necessary for the maintenance of cell growth and development. An overview of zinc metabolism is presented in Appendix Ic. Because prematurely born infants will miss the peak zinc accumulation in the third trimester of pregnancy they will have low zinc stores at birth (Zlotkin & Cherian 1988), which could easily become depleted if fed a diet low in zinc. The importance of zinc for the preterm infant was demonstrated by findings that preterm infants had lower hair zinc concentrations when compared to term born infants in the first year of life (Friel et al. 1984) and zinc intake was demonstrated to predict long-term growth in preterm infants (Friel et al. 1985). Because of the relatively low zinc content in mother's milk, in addition to immaturity of the gastrointestinal tract which results in difficulties of absorption (Dauncey et al. 1976, Higashi et al. 1988), it is likely that the breast-fed preterm infant is more vulnerable to develop zinc deficiency. Nutritional zinc deficiency has been observed in preterm infants receiving their mother's milk (Atkinson et al. 1989). Delayed growth and wound healing, perioral and perineal acrodermatitis, irritability, hypozincemia and low plasma alkaline phosphatase were observed in these infants. The etiology of the zinc deficiency in these infants, however, was the abnormally low zinc concentration of the mothers' milk and such clear manifestations of zinc deficiency in preterm infants are rare.

Current recommendations imply that fetal accretion rates can theoretically be attained with mother's milk and supplemental zinc is not recommended (AAP 1985).
**Zinc absorption and retention** - Negative zinc balances have been observed in preterm infants fed human milk as a result of endogenous zinc losses (Dauncey et al. 1976). Supplemental zinc in the form of a multi-nutrient fortifier for mother's milk may restore depleted zinc stores in premature infants. Multi-nutrient fortification of mother's milk resulted in zinc retention exceeding the intrauterine reference values (Table 3). Only three studies in a limited number of infants have measured zinc absorption and retention in relation to mother's milk supplementation.

Several investigations in animals, adults and preterm infants have demonstrated that calcium and or phosphorus reduced fractional zinc absorption (Spencer et al. 1984, Atkinson et al. 1990, Atkinson et al. 1993). Addition of a powdered multi-nutrient fortifier to mother's milk appeared to reduce fractional zinc absorption. The number of infants, however, studied in these investigations were likely too small to detect significant differences in fractional zinc absorption.

The optimal zinc content of multi-nutrient fortifiers will be dependent on potential interactions between calcium and zinc. By providing lower amounts of calcium and phosphorus intakes, in the form of CaGP, the potential for calcium:zinc interactions may be reduced. *The absorption and retention of zinc in preterm infants fed mother's milk supplemented with either MNF or CaGP alone, as well as the antagonistic effect of calcium on fractional zinc absorption are investigated using stable isotope tracers, by the research described in this thesis.*
Table 3. Zinc balance in preterm infants fed mother's milk with or without a multi-nutrient supplement

<table>
<thead>
<tr>
<th>Reference</th>
<th>Diets and subjects (n)</th>
<th>Zn intake (µmol/kg.d⁻¹)</th>
<th>Zn absorption (%)</th>
<th>Zn retention (µmol/kg.d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Randomized controlled trial</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ehrenkranz et al. 1984</td>
<td>MM (4)</td>
<td>10.11</td>
<td>66*</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>MM+LF (4)</td>
<td>14.5</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td><strong>Descriptive studies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ehrenkranz et al. 1989b</td>
<td>MM (7)</td>
<td>10.05</td>
<td>63*</td>
<td>5.21</td>
</tr>
<tr>
<td></td>
<td>MM+PF (5)</td>
<td>28.29</td>
<td>48</td>
<td>9.00</td>
</tr>
<tr>
<td>Ehrenkranz et al. 1989a</td>
<td>MM+PF (16)</td>
<td>27.92</td>
<td>40</td>
<td>10.49</td>
</tr>
</tbody>
</table>

MM= Mother's milk, PF= Powdered multi-nutrient fortifier, LF= Liquid multi-nutrient fortifier, NA= Not available.

* Percent absorption determined with stable isotope tracer
: Significantly different from comparison group

Recommended intake: 7.6 µmol/100 KCal [approximately 10.0 µmol/kg.d⁻¹] (AAP 1985).

Intrauterine accretion rate: 21.7-5.4 µmol/kg.d⁻¹ from 24 to 34 weeks gestational age (Widdowson et al. 1988).
Biochemical indices of zinc status - Zinc deficiency can only be diagnosed by clinical and biochemical presentations and by responses to zinc supplementation (King 1990). More commonly used indices of zinc status such as serum or plasma zinc do not represent whole body zinc stores or long term zinc status and are susceptible to circadian variations. Newer indices such as red blood cell zinc and leucocyte or neutrophil concentrations do reflect body zinc stores, but are difficult to analyze and red blood cell zinc has demonstrated inconsistent responses to zinc depletion and supplementation studies (King 1990). Furthermore, such indices require blood sampling. Hair zinc concentrations have shown to reflect redistribution and changing zinc status in infants as well as dietary zinc over a prolonged period of time and are not influenced by circadian variations and pathological conditions (Gibson 1980, Klevay et al. 1987, Prasad 1988). The research presented in this thesis describes zinc status, as measured by hair zinc concentrations in preterm infants fed mother's milk supplemented with either MNF or CaGP alone in hospital and mother's milk or formula post hospital discharge.

1.3.4 Iron

Iron deficiency in humans is most prevalent among six to 24 months old infants, coinciding with the latter part of brain growth spurt and with the development of fundamental mental and motor processes (Lozoff 1992). It has been suggested that iron deficiency can alter the normal development of the central nervous system (Lozoff 1992). An overview of iron metabolism is presented in Appendix Id.
Although premature birth interrupts intrauterine accretion of iron, the total body iron content per kilogram body weight of preterm infants at birth is similar to term infants. The major reserve of iron for preterm infants at birth is the haemoglobin mass. Nutritional iron deficiency is unlikely to play a role in the anaemia of prematurity during the first two months of life and supplemental dietary iron will not prevent this "physiologic anaemia" observed when erythropoiesis is still suppressed in early neonatal life (Ehrenkranz 1993). However, once active erythropoiesis begins six to eight weeks postnataally, preterm infants require supplemental iron when fed mother's milk or when bottle-fed an iron fortified formula in order to maintain haemoglobin production and to ensure normal brain development (AAP 1985).

Preterm infants absorb iron well (Ehrenkranz et al. 1992). Iron absorption appears to be related to postnatal age, growth rate, haemoglobin concentration and type of feeding (Osaki 1985, Dallmann 1988). Several studies in animals and humans have demonstrated that high calcium intake interfered with fractional iron absorption and iron retention from the diet (Barton et al. 1983, Dawson-Hughes et al. 1986, Hallberg et al. 1992, Atkinson et al. 1993). Thus, the early physiological decline of iron status in preterm infants may coincide with nutritional practices that deliver additional amounts of calcium and phosphorus which may further compromise iron status. Although it has been proposed that infants fed a diet with high calcium content may be at risk for developing iron deficiencies (Barton et al. 1983, Hallberg et al. 1992) it is questionable whether the findings from adult human and animal studies can
be extrapolated to preterm infants.

The investigation of iron absorption in preterm infants is difficult for several reasons. First, cellular mechanisms within the enterocyte are responsible for the maintenance of iron homeostasis (Halliday 1992). Therefore, when measuring iron absorption by mass balance techniques, stool collections will need to be performed over a prolonged period of time to account for "trapped" unabsorbed iron in sloughed mucosal cells. This can be circumvented by the use of isotopic tracers and the measurement of their incorporation in red blood cells (Oettinger et al. 1954). Repeated blood sampling may, however, not be appropriate in healthy preterm infants. Secondly, there are substantial endogenous losses in the first 30 days of life (Dauncey et al. 1976), which will reduce the accuracy of the measurement of iron absorption when applying mass balance techniques or isotopic tracers with subsequent stool collection. Further, iron absorption may be influenced by many factors, such as blood transfusions and health status (Ehrenkranz 1993).

The infant-piglet model can provide a suitable alternative to the preterm infant and has previously been used as a model for preterm infants to study mineral metabolism (Atkinson et al. 1993). The infant-piglet is a suitable model to study specifically calcium:iron interactions. Firstly, similar to preterm infants, infant-piglets are born with low iron stores and have a similar postnatal decline in circulating hemoglobin. Secondly the anatomical and physiological functions of the gastrointestinal tract are similar and many nutrients required by humans are essential
to pigs as well (Miller & Ullrey 1987). *The effect of a diet high in calcium and phosphorus on iron bioavailability, as measured in-vivo, in-situ and in-vitro models of the infant-piglet, is investigated by the research described in this thesis.*

### 1.3.5 Functional responses to multi-nutrient supplementation of mother's milk

Responses of growth and bone mineral mass are often not specific to one mineral, therefore, the different functional responses in this literature review are not categorized by mineral.

*Bone mineral mass* - Several investigations have measured whether additions of multi-nutrient supplements to mother's milk improved BMC as measured in a single bone using SPA (summarized in Table 4). In two studies a response to supplementation of mother's milk in radial BMC and BMD was observed (Greer & McCormick 1988, Pettifor et al. 1987), while others did not show a response in BMC in either the radius or humerus. BMC attained in these studies was reported to be lower than intrauterine reference values (Gross et al. 1987, Greer & McCormick 1988). A more recent study, however, demonstrated that a BMC similar to intrauterine values was attained by addition of a powdered multi-nutrient fortifier to mother's milk (Moyer-Milleur et al. 1992). From these randomized controlled trials, however, it cannot be concluded that multi-nutrient fortification of mother's milk resulted in greater BMC in comparison with preterm infants fed unsupplemented mother's milk. Differences in dietary calcium and phosphorus bioavailability from different multi-nutrient supplements, the lack of accurate methodology to measure
BMC, as well as nutrient composition regarding protein and other minerals may have accounted for these inconsistent results. To date there have been no randomized controlled trials which measure whole body BMC with DXA techniques in response to dietary interventions in preterm infants fed mother's milk. *In this thesis whole body BMC at term age is described in preterm infants fed mother's milk supplemented with either MNF or CaGP alone.*
### Table 4. Bone mineral mass in preterm infants fed mother's milk with or without a multi-nutrient supplement

<table>
<thead>
<tr>
<th>Reference</th>
<th>Diets, subjects (n) and duration supplement [weeks]</th>
<th>Measurement site</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Randomized controlled trials</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Modanlou et al. 1986</td>
<td>MM (10) MM+PF (8) [3 - 4]</td>
<td>midpoint humerus</td>
<td>*BMC and BW no differences</td>
</tr>
<tr>
<td>Venkataraman &amp; Blick 1988</td>
<td>MM (8) MM+PF (8) [3 - 4]</td>
<td>one third distal radius</td>
<td>*BMC no differences</td>
</tr>
</tbody>
</table>
| Gross et al. 1987   | HM (10) HM+LF (9) HM+PF (8) [5]                    | midpoint humerus  | *BMC no difference  
|                    |                                                   |                  | *BMC was significantly lower than values from term born infants |
| Greer & McCormick 1988 | MM (10) MM+PF (10) [6]                           | one third distal radius | *BMC no differences  
|                    |                                                   |                  | *BMD no differences  
|                    |                                                   |                  | *ΔBMC greater in MM+PF  
|                    |                                                   |                  | *BMC was significantly lower than intrauterine values |
| Pettifor et al. 1987 | MM (30) MM+PF (29) [4 - 6]                        | midpoint radius  | *BMC greater in MM+PF  
|                    |                                                   |                  | *BMD greater in MM+PF |
| Moyer-Milleur et al. 1992 | MM+PF (18) MM+LF (17) [4]                       | one third distal radius | *BMC greater in powdered MM+PF at 2 and 4 weeks  
|                    |                                                   |                  | *BMC in MM+PF similar to 50th percentile of intrauterine values |

MM= Mother's milk, HM= Human donor milk (term or preterm), PF= Powdered multi-nutrient fortifier, LF= Liquid multi-nutrient fortifier, BMC= Bone mineral content, BW= Bone width, BMD= Bone mineral density.
Growth and quality of growth - Greater weight gain in preterm infants fed fortified mother's milk compared to infants fed unfortified mother's milk was observed in five out of six randomized controlled trials (Table 5). Intrauterine weight gain was achieved consistently by multi-nutrient fortification of mother's milk. The greater weight gain in preterm infants fed fortified mother's milk was likely associated with the increase in protein intake with multi-nutrient fortification (Lucas 1993). No clear consistent improvement of length and head circumference increments were demonstrated with multi-nutrient fortification of mother's milk. Intrauterine length gain was only achieved in two studies. Differences in the dietary mineral bioavailability from multi-nutrient supplements or interactions with other minerals (such as magnesium, zinc) important for length and skeletal growth may account for slower length gains in comparison to intrauterine length gain. Because length gain in part may represent skeletal growth, the lack of response in length growth to multi-nutrient supplementation is consistent with findings of lack of response in an increase in radial or humeral BMC.

The optimal growth for preterm infants is thought to be similar to the intrauterine fetus (AAP 1985). Intrauterine weight growth is estimated to be 13 g/kg.d\(^{-1}\) and length gain 1.1-1.2 cm/wk (Ziegler et al. 1976, Blidner et al. 1984). Preterm infants fed a preterm formula, however, achieve greater weight and length gains (Carey et al. 1987, Raschko et al. 1989). Whether it is desirable for the preterm infant to achieve a greater weight gain than its intrauterine counterparts is not
Because of the lack of noninvasive methods to accurately measure body composition, few studies have investigated body composition in preterm infants fed fortified mother's milk. Kashyap et al. (1990) reported no response in skin fold thickness at various sites in preterm infants fed fortified mother's milk in comparison to unsupplemented mother's milk. This method, however, is not very accurate nor precise. With the new DXA technique, whole body lean and fat mass can be determined. In the research in this thesis short-term growth and lean and fat mass at term age are described in preterm infants fed mother's milk supplemented with either MNF or CaGP alone.
Table 5. Short-term growth in preterm infants fed mother’s with or without a multi-nutrient supplement

<table>
<thead>
<tr>
<th>Reference</th>
<th>Diets and subjects (n)</th>
<th>weight (g/kg.d(^{-1})) [g/d]</th>
<th>Length (cm/wk)</th>
<th>Head circumference (cm/wk)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Randomized controlled trials</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Modanlou et al. 1986</td>
<td>MM (10)</td>
<td>[25.2]</td>
<td>0.8</td>
<td>0.8 :</td>
</tr>
<tr>
<td></td>
<td>MM+PF (8)</td>
<td>[29.7]</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Carey et al. 1987</td>
<td>MM (9)</td>
<td>13.9 :</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>MM+PF (6)</td>
<td>19.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gross 1987</td>
<td>HM (10)</td>
<td>[22.7]</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>HM+LF (9)</td>
<td>[27.0]</td>
<td>0.8</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>HM+PF (8)</td>
<td>[33.0]</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>Greer &amp; McCormick 1988</td>
<td>MM (10)</td>
<td>13.4</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>MM+PF (10)</td>
<td>17.3</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Pettifor et al. 1987</td>
<td>MM (30)</td>
<td>15.8</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>MM+PF (29)</td>
<td>16.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kashyap et al. 1990</td>
<td>MM (41)</td>
<td>16.5</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>MM+PF (13)</td>
<td>20.5</td>
<td>1.3</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>HM+PF (15)</td>
<td>18.2</td>
<td>1.1</td>
<td>0.9</td>
</tr>
</tbody>
</table>

MM= Mother’s milk, HM= Human donor milk (term or preterm), PF= Powdered multi-nutrient fortifier, LF= Liquid multi-nutrient fortifier, NA= Not available, :
Significantly different from comparison group(s).
Intrauterine references for weight gain: 13 g/kg.d\(^{-1}\) and length gain: 1.1-1.2 cm/wk between 27 and 40 weeks post-menstrual age (Ziegler et al. 1976, Blidner et al. 1984).
1.4 Long-term outcomes of growth and bone mineral mass in preterm infants in relation to nutritional management in hospital

In comparison with short-term outcomes of growth and radial BMC in preterm infants, no studies have examined the long-term outcomes of growth, bone mass and lean and fat mass among preterm infants fed mother's milk with a multi-nutrient supplement in hospital in a randomized controlled trial. Several studies have investigated the importance of post-hospital discharge nutrition on long-term growth and bone mineral mass (Lucas et al. 1992b, Schanler et al. 1992, Bishop et al. 1993, Chan 1993). These studies demonstrated that feeding preterm infants nutrient-enriched formulas resulted in greater BMC as measured in a single bone as well as long-term length and weight growth compared to infants fed a standard term formula. Because mother's milk feeding is becoming more prevalent in preterm infants, it is of great importance to determine whether their nutritional needs can be met by mother's milk alone after hospital discharge. No information is available on long-term bone mineral, lean and fat mass determined with appropriate tools such as DXA. The research described in this thesis reports on long-term outcomes of growth, bone mineral, lean and fat mass in preterm infants fed mother's milk supplemented with either MNF or CaGP alone in hospital and mother's milk or formula post-hospital discharge.
Chapter Two

CALCIUM, MAGNESIUM AND ZINC BIOAVAILABILITY FROM PRETERM MOTHER'S MILK FORTIFIED WITH A NEW MULTI-NUTRIENT FORTIFIER DETERMINED BY STABLE ISOTOPE TRACERS AND MASS BALANCE TECHNIQUES

*This chapter represents a manuscript that is prepared under the auspices of the Journal of Pediatric Gastroenterology and Nutrition.

This research will be presented at the 16th International Congress of Nutrition, July-August 1997, Montreal, Canada.
Calcium, Magnesium and Zinc Bioavailability From Preterm Mother's Milk Fortified
With a New Multi-Nutrient Fortifier Determined by Stable Isotope Tracer and Mass
Balance Techniques

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Department of Pediatrics, McMaster University and The Children's Hospital, Hamilton
Health Sciences Corporation, Hamilton, Canada

Running Title: Bioavailability of Ca, Mg and Zn from preterm infants' diets

Pages: 30
Tables: 5
Figures: 2

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Abstract: We investigated the bioavailability of calcium, magnesium and zinc from a new multi-nutrient fortifier for mother's milk. Absorption and retention of calcium, magnesium and zinc were determined with the extrinsic stable isotope tracers $^{44}$Ca, $^{25}$Mg and $^{65}$Zn. Absorption and retention of these minerals were measured simultaneously by mass balance techniques in a total 37 preterm infants [birth weight 1.3±0.3 kg, gestational age 30±2 wk (mean±SD)]. Preterm infants receiving their mother's milk (MM) were randomly allocated to receive the new multi-nutrient fortifier (MM+MNF) or calcium and phosphorus alone (MM+CaGP). Preterm infants fed a preterm formula (PTF) served as a comparison group. Addition of whey-protein, lactose, minerals and trace elements, as MNF, to mother's milk in comparison to supplementation with calcium and phosphorus alone did not appear to reduce fractional absorption of calcium (MM+MNF: 76±16% and MM+CaGP: 79±17%) or magnesium (MM+MNF: 77±14% and MM+CaGP: 84±9%) as determined by stable isotope tracers. Fractional absorption of zinc was similar between MM+MNF (43±18 %) and MM+CaGP (44±27 %) groups, but was lower in comparison with reported values from unsupplemented mother's milk (63-68%). Fractional mineral absorption in PTF was similar to MM+MNF and MM+CaGP for calcium (72±16%) and zinc (31±17%) but significantly lower for magnesium (67±19%) when compared to MM+CaGP (p<0.05). The mass balance technique compared to the isotope tracer technique underestimated fractional mineral absorption by 11±12 % for calcium, 11±11 % for magnesium and 18±27 % for zinc. Results from mass balance
techniques should be used with foremost caution when evaluating mineral bioavailability from the diet. Future studies are required to investigate the optimal amount and ratios of minerals to be provided in a multi-nutrient fortifier for preterm infants fed mother's milk. **Key Words:** Multi-nutrient fortifier--Preterm infants--Mother's milk--Mineral bioavailability.

**INTRODUCTION**

Mother's milk for preterm infants has many advantages over formula such as better neurocognitive development and lower incidence of necrotizing enterocolitis (1). One of the nutritional benefits of mother's milk is the high bioavailability of minerals from mother's milk (2,3). Despite these properties, mother's milk may not provide for the estimated nutrient needs of preterm infants for several nutrients such as protein, minerals and trace elements (4). Therefore, multi-nutrient supplementation of mother's milk for preterm infants is recommended to meet the premature infants' required nutrient intakes for support of somatic and skeletal growth (5).

The addition of nutrients to mother's milk in the form of a multi-nutrient fortifier (containing protein, carbohydrates, minerals, trace elements and sometimes vitamins) may decrease the bioavailability of minerals in mother's milk by changing their intestinal solubility or by competing for uptake at absorption sites, which may occur between minerals whose physical and chemical properties are similar (6). Since
nutrient and mineral interactions mainly occur at the level of absorption, the fractional absorption of a mineral is a measure of its dietary bioavailability. Bioavailability is defined as the fraction of an ingested mineral that is both absorbed and utilized by the body. Therefore, net retention of a mineral should be considered in addition to absorption (2,6).

Many investigations in preterm infants have measured apparent mineral absorption and retention from different diets with mass balance techniques. However, non-random collection errors of feces and urine, and the inability to distinguish unabsorbed dietary minerals from endogenous mineral secretions can result in inaccurate estimations of mineral absorption and retention (7). The use of stable isotope tracers combined with accurate collection techniques can minimize these errors (8). Stable isotope tracers added as extrinsic labels to diets for preterm infants have been successfully applied to measure true calcium, magnesium and zinc absorption from fortified mother's milk (9-11). None of these studies, however, have measured the effect of supplementing mother's milk with a multi-nutrient fortifier on true mineral absorption and retention in a randomized study design.

Our objectives were 1) to investigate, in a randomized controlled trial, the fractional absorption of calcium, magnesium and zinc from mother's milk supplemented with a new multi-nutrient fortifier or supplemented with calcium and phosphorus alone, 2) to compare the fractional absorption of calcium, magnesium and zinc from supplemented mother's milk to unsupplemented mother's milk from data
available in the literature and 3) to compare outcomes of fractional mineral absorption as determined by stable isotope tracers to outcomes obtained by mass balance techniques.

MATERIALS AND METHODS

Subjects and Diets

Preterm infants were recruited from the Neonatal Intensive Care Units of the Children's Hospital of the Hamilton Health Sciences Corporation (formerly Chedoke-McMaster Hospital and St Joseph's Hospital) in Hamilton, Canada. The inclusion criteria for enrolment in the study were: birth weight below 1800 g; appropriate for gestational age; postnatal age of greater than one week; on full oral feeds which have been tolerated for at least five days and gaining weight more than 10 g/kg.d\(^{-1}\); absence of severe congenital malformation or chromosomal abnormalities and absence of gastrointestinal disease.

Infants whose mothers supplied milk for more than 80 % of their total enteral intake were randomly allocated, by block randomization, to receive either a new multi-nutrient fortifier (MNF; produced to our design specifications by Wyeth-Ayerst, Canada) (MM+MNF) or calcium and phosphorus alone in the form of calcium glycerophosphate (CaGP) (C\(_3\)H\(_2\)CaO\(_6\)P; Paul Lohman Chemicals, Emmerthal, Germany) (MM+CaGP). CaGP was given to provide approximately 3 mmol/kg.d\(^{-1}\) of
calcium and phosphorus. A group of preterm infants fed solely
unsupplemented mother's milk was not studied, because of the risk of developing
osteopenia in such infants (12). Infants whose parent(s) had decided to formula feed
were matched as closely as possible by gestational age and birth weight to the infants
in either MM+MNF or MM+CaGP. These preterm infants fed a preterm formula
(PTF) and served as a comparison group. The infants in the PTF group received
Preemie SMA (Wyeth-Ayerst, Canada).

Whole body BMC was the main outcome of interest in this randomized
controlled trial as was described elsewhere (13). The sample size was based on
observed differences in whole body BMC between term infants at birth and preterm
infants at term corrected age (13). Of 44 infants recruited, seven discontinued the
study due to intolerance to the introduction of MNF (two infants), development of
chronic lung disease (three infants), insufficient breast milk (one infant) and one infant
developed metabolic acidosis. Results of 37 infants are presented.

The composition of MNF for the main nutrients and minerals is shown in
Table 1. The mother’s milk supplementation protocol was described elsewhere (13).

**Ethical Consideration**

Ethical approval was obtained from the Research Advisory Committee at the
Children's Hospital of the Hamilton Health Sciences Corporation in Hamilton, Canada.
Informed and written consent was obtained from the parent(s).
Preparation and Administration of the Stable Isotope Tracers

When the infants had received MM+MNF or MM+CaGP for at least 10 days, true calcium, magnesium and zinc absorption and retention were determined using the stable isotope tracers $^{44}\text{Ca}$, $^{25}\text{Mg}$ and $^{70}\text{Zn}$. Simultaneously, a mass balance was performed to determine apparent calcium, magnesium and zinc absorption and retention as previously described (14). The stable isotopes were obtained from Oakridge National Laboratories (Oakridge, TN) as 100 mg $^{44}\text{CaCO}_3$ (97.10 atom % enrichment), 100 mg $^{25}\text{MgO}$ (64.40 atom % enrichment) and 12.4 mg $^{70}\text{ZnO}$ (88.17 % atom enrichment). The isotopes were dissolved in the smallest possible volume of hydrochloric acid and diluted with deionized water to obtain stock solutions of 1.90 mg/mL $^{44}\text{Ca}$, 1.90 mg/mL $^{25}\text{Mg}$ and 99.33 µg/mL $^{70}\text{Zn}$. The solutions were filtered through a sterile 0.45 µm Acrodisc (Gelman Sciences, Ann Arbor, MI).

For preparation of the labelled diets for the absorption studies, mother's milk was collected from the infants' mothers using an electrical breast pump (Medela Inc. McHenry, IL). Approximately 12 hours prior to the isotope dosing, a weighed volume of breast milk or formula for three feedings was warmed to 37°C and MNF was added for those infants randomized to MM+MNF. An accurate weighed amount of all three isotope stock solutions was added to the mother's milk, mother's milk with MNF or formula to deliver to the infant an isotope dose of approximately 2.0 mg/kg $^{44}\text{Ca}$, 2.0 mg/kg $^{25}\text{Mg}$ and 0.2 mg/kg $^{70}\text{Zn}$. This "isotope cocktail" was equilibrated overnight by gently stirring for 12 to 16 hours at 4°C. On the morning of the isotope dosing the
"isotope cocktail" was rewarmed to 37°C and portioned out for three consecutive feeds in graduated nurser. The graduated nurser with the "isotope cocktail" were weighed pre-and post-administration to the infant to determine total isotope dose. The "isotope cocktail" was administered by gavage when infants were gavage-fed or by bottle when infants were bottle-fed. An ashless filter paper was placed under the infants head during feeding to collect any regurgitation. The isotopes were administered in three consecutive feeds to minimize the elevation of mineral intake by administration of all three isotopes in one feed. With the first and third isotope dose polyethylene glycol (PEG) was given at 0.2 g/kg body weight which was intended to serve as a fecal marker (15).

Sample Collection and Analysis

Prior to the isotope dose a baseline stool sample was collected to determine background isotopic ratios of $^{44}$Ca/$^{42}$Ca, $^{25}$Mg/$^{24}$Mg and $^{70}$Zn/$^{67}$Zn for each subject. Following the first isotope dose, stools were collected for five consecutive days using plastic-lined ashless filter papers (Whatman, Maidstone, England) which were placed in pre-weighed diapers. In case of stool losses in the diaper the nursing staff weighed the diaper and recorded the losses. Stools were placed in plastic ziplock bags and stored in a - 4°C freezer. The mass balance was timed from the first isotope dose. Mother's milk, fortified mother's milk and formula intakes for MM+MNF, MM+CaGP and PTF were prepared by the researchers for three days in order to record accurate
intakes of calcium, magnesium and zinc. Urine was collected for 3 days as described before (14). Samples of mother's milk, formula and stools were lyophilized (Flexidyne microprocessor, FTS Systems, Stone Ridge, NY) and acid digested (MDS 2000, CEM microwave sample preparation system, Matthews, NC) with ultrapure nitric acid. Filter papers were soaked in 10 % HCl acid for three days. Total calcium, magnesium and zinc content in milk, formula, stools, urine and filter papers were determined by flame atomic absorption spectrometry (model 703; Perkin-Elmer, Norwalk, CT).

Mass Spectrometry

Isotopic ratios of $^{70}\text{Zn}/^{67}\text{Zn}$ and $^{25}\text{Mg}/^{24}\text{Mg}$ in faecal samples were determined with inductively coupled plasma mass spectrometry (ICP-MS) (Perkin Elmer SCIEX ELAN 5000, Thornhill, Canada) after dilution of digested samples with deionized distilled water to achieve magnesium and zinc concentrations of 0.10-0.20 mg/L. These solutions were pumped into the ultrasonic nebulizer of the ICP-MS instrument. Peaks of 67 and 70 for zinc and of 25 and 24 for magnesium were monitored for determination of the $^{70}\text{Zn}/^{67}\text{Zn}$ and $^{25}\text{Mg}/^{24}\text{Mg}$ ratios. The coefficient of variation for the measurement of the isotopic ratios of $^{70}\text{Zn}/^{67}\text{Zn}$, $^{25}\text{Mg}/^{24}\text{Mg}$ were in the range of 0.6-3.0 % and 0.5-0.8 %, respectively. The accuracy of the respective isotope ratios were in the range of 3.5-5.0 % and 2.2-3.4 %. Isotopic ratio of $^{44}\text{Ca}/^{42}\text{Ca}$ in faecal samples was determined with thermal ionization mass spectrometry (TIMS) (VG 354, Micromass, Winsford, UK). Calcium in lyophilized and digested
fecal samples was extracted by calcium oxalate precipitation by the method of Yergey (16). Reconstituted samples were purified by cation exchange chromatography using AG 50WX8 cation exchange resin, 100-200 mesh (Bio-Rad). One mL of the purified samples with concentrations between 10 and 15 ppm was evaporated to dryness, and the white crystalline substance was dissolved in 1-2 µl 0.2 M sulphuric acid and loaded onto a tantalum filament. A current of 2 to 3 ampere was sent through the filament for one minute to remove the water of the solution, which leaves the sample firmly adhered to the filaments. Beads with the tantalum filaments were loaded into the TIMS instrument and ions were produced by heating the ionization filament. Peaks of 44 and 42 for calcium were monitored for determination of the $^{44}\text{Ca}/^{42}\text{Ca}$ isotopic ratio. The coefficient of variation for the measurement of the isotopic $^{44}\text{Ca}/^{42}\text{Ca}$ ratio measurements was < 0.5 %. The accuracy was between -0.7 and +1.1 %.

**Calculations**

Absorption of $^{44}\text{Ca}$, $^{25}\text{Mg}$ and $^{70}\text{Zn}$ was determined by the method of Schuette et al (17). Briefly, the expression to calculate absorption of $^{44}\text{Ca}$, $^{25}\text{Mg}$ and $^{70}\text{Zn}$ is shown in the following equation:
Tracer absorption (mg) = ID - \[ \frac{M \times (IR-I\bar{R}o)}{1/na + (IR-I\bar{R}o)} \]

ID = amount of the administered dose of stable isotope; M = the total amount of mineral in faecal sample; IR = isotopic ratio of the stool sample; I\bar{R}o = isotopic ratio of the baseline stool sample; and na = natural abundance of the denominator isotope.

Absolute absorption of calcium, magnesium and zinc was determined by multiplying the fractional tracer absorption (percent of tracer absorbed) by total mineral intake. Absolute retention was determined by subtracting calcium, magnesium and zinc in urine from absolute mineral absorption.

PEG was intended to serve as a faecal marker, however, urinary PEG excretions were observed in several infants, indicating that PEG was absorbed, and could therefore not be used as a quantitative faecal marker. As an alternative method, a stool sample was considered to be enriched when the isotopic ratio, as determined by mass spectrometry analysis, was greater than three standard deviations above the baseline stool sample.

Statistics

Differences in mineral absorption and retention between diet groups were determined by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls multiple means test. Correlations and ANOVA analysis were performed with
SigmaStat software (SigmaStat, Jandel Scientific, San Rafael, CA). Comparison between fractional mineral absorption as determined by mass balance and stable isotope techniques were determined by the method of Bland & Altman (18).

RESULTS

The infants characteristics are demonstrated in Table 2. The kinetics of daily $^{44}$Ca, $^{25}$Mg and $^{70}$Zn excretion in faecal samples are illustrated for one infant in Fig. 1. In most infants the five-day stool collection was adequate to collect all unabsorbed tracers. In eleven infants, however, the faecal isotopic enrichment had not completely returned to baseline values after the five day stool collection, indicating that not all unabsorbed tracer had been excreted. We assumed, however, that additional stool collections would not have been significantly enriched with the tracers as fecal transit time in previous studies in preterm infants of similar gestational age fed similar diets was on average 22 to 39 hours (10).

Dietary bioavailability of calcium, magnesium and zinc from preterm infants’ diets. No significant differences existed between diet groups in fractional calcium and zinc absorption. Only when determined by stable isotope tracers, fractional magnesium absorption was significantly greater in MM+CaGP when compared to PTF, while values for MM+MNF were intermediary between MM+CaGP and PTF (Table 3). There were no differences in fractional mineral retention as determined by stable
isotope tracers and mass balance techniques between diet groups (Table 3). Because absolute mineral retention was a function of mineral intake (Table 4), significant differences in absolute mineral retention were present as mineral intakes differed among diet groups (Table 3).

In Table 5, fractional mineral absorption from mother's milk supplemented with either MNF or CaGP are compared to fractional mineral absorption from unsupplemented milk from studies in the literature. No studies, however, have reported fractional magnesium absorption as determined by stable isotopes from preterm mother's milk. Therefore, a comparison of our results were made with a value of fractional absorption as determined by mass balance in one study.

Comparison of stable isotope tracer and mass balance techniques.

Significant correlations existed between the measurement of fractional absorption determined by mass balance and stable isotope techniques (calcium: \( R=0.78, p<0.001 \); magnesium: \( R=0.80, p<0.001 \); and zinc: \( R=0.47, p=0.003 \)). However, a lack of agreement could be observed when a comparison was made by the method of Bland & Altman (1986) (Fig. 2). The mean difference in the measurement of fractional absorption between mass balance techniques and stable isotope tracers were \( 11\pm12\% \) for calcium, \( 11\pm11\% \) for magnesium and \( 18\pm27\% \) for zinc. The intervals of agreement (mean \( \pm 2SD \)) were wide (calcium: +12 to -36\%, magnesium: +11 to -34\% and zinc: +36 to -73\%) (Fig. 2).
**TABLE 1.** Composition, per 44 g powder added to 1 L of mother's milk, of the multi-nutrient fortifier MNF

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (as whey) [g]</td>
<td>3.7</td>
</tr>
<tr>
<td>Carbohydrate (as lactose) [g]</td>
<td>34.7</td>
</tr>
<tr>
<td>Calcium (as CaGP) [mmol]</td>
<td>15.2</td>
</tr>
<tr>
<td>Phosphorus (as CaGP) [mmol]</td>
<td>14.1</td>
</tr>
<tr>
<td>Magnesium [mmol]</td>
<td>1.0</td>
</tr>
<tr>
<td>Zinc [μmol]</td>
<td>211</td>
</tr>
<tr>
<td>Copper [μmol]</td>
<td>29.9</td>
</tr>
<tr>
<td>Manganese [μmol]</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Nutrient composition as analyzed by the manufacturer.
TABLE 2. Infant characteristics of preterm infants fed mother’s milk randomized to MNF and CaGP and of preterm infants fed PTF

<table>
<thead>
<tr>
<th></th>
<th>MM+MNF n=12</th>
<th>MM+CaGP n=13</th>
<th>PTF n=12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (males/females)</td>
<td>7/5</td>
<td>10/3</td>
<td>7/5</td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>1.4±0.2\textsuperscript{a}</td>
<td>1.3±0.2\textsuperscript{a,b}</td>
<td>1.2±0.2\textsuperscript{b}</td>
</tr>
<tr>
<td>Gestational age (wk)</td>
<td>29.9±1.9</td>
<td>30.1±1.5</td>
<td>29.7±1.7</td>
</tr>
<tr>
<td>Post-menstrual age at start of MNF or CaGP (wk)</td>
<td>33.0±1.6</td>
<td>33.1±1.2</td>
<td></td>
</tr>
<tr>
<td>Duration of supplementation of MNF or CaGP (wk)</td>
<td>4.7±1.5</td>
<td>5.3±1.6</td>
<td></td>
</tr>
<tr>
<td>Post-menstrual age at start of absorption study (wk)</td>
<td>34.7±1.2</td>
<td>34.9±1.2</td>
<td>34.8±0.9</td>
</tr>
</tbody>
</table>

Results are presented as mean ± SD; different superscripts indicate p<0.05 (ANOVA).
TABLE 3. Absorption and retention of calcium, magnesium and zinc in preterm infants fed mother’s milk randomized to MNF or CaGP and in preterm infants fed PTF as determined by mass balance (MB) and stable isotope tracer technique (SIT)

<table>
<thead>
<tr>
<th>Measurement</th>
<th>MM+MNF</th>
<th>MM+CaGP</th>
<th>PTF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Calcium: Intake (mmol/kg.d⁻¹)</strong></td>
<td>2.71±0.32ᵃ</td>
<td>3.26±0.60ᵇ</td>
<td>2.56±0.33ᵃ</td>
</tr>
<tr>
<td><strong>MB:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fractional absorption (%)</td>
<td>66±20</td>
<td>72±18</td>
<td>58±16</td>
</tr>
<tr>
<td>Fractional retention (%)</td>
<td>58±17</td>
<td>66±16</td>
<td>50±13</td>
</tr>
<tr>
<td><strong>SIT:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fractional absorption (%)</td>
<td>77±15</td>
<td>79±16</td>
<td>73±15</td>
</tr>
<tr>
<td>Absolute absorption (mmol/kg.d⁻¹)</td>
<td>2.07±0.45ᵃ</td>
<td>2.55±0.59ᵇ</td>
<td>1.83±0.37ᵃ</td>
</tr>
<tr>
<td>Urine (mmol/kg.d⁻¹)</td>
<td>0.19±0.16ᵃ</td>
<td>0.34±0.16ᵇ</td>
<td>0.19±0.12ᵃᵇ</td>
</tr>
<tr>
<td>Fractional retention (%)</td>
<td>70±13</td>
<td>69±16</td>
<td>65±14</td>
</tr>
<tr>
<td>Absolute retention (mmol/kg.d⁻¹)</td>
<td>1.88±0.41ᵃᵇ</td>
<td>2.21±0.60ᵇ</td>
<td>1.64±0.39ᵇ</td>
</tr>
<tr>
<td><strong>Magnesium: Intake (mmol/kg.d⁻¹)</strong></td>
<td>0.27±0.02ᵃ</td>
<td>0.22±0.03ᵃ</td>
<td>0.55±0.10ᵇ</td>
</tr>
<tr>
<td><strong>MB:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fractional absorption (%)</td>
<td>67±19</td>
<td>73±9</td>
<td>56±21</td>
</tr>
<tr>
<td>Fractional retention (%)</td>
<td>47±17</td>
<td>54±12</td>
<td>42±20</td>
</tr>
<tr>
<td><strong>SIT:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fractional absorption (%)</td>
<td>77±14ᵃᵇ</td>
<td>84±9ᵇ</td>
<td>67±19ᵃ</td>
</tr>
<tr>
<td>Absolute absorption (mmol/kg.d⁻¹)</td>
<td>0.21±0.04ᵃ</td>
<td>0.19±0.03ᵃ</td>
<td>0.36±0.11ᵇ</td>
</tr>
<tr>
<td>Urine (mmol/kg.d⁻¹)</td>
<td>0.05±0.02ᵃ</td>
<td>0.04±0.02ᵃ</td>
<td>0.07±0.02ᵇ</td>
</tr>
<tr>
<td>Fractional retention (%)</td>
<td>57±11</td>
<td>65±11</td>
<td>53±18</td>
</tr>
<tr>
<td>Absolute retention (mmol/kg.d⁻¹)</td>
<td>0.15±0.03ᵃ</td>
<td>0.15±0.03ᵃ</td>
<td>0.29±0.10ᵇ</td>
</tr>
<tr>
<td><strong>Zinc: Intake (µmol/kg.d⁻¹)</strong></td>
<td>33.84±3.40ᵃ</td>
<td>11.64±1.75ᵇ</td>
<td>23.45±3.46ᶜ</td>
</tr>
<tr>
<td><strong>MB:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fractional absorption (%)</td>
<td>28±24</td>
<td>19±35</td>
<td>17±26</td>
</tr>
<tr>
<td>Fractional retention (%)</td>
<td>23±25</td>
<td>9±41</td>
<td>12±25</td>
</tr>
<tr>
<td><strong>SIT:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fractional absorption (%)</td>
<td>43±18</td>
<td>44±27</td>
<td>31±17</td>
</tr>
<tr>
<td>Absolute absorption (µmol/kg.d⁻¹)</td>
<td>14.53±3.06ᵃ</td>
<td>5.18±3.54ᵇ</td>
<td>7.00±3.95ᵇ</td>
</tr>
<tr>
<td>Urine (µmol/kg.d⁻¹)</td>
<td>1.55±0.70</td>
<td>1.21±0.98</td>
<td>1.16±0.74</td>
</tr>
<tr>
<td>Fractional retention (%)</td>
<td>39±18</td>
<td>33±30</td>
<td>25±17</td>
</tr>
<tr>
<td>Absolute retention (µmol/kg.d⁻¹)</td>
<td>12.99±3.07ᵃ</td>
<td>3.97±3.92ᵇ</td>
<td>5.83±4.07ᵇ</td>
</tr>
</tbody>
</table>

Results are presented as mean ± SD; different superscript indicate p<0.05 (ANOVA). Intraterine accretion rates are for calcium 2.3-3.0 mmol/kg.d⁻¹; for magnesium 0.10-0.14 mmol/kg.d⁻¹; and for zinc 5.5-13.2 µmol/kg.d⁻¹ (19,20).
TABLE 4. **Calcium, magnesium and zinc absorption and retention as a function of intake for all preterm infants combined**

<table>
<thead>
<tr>
<th></th>
<th>All infants (n=37)</th>
<th>Slope</th>
<th>Intercept</th>
<th>R</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Calcium</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absorption</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(absolute)</td>
<td>0.61</td>
<td>0.42</td>
<td>0.58</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>(fractional)</td>
<td>-5.30</td>
<td>91.42</td>
<td>0.18</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Retention</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(absolute)</td>
<td>0.54</td>
<td>0.38</td>
<td>0.55</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>(fractional)</td>
<td>-4.80</td>
<td>81.44</td>
<td>0.17</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td><strong>Magnesium</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absorption</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(absolute)</td>
<td>0.54</td>
<td>0.07</td>
<td>0.81</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>(fractional)</td>
<td>-41.75</td>
<td>90.16</td>
<td>0.41</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Retention</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(absolute)</td>
<td>0.47</td>
<td>0.04</td>
<td>0.79</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>(fractional)</td>
<td>-25.51</td>
<td>67.34</td>
<td>0.29</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td><strong>Zinc</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absorption</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(absolute)</td>
<td>0.38</td>
<td>0.15</td>
<td>0.60</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>(fractional)</td>
<td>-1.54</td>
<td>41.54</td>
<td>0.05</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Retention</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(absolute)</td>
<td>0.37</td>
<td>-0.89</td>
<td>0.62</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>(fractional)</td>
<td>2.25</td>
<td>28.66</td>
<td>0.07</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

R = correlation coefficient; NS = not significant.
### TABLE 5. Comparison of fractional calcium, magnesium and zinc absorption with literature values, determined with stable isotope tracers, from previous reports

<table>
<thead>
<tr>
<th></th>
<th>Literature values:</th>
<th>MM+CaGP</th>
<th>MM+MNF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Preterm mother's milk</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>68±5 [5] (22)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[n] = number of subjects

*As determined by mass balance technique
Fig. 1. Illustration of isotopic enrichments of stools from one infant for $^{44}\text{Ca} / ^{42}\text{Ca}$ (■), $^{25}\text{Mg} / ^{24}\text{Mg}$ (○) and $^{70}\text{Zn} / ^{67}\text{Zn}$ (▼).
Fig. 2. Difference in fractional absorption by mass balance technique (MB) and stable isotope tracer (SI) as a function of mean fractional absorption of both techniques (18). Hatched lines represent mean ± 2 SD.
DISCUSSION

Our study demonstrated that the addition of whey-protein, lactose, CaGP and trace elements, in the form of MNF, to mother's milk in comparison with supplementation with CaGP alone did not compromise the dietary bioavailability of calcium, magnesium or zinc.

Zinc was the only trace element added in significant amounts to MNF. Zinc has been demonstrated to interact with minerals such as calcium, iron and copper (23,24). The additional zinc in MNF did not appear to decrease the fractional absorption of calcium or magnesium. Animal and human studies have shown that the presence of glucose polymers and lactose in the diet enhances calcium and magnesium absorption (25). Thus it was possible that the presence of lactose in MNF may have counteracted potential mineral interactions. The nature of protein in the diet for preterm infants will also play a role in mineral bioavailability. The majority of minerals in human milk are bound to whey proteins (3), while in cow's milk to casein micelles or proteins (26). This is nutritionally relevant for the preterm infant. As acid secretion is low in the early neonatal period, a proportion of the minerals in cow's milk based formulas or casein-based fortifiers may not be released from casein and its curds and may thus not be available to the intestine for absorption (27,28). MNF contained whey-protein and would therefore likely not have reduced mineral bioavailability from mother's milk supplemented with MNF. The differences in
mineral-ligand binding between cow's milk and human milk (26) was also observed by the findings of a somewhat lower (but not significant) fractional calcium, magnesium and zinc absorption observed in preterm infants fed PTF.

Because of the recognized needs of the premature infant for calcium and phosphorus to prevent osteopenia (12), we did not include a comparison group of preterm infants fed only mother's milk in our study design. For this reason we compared our results with previous studies that determined fractional mineral absorption, using stable isotopes, in preterm infants fed un supplemented mother's milk. Taking into account differences in study protocols (e.g. different equilibration times of stable isotopes with mother's milk and the duration of stool collection), supplementation of mother's milk with either CaGP or MNF did not appear to reduce dietary calcium or magnesium bioavailability. Fractional zinc absorption, however, appeared to be considerably lower from supplemented mother's milk compared to unsupplemented milk. Because the fractional zinc absorption between MM+CaGP and MM+MNF was similar, it can be implied that calcium and/or phosphorus were the main contributors to reduce the fractional zinc absorption. This supposition is consistent with other studies in adults (29) and infants (30,31). Also, in-vitro observations from our laboratory in piglet brush border membrane vesicles showed that calcium and zinc compete for uptake at the intestinal brush border membrane by a multi- cation channel (32).

Although it was reported that a high calcium and/or phosphorus content in
premature infant diets reduced fractional magnesium absorption (33,34), the fractional magnesium absorption in our study was high despite additions of MNF and CaGP to mother's milk. Studies in animal models have demonstrated that calcium, magnesium and phosphorus can form an insoluble complex and decrease magnesium bioavailability for absorption (35). Thus, unlike the interaction between calcium and zinc, the interaction between calcium/phosphorus and magnesium may not be at uptake sites in the intestine but rather within the intestinal lumen itself. Although studies in rat pups and infants have demonstrated that magnesium bioavailability from formula and mother's milk is comparable (36), it is possible that, in comparison with a formula, addition of calcium and phosphorus to mother's milk would not result in the formation of an insoluble calcium magnesium phosphate complex as a result of different ligand binding of these minerals in mother's milk.

For the preterm infant, the optimal quantity of mineral retained by the body is believed to represent the amount that is accumulated during the third trimester of pregnancy (37). Thus, although the addition of supplemental nutrients to mother's milk may have reduced the efficiency of zinc absorption, when intake was three-fold higher, intrauterine accretion rates were attained as was shown for MM+MNF. Calcium retention was below intrauterine accretion rates in all infants. Greater absolute retention could theoretically have been achieved with greater calcium intakes, since absolute calcium retention was shown to be a function of calcium intake. However, adverse effects on mineral homeostasis and bone turnover have been
observed with high calcium intakes in premature infants (38). The multi-nutrient fortifier studied in our report was designed to contain less amounts of protein, calcium and phosphorus in comparison to currently available fortifiers in order to minimize adverse effects. Despite that calcium retention rates were below the intrauterine values, we have demonstrated elsewhere that mineral homeostasis as measured by serum calcium and phosphorus concentrations and whole body bone mineral were adequate in all infants in this study (13).

As the mass balance technique cannot account for endogenous mineral losses, the difference between fractional mineral absorption determined by mass balance techniques and stable isotopes techniques should theoretically represent these endogenous losses. To measure endogenous losses, when determining true mineral absorption, a dual isotope technique can be employed (39,40). This technique, however, requires intravenous administration of stable isotopes, which was not feasible in our healthy preterm infant population. Endogenous losses, determined by dual isotope techniques, for calcium and zinc have been shown to account for approximately 10 % of calcium intake (39,40) and approximately 13 % of zinc intake (41). With the use of a single isotope tracer to determine mineral absorption, as has been described in this study one has to assume that reentry of the absorbed stable isotope in the intestinal lumen as endogenous secretion is negligible. The differences between fractional mineral absorption determined by mass balance and stable isotopes in our study were similar to the reported percent endogenous losses for calcium and
zinc as described above. Based on this information we assumed that the reentry of absorbed isotopes in the intestinal lumen was likely small.

A potential limiting factor to the use of stable isotope tracer methods is that it is inferred that the extrinsic tracer added to the diet acts biologically similar as the native mineral. Liu et al. (11) demonstrated in preterm infants that intrinsic and extrinsic tracers for calcium and magnesium were absorbed with equal efficiency from fortified mother's milk. Studies investigating whether zinc absorption from intrinsic and extrinsic tracers occurs with similar efficiency in rats have demonstrated that the extrinsic zinc tracer was absorbed with less efficiency than an intrinsic zinc tracer (42,43). In studies more relevant to infant diets, the distribution of an extrinsic zinc tracer among the different milk fractions of human milk was similar to the distribution of native zinc (44). In term infants percent zinc absorption from intrinsic and extrinsic stable isotope tracers was similar thus validating the use of the extrinsic zinc tracer.

As stable isotopes of magnesium have a relative high abundance, the stable isotope tracer dose elevated daily magnesium intake by approximately 30 % and 38 % for preterm infants fed mother's milk supplemented with CaGP and MNF, respectively and by approximately 15 % for preterm infants fed PTF. In contrast to calcium and zinc, percent magnesium absorption is negatively associated with magnesium intake as was demonstrated in Table 4. Thus it is possible that percent magnesium absorption from mother's milk supplemented with either CaGP or MNF was underestimated in comparison to PTF.
In summary, supplementation of whey-protein, lactose and trace elements in addition to calcium and phosphorus in a multi-nutrient fortifier did not decrease the bioavailability of calcium, magnesium or zinc. Addition of calcium and phosphorus, however, may have reduced zinc bioavailability but supplemental zinc in MNF compensated for the reduced zinc bioavailability as it resulted in a greater absolute zinc retention. The use of a single isotope tracer is a suitable approach to measure dietary mineral bioavailability. Further, stable isotope tracers provide better accuracy in comparison to mass balance techniques. Interpretation of results derived from mass balance techniques should be regarded with caution and should not be used to determine dietary mineral bioavailability.

It should be clear that addition of any mineral or nutrient to mother's milk may alter the delicate balance between its native minerals and may thereby decrease their bioavailability. Therefore, future studies using stable isotope tracers should also investigate the impact of supplementing mother's milk on the fractional absorption of other minerals, such as copper and iron, which are important for growth and development. This would give further information for the optimal ratios of minerals to be provided in a multi-nutrient fortifier.

**Acknowledgements:** We thank Michelle Whelan RN for her assistance with the sample collections and Medela Inc. for making breast pumps available to the mothers in the study. This project was supported by a grant from the Dairy Farmers of Canada and
an in-kind donation of the powdered fortifier from Wyeth-Ayerst, Canada.

REFERENCES


Addendum to Chapter Two

DISTRIBUTION OF NATIVE CALCIUM AND MAGNESIUM AND EXTRINSIC

\(^{44}\text{Ca} \text{ AND } ^{65}\text{Zn} \text{ TRACERS AMONG DIFFERENT FRACTIONS OF PRETERM}

MOTHER'S MILK AND FORTIFIED PRETERM MOTHER'S MILK
Distribution of Native Calcium and Zinc and Extrinsic $^{45}$Ca and $^{65}$Zn Tracers Among Different Fractions of Preterm Mother's Milk and Fortified Preterm Mother's Milk

Ine PM Wauben, Vinay Bhide and Stephanie A Atkinson

Abstract. The validity of using extrinsic isotope tracers to determine dietary mineral bioavailability in preterm infants was investigated in preterm mother's milk (MM). The extrinsic tracers $^{45}$Ca and $^{65}$Zn were added to MM and fortified MM (MM+MNF) and equilibrated for 12 to 16 hours. The milk samples were subsequently subjected to differential centrifugation and ultrafiltration to yield a fat, casein, whey protein and low molecular weight (LMW) fraction. Total elemental calcium and zinc and $^{45}$Ca and $^{65}$Zn in the milk fractions were determined and compared. The distribution of $^{45}$Ca among the milk fractions of MM was comparable to native calcium. Distribution of $^{65}$Zn among the milk fractions of MM and MM+MNF compared to native zinc was significantly different with less of the extrinsic tracers present in the casein fraction and more in fat and LMW fractions, but was similar for the total soluble whey fraction (whey protein and LMW). The distribution of $^{45}$Ca among the milk fractions of MM+MNF demonstrated the greatest difference with the native mineral in the milk fractions. The results presented in this experiment demonstrated that a 12 to 16 hours equilibration period with extrinsic tracers did not result in equal distribution among milk fractions compared to the native minerals especially for fortified preterm mother's
milk. Therefore, caution is implicated when interpreting results from studies comparing mineral absorption from different milks using extrinsic isotope tracers. It remains speculative whether the differences observed will result in reduced or increased dietary mineral bioavailability in healthy preterm infants. Therefore, in-vivo studies are needed to validate whether the efficiency of absorption of extrinsic and intrinsic tracers are similar for the specific diets described in this experiment.

Introduction

The use of stable isotopes to study dietary mineral bioavailability from premature infants' diets has many advantages over conventional methods such as mass balance techniques (Weaver 1988). Stable isotopes can be used as intrinsic tracers or extrinsic tracers. As the use of intrinsic tracers are laborious and expensive, stable isotopes have been commonly applied as extrinsic tracers in diets for preterm infants (Ehrenkranz et al. 1989, Liu et al. 1989, Hillman et al. 1993). When using stable isotopes as an extrinsic tracers it is inferred that the extrinsic tracer added to the diet will be distributed among the respective milk fractions in a similar way as its native counterpart. Several studies have investigated the validity of this assumption (Sandstrom et al. 1983, Serfass et al. 1989, Abrams et al. 1990, Fairweather-Tait et al 1991, Lonnerdal et al. 1993, Boza et al. 1995), but findings have been inconsistent. This, however, can be explained by differences in study designs (animal studies versus investigations in infants), diets used (solid foods versus formula and/or human milk)
and equilibration time allowed for the extrinsic tracers to equilibrate with native minerals.

To assist in the interpretation of our studies measuring dietary mineral bioavailability from preterm infants' diets using extrinsic stable isotope tracers as presented in chapter two, the objective of this experiment was to investigate whether the 12 to 16 hour equilibration period allowed for the extrinsic tracers to equilibrate with the diets, resulted in a similar distribution among milk fractions as the native minerals.

Materials and Methods

The diets that were chosen for this experiment were similar to the diets described in chapter two. Donor breast milk was obtained from several mother's who had delivered preterm infants at the Children's Hospital of the Hamilton Health Sciences Corporation (formerly Chedoke McMaster Hospital and St Joseph's Hospital) in Hamilton, Canada. The breast milk was obtained at approximately six to ten weeks of lactation. All breast milk was pooled and the multi-nutrient fortifier (MNF) described in chapter two and four was added to a portion of the breast milk. All milk was frozen in 50 mL portions for latter use.

The radioisotopes \(^{45}\text{Ca}\) and \(^{65}\text{Zn}\) were obtained from Amersham (Mississauga, ON). The use of radioisotopes was chosen in this experiment as their preparation and detection is simple and inexpensive. Magnesium was not studied due
to high cost of its radioisotope.

Approximately 25 mL of mother's milk (MM), fortified mother's milk (MM+MNF) and preterm formula (PTF) were labelled with 2.5 μCi ⁶⁵Zn or ⁴⁵Ca and equilibrated while gently stirring for 12 to 16 hours at 4°C. This labelling method was similar to the methods as described in chapter two. After equilibration, the labelled milk was warmed to 37°C and subjected to differential centrifugation and filtration as illustrated in Figure 1 to render the different milk fractions. The fractionation procedure was performed at 37°C to mimic the presence of the extrinsic tracers in the different milk fractions under physiological conditions (in the gastrointestinal tract). Approximately 20 mL of the labelled milk and formula samples were fractionated into fat and skim milk by centrifugation (Sorvall RC-5B superspeed centrifuge, Dupont, Canada) at 4,000 g for 30 minutes at room temperature. The fat layer was removed with a spatula. The skim milk was fractionated by ultracentrifugation (L8-M Ultracentrifuge, Beckman, Canada) at 150,000 g for 60 minutes at 37°C into a casein pellet and supernatant containing the soluble whey fraction. The whey fraction was subsequently ultrafiltered using a low molecular weight cut-off filter (Mandell Scientific, Canada) to obtain a fraction with molecular weight less than 10,000 (low molecular weight fraction [LMW]) and a whey protein fraction. The fractions were counted for ⁴⁵Ca, after addition of 15 mL of liquid scintillation cocktail (Ready gel, Beckman, Canada) using a Phillips PW4700 scintillation counter (Phillips, The Netherlands) with an efficiency of approximately 95
% of the fractions for $^{65}$Zn were counted in a $\gamma$-counter (Minimax, auto-$\gamma$, Packard-Cranberra, Canada). The counts per minute (cpm) obtained for the different milk fractions were expressed as a percentage of the cpm obtained from the whole milk sample.

Unlabelled samples of MM, MM+MNF and PTF were subjected to the same fractionation procedure. The different fractions were acid digested (MDS 2000, CEM microwave sample preparation system, Matthews, NC). The digested samples were diluted and total elemental calcium and zinc in whole milk, and the different fractions were determined by flame atomic absorption spectrometry (Perkin-Elmer, Norwalk, CT).

Differences between distribution of extrinsic tracers and native minerals among milk fractions were tested by Students T-Test or Mann-Whitney U-Test if data were not normally distributed (Sigmastat, Jandell Scientific, San Rafael, CA).

Results

The fractionation of the respective milk fractions from preterm formula was unsuccessful due to the use of emulsifiers in preterm formula. Therefore, results for preterm formula are not reported.

The distribution of the $^{45}$Ca and $^{65}$Zn in comparison to the native minerals for calcium and zinc in the respective milk fractions are presented in Table 1 and Figure 2. The distribution of $^{45}$Ca in fractions of MM was similar to native calcium,
with the exception of the fat fraction. In MM+MNF, $^{45}\text{Ca}$ was distributed differently among all fractions in comparison to native calcium. $^{65}\text{Zn}$ was present in greater amounts in the fat and LMW fraction and lesser amounts in the casein fractions for both MM and MM+MNF, but $^{65}\text{Zn}$ present in the total soluble whey fraction (whey protein and LMW) was similar to native zinc.
Table 1. Distribution of the extrinsic tracer $^{45}$Ca and $^{65}$Zn and native calcium and zinc in the milk fractions of MM and MM+MNF

<table>
<thead>
<tr>
<th>Milk fraction (%) of total</th>
<th>MM extrinsic tracer (n=8)</th>
<th>MM native mineral (n=8)</th>
<th>MM+MNF extrinsic tracer (n=8)</th>
<th>MM+MNF native mineral (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat</td>
<td>17.9±1.5$^a$</td>
<td>23.0±2.3$^b$</td>
<td>3.4±0.6$^a$</td>
<td>6.5±0.5$^b$</td>
</tr>
<tr>
<td>Casein</td>
<td>6.3±1.2</td>
<td>5.9±0.5</td>
<td>1.4±0.2$^a$</td>
<td>17.8±1.4$^b$</td>
</tr>
<tr>
<td>Whey protein</td>
<td>44.6±9.0</td>
<td>40.0±7.3</td>
<td>47.3±5.6$^a$</td>
<td>77.4±9.7$^b$</td>
</tr>
<tr>
<td>LMW</td>
<td>31.1±7.7</td>
<td>31.3±8.0</td>
<td>47.9±5.8$^a$</td>
<td>-1.7±9.3$^b$</td>
</tr>
<tr>
<td>Zinc</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat</td>
<td>20.3±5.0$^a$</td>
<td>6.4±0.7$^b$</td>
<td>28.2±2.3$^a$</td>
<td>7.4±1.7$^b$</td>
</tr>
<tr>
<td>Casein</td>
<td>24.5±2.6$^a$</td>
<td>37.5±5.1$^b$</td>
<td>24.1±2.1$^a$</td>
<td>49.2±6.3$^b$</td>
</tr>
<tr>
<td>Whey protein</td>
<td>42.8±3.5</td>
<td>56.3±15.0</td>
<td>17.2±0.8</td>
<td>17.1±2.4</td>
</tr>
<tr>
<td>LMW</td>
<td>12.3±2.2$^a$</td>
<td>1.1±5.5$^b$</td>
<td>32.6±5.3$^a$</td>
<td>26.2±7.7$^b$</td>
</tr>
</tbody>
</table>

Values represent mean ± SD
Different superscripts indicate p<0.05 for extrinsic tracers compared to native mineral
**Figure 1:** Fractionation procedure
Figure 2: Distribution of calcium (a) and zinc (b) as extrinsic tracers (A) and native mineral (B) among the fat, casein, whey protein and LMW fractions of MM and MM+MNF.
Discussion

The results presented in this investigation demonstrated that an equilibration period of 12 to 16 hours of milk with extrinsic tracers of calcium and zinc did not achieve a similar distribution as native calcium and zinc among the different milk fractions of preterm mother's milk supplemented with a multi-nutrient fortifier.

The distribution of the extrinsic tracers for calcium and zinc among the different milk fractions of unsupplemented mother's milk were comparable to previous studies (Sandstrom et al. 1983, Abrams et al. 1990) with less of the extrinsic tracers present in the casein fraction and more in the fat fraction when compared to native the minerals. This difference, however, was relatively small. The percent of the extrinsic tracers and the native minerals in the soluble whey fraction (whey protein and LMW) were comparable.

The difference in distribution between extrinsic tracers and the native minerals in the milk fractions of fortified mother's milk were considerably greater. With the addition of the multi-nutrient whey-based fortifier, significantly less extrinsic calcium and zinc tracers were present in the casein fraction compared to native calcium and zinc. In-vivo, the bioavailability of calcium and zinc bound to casein is lower than from whey protein (Mason 1968, Abrams et al. 1990). Therefore, mineral absorption from fortified mother's milk as determined with extrinsic tracers could be overestimated. Furthermore, extrinsic tracers were found to be present in the LMW fraction compared to native minerals, especially for calcium. Binding to the soluble
whey fraction, especially the LMW fraction, in human milk contributes to greater bioavailability of minerals (Sandstrom et al. 1983). In vivo studies, however, demonstrated a similar efficiency of absorption from intrinsic and extrinsic calcium tracers from fortified mother's milk (Liu et al. 1989). No studies have determined the efficiency of absorption of extrinsic and intrinsic zinc tracers from fortified mother's milk. Serfass et al (1989) did not observe differences in the efficiency of absorption of extrinsic and intrinsic zinc tracers from formula in infants.

The distribution of native calcium and zinc among the major fractions of mother's milk as determined in this experiment was different from previously reported values (Fransson & Lonnerdal 1982, Fransson & Lonnerdal 1983). In our experiment greater proportions of native calcium and zinc were found in the fat fraction of mother's milk. This could be a result of repeated freezing and thawing of the mother's milk during collection and experimental procedures. Freezing will damage the fat globule membrane and will increase mineral binding to free fatty acids (Neville et al. 1995). Native zinc was present in greater amounts in casein compared to one previous study (Fransson & Lonnerdal 1982). Other investigations have found higher values, which were still lower, however, compared to our study (Keenan & Patton 1995). An explanation for this may be that our study applied the fractionation procedure at a higher temperature. At high temperatures casein is less soluble, thus when fractionation is performed at a lower temperature as previous studies have applied not all of the casein may have precipitated during ultracentrifugation (Abrams
et al. 1990). This could explain why more native zinc was found to be present in the casein fraction in our experiment. Further, difficulties in the absolute separation of different fractions (e.g. casein from skim milk membranes) can also contribute to the discrepancies found in our study compared to previous reports in distribution of minerals among milk fractions.

Despite the differences in the distribution among milk fractions of extrinsic tracers compared to native minerals, it may be argued that extrinsic tracers may still be an acceptable alternative to intrinsic tracers in the assessment of dietary mineral bioavailability as described in chapter two. The distribution of extrinsic tracers and native minerals among the total whey fraction was similar. Further, previous studies in infants demonstrated that intrinsic tracers and extrinsic tracers were absorbed with similar efficiency.

Future studies, however, are needed to confirm that the extrinsic tracers of calcium and zinc are absorbed with similar efficiency as native minerals in-vivo in preterm infants fed the specific diets tested in our experiment. The findings in this investigation further indicate that the sample handling (e.g. freezing and thawing of milk samples) and the fractionation procedure itself (e.g. temperature, separation of milk fractions), can have an effect on the results generated on mineral distribution in the milk fractions; thus such experiments should to be designed and performed with specific emphasis on the techniques applied to separate different milk fractions.
References


Chapter Three

**CALCIUM DOES NOT INHIBIT IRON ABSORPTION IN INFANT-PIGLETs FED A HIGH CALCIUM DIET**

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Calcium Does Not Inhibit Iron Absorption in Infant-Piglets Fed
a High Calcium Diet\textsuperscript{1,2}

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ABSTRACT

We investigated whether a dietary calcium:iron ratio similar to that provided to preterm infants inhibited iron absorption in the infant-piglet model after adaptation to a high calcium diet. Male Yorkshire piglets were randomized at 3 to 4 days of age to a high calcium diet (Hi-Ca) or normal diet (N-Ca) and fed for 2 to 2.5 weeks. Iron dextran injection was administered in amounts to achieve a marginal state of iron repletion to simulate iron status of preterm infants. In-vivo iron absorption from the diet was determined using the tracers $^{55}$Fe and $^{59}$Fe and whole body counting. Calcium:iron interactions at absorption sites of piglets fed Hi-Ca and N-Ca, were investigated by measurements of time dependent $^{59}$Fe uptake in response to different calcium:iron ratios injected in-situ into ligated loops of the jejunum and in-vitro in brush border membrane vesicles (BBMV). In-vivo iron absorption from the diet was similar between diet groups [57±8 % versus 55±17 % (mean±SD) for N-Ca and Hi-Ca, respectively]. In-situ iron uptake at different calcium concentrations in ligated loops was not significantly different between N-Ca and Hi-Ca piglets. In-vitro iron uptake in BBMV was significantly higher in Hi-Ca piglets compared to N-Ca piglets at a molar calcium:iron ratio of 65, but not at higher ratios. Iron status and iron content in spleen, liver, intestine, kidney and heart were similar between diet groups. In infant piglets, iron absorption was not altered when they were adapted to a diet high in calcium. The higher iron uptake in BBMV from Hi-Ca piglets suggests that adaptive mechanisms at the brush border membrane counteract the inhibitory effects of
calcium on iron absorption. At amounts currently used, calcium added to diets for preterm infants will likely not compromise iron status in early neonatal life.

INDEXING KEY WORDS: • infant-piglet • iron absorption
• calcium:iron interactions • adaptation • high calcium diet.

INTRODUCTION

Previous studies in animals and humans have demonstrated that calcium inhibited iron absorption (Dawson-Hughes et al. 1986, Hallberg et al. 1991, Hallberg et al. 1992a, Cook et al. 1991, Barton et al. 1983). Based on these findings, it has been suggested that infants fed a diet with a high calcium content may be at risk for developing iron deficiencies (Barton et al. 1983, Hallberg et al. 1992a).

The issue of calcium:iron interactions is important for the preterm infant population. Supplementation of calcium and phosphorus is common practice for preterm infants fed their mother’s milk or preterm formulas during the early neonatal period in order to prevent osteopenia. Furthermore, supplementation with calcium and phosphorus of formulas intended for feeding of preterm infants after discharge from hospital is gaining interest in order to optimize growth and bone mineralization. In addition, the preterm infant has a marginal iron status at birth and is more vulnerable to becoming iron deficient in comparison to an infant born at term.

Interpretation of results from adult human or animal studies on calcium:iron
interactions require caution in extrapolating them to preterm infants for several reasons. Infants consume liquid formulas or breast milk in early neonatal life. Therefore, it is possible that other factors in adult human or animal diets may partially account for inhibiting iron absorption. The subjects in adult studies were in an iron replete status. Preterm infants have high iron needs and thus might have a greater efficiency of absorption in comparison to adults. Further, most of the reported studies were performed with single test meals, ignoring the possibility that an individual may adapt to a diet high in calcium. Finally, the source of calcium supplementation may be of importance (Prather and Miller 1992). The reported studies in adults have used calcium-chloride (Hallberg et al. 1992a), calcium-carbonate, calcium-citrate, hydroxyapatite or calcium-phosphate (Hallberg et al. 1991, Cook et al. 1991, Dawson-Hughes et al. 1986). Preterm infants, however, are usually supplemented with calcium tribasic or calcium gluconate and potassium phosphate. More recently, calcium glycerophosphate (CaGP) has been used in mother's milk fortifiers (Wauben et al. 1995, Shanler and Abrams 1995). CaGP has been shown to have greater solubility (Hanning et al. 1989) and does not appear to inhibit magnesium and zinc absorption when incorporated into a mother's milk fortifier (Wauben et al. 1994).

There is no information as to whether calcium supplementation does inhibit iron absorption or alter iron status in infant populations. Since calcium supplementation in preterm infants is maintained over a prolonged period of time, it is important to determine if continuous exposure to a high calcium diet inhibits iron
absorption. In previous studies the infant-piglet has been used as a model for preterm infants to study mineral metabolism (Atkinson et al. 1993). The benefits of using the piglet model is that the anatomy and functions of the gastrointestinal tract are similar to the human infant. Further, many nutrients required by human infants are essential to piglets as well (Pond 1991).

To address the issue of calcium:iron interactions in infant populations, we hypothesized that, when consumed over a prolonged period of time, a diet high in calcium will not inhibit iron absorption or compromise iron status in the infant-piglet. Our objectives were to study the influence of a diet high in calcium on: 1) in-vivo iron absorption; 2) iron uptake at absorption sites; and 3) iron status and iron stores.

MATERIALS AND METHODS

Animals. Eighteen three to five day old male Yorkshire piglets were removed from the sow at the Swine Research Facility, Arkell Farms (Guelph, Ontario, Canada) and transported to the McMaster University Central Animal Facility. All procedures were in agreement with the Guide for the care and use of laboratory animals (Canadian Council on Animal Care 1993). Upon arrival the piglets were block randomized to a regular piglet formula (N-Ca) or to a high calcium piglet formula (Hi-Ca). At this time an iron dextran injection providing 100 mg elemental iron was administered intramuscularly. Iron content in the experimental diets was adjusted in combination with the modified iron dextran dose to achieve a marginal state of iron repletion to
simulate iron status of the preterm infant.

**Experimental diets.** The composition of the liquid piglet formulas are shown in Table 1. The high calcium content in Hi-Ca was achieved by addition of CaGP to the diet. The increase in the molar calcium:iron ratio in Hi-Ca from N-Ca was similar to the increase in molar calcium:iron ratio in preterm infants fed mother's milk with additional calcium but was somewhat greater than the increase in calcium:iron ratio when term infant formulas are supplemented with calcium (Table 2).

**Study Design and Analysis.** After arrival at our Central Animal Facilities the piglets were initially weaned to the regular liquid piglet formula (N-Ca) within 12 hours. At four to five days of age when the piglets tolerated feeds of 400 mL/kg⁻¹.d⁻¹ Hi-Ca was introduced to piglets assigned to this group. Weaning to the experimental diets was achieved at latest at seven days of age. The experimental diets were then fed for 2 to 2.5 weeks and piglets were killed at 21 to 24 days of age.

**Growth and iron status.** Piglets were weighed prior to each morning feeding. Weight was determined to one gram (Sartorius, Goettingen, Germany). Length was measured at three time points during the protocol by measuring snout to rump length when piglets were anaesthetized for blood sampling. Length was measured with a non-stretchable tape measure. Blood samples from fasted piglets were collected in dry heparin by using an internal jugular blind-stab technique while piglets were under light anaesthesia (isofluorane gas Aerrane; Anaquest, Ontario) at 6, 12 and 21 days of age in order to determine hemoglobin (Hb) and haematocrit (Hct)
concentrations (Kodak Ektachem Analyzer, model 700XR, Rochester, NY). Prior to necropsy, while piglets were anaesthetized, blood was collected by cardiac puncture to determine serum iron concentrations.

**In vivo iron absorption.** When the experimental diets had been fed for a minimum of five days, iron absorption from the experimental diets was determined over a six day period as follows. Iron absorption was determined in the absence of calcium from a 5 mL aqueous solution containing 3 mg ferrous sulphate, 30 mg ascorbic acid and 3 μCi $^{55}$Fe. $^{55}$Fe absorption served as a reference in order to take individual variability in iron absorption into account. $^{55}$Fe absorption was calculated from incorporation of $^{55}$Fe into red blood cells six days post dosing adjusting for isotopic decay, assuming a blood volume of 90 mL/kg body weight (Talbot & Swenson, 1970) and assuming that 80 % of absorbed iron was incorporated into red blood cells (Davidson et al. 1990). $^{55}$Fe was quantified in whole blood samples after digestion and decolorization by the modified method of Eakins and Brown (1966). The samples were counted using a Phillips PW4700 scintillation counter (Phillips, The Netherlands) with an efficiency of approximately 20 %.

Iron absorption from the experimental diets was determined with the tracer $^{59}$Fe. N-Ca or Hi-Ca formula (25 mL) was labelled with 2.0 to 4.0 μCi $^{59}$Fe and equilibrated over a three hour period. $^{59}$Fe absorption from the diet was determined six days post dosing by whole body counting using sodium iodide crystals (Engineered by the Department of Nuclear Medicine, McMaster University, Hamilton, ON). Both
the $^{59}$Fe and $^{59}$Fe doses were administered by oral gavage after a period of 9 hours where food was withheld and followed by a 3 hour period where food was withheld. 

**In-situ iron uptake in ligated loops of the jejunum.** Prior to the ligated loop experiments food was withheld for 12 to 16 hours. The ligated loop experiments were performed prior to necropsy in seven piglets from each diet group. Piglets were anaesthetized with isofluorane and the proximal end of the intestine was isolated. To maintain tissues under physiological conditions, they were covered with gauze pads soaked with warm saline (37°C). Although the duodenum is the main site for iron absorption (Underwood 1977), it was too short for the loop experiments. Therefore, only the distal end of the jejunum adjacent to the duodenum was used for ligated loop experiments. Initial experiments in ligated loops of pilot piglets were performed to determine the iron concentration to be used to obtain optimal iron uptake in the ligated loops. Per piglet three segments of 10 cm in length were tied off, and randomly injected with a 5 mL solution with molar calcium:iron ratios of either 0, 230 or 540 (0-117 mmol/L CaCl$_2$, 0.2 mmol/L FeCl$_2$, 1.0 µCi $^{59}$Fe, 10 mmol/L tris-HEPES, 100 mmol/L D-Mannitol, 5 mmol/L glucose, 112 mmol/L NaCl, 3 mmol/L KCl, 0.7 mmol/L ascorbic acid at pH 6.8). After 1, 5, 10, 15 and 20 minutes incubation, 100 µL was redrawn from the loop and $^{59}$Fe activity was counted in a $\gamma$-counter (Minimax, auto-gamma, Packard-Canberra, Canada) to determine percent disappearance from the lumen, from which apparent iron uptake was calculated.
In-vitro iron uptake in brush border membrane vesicles. After the in-situ iron uptake experiments and necropsy the remaining circa 60 cm segment of the jejunum was removed separately. Segments were rinsed and scraped, while on ice, to obtain mucosa for the preparation of brush border membrane vesicles (BBMV). Intestinal BBMV were obtained by employing a magnesium precipitation/differential centrifugation method (Kessler et al 1978, Davidson and Lonnerdal 1988). Briefly, mucosa were homogenized in a hypo-osmotic buffer, basolateral membranes and intracellular components were precipitated with MgCl₂, and differential centrifugation produced a final pellet of purified brush border membrane fragments. This final pellet was resuspended in an incubation buffer (112 mmol/L NaCl, 100 mmol/L D-mannitol, 10 mmol/L tris-HEPES at pH 6.8). The prepared BBMV of five piglets in each diet group were used in ⁵⁹Fe uptake studies the same day. To assess purity of BBMV, the specific activity of sucrase was measured in the initial preparation and compared to the final preparation which contains BBMV (Davidson and Lonnerdal, 1988, Dahlqvist 1968). Protein content of the purified BBMV was measured by the colorimetric method of Bradford (1976).

To perform iron uptake experiments a modification of the procedure outlined by Muir et al. (1984) was used. Initial experiments were performed to determine optimal pH, protein concentration and iron concentration conditions for iron uptake by BBMV of piglets. Iron uptake in response to different calcium:iron ratios in the BBMV derived from piglets fed Hi-Ca or N-Ca was determined as follows: 500 µL of
BBMV (containing between 450 and 750 μg protein) was added to a 500μL incubation solution (0 - 117 mmol/L CaCl₂, 0.25 mmol/L FeSO₄, 1μCi ⁵⁹Fe and 5 mmol/L ascorbic acid at pH 6.8). A 20-fold molar excess of ascorbate was present in the incubation solution to ensure that iron was maintained in the iron(II) form. Isoosmolarity was maintained by adjustment of NaCl concentration in the incubation solution. The maximum concentration of CaCl₂ that could be used to maintain isoosmolarity was 117 mmol/L. Thus the final calcium:iron ratios to which BBMV were exposed were 0, 56, 112 and 235. After 1, 5, 10, 15 and 20 minutes incubation, 50 μL of the BBMV-incubation solution was transferred in triplicate to a Millipore filter (Type HA, 0.45μm, diameter 25 mm, Millipore, Groton, CT) and ⁵⁹Fe uptake was stopped by addition of 100 μL ice cold stop solution (0.1 mmol/L FeCl₃, 100 mmol/L citrate at pH 7.0). Then the BBMV were collected on the Millipore filters using a vacuum. The filters were rinsed with 5 mmol/L EDTA to remove extracellular bound ⁵⁹Fe. To determine iron uptake the filters were counted using a γ-counter.

**Necropsy.** Piglets were killed by a lethal cardiac injection of sodium pentobarbital. Iron content in the body were determined by excising liver, spleen, kidney, intestine and heart at necropsy. Organs were washed with saline and homogenized, lyophilized, ashed at 500°C (Thermolyne Furnace 30400, Sybron/Thermolyne Corporation, IW) and reconstituted in 10 % nitric acid. Total iron in wet digested serum and in organs were determined by flame atomic absorptionmetry (Perkin Elmer, Norwalk, CT).
Statistical analysis. The Student's T-Test was used to determine differences between diet groups (SigmaStat Software, Jandel Scientific, San Rafael, CA). Data are expressed as the mean ± one standard deviation (SD) unless stated otherwise. The level of significance for all tests was p<0.05.

RESULTS

Growth and iron status. Body size and growth during the protocol were similar between diet groups as shown in Table 3.

The iron dextran dose with modified iron content in the liquid resulted in a marginal iron repletion state. As shown in Figure 1, the Hb concentration of all piglets was below the normal range but above iron deficiency concentrations (Underwood 1977) after adaptation to the experimental diets. No differences between diet groups were observed in Hct concentrations at all time points. Similarly, no differences were observed in serum iron at necropsy (1.37±0.82 and 1.59±0.72 μg/mL for N-Ca and Hi-Ca, respectively), but average values were in the low normal range (0.99-2.97 μg/mL, Pound and Houpt 1978) with 3 piglets in N-Ca and two in Hi-Ca being below the normal range.

Iron content in spleen, liver, intestine, heart and kidney was similar between diet groups (Figure 2).

In-vivo iron absorption. There were no significant differences in measurements of iron absorption from the diet between N-Ca and Hi-Ca piglets (55±7
and 57±17 %, respectively). In order to control for individual variability in iron absorption in the piglets, $^{59}$Fe absorption from the diet was expressed as a ratio of $^{55}$Fe absorption. There were no differences between diet groups in $^{59}$Fe/$^{55}$Fe ratio which was 0.66±0.15 and 0.66±0.17 for N-Ca and Hi-Ca, respectively (Figure 3).

**In-situ iron uptake in ligated loops** To determine the optimal iron concentration for in-situ iron uptake experiments, loops were injected with iron concentrations of 0.008, 0.04, 0.02 and 0.2 mmol/L. Iron uptake after 20 minutes was 52±2, 54±7, 52±12 and 49±2 %. Since iron uptake was similar at all iron concentrations we choose to use the concentration of 0.2 mmol/L as this was similar to the iron content of the diet. Calcium and ascorbate concentrations used in the ligated loop experiments were similar to those provided in the Hi-Ca and N-Ca diets as well. Iron uptake in the presence of calcium, at calcium:iron ratios of 230 and 540 was similar between Hi-Ca and N-Ca. It appeared that iron uptake in Hi-Ca piglets was less affected by addition of calcium compared to N-Ca piglets (Figure 4); however, this was not statistically significant.

**In-vitro iron uptake in brush border membrane vesicles** Initial experiments determined that iron uptake in BBMV was mediated by a facilitated transport mechanism (Figure 5a). Other conditions for optimal iron uptake in BBMV were; pH: 6.75-7.00 (Figure 5b), protein concentration: 500-750 μg/500 μL incubation solution (Figure 5c), and optimal iron concentration: 0.10-0.50 mmol/L incubation solution (Figure 5d). Iron uptake in BBMV of piglets fed N-Ca and Hi-Ca in response to
molar calcium:iron ratios of 0, 56, 112 and 235 is shown in Figure 6. Iron uptake was significantly suppressed in both diet groups by calcium at all ratios irrespective of adaptation to a diet high in calcium. However at 15 and 20 minute incubation times at the calcium:iron ratio of 56, iron uptake was significantly greater in Hi-Ca piglets (Figure 6b) compared to N-Ca piglets (Figure 6a) (p<0.05). At the two highest calcium:iron ratios iron uptake was suppressed to a similar degree (Figure 6a and 6b).
### TABLE 1 The composition of the experimental diets

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>N-Ca&lt;sup&gt;a&lt;/sup&gt;/Hi-Ca&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein, g/L</td>
<td>42</td>
</tr>
<tr>
<td>Whey powder&lt;sup&gt;1&lt;/sup&gt;</td>
<td>6</td>
</tr>
<tr>
<td>Skim-milk powder&lt;sup&gt;2&lt;/sup&gt;</td>
<td>36</td>
</tr>
<tr>
<td>Fat, g/L&lt;sup&gt;3&lt;/sup&gt;</td>
<td>36</td>
</tr>
<tr>
<td>Carbohydrate, g/L&lt;sup&gt;4&lt;/sup&gt;</td>
<td>76</td>
</tr>
<tr>
<td>Energy, kJ/L</td>
<td>3308</td>
</tr>
<tr>
<td>Calcium, mg/L&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1996&lt;sup&gt;a&lt;/sup&gt;/4666&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phosphorus, mg/L&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1422&lt;sup&gt;a&lt;/sup&gt;/3485&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Iron, mg/L&lt;sup&gt;7&lt;/sup&gt;</td>
<td>11.8</td>
</tr>
<tr>
<td>Zinc, mg/L&lt;sup&gt;8&lt;/sup&gt;</td>
<td>6.24</td>
</tr>
<tr>
<td>Copper, μg/L&lt;sup&gt;9&lt;/sup&gt;</td>
<td>780</td>
</tr>
<tr>
<td>Vitamin mixture&lt;sup&gt;10&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Methyl mixture, g/L&lt;sup&gt;11&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td>Molar calcium:iron ratio</td>
<td>230&lt;sup&gt;a&lt;/sup&gt;/540&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>Electrodialyzed whey (53 g/L), Wyeth Canada Ltd, Windsor, ON, Canada.
<sup>2</sup>Skim milk powder (82 g/L), Wyeth Canada Ltd.
<sup>3</sup>30% Corn oil, Best Foods Canada, Inc Etobicoke, ON and 70% Canola oil.
<sup>4</sup>Lactose from skim milk.
<sup>5</sup>CaGP, Paul Lohmann, Emmerthal, Germany and CaCO<sub>3</sub>, Fischer Scientific, Toronto, ON, Canada.
<sup>6</sup>Phosphorus derived from native elements in skim milk and whey powders plus calcium glycerophosphate (as detailed in footnote <sup>5</sup>).
<sup>7</sup>Iron from skim milk and whey powders plus added as FeSO<sub>4</sub>.
<sup>8</sup>Zinc from skim milk and whey powders plus added as ZnSO<sub>4</sub>.
<sup>9</sup>Copper from skim milk and whey powders and added as CuSO<sub>4</sub>.
<sup>10</sup>Additions provided the following per L of liquid formula: 0.8 mg retinol, 0.6 mg thiamin, 8.8 mg niacin, 4 mg pantothenic acid, 0.5 mg vitamin B6, 0.2 mg folacin, 8 μg vitamin B12, 40 μg D-Biotin, 120 mg ascorbate, 0.6 g choline chloride, 2 mg all-rac-α-tocopherol acetate and 63 IU vitamin D.
<sup>11</sup>Methyl cellulose, BDH Chemicals Ltd, Prole, UK.
TABLE 2 Comparison of calcium and iron content and calcium:iron ratios in diets for preterm infants and piglets

<table>
<thead>
<tr>
<th>Diet</th>
<th>calcium (mmol/L)</th>
<th>iron (μmol/L)</th>
<th>calcium:iron ratio (molar)</th>
<th>Relative increase in calcium:iron ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infants</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preterm MM (4 to 8 weeks)</td>
<td>6.4</td>
<td>16</td>
<td>400</td>
<td></td>
</tr>
<tr>
<td>Preterm MM plus calcium</td>
<td>21.5</td>
<td>23</td>
<td>935</td>
<td>2.3</td>
</tr>
<tr>
<td>Term formula</td>
<td>11.9</td>
<td>22</td>
<td>541</td>
<td></td>
</tr>
<tr>
<td>Term formula plus calcium</td>
<td>17.2</td>
<td>22</td>
<td>782</td>
<td>1.4</td>
</tr>
<tr>
<td>Piglets</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Ca</td>
<td>49.8</td>
<td>216</td>
<td>231</td>
<td>2.3</td>
</tr>
<tr>
<td>Hi-Ca</td>
<td>116.4</td>
<td>216</td>
<td>539</td>
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</tbody>
</table>

MM = mother's milk
**TABLE 3 Weight and length of piglets randomized to N-Ca and Hi-Ca**

<table>
<thead>
<tr>
<th>Measurement</th>
<th>N-Ca n=9 piglets</th>
<th>Hi-Ca n=9 piglets</th>
</tr>
</thead>
<tbody>
<tr>
<td>At Randomization (d 5):</td>
<td></td>
<td></td>
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<tr>
<td>Weight, kg</td>
<td>2.04±0.40</td>
<td>2.09±0.37</td>
</tr>
<tr>
<td>Length, cm</td>
<td>39.9±2.5</td>
<td>39.2±1.9</td>
</tr>
<tr>
<td>Adapted to diet (d 12):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight, kg</td>
<td>3.00±0.66</td>
<td>2.95±0.69</td>
</tr>
<tr>
<td>Length, cm</td>
<td>44.0±3.0</td>
<td>44.6±3.4</td>
</tr>
<tr>
<td>Necropsy (d 21):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight, kg</td>
<td>5.22±1.14</td>
<td>4.94±1.45</td>
</tr>
<tr>
<td>Length, cm</td>
<td>51.7±4.0</td>
<td>51.3±4.5</td>
</tr>
<tr>
<td>Weight gain, g/d</td>
<td>202±44</td>
<td>178±59</td>
</tr>
</tbody>
</table>

1Values are group means±SD.
FIGURE 1 Hemoglobin concentrations in piglets fed N-Ca (■) and Hi-Ca (●). Shaded area represents normal range and dotted line indicates iron deficiency anemia in piglets (Underwood 1977)
FIGURE 2 Iron content in the various tissues of piglets fed N-Ca (shaded bars) and Hi-Ca (solid bars)
FIGURE 3 Ratio of $^{59}$Fe to $^{55}$Fe absorption in N-Ca (■) and Hi-Ca (●) fed piglets
**FIGURE 4** Time dependent iron uptake (mean±SEM) in ligated loops of piglets fed N-Ca (a) and Hi-Ca (b) in response to calcium:iron ratios of 0 (○), 230 (●) and 540 (▲)
FIGURE 5 Iron uptake a) at various time intervals at 4°C (○) and 37°C (■) [iron concentration of 1.45 mmol/L], b) at various pH [iron concentration 1.45 mmol/L]
FIGURE 5 Iron uptake c) at various protein (BBMV) concentrations [iron concentration 0.5 mmol/L] and d) at various concentrations of iron
**FIGURE 6** Time dependent iron uptake (mean±SEM) in BBMV of piglets fed N-Ca (a) and Hi-Ca (b) in response to calcium:iron ratios of 0 (○), 56 (■), 112 (★) and 235 (●). * P < 0.05 at calcium:iron of 56 for Hi-Ca versus N-Ca. # Calcium added to the incubation medium decreased iron uptake at all calcium:iron ratios compared to no addition of calcium.
DISCUSSION

We have demonstrated that a diet high in calcium, fed for a two week period did not inhibit iron absorption or compromise iron status in the rapidly growing piglets which were in a marginal iron status. This is in contrast to other reports in adult humans and animals which have demonstrated a distinct effect of calcium on both haem and non-haem iron absorption (Barton et al. 1983, Dawson-Hughes et al. 1986, Hallberg et al. 1991, Hallberg et al. 1992a, Cook et al. 1991, Gleerup et al. 1995). Only two of these previous studies were relevant for infant nutrition (Hallberg et al. 1992a, Barton et al. 1983). In these studies human milk with and without added calcium was fed to adult humans (Hallberg et al. 1992) and to young rats (Barton et al. 1983). Calcium was added to human milk to simulate the higher calcium content in cow’s milk. As a result the increase in the molar calcium:iron ratio from addition of calcium to human milk was 5-fold, from 600 to 3000. In both these studies iron absorption was significantly reduced by addition of calcium to human milk. In contrast, the study of Atkinson et al. (1993) demonstrated that a 6-fold increase in calcium:iron ratio of the diet for piglets, which represent a better model for the human neonate, did not significantly reduce iron absorption. It is difficult to extrapolate any of these studies to the human neonate. A five- to six-fold increase in calcium:iron ratio by supplementing calcium to the diet is greater than would be observed in clinical practice by the addition of calcium to formulas or breast milk.

During infancy the need for iron is high, and it was shown that the
efficiency of iron absorption from human milk was greater in infants (49 %) than in adults (21-23 %) (McMillan et al. 1976, Oski and Landaw 1980, Saarinen et al. 1977). Thus an increased efficiency of iron absorption in our infant-piglets may explain in part why the inhibitory effect of calcium on iron absorption was not observed.

Our findings further suggest that an adaptive mechanism was present in Hi-Ca piglets at the brush border membrane to counteract the inhibition of Hi-Ca diet on iron uptake. Previous studies which have investigated calcium:iron interaction using single test meals, have ignored the possibility of the presence of an adaptation in iron uptake in response to continuous depression by calcium on iron uptake. In our study we achieved a marginal iron status in the piglets which would give us the opportunity to observe calcium:iron interactions effects but as well possible adaptive mechanisms to counteract calcium:iron interactions.

The location(s) within the enterocyte where calcium interacts with iron uptake remains speculative. Previous studies have suggested that the inhibitory mechanism of calcium on iron absorption involves transfer from the mucosal cell into the circulation (Hallberg et al. 1991b, Prather and Miller 1992, Barton et al. 1993). However, a more recent study in rats demonstrated that calcium competes for iron binding sites on the intestinal shuttle protein mobilferrin which could interfere with intestinal iron uptake (Conrad et al. 1993, Wolf and Wesling-Resnick 1994). Our study supported that calcium and iron compete for uptake by the enterocyte, however, other possible sites of calcium:iron interactions were not investigated in our study.
design.

The specific cellular mechanisms responsible for control of iron absorption are not well understood; although it is well established that there is a rapid response to iron deficiency by an appropriate increase in the uptake of iron from the intestinal lumen (Halliday 1992). A proposed mechanism for adaptation to a Hi-Ca diet would involve an initial depression of iron absorption by calcium, resulting in a compromised iron status. In response, iron uptake by the mucosal cell would be enhanced to compensate for this.

In the ligated loop of the jejunum, iron uptake was not significantly decreased by calcium even at high calcium:iron ratios, while in BBMV iron uptake was significantly decreased by Ca in both diet groups. It has been suggested that uptake of iron consists of a two step process (Muir et al. 1984, Wolf and Wesling-Resnick 1994). The first step is the binding of iron to apparent high affinity sites on the membrane surface and the second step is carrier mediated transport across the membrane. This latter step is believed to be under regulatory control (Muir et al. 1984). In the ligated loop experiments apparent iron uptake included both binding to the membrane surface and transport across the membrane and therefore did not represent true iron uptake. In the BBMV experiments extracellular bound iron was removed from the BBMV with EDTA and thus represented true iron uptake. This could partially explain why inhibition of iron uptake was not observed in ligated loop experiments. A similar trend, however, was observed in the ligated loops. Suppression
of iron uptake by calcium was less pronounced in those piglets adapted to a high
calcium diet compared to piglets fed the regular diet.

In summary this is the first report addressing calcium:iron interaction in an
appropriate infant-animal model for extrapolation to the issues related to nutrition of
term and preterm infants. A diet high in calcium did not inhibit iron absorption since
the infant-piglet appeared to be capable of adjusting iron absorption to meet their
increased iron needs. Thus, at amounts currently used in preterm infant diets calcium
supplementation will likely not compromise iron status in early neonatal life.

Acknowledgements: We acknowledge Dr CE Webber for his assistance with the whole
body counter and Filomina Inciti for her contribution to method development.

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Chapter four

MODERATE NUTRIENT SUPPLEMENTATION TO MOTHER'S MILK FOR PRETERM INFANTS SUPPORTS ADEQUATE BONE MASS AND SHORT-TERM GROWTH: A RANDOMIZED CONTROLLED TRIAL.*

* This chapter represents a manuscript submitted to the American Journal of Clinical Nutrition, December 1996. Revised manuscript submitted May 1997.

Moderate nutrient supplementation to mother's milk for preterm infants supports adequate bone mass and short-term growth: A randomized controlled trial\textsuperscript{1-3}

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\textbf{Running title:} Mother's milk fortification, growth and BMC
ABSTRACT  Our objectives were: 1) to determine whether moderate amounts of supplementation to mother's milk with protein, calcium and phosphorus but additional trace elements and vitamins, in the form of a new multi-nutrient fortifier (MNF) would improve short-term growth and bone mineral mass when compared to supplementation with calcium and phosphorus alone; 2) to investigate whether moderate calcium and phosphorus intakes, in the form of calcium glycerophosphate (CaGP) either from MNF or added to mother's milk alone resulted in a bone mineral mass similar to term born infants at term corrected age. Thirty seven preterm infants were studied with a mean gestational age of 29.9±1.7 wk and a mean birth weight of 1.31±0.24 kg. Twenty five infants fed their mother's milk (MM) for more than 80 % of their enteral intake were randomized to receive either MM+MNF or MM+CaGP from 33 to 38 wk post-menstrual age. A third group consisting of 12 preterm infants fed preterm formula (PTF) served as a comparison group. Whole body bone mineral content (BMC) and lean and fat mass were determined by Dual Energy X-ray Absorptiometry (DXA) at term age. Nitrogen retention and calcium, phosphorus and zinc intakes were determined using the mass balance technique. Nitrogen retention was significantly lower in MM+CaGP when compared to PTF as was both weight and length gain (weight gain:16.6±1.6, 14.2±2.0 and 16.1±2.9 g/kg.d⁻¹; length gain:1.1±0.2, 0.9±0.2 and 1.1±0.3 cm/wk for MM+MNF, MM+CaGP and PTF, respectively). Growth in all diet groups was similar to intrauterine reference values, except for mean length gain in MM+CaGP which was below the 50th percentile of reference values. Whole body
BMC in infants fed MM+MNF, MM+CaGP and PTF was similar and within the normal range for term infants at birth (58±6, 55±8 and 60±10 gram for MM+MNF, MM+CaGP and PTF respectively, normal range: 47-100 gram) with normal biochemical indices for mineral status and bone turnover. Nutrient addition to mother's milk in conservative amounts may have the benefit of minimizing stress on the developing metabolic systems of preterm infants. We demonstrated that moderate calcium and phosphorus intakes, as CaGP, resulted in adequate bone mineral mass at term corrected age. Moderate amounts of protein, calcium, phosphorus and additional trace elements, added to mother's milk in the form of MNF, resulted in improved linear growth but did not provide any advantages to bone mineral mass when compared to supplementation with calcium and phosphorus alone.

**KEY WORDS:** Preterm infants, mother's milk, multi-nutrient fortifier, bone mineral mass, growth

**INTRODUCTION**

Mother's milk is unique in that its cellular and nutritional factors provide for optimal growth and development in term born infants. For prematurely born infants, mother's milk is not considered to be nutritionally adequate to support optimal growth (1,2). For this reason, supplementation of mother's milk with calcium and phosphorus salts and more recently multi-nutrient fortifiers (containing calcium, phosphorus,
protein, trace elements and sometimes vitamins) has been standard practice in many neonatal intensive care units. These supplements are added with the clinical goal to support intrauterine accretion rates of nutrients and to improve functional outcomes of bone mineral mass and growth.

The optimal quantity and quality of nutrient supplementation for preterm infants fed their mother's milk has not been well established. Inconsistent results have emerged from several studies which evaluated the beneficial effects derived by addition of multi-nutrient fortifiers to mother's milk on growth and bone mineralization (3). These variable results can be explained by factors such as: dissimilarities between different multi-nutrient fortifiers in chemical (liquid versus powdered fortifiers) and compositional properties (protein, calcium, phosphorus and trace element quantity and quality); methodological limitations in measurements, particularly of bone mineral mass; differences in study design; duration of intervention; and the specific normative reference values used.

It was stated by Schanler (1) that, "Concerns remain that fortified human milk-fed low birth weight infants manifest slower growth rates than their formula-fed counterparts". This observation, however, parallels that of term born breast-fed infants who demonstrate slower growth compared to term born formula-fed infants (4). The issue of whether slower growth rates in breast-fed preterm infants compared to formula-fed infants is of clinical importance remains unanswered. To date there is little information on the effect of early nutritional practices on the quality of growth as
measured by short- and long-term outcomes of whole body bone mineral content (BMC) and lean and fat mass accretion in preterm infants by appropriate tools such as Dual Energy X-ray Absorptiometry (DXA).

Protein added to multi-nutrient fortifiers for mother's milk is likely the most important nutrient for the observed improvement in short-term growth in preterm infants (5). A negative consequence of a higher protein intake, however, was observed by findings of both higher plasma amino acids and urea concentrations in preterm infants with protein supplementation in comparison to mother's milk feeding alone (5,6,7). This is perhaps secondary to an incomplete development of metabolic pathways in the preterm infants. Previous fortifiers studied provided approximately 7 g per L of mother's milk (5,6,7). Thus, a lower protein intake from a whey-based multi-nutrient supplement may reduce the risk of nitrogen overload and its subsequent metabolic stress and as well be beneficial to short-term growth.

Excessive or imbalanced administration of calcium and phosphorus can result in abnormal mineral homeostasis in preterm infants (8). The quality as well as the quantity of calcium and phosphorus salts added to mother's milk or multi-nutrient fortifiers will determine the optimal amount of calcium and phosphorus supplementation. Different calcium and phosphorus salts, such as calcium tribasic, calcium gluconate and potassium phosphate, and more recently calcium glycerophosphate (CaGP) have been used in multi-nutrient fortifiers (9,10). In vitro studies have demonstrated that CaGP has greater solubility than calcium gluconate and
potassium phosphate in total parenteral nutrition solutions for preterm infants (11). If CaGP in enteral feeds is more available for absorption than other salts then it should allow for reduced amounts of calcium and phosphorus added to multi-nutrient fortifiers.

In order to address these issues related to fortification of mother's milk for preterm infants, a new whey-based powdered multi-nutrient fortifier (MNF) for mother's milk was developed with a moderate protein and Ca/P content, the latter in the form of CaGP. Our objectives were first to determine whether moderate supplementation with protein, calcium and phosphorus but additional trace elements and vitamins to mother's milk, in the form of a new multi-nutrient fortifier would improve short-term growth and bone mineral mass when compared to supplementation with calcium and phosphorus alone, which represents standard clinical practice in our neonatal intensive care units. Secondly, we investigated whether moderate calcium and phosphorus intakes, in the form of CaGP, from MNF or added to mother's milk alone, would result in a bone mineral mass similar to term born infants at term corrected age.

SUBJECTS AND METHODS

Subjects

Preterm infants were recruited from the neonatal intensive care units of the Children's Hospital of the Hamilton Health Sciences Corporation (formerly Chedoke-
McMaster and St Joseph's Hospitals) in Hamilton, Canada. Ethical approval was obtained from the Research Advisory Committees at the Children's Hospital, Hamilton Health Sciences Corporation. Informed and written consent was obtained from the parent(s). The inclusion criteria for enrollment in the study were: birth weight below 1800 g; appropriate for gestational age; postnatal age of greater than one week; on full oral feeds which have been tolerated for at least five days and gaining weight more than 10 g/kg.d\(^{-1}\); absence of severe congenital malformation or chromosomal abnormalities and absence of gastrointestinal disease.

Supplementation of mother's milk (MM) was started when mothers were supplying expressed breast milk for more than 80% of their infants' total enteral intake. The infants were randomly allocated, by block randomization, to receive the experimental fortifier (MM+MNF) or calcium and phosphorus alone in the form of CaGP (MM+CaGP). Because of the nature of the supplements the study could not be blinded. Infants whose parent(s) had decided to formula feed and who matched by gestational age and/or birth weight to the infants enrolled in either MM+MNF or MM+CaGP were recruited and served as a comparison group (PTF).

The sample size was based on observed differences in whole body BMC between term infants at birth and preterm infants at term corrected age (12). Our goal was to obtain a whole body BMC in our preterm infants similar to term infants at birth, as well as to be able to detect significant differences among diet groups. The observed standard deviation of 13 gram in whole body BMC in 36 term born infants
at birth as well as the observed standard deviation of 10 gram in preterm infants at
term corrected age (12) was used for the sample size calculation. With a mean
difference of 15 gram, an expected standard deviation in preterm infants of 10 grams
and using $\alpha=0.05$ and $\beta=0.1$, 12 preterm infants per diet group were needed. To take
into account an expected drop-out rate of approximately 20 %, 44 infants were
recruited. Seven infants dropped out of the study. Two infants in MM+MNF
presented intolerance to introduction of MNF. One infant in the PTF group and two
in the MM+CaGP group developed chronic lung disease and thus the feeding protocol
could not be maintained. One infant in MM+MNF discontinued the study due to
insufficient breast milk and one infant in MM+CaGP developed metabolic acidosis.
Because this was not an intention to treat clinical trial, the infants that dropped out of
the study were not followed.

**Diets**

MNF (produced, to our design specification, by Wyeth-Ayerst, Canada) was
introduced into infants' feeds in concentrations of: 5 g/L of mother's milk for 12 hours,
then fortification was increased to 11, 22, 33 and 44 g/L mother's milk every 24 hours,
or as tolerated. The composition of MNF is shown in Table 1. CaGP ($C_3H_7CaO_4P$,
Paul Lohmann Chemicals, Emmerthal, Germany) was given to provide a total intake
of approximately 3.5 mmol/kg.d$^1$ of calcium and phosphorus which would be similar
to calcium and phosphorus intakes from mother's milk supplemented with MNF at
volume intakes of 180 mL/kg.d$^1$. CaGP was introduced in a similar fashion as MNF,
starting at 1/4 of full dose working up to 1/2, 3/4 and full dose every 24 hours or as tolerated. The formula-fed infants received Preemie SMA (Wyeth-Ayerst, Canada)

Trained nursing staff mixed pre-weighed portions of MNF with mother's milk every morning and mother's milk with MNF was portioned out in graduated nursers for the feedings of the following 24 hour period and refrigerated. CaGP was prepared for a three day period by the researchers and drawn up in oral dispensers (SoloPak Laboratories, Franklin Park, IL). CaGP was given every feeding, by gavage when infants were gavage-fed or mixed with mother's milk when infants were bottle-fed. MNF and CaGP were randomly tested for contamination, but no bacterial growth was found. Infants in the MM+CaGP group received 600 IU oral vitamin D supplement and infants in PTF received 400 IU oral vitamin D supplement. As MNF provided 472 IU vitamin D added to 100 mL mother's milk vitamin D intake was higher in MM+MNF but within the range of 1200-2000 IU/day which has been suggested previously for preterm infants at risk for vitamin D deficiency (13).

When infants were ready to suckle from the breast, CaGP and MNF supplements were not administered, but no more than two breast feedings per day were given by mothers in both groups until discontinuation of supplementation. Supplementation of mother's milk with MNF or CaGP was discontinued when the infants were discharged if their post-menstrual age was greater than 38 weeks. If post-menstrual age was less than 38 weeks, supplementation was continued at home until infants reached 38 weeks post-menstrual age. After discharge from the neonatal
intensive care unit all infants received standard vitamin supplements.

**Data Collection**

*Bone mineral, lean and fat mass.* At term corrected age, whole body BMC, fat and lean mass were determined by DXA (Hologic QDR1000W\textsuperscript{8}, Hologic Inc, Waltham, MA). The principles of dual photon absorptiometry to determine body composition are described in detail elsewhere (14). The DXA scans were analyzed using pediatric software version 5.63 (Hologic Inc, Waltham, MA).

Whole body BMC, lean and fat mass reference values from term born infants were obtained from an ongoing trial (12). In this trial, thirty six healthy term born infants (birthweight 3.4±0.4 kg, gestational age 39.5±1.1 weeks) were recruited at birth from Chedoke McMaster Hospital and a DXA scan was obtained at 3.2±1.4 days postnatal age.

At approximately 36 weeks post-menstrual age, a venous blood sample was obtained to determine serum calcium, phosphorus, alkaline phosphatase (Kodak Ektachem Analyzer, model 700XR, Rochester, NY) and plasma osteocalcin (\textsuperscript{125}I-RIA, Incstar Corporation, Stillwater, MN) as biochemical indices for mineral status and bone turnover.

*Anthropometry.* Growth was monitored by biweekly assessments of weight, length and head circumference from recruitment to discharge. Weight was determined to one gram (Sartorius, Goettingen, Germany). Length was measured on a plexiglass board (Ellard Scientific, Seattle DC), and head circumference was measured with an
unstretchable tape (Wyeth-Ayerst, Toronto, ON). Anthropometric measurements were performed in triplicate by the same researchers throughout the study.

Nitrogen retention. When the infants received MNF or CaGP at full strength for at least five days, a mass balance was performed to determine apparent nitrogen retention. Feeds for MM+MNF, MM+CaGP and PTF for the balance study were prepared by the researchers in order to ascertain accurate intakes. An ashless filter paper was placed under the infants' head during feeding to collect any regurgitation. Urine was collected for three days using mineral free, sterile adhesive condom-like urine collector as described before (15). Stools were collected using plastic-lined ashless filter papers (Whatman, Maidstone, England) which were placed in pre-weighed diapers. In the case of urine or stool leakage the nursing staff weighed the diaper and recorded the losses. Stools were collected for three days, placed in plastic ziplock bags and stored in a -4 °C freezer. Nitrogen content in milk, urine and stool samples were determined by the Kjeldahl procedure (model 1026; Kjeltec system, Brinkman, Toronto, ON) from lyophilized stool samples (Flexidry microprocessor, FTS Systems, Stone Ridge, NY) and wet milk and urine samples.

Nutrient intake. Calcium and zinc intakes were calculated from measured volume intake and milk or formula analysis during the balance period. During the balance period the infants were not allowed to suckle at the mothers' breast. Calcium and zinc concentration in milk or formula samples were determined by flame atomic absorption spectrometry (model 703; Perkin-Elmer, Norwalk, CT), after lyophilization
and microwave acid digestion (MDS 2000, CEM microwave sample preparation system, Matthews, NC). Phosphorus from digested samples was determined by colorimetric photospectrometry by the modified method of Fiske and Subbarow (16). **Statistical analysis.**

Differences between diet groups were determined by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls multiple mean test. The Dunn's multiple mean test was used if the data were not normally distributed (SigmaStat, Jandell Scientific, San Rafael, CA). For analysis of growth, analysis of covariance (ANCOVA) was performed using the covariable birth weight (BMDP Statistical Software Inc. Los Angelas, CA). The level of significance for all tests was $p<0.05$.

**RESULTS**

Infants in the study groups had similar characteristics at birth and other time points during the study protocol with the exception of birth weight which was lower in PTF when compared to MM+MNF (Table 2). Therefore, birth weight was entered as a covariable for the statistical analysis of growth.

**Bone mineral mass.** Whole body BMC was expressed as a function of length and lean mass (Figure 1). Whole body BMC at term age was not different amongst study groups (for MM+MNF, MM+CaGP and PTF: 58±6, 55±8 and 60±10 gram, respectively) and was within the normal range of reference values obtained from
healthy term born infants except for two infants in MM+CaGP and one infant in PTF. Whole body BMC of term infants at birth was 74±13 gram (12).

Biochemical indices for mineral status and bone turnover are presented in Table 3. CaGP added to mother's milk or incorporated into MNF resulted in a higher serum calcium concentration compared to PTF. The serum phosphorus concentration was greater in MM+CaGP when compared to MM+MNF and PTF. No hypocalcemia (<2.2 mmol/L), hypercalcemia (>2.7 mmol/L) or hypophosphatemia (<2.0 mmol/L) were observed in any of the infants in the three diet groups. Serum alkaline phosphatase was similar between diet groups but plasma osteocalcin was significantly greater in MM+MNF when compared to MM+CaGP and PTF (Table 3).

Anthropometry. Mean weight and length gain were not significantly different between MM+MNF and MM+CaGP, but were significantly less in MM+CaGP when compared to PTF (Table 4). Mean weight gain in all groups was greater than or similar to the 50th percentile of intrauterine reference values based on size-at-birth data of infants born between 33-38 wk post-menstrual age (17). Mean length was lower than the 50th percentile of intrauterine reference values for the MM+CaGP group only (Table 4).

Despite the small variations in growth, no significant differences between diet groups were present in absolute body size at term corrected age. Nutritional management in all infant groups resulted in a body weight (MM+MNF: 3.3±0.3 kg; MM+CaGP: 3.2±0.4 kg and PTF: 3.5±0.4 kg) between the 25th and 50th percentile
(3.2-3.5 kg) of term reference values at term corrected age. However, average length achieved (MM+MNF: 49.6±1.3 cm; MM+CaGP: 48.4±1.8 cm and PTF: 49.4±2.3 cm) was below the 25th percentile (50.2 cm) for MM+MNF and PTF and at the 5th percentile (48.3) of term reference values for MM+CaGP (17). Figure 2 demonstrates lean and fat mass attained by the preterm infants by term corrected age compared to published reference data. No differences existed amongst study groups in absolute and percent lean and fat mass, but percent lean and fat mass were different when compared to reference data from the reference fetus (18) or term born infants at birth (12).

Nitrogen retention. Apparent nitrogen retention was 26±4, 22±3 and 27±6 mmol/kg.d\(^{-1}\) for MM+MNF, MM+CaGP and PTF, respectively. Apparent nitrogen retention was significantly lower in MM+CaGP when compared to PTF. The intrauterine accretion rate for nitrogen (at a similar post-menstrual age) of approximately 20 mmol/kg.d\(^{-1}\) (18) was achieved in 12/12, 8/13 and 11/12 infants in MM+MNF, MM+CaGP and PTF, respectively.

Nutrient intake. Small but significant differences in calcium, phosphorus and zinc intakes existed during the three-day balance period. Differences in calcium and phosphorus intakes between MM+MNF and MM+CaGP were a result of different volume intake (Table 2) due to differences in individual management of the infants by our neonatologists. Intakes of the four nutrients presented in Table 5 were not consistently within the range of recently published recommended nutrient intakes for preterm infants (19).
TABLE 1

Nutrient composition, per 44 g powder added to 1 L of mother's milk, of the powdered whey-based fortifier MNF

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>MNF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (g)</td>
<td>3.7</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>34.7</td>
</tr>
<tr>
<td>Sodium (mmol)</td>
<td>2.4</td>
</tr>
<tr>
<td>Potassium (mmol)</td>
<td>5.0</td>
</tr>
<tr>
<td>Chloride (mmol)</td>
<td>2.2</td>
</tr>
<tr>
<td>Calcium (mmol)</td>
<td>15.2</td>
</tr>
<tr>
<td>Phosphorus (mmol)</td>
<td>14.1</td>
</tr>
<tr>
<td>Magnesium (mmol)</td>
<td>1.0</td>
</tr>
<tr>
<td>Zinc (μmol)</td>
<td>211</td>
</tr>
<tr>
<td>Copper (μmol)</td>
<td>29.9</td>
</tr>
<tr>
<td>Manganese (μmol)</td>
<td>2.1</td>
</tr>
<tr>
<td>Vitamin D (IU cholecalciferol acetate)</td>
<td>4720</td>
</tr>
<tr>
<td>Vitamin A (μg retinol palmitate)</td>
<td>1034</td>
</tr>
<tr>
<td>Vitamin E (μg tocopherol acetate)</td>
<td>64.2</td>
</tr>
<tr>
<td>Vitamin K (μg phytonadione)</td>
<td>34.9</td>
</tr>
<tr>
<td>Biotin (μg)</td>
<td>27.6</td>
</tr>
<tr>
<td>Ascorbic acid (mg)</td>
<td>450</td>
</tr>
</tbody>
</table>

¹Nutrient composition as provided by the manufacturer.
Nutrient composition of Enfamil human milk fortifier (Mead Johnson, US) added per L of human milk: protein: 7g; carbohydrate: 27.0 g; sodium: 3.0 mmol; potassium: 4.0 mmol; calcium: 22.4 mmol; phosphorus: 14.5 mmol; magnesium: 0.4 mmol; zinc 109 μmol; copper 9.8 μmol; manganese: 0.9 μmol; vitamin D: 2100 IU; vitamin A: 1900 μg; vitamin E: 4.61 IU; vitamin K 44.0 μg; biotin 0 μg, ascorbic acid: 120 μg.
TABLE 2

Infant characteristics of the three diet groups\(^1\).

<table>
<thead>
<tr>
<th></th>
<th>MM+MNF n=12</th>
<th>MM+CaGP n=13</th>
<th>PTF n=12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (males/females)</td>
<td>7/5</td>
<td>10/3</td>
<td>7/5</td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>1.4±0.2(^a)</td>
<td>1.3±0.2(^ab)</td>
<td>1.2±0.2(^b)</td>
</tr>
<tr>
<td>Gestational age (wk)</td>
<td>29.9±1.9</td>
<td>30.1±1.5</td>
<td>29.7±1.7</td>
</tr>
<tr>
<td>Post-menstrual age at start of MNF or CaGP (wk)</td>
<td>33.0±1.6</td>
<td>33.1±1.2</td>
<td></td>
</tr>
<tr>
<td>Post-menstrual age at start of balance (wk)</td>
<td>34.7±1.2</td>
<td>34.9±1.2</td>
<td>34.8±0.9</td>
</tr>
<tr>
<td>Weight at start of balance (kg)</td>
<td>2.0±0.2(^a)</td>
<td>1.7±0.2(^b)</td>
<td>1.9±0.2(^a)</td>
</tr>
<tr>
<td>Length at start of balance (cm)</td>
<td>43.8±1.3(^a)</td>
<td>42.5±1.7(^b)</td>
<td>42.5±2.0(^ab)</td>
</tr>
<tr>
<td>Milk intake (mL/kg.d(^1))</td>
<td>164±9(^a)</td>
<td>177±12(^b)</td>
<td>160±19(^a)</td>
</tr>
<tr>
<td>Duration of supplementation of MNF or CaGP (wk)</td>
<td>4.7±1.5</td>
<td>5.3±1.6</td>
<td></td>
</tr>
<tr>
<td>Post-menstrual age at discharge (wk)</td>
<td>37.0±1.2</td>
<td>37.6±0.6</td>
<td>37.5±1.7</td>
</tr>
<tr>
<td>Weight at discharge (kg)</td>
<td>2.4±0.3(^a)</td>
<td>2.2±0.2(^b)</td>
<td>2.4±0.1(^a)</td>
</tr>
<tr>
<td>Length at discharge (cm)</td>
<td>46.1±1.4(^a)</td>
<td>44.4±1.3(^b)</td>
<td>44.7±1.2(^b)</td>
</tr>
</tbody>
</table>

\(^1\)Mean ± SD.
Values with different superscripts are significantly different with p<0.05 (ANOVA).
**TABLE 3**

Biochemical indices for mineral status and bone turnover after approximately three weeks on intervention

<table>
<thead>
<tr>
<th></th>
<th>MM+MNF</th>
<th>MM+CaGP</th>
<th>PTF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post-menstrual age (wk)</td>
<td>35.5±1.7</td>
<td>35.7±1.2</td>
<td>36.2±1.3</td>
</tr>
<tr>
<td>Serum calcium (mmol/L)</td>
<td>2.46±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.53±0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.38±0.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum phosphorus (mmol/L)</td>
<td>2.22±0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.43±0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.23±0.13&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum alkaline phosphatase (U/L)</td>
<td>388±99</td>
<td>371±92</td>
<td>347±96</td>
</tr>
<tr>
<td>Plasma osteocalcin (nmol/L)</td>
<td>6.74±2.58&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.38±1.71&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.87±1.23&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>Mean ± SD.  
Values with different superscripts are significantly different with p<0.05 (ANOVA).
<table>
<thead>
<tr>
<th></th>
<th>MM+MNF</th>
<th>MM+CaGP</th>
<th>PTF</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g/kg.d⁻¹)</td>
<td>16.6±1.6ᵃᵇ</td>
<td>14.2±2.0ᵇ</td>
<td>16.1±2.9ᵃ</td>
<td>13</td>
</tr>
<tr>
<td>Length (cm/wk)</td>
<td>1.1±0.2ᵃᵇ</td>
<td>0.9±0.2ᵇ</td>
<td>1.1±0.3ᵃ</td>
<td>1.1</td>
</tr>
<tr>
<td>Head circumference (cm/wk)</td>
<td>1.0±0.1</td>
<td>0.9±0.2</td>
<td>1.0±0.1</td>
<td>0.6</td>
</tr>
</tbody>
</table>

¹Mean ± SD.
²50th percentile of intrauterine reference values derived from Blidner et al (17).
Values with different superscripts are significantly different with p<0.05 (ANCOVA).


**TABLE 5**

Mineral and nitrogen intake during the 72-hour mass balance compared to recommended intakes for preterm infants during the stable growing period prior to hospital discharge\(^1\).

<table>
<thead>
<tr>
<th></th>
<th>MM+MNF</th>
<th>MM+CaGP</th>
<th>PTF</th>
<th>P-RNI(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (mmol/kg.d(^{-1}))</td>
<td>2.71±0.32(^a)</td>
<td>3.26±0.60(^b)</td>
<td>2.56±0.33(^a)</td>
<td>4.0-6.0</td>
</tr>
<tr>
<td>Phosphorus (mmol/kg.d(^{-1}))</td>
<td>3.27±0.27(^a)</td>
<td>3.37±0.41(^a)</td>
<td>2.18±0.35(^b)</td>
<td>2.5-3.8</td>
</tr>
<tr>
<td>Zinc (μmol/kg.d(^{-1}))</td>
<td>33.84±3.40(^a)</td>
<td>11.64±1.75(^b)</td>
<td>23.45±3.46(^c)</td>
<td>7.7-12.3</td>
</tr>
<tr>
<td>Nitrogen (mmol/kg.d(^{-1}))</td>
<td>37.02±3.68</td>
<td>33.55±2.20</td>
<td>35.11±5.11</td>
<td>34.0-40.9</td>
</tr>
</tbody>
</table>

\(^1\)Mean ± SD.  
\(^2\)P-RNI = Premature Recommended Nutrient Intake (19).  
Values with different superscripts are significantly different with p<0.05 (ANOVA).
FIGURE 1. Whole BMC at term corrected age, expressed as a function of length (a) and of lean mass (b) for preterm infants receiving MM+MNF (●), MM+CaGP (○) and PTF (■). Dotted lines represent mean and 95% confidence interval for healthy term born infants measured within 5 days after birth (12).
FIGURE 2. Percent lean (open bars) and fat mass (hatched bars) of body weight at term corrected age, compared to the reference fetus at term age (18) and to term born infants (12) measured at birth and at two months of age.
DISCUSSION

In healthy preterm infants fed mother's milk, calcium and phosphorus intakes of approximately 3 mmol/kg.d\(^{-1}\) supported adequate bone mass accretion as judged by the comparison with whole body BMC observed in term born infants. There appeared to be no benefit to bone mass accretion to term corrected age by addition of protein, minerals and trace elements to mother's milk in comparison to addition of calcium and phosphorus alone. In other randomized controlled trials, which determined BMC in a single bone using single photon absorptiometry, there was also no benefit of a multi-nutrient fortifier to BMC of the radius (20,21) or humerus (22) in preterm infants fed mother's milk, with the exception of one study (23). The present study differs from these previous reports as it is the only which used whole body BMC as determined by DXA rather than the measurement of BMC by the single photon absorptiometry, a technique which lacks precision (24). DXA has been validated for the measurement of whole body BMC in infants (25,26), and our study is the first to present whole body BMC measurements in mother's milk fed infants in relation to early nutritional intervention in a randomized controlled trial.

The intakes of calcium and phosphorus in our infants who obtained adequate bone mass were lower, in some instances by half, than intakes of previous studies (5,9). In addition, these intakes were lower than the currently recommended nutrient intakes for preterm infants (19). Furthermore, all biochemical indices of mineral status measured were within normal ranges. Higher intakes of calcium and phosphorus have
actually been associated with biochemical indices of phosphorus depletion (5,27). The clinical significance of such findings remains to be evaluated. It is possible that a lower bioavailability of mineral salts used in other fortifiers or an inappropriate calcium:phosphorus ratio may have contributed to these findings. From the findings of our study it appears that when giving a bioavailable source of calcium and phosphorus, only moderate intakes of calcium and phosphorus relative to current recommendations were sufficient to provide for adequate bone mass accretion in early life.

Nutrients other than calcium and phosphorus may also stimulate bone growth and mineralization. In a study of infants similar to our population, feeding fortified pasteurized human milk resulted in whole body BMC lower than we observed at term age in our study (28). Since the amount and source (CaGP) of calcium and phosphorus in the multi-nutrient supplement used in the latter study were similar to our study, differences in the provision of other nutrients such as, zinc or vitamin D may have contributed to the greater bone mineral mass we observed.

Interestingly we observed a greater plasma osteocalcin concentration, which is a specific marker for osteoblastic activity, in infants fed mother's milk supplemented with multi-nutrients. This may be related to the greater vitamin D intake which is known to act indirectly on the osteoblast (29). The greater plasma osteocalcin concentration in MM+MNF was, however, not reflected in greater whole body BMC. Perhaps the response of bone formation to supplementation of mother's milk with MNF was transient. Alternatively, although bone matrix was apparently
being formed in our preterm infants, mineral deposition apparently lagged behind.

Although reduced length growth may be associated with bone mineral deficiency (30), there were no significant correlations between whole body BMC and length gain during the study period, however, an association between absolute length attained at term corrected age and whole body BMC was observed. Protein intake is of importance for both weight and linear growth. As previously observed (6,7,21,27), preterm infants fed mother's milk without protein supplementation achieved intrauterine weight but not length growth. Consistent with these findings infants in MM+CaGP did not achieve intrauterine length growth during the supplementation period.

The optimal amount of protein to be added to multi-nutrient fortifiers for mother's milk is difficult to establish. Concerns exist that a fixed addition of protein to breast milk may give some infants more than the recommended protein intake resulting in nitrogen overload, while others may receive less than desirable intakes resulting in slower growth rates (1). Our study demonstrated that with moderate protein supplementation, in the form of whey protein, adequate growth rates can be achieved.

Based on DXA measurements which have demonstrated to be accurate and precise measures of lean and fat mass in infants (26,31), our infants had a mean percent lean and fat mass intermediary between reference values from term born infants at birth and two months postnatal age. The composition of weight gain is
dependent on the protein to energy ratio in the diet, with a greater contribution of lean compared to fat mass when the protein intake increases (32). Since the extrauterine environment and nutrition for the preterm infant is very different from that in utero, it is questionable if one should aim for lean and fat mass accretion similar to the reference fetus. A moderate protein addition to MNF resulted in a small increase in the protein to energy ratio in the diet for infants in MM+MNF when compared to mother's milk alone (from approximately 9.7 % to 10.7 %). Since percent lean and fat mass in our study groups were more similar to term born infants of similar postnatal rather than term infants at birth, the postnatal age matched infants may be a more appropriate reference model for the preterm infant.

Interpretation of our study must be done with recognition of some limitations. The subjects were stable growing infants of relatively large birth weight. Therefore, our findings cannot be extrapolated to smaller and sicker babies. Unexpectedly, the volume of milk intake was slightly greater in MM+CaGP compared to MM+MNF. This may have obscured subtle differences in growth and whole body BMC between MM+MNF and MM+CaGP.

Using DXA technology, we have confirmed that body composition of the preterm infant is different from the fetus of term gestation. The whole body BMC in the low normal range and differences in lean and fat mass in our preterm infants when compared to term infants most likely reflects the fact that premature birth interrupts the period of most rapid in utero nutrient accretion. The nutritional and metabolic
maturational events in preterm infants necessary for adaptation to extrauterine life may further compromise postnatal growth. Thus our findings appear to represent a characteristic of the preterm infant rather than the lack of influence of nutritional intervention.

Based on our findings and outcomes of other recent studies (5,28), it is clear that the optimal nutrient composition for mother's milk fortifiers has not been well defined. We speculated that preterm infants may benefit from more conservative multi-nutrient supplementation which would place less stress on the developing digestive and metabolic systems of the healthy preterm infant. Our study demonstrated that moderate calcium and phosphorus intakes at less than current recommendations (19), when provided as either MNF or CaGP, resulted in adequate whole body bone mass. Furthermore, moderate multi-nutrient addition to mother's milk with MNF resulted in growth rates similar to the intrauterine reference values.

Future investigations should address long term outcomes of growth, lean and fat mass and bone mineral mass in relation to mother's milk fortification. This would provide information on the optimal amounts and type of nutrient supplementation to mother's milk to provide for both adequate short- and long term growth and development.

Acknowledgements: We thank Michelle Whelan, R.N. for assisting with the performance of anthropometric measurements and metabolic balance studies.
REFERENCES


Chapter Five

BODY COMPOSITION OF PREMATURE INFANTS: INFLUENCE OF NUTRIENT FORTIFICATION OF MOTHER'S MILK IN HOSPITAL AND BREAST FEEDING POST-HOSPITAL DISCHARGE*

*This chapter represents a manuscript submitted to Acta Paediatrica, July 1997.

Growth and body composition of preterm infants: Influence of nutrient fortification of mother's milk in hospital and breast feeding post-hospital discharge

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Short title: Breast feeding, growth and body composition in preterm infants
Abstract

We examined the influence of multi-nutrient fortification of mother's milk (MM+MNF) compared to supplementation with calcium and phosphorus (MM+CaGP) alone in hospital, and of breast feeding (Post-MM) compared to formula feeding (Post-FF) after hospital discharge on growth and body composition to one year corrected age in preterm infants. Anthropometry, nutrient intakes and whole body bone mineral content, lean and fat mass were determined at four time points in the first year after term corrected age. Body composition was determined with dual energy x-ray absorptiometry. MM+MNF compared to MM+CaGP for preterm infants in the early neonatal period did not influence growth or body composition in the first year. Growth in Post-MM and Post-FF groups was similar and within the normal range of growth references derived from term infants fed mother's milk. Post-MM infants had lower whole body bone mineral content (132.3±10.4 g) at six months corrected age when compared to Post-FF infants (159.4±14.1 g) and greater percent fat mass to 12 months corrected age. These findings appeared to be associated with lower calcium, phosphorus and protein intakes in Post-MM compared to Post-FF infants. Our findings demonstrate that dietary practices after hospital discharge have a greater impact on body composition in prematurely born infants than dietary practices in hospital. Whether the observed differences in body composition between breast-fed and formula-fed preterm infants have any long-term consequences requires further investigation. □ Body composition, mother's milk, multi-nutrient supplementation,
preterm infants

Introduction

Providing mother's milk to preterm infants has been associated with better neurodevelopmental outcomes (1,2). Mother's milk, however, is not considered adequate to meet all of the preterm infants' nutrient requirements in early neonatal life (3). Supplementation of mother's milk with calcium and phosphorus salts, powdered or liquid fortifiers or preterm formula is, therefore, common practice in many neonatal intensive care units. The use of multi-nutrient fortifiers added to mother's milk rather than supplementation with calcium and phosphorus alone has allowed preterm infants fed mother's milk to achieve intrauterine growth rates (4-6). Investigations to determine whether multi-nutrient fortification of mother's milk improves bone mineral mass during the early neonatal period, as measured in a single bone, have been inconclusive (7-10). We recently demonstrated that preterm infants fed mother's milk with a multi-nutrient fortifier (MNF) or calcium and phosphorus alone, in the form of calcium glycerophosphate (CaGP), resulted in a whole body BMC at term corrected age, as measured by dual energy x-ray absorptiometry (DXA), in the low normal range of healthy term born infants at birth (6).

The beneficial effects derived from multi-nutrient fortification of mother's milk in hospital on growth and bone mineral mass after discharge from hospital has received little attention in the literature. Recently, Lucas et al. (4) demonstrated in a
multi-centred trial that no differences in weight and length existed at nine and 18 months corrected age between preterm infants fed mother's milk supplemented with a human milk fortifier or mother's milk supplemented with phosphorus and vitamins alone. In that study, however, infants consumed only 50 percent of their total enteral intake as breast milk and the rest as preterm formula. Although this may represent clinical practice, it is possible that the use of up to 50 % formula feeding in human milk-fed infants may have biased these results.

While nutrient supplementation of mother's milk in hospital is standard practice, supplementation of mother's milk (with the exception of iron and vitamins) is usually discontinued when breast feeding is established. No information is available on whole body bone, lean and fat mass in the first year, measured with appropriate techniques such as DXA, in preterm infants who are fed mother's milk for a prolonged period of time after hospital discharge.

This report describes long-term outcomes of whole body BMC, lean and fat mass and body size in preterm infants fed mother's milk who participated in a randomized controlled trial receiving either a multi-nutrient fortifier or calcium and phosphorus alone in the early neonatal period. Because about one third of these preterm infants continued to be breast-fed to six months corrected age, we performed a post-hoc analysis to evaluate the influence of post-hospital discharge breast feeding on these long-term outcomes of growth and body composition.
Subjects and methods

Diet groups pre-hospital discharge

We studied 37 preterm infants who participated in a randomized controlled trial which has been described elsewhere (6). Briefly, preterm infants whose mothers' chose to provide breast milk were allocated, by block randomization, to receive either a new multi-nutrient fortifier (MM+MNF) (n=12, birthweight: 1.4±0.2 kg, post-menstrual age: 29.9±1.9 wk) (produced, to our design specification, by Wyeth-Ayerst, Canada) or calcium and phosphorus alone, providing approximately 3 mmol/kg.d of calcium and phosphorus, in the form of CaGP (MM+CaGP) (n=13, birth weight: 1.3±0.2 kg, post-menstrual age: 30.1±1.5 wk) (C₃H₇CaO₆P, Paul Lohmann Chemicals, Emmerthal, Germany). MNF added 3.7 g protein; 34.7 g carbohydrate; 15.2 mmol calcium; 14.1 mmol phosphorus; and 4730 IU vitamin D per litre of mother's milk. Infants whose parent(s) elected to formula feed served as a comparison group (PTF) (n=12, birth weight: 1.2±0.2 kg, post-menstrual age 29.7±1.7 wk). The formula-fed infants received Preemie SMA (Wyeth-Ayerst, Canada).

Diet group post-hospital discharge

After hospital discharge feeding practices were by parental choice. Of 25 infants receiving mother's milk in hospital with either MNF or CaGP, ten infants (five from each group) discontinued breast-feeding after hospital discharge and before reaching term corrected age. Once receiving exclusive standard formula feeding these infants were designated as the Post-FF group. In MM+MNF, breast feeding continued in
seven infants at term age, four infants at three months corrected age and four infants at six months corrected age. In MM+CaGP breast feeding continued in eight infants at term age, six infants at three months corrected age and three infants at six months corrected age. These infants from MM+MNF and MM+CaGP groups were combined and were designated as the Post-MM group. Breast feeding in Post-MM was defined as receiving over 60% of enteral intake as breast milk. The seven infants who were fed mother's milk to six months corrected age were followed to 12 months corrected age and thus remained in the Post-MM group. After hospital discharge all preterm infants received approximately 400 IU supplemental vitamin D and when breast fed or receiving no iron-fortified formula, supplemental iron was provided at approximately 10 mg daily to about six months corrected age.

Growth and body composition measurements

After hospital discharge, infants returned for follow-up visits at the Children's Hospital of the Hamilton Health Sciences Corporation (formerly Chedoke-McMaster Hospital) at term age and at three, six and 12 months corrected age. During these follow-up visits growth was measured by weight, length and head circumference as previously described (6). Whole body BMC and lean and fat mass were measured using DXA (Hologic QDR1000W®, Hologic Inc, Waltham, MA) at each visit. The DXA scans were performed while the infants were sleeping (without sedation) wrapped in a blanket wearing only a diaper while being scanned. The individual scans were analyzed using the Pediatric Whole Body Software version 5.63 (Hologic Inc.
Waltham, MA). If a good scan could not be obtained, parents were asked to return for a second scan; however, it was not possible to obtain scans from infants at all follow-up visits.

**Nutrient intake**

After each visit parents were asked to keep a five day food intake record of their infants. If infants were breast-fed, parents were given a scale (Sartorius, Goettingen, Germany) to test-weigh their infant before and after breast feeding to measure breast milk intake. Food intake records were analyzed using Nutrient Analysis Software (Nutrient Analysis Programme, E. Warwick, PEI, Canada).

**Data analysis**

Differences between MM+MNF, MM+CaGP and PTF were determined by one-way analysis of variance (ANOVA) (Sigmastat, Jandell Scientific, San Rafael, CA) followed by Student-Newman-Keuls multiple mean test. A Dunn's multiple mean test was performed if the data were not normally distributed. In order to determine the influence of post-hospital nutrition only comparisons between Post-MM and Post-FF were performed using the Students T-Test or Mann-Whitney U-Test if data were not normally distributed. The level of significance for all tests was p<0.05.

**Results**

*Outcomes related to pre-hospital discharge nutrition*

No significant differences were present between MM+MNF, MM+CaGP and PTF
groups in whole body BMC, lean and fat mass and growth at four time points over the first year (Table 1). The group means for protein, energy, calcium and phosphorus intakes for MM+MNF, MM+CaGP and PTF were similar at all follow-up visits (Table 2). There were no significant differences in the time of introduction of solids between MM+MNF, MM+CaGP and PTF (3.5±1.1, 3.7±1.0 and 2.9±1.1 months, respectively).

Outcomes related to post-hospital discharge nutrition

Weight and length were similar between Post-MM and Post-FF infants and were within the normal range of WHO reference standards generated from breast-fed infants born at term (11) (Figure 1). Whole body BMC for Post-MM was significantly lower at six months corrected age when compared to Post-FF. When whole body BMC was expressed per lean mass this difference disappeared (Figure 2). The increase in BMC between three and six months corrected age was less (p<0.05) in Post-MM infants (1.4 g/wk) compared to Post-FF infants (3.9 g/wk). Post-MM infants demonstrated a significant greater percent fat mass at three, six and 12 months corrected age when compared to Post-FF (Figure 3).

No differences were present between Post-MM and Post-FF (3.7±1.0 versus 3.7±1.2 months, respectively) in the introduction of solids. Intakes of protein, energy, calcium and phosphorus for the preterm infants in Post-MM and Post-FF are shown in Table 3.
Table 1. Body size, whole body BMC and percent lean and fat mass at term, three, six and 12 months corrected age of preterm infants fed MM+MNF, MM+CaGP and PTF prior to hospital discharge.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>MM+MNF</th>
<th>MM+CaGP</th>
<th>PTF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Term</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>3.3±0.3</td>
<td>3.2±0.4</td>
<td>3.5±0.4</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>49.6±1.3</td>
<td>48.4±1.8</td>
<td>49.4±2.3</td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>36.3±1.4</td>
<td>36.2±1.0</td>
<td>36.6±1.1</td>
</tr>
<tr>
<td>Successful DXA scans (n)</td>
<td>11</td>
<td>13</td>
<td>11</td>
</tr>
<tr>
<td>Whole body BMC (g)</td>
<td>58.0±5.8</td>
<td>55.3±1.8</td>
<td>60.0±10.5</td>
</tr>
<tr>
<td>Lean mass (%)</td>
<td>77.3±4.6</td>
<td>76.4±6.7</td>
<td>78.8±5.4</td>
</tr>
<tr>
<td>Fat mass (%)</td>
<td>21.9±2.5</td>
<td>22.2±3.4</td>
<td>19.6±5.4</td>
</tr>
<tr>
<td><strong>3 months</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>5.6±0.6</td>
<td>5.7±0.5</td>
<td>5.8±0.7</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>59.8±2.0</td>
<td>60.5±3.1</td>
<td>60.5±2.7</td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>41.1±1.1</td>
<td>41.5±0.7</td>
<td>41.5±1.7</td>
</tr>
<tr>
<td>Successful DXA scans (n)</td>
<td>7</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Whole body BMC (g)</td>
<td>113.5±20.9</td>
<td>106.1±13.6</td>
<td>119.0±28.0</td>
</tr>
<tr>
<td>Lean mass (%)</td>
<td>66.5±7.8</td>
<td>69.8±4.6</td>
<td>63.4±22.4</td>
</tr>
<tr>
<td>Fat mass (%)</td>
<td>31.9±7.8</td>
<td>28.6±4.7</td>
<td>25.5±8.8</td>
</tr>
<tr>
<td><strong>6 months</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>7.0±0.7</td>
<td>7.1±0.7</td>
<td>7.4±1.1</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>66.3±2.5</td>
<td>66.5±2.7</td>
<td>66.4±2.9</td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>44.3±1.0</td>
<td>44.1±1.3</td>
<td>44.0±1.5</td>
</tr>
<tr>
<td>Successful DXA scans (n)</td>
<td>9</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Whole body BMC (g)</td>
<td>146.2±19.4</td>
<td>155.2±19.9</td>
<td>163.2±31.5</td>
</tr>
<tr>
<td>Lean mass (%)</td>
<td>70.2±5.7</td>
<td>69.8±3.7</td>
<td>69.5±8.3</td>
</tr>
<tr>
<td>Fat mass (%)</td>
<td>27.7±5.7</td>
<td>28.1±3.7</td>
<td>28.4±8.1</td>
</tr>
<tr>
<td><strong>12 months</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>9.0±0.9</td>
<td>9.0±0.9</td>
<td>9.6±1.4</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>74.9±3.7</td>
<td>75.9±2.5</td>
<td>76.0±2.8</td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>46.9±3.9</td>
<td>46.8±1.3</td>
<td>46.6±2.0</td>
</tr>
<tr>
<td>Successful DXA scans (n)</td>
<td>7</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Whole body BMC (g)</td>
<td>209.9±27.1</td>
<td>223.0±29.2</td>
<td>243.9±40.6</td>
</tr>
<tr>
<td>Lean mass (%)</td>
<td>74.0±5.0</td>
<td>73.3±4.7</td>
<td>73.8±5.6</td>
</tr>
<tr>
<td>Fat mass (%)</td>
<td>23.6±5.0</td>
<td>24.3±4.7</td>
<td>23.9±5.6</td>
</tr>
</tbody>
</table>

*Mean ± SD.
Table 2. Nutrient intakes at term, three, six and 12 months corrected age for preterm infants fed MM+MNF, MM+CaGP and PTF prior to hospital discharge.

<table>
<thead>
<tr>
<th>Nutrient Intakesb</th>
<th>MM+MNF</th>
<th>MM+CaGP</th>
<th>PTF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Term</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (g/kg.d⁻¹)</td>
<td>2.0±0.5c</td>
<td>2.7±0.6</td>
<td>2.9±0.5c</td>
</tr>
<tr>
<td>Energy (kcal/kg.d⁻¹)</td>
<td>114±13</td>
<td>136±14</td>
<td>133±25</td>
</tr>
<tr>
<td>Calcium (mmol/kg.d⁻¹)</td>
<td>1.6±0.4d</td>
<td>1.9±0.4</td>
<td>2.1±0.4</td>
</tr>
<tr>
<td>[mmol/d]</td>
<td>[5.2±1.5]</td>
<td>[6.2±1.2]</td>
<td>[7.1±1.4]</td>
</tr>
<tr>
<td>Phosphorus (mmol/kg.d⁻¹)</td>
<td>1.1±0.5d</td>
<td>1.5±0.5</td>
<td>1.8±0.3</td>
</tr>
<tr>
<td>[mmol/d]</td>
<td>[3.7±1.8]</td>
<td>[4.7±1.5]</td>
<td>[6.4±1.0]</td>
</tr>
<tr>
<td><strong>3 months</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (g/kg.d⁻¹)</td>
<td>2.0±0.8</td>
<td>2.1±1.0</td>
<td>2.4±0.5</td>
</tr>
<tr>
<td>Energy (kcal/kg.d⁻¹)</td>
<td>89±21</td>
<td>103±38</td>
<td>107±17</td>
</tr>
<tr>
<td>Calcium (mmol/kg.d⁻¹)</td>
<td>1.7±0.8</td>
<td>1.7±0.7</td>
<td>1.9±0.4</td>
</tr>
<tr>
<td>[mmol/d]</td>
<td>[9.4±4.1]</td>
<td>[9.3±3.3]</td>
<td>[10.8±2.6]</td>
</tr>
<tr>
<td>Phosphorus (mmol/kg.d⁻¹)</td>
<td>1.5±0.9</td>
<td>1.4±0.8</td>
<td>1.8±0.4</td>
</tr>
<tr>
<td>[mmol/d]</td>
<td>[8.1±4.4]</td>
<td>[7.7±3.6]</td>
<td>[10.0±2.8]</td>
</tr>
<tr>
<td><strong>6 months</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (g/kg.d⁻¹)</td>
<td>1.9±0.5</td>
<td>2.3±0.6</td>
<td>2.1±0.4</td>
</tr>
<tr>
<td>Energy (kcal/kg.d⁻¹)</td>
<td>85±19</td>
<td>97±27</td>
<td>90±12</td>
</tr>
<tr>
<td>Calcium (mmol/kg.d⁻¹)</td>
<td>2.0±0.7</td>
<td>2.0±0.7</td>
<td>1.9±0.5</td>
</tr>
<tr>
<td>[mmol/d]</td>
<td>[13.7±5.1]</td>
<td>[13.9±3.2]</td>
<td>[13.8±5.2]</td>
</tr>
<tr>
<td>Phosphorus (mmol/kg.d⁻¹)</td>
<td>2.0±0.7</td>
<td>2.0±0.7</td>
<td>2.0±0.7</td>
</tr>
<tr>
<td>[mmol/d]</td>
<td>[14.1±5.8]</td>
<td>[13.9±3.2]</td>
<td>[14.7±6.6]</td>
</tr>
<tr>
<td><strong>12 months</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (g/kg.d⁻¹)</td>
<td>4.5±1.1c</td>
<td>3.5±0.8</td>
<td>5.2±0.5c</td>
</tr>
<tr>
<td>Energy (kcal/kg.d⁻¹)</td>
<td>123±32</td>
<td>110±18</td>
<td>131±29</td>
</tr>
<tr>
<td>Calcium (mmol/kg.d⁻¹)</td>
<td>4.0±2.1</td>
<td>2.7±1.1</td>
<td>4.0±0.7</td>
</tr>
<tr>
<td>[mmol/d]</td>
<td>[37.0±19.4]</td>
<td>[23.4±11.4]</td>
<td>[37.0±6.8]</td>
</tr>
<tr>
<td>Phosphorus (mmol/kg.d⁻¹)</td>
<td>4.9±2.3</td>
<td>3.2±1.4</td>
<td>4.7±0.8</td>
</tr>
<tr>
<td>[mmol/d]</td>
<td>[45.1±21.1]</td>
<td>[28.0±13.9]</td>
<td>[44.4±9.2]</td>
</tr>
</tbody>
</table>

*Mean ± SD.

*Recommended intakes for preterm infants one year following discharge from hospital are: for protein: 2.2 g/kg.d⁻¹; for energy: 100-120 kcal/kg.d⁻¹; for calcium 6.3-9.4 mmol/d; and for phosphorus 3.4-8.8 mmol/d (12).

*cSignificantly different from MM+CaGP, p<0.05.

*dSignificantly different from PTF, p<0.05.
Table 3. Nutrient intakes for preterm infants assigned to Post-MM and Post-FF post-hospital discharge.

<table>
<thead>
<tr>
<th>Nutrient Intakes b</th>
<th>Post-MM</th>
<th>Post-FF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Term</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (g/kg.d⁻¹)</td>
<td>1.9±0.4 c</td>
<td>2.9±0.4</td>
</tr>
<tr>
<td>Energy (kcal/kg.d⁻¹)</td>
<td>121±17</td>
<td>130±17</td>
</tr>
<tr>
<td>Calcium (mmol/kg.d⁻¹)</td>
<td>1.4±0.3 c</td>
<td>2.1±0.2</td>
</tr>
<tr>
<td>[mmol/d]</td>
<td>[4.6±1.0]</td>
<td>[7.0±0.5]</td>
</tr>
<tr>
<td>Phosphorus (mmol/kg.d⁻¹)</td>
<td>0.9±0.2 c</td>
<td>1.8±0.2</td>
</tr>
<tr>
<td>[mmol/d]</td>
<td>[2.8±0.7]</td>
<td>[5.9±0.6]</td>
</tr>
<tr>
<td><strong>3 months</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (g/kg.d⁻¹)</td>
<td>1.6±0.9 c</td>
<td>2.3±0.8</td>
</tr>
<tr>
<td>Energy (kcal/kg.d⁻¹)</td>
<td>91±22</td>
<td>100±36</td>
</tr>
<tr>
<td>Calcium (mmol/kg.d⁻¹)</td>
<td>1.5±0.9 c</td>
<td>1.9±0.6</td>
</tr>
<tr>
<td>[mmol/d]</td>
<td>[7.9±4.2]</td>
<td>[10.3±3.0]</td>
</tr>
<tr>
<td>Phosphorus (mmol/kg.d⁻¹)</td>
<td>1.1±0.9 c</td>
<td>1.7±0.6</td>
</tr>
<tr>
<td>[mmol/d]</td>
<td>[5.8±4.5]</td>
<td>[9.2±3.0]</td>
</tr>
<tr>
<td><strong>6 months</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (g/kg.d⁻¹)</td>
<td>1.7±0.4 c</td>
<td>2.2±0.5</td>
</tr>
<tr>
<td>Energy (kcal/kg.d⁻¹)</td>
<td>80±21</td>
<td>96±24</td>
</tr>
<tr>
<td>Calcium (mmol/kg.d⁻¹)</td>
<td>1.6±0.5</td>
<td>2.1±0.7</td>
</tr>
<tr>
<td>[mmol/d]</td>
<td>[10.5±2.9]</td>
<td>[15.2±4.1]</td>
</tr>
<tr>
<td>Phosphorus (mmol/kg.d⁻¹)</td>
<td>1.5±0.5 c</td>
<td>2.2±0.7</td>
</tr>
<tr>
<td>[mmol/d]</td>
<td>[10.0±3.2]</td>
<td>[15.6±4.3]</td>
</tr>
</tbody>
</table>

aMean ± SD.
bRecommended intakes for preterm infants one year following discharge from hospital are: for protein: 2.2 g/kg.d⁻¹; for energy: 100-120 kcal/kg.d⁻¹; for calcium 6.3-9.4 mmol/d; and for phosphorus 3.4-8.8 mmol/d (12).
cSignificantly different from Post-FF, p<0.05.
Figure 1. Weight (a) and length (b) of preterm infants fed Post-MM (●) and Post-FF (■) to one year corrected age in comparison to growth curves derived from healthy term infants fed mother's milk. Dotted lines represent mean and 3rd to 97th percentile (11).
Figure 2. Whole body BMC (a) and whole body BMC expressed per kg lean mass (b) to one year corrected age for Post-MM (●) and Post-FF (■). p<0.05 Post-MM versus Post-FF. Bone mass accretion between three and six months corrected age for Post-MM: 1.4 g/wk and Post-FF: 3.9 g/wk, p<0.05.
Figure 3. Percent lean and fat mass at term, three, six and 12 months corrected age in Post-MM (solid bars) and Post-FF (hatched bars). * p<0.05 Post-MM versus Post-FF.
Discussion

Our data suggest that dietary practices over the first year after hospital discharge have a greater impact on body composition in prematurely born infants than nutritional interventions in hospital. In agreement with our findings, others have also suggested that post-hospital discharge nutrition is more important for both long-term growth and bone mineral mass than early neonatal nutrition (4,13,14). Bishop et al. (14) demonstrated that at three months corrected age, the diet after hospital discharge was the only clinical factor that was related to radial BMC. Likewise, a nutrient enriched formula for preterm infants post-hospital discharge resulted in improved growth to nine months corrected age (15-17).

The observation that preterm infants fed breast-milk had a lower BMC compared to formula-fed preterm infants was not consistent with observations in term born infants. Investigations of radial and humeral BMC in term infants fed mother's milk and formula revealed no differences in bone mineral mass to one year of age (18,19). However, measurements of BMC in a single bone using single photon absorptiometry techniques may not be representative of whole body bone mass.

The lower whole body BMC in Post-MM compared to Post-FF infants at six months corrected age was associated with lower calcium and phosphorus intakes in Post-MM infants. Reduced longitudinal linear growth has been associated with a high alkaline phosphatase activity in early neonatal life which is presumably a reflection of calcium and phosphorus deficiency and subsequent compromised bone mineralization
(20). Other investigators have observed lower bone mass, as measured in a single bone, in preterm infants fed mother's milk compared to preterm infants fed formula at four months and one year corrected age (13,15). However, catch-up in radial BMC was demonstrated in a small group of human milk-fed preterm infants before they reached two years corrected age (13). In Post-MM infants catch-up in whole body BMC, expressed per lean mass, to Post-FF infants could be observed between six and 12 months corrected age (Figure 2b).

The finding of higher percent body fat in breast-fed compared to formula-fed preterm infants was consistent with findings in a study in term infants where lean and fat mass were measured in term infants studied at four months of age using $^{18}$O dilution and total body electrical conductivity techniques (21). In contrast, Dewey et al. (22) reported lower body fatness in term infants fed mother's milk. That study, however, measured body fatness by skinfold thickness, which assumes that subcutaneous fat is representative of internal fatness and these results may not have been an accurate reflection of whole body fat mass.

The difference in percent fat mass from three to 12 months corrected age between Post-MM and Post-FF infants may be explained by a lower protein intake in post-MM infants compared to Post-FF infants in the first six months past term. It has been suggested previously that the ratio of protein to energy in the diet will influence body composition (23,24). The protein to energy ratio in human milk is less than in formula. A lower protein intake will result in less lean mass deposition while the
remainder of energy will be deposited as fat mass.

It has been recognized that term breast-fed infants grow more slowly than their formula-fed counterparts (22). A similar tendency could be observed in our study. However, the possibility of a type-II error could be responsible for finding no significant differences in body size between Post-MM and Post-FF infants. Despite the fact that achieved weight and length in Post-MM infants appeared somewhat less compared to Post-FF infants, their growth was within the normal range of growth curves generated from healthy term breast-fed infants (11). As no appropriate reference data for body composition is available derived from term infants fed breast milk, it is difficult to speculate if the apparently different body composition in preterm infants fed mother's milk is of any clinical significance. Interestingly, Bishop et al. (25) found that intake of human milk in preterm infants was positively associated with a greater radial BMC at five years of age and suggested that either low bone mineral content in early infancy programmes bones to be conservative with bone mineral or that growth factors in mother's milk may stimulate later bone mass accretion. The findings in that study emphasize the importance of long-term follow-up in preterm infants past one year corrected age.

We realize that our study is limited because of the small number of infants, and our analysis of the influence of post-hospital discharge nutrition on long-term outcomes was a post-hoc analysis. Nevertheless we have identified that breast feeding in the preterm infant resulted in a lower whole body bone mass to six months
corrected age and a greater percent fat mass to one year corrected age when compared to formula-fed preterm infants.

In summary, although supplementation of mother's milk with human milk fortifiers has been shown to improve short-term growth, there appears to be no benefit for long-term outcomes of growth and body composition. Post-hospital discharge nutrition has a greater impact on long-term body composition than nutrition in early neonatal life. Our study implicates an urgent need to define appropriate reference values for whole body BMC and lean and fat mass derived from term infants fed mother's milk. Only then can the clinical significance of long-term outcomes of body composition of preterm infants fed mother's milk be evaluated.

Acknowledgement: We thank Michelle Whelan, R. N. for her assistance during the follow-up visits. This research was supported by a grant from the Dairy Farmers of Canada and an in-kind donation of the powdered fortifier from Wyeth-Ayerst, Canada.

References

Chapter Six

PRETERM INFANTS FED THEIR MOTHER'S MILK UP TO SIX MONTHS CORRECTED AGE DEMONSTRATE ADEQUATE GROWTH AND ZINC STATUS IN THE FIRST YEAR

*This chapter represents a manuscript prepared under the auspices of Early Human Development.

Premature infants fed mothers' milk to six months corrected age demonstrate adequate growth and zinc status in the first year

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Abstract

The objective of this investigation was to describe zinc status to 12 months corrected age in premature infants fed their mother's milk in relation to nutritional management in hospital and post-hospital discharge. Twenty-five premature infants fed their mother's milk in hospital were randomized to receive either a multi-nutrient fortifier (MNF), providing protein, calcium, phosphorus and zinc (MM+MNF) or calcium and phosphorus alone (MM+CaGP). Twelve preterm infants fed a preterm formula (PTF) served as a comparison group. At 35 weeks post-menstrual age a mass balance was performed to determine zinc retention. After hospital discharge infants in MM+MNF and MM+CaGP were designated to a breast-feeding group to six months corrected age (Post-MM) or formula feeding group (Post-FF) based on feeding practices. Anthropometry was performed at term, three, six and 12 months corrected age. At six and 12 months corrected age a hair sample was obtained to determine hair zinc concentrations. Preterm infants receiving supplemental zinc in hospital, as MNF, had significantly greater zinc retention in hospital compared to MM+CaGP but not greater hair zinc concentrations at six or 12 months corrected age. Despite significantly lower zinc intakes to six months corrected age, Post-MM had significantly greater hair zinc concentrations at six months (median[25th-75th percentile]: 146[106-190] compared to PTF: 85[54-91] µg/g, p<0.05). Hair zinc in Post-FF (124[77-163] µg/g) was lower than Post-MM, but this was not significant (p=0.09). Only in Post-MM were hair zinc concentrations above the median of
reference values from term born infants at 12 months corrected age. Growth was not significantly different between diet groups and was between the 3rd and 97th percentile derived from WHO growth references of breast-fed term infants. Our findings suggest that supplemental zinc either in hospital or post-hospital discharge does not appear to be required for preterm infants fed their mother's milk.

Keywords: Preterm infants; mother's milk; Zinc status

1. Introduction

Most of the zinc in the infant born at term is accumulated during the third trimester of pregnancy. Consequently, prematurely born infants will have low zinc stores at birth [1] which could easily become depleted if dietary zinc supplies are inadequate. In addition, for the preterm infant, the maintenance of a positive zinc balance in early neonatal life is difficult because of their immature gastrointestinal tract, which results in excessive endogenous losses and perhaps limitations in absorption [2]. Suboptimal zinc status in the first year of life of premature infants has been confirmed by findings of low hair zinc concentrations compared to term born infants [3]. For these reasons, an adequate postnatal zinc supply for premature infants is critical to both restore and maintain zinc stores. Zinc is essential for growth and development and dietary zinc has been positively associated with growth in both term [4] and preterm infants [5,6]. Because human milk has a relatively low zinc
concentration which declines rapidly post-partum [7], it has been suggested that premature infants fed mother's milk may be at risk of developing zinc deficiency [8].

Earlier studies describing suboptimal zinc status in preterm infants [3,5,6], cannot be extrapolated to mother's milk-fed infants since they did not distinguish between infants fed breast milk or formula. Zinc from mother's milk has a greater bioavailability when compared to infant formulas [9]. McDonald et al [10] demonstrated that term born infants fed mother's milk had greater hair zinc concentrations when compared to term born infants fed formula at six months of age despite a significantly lower zinc intake in the former group. Zinc status in preterm infants who are fed mother's milk for a prolonged period of time has not been described to date. The objectives of this study were firstly to determine whether zinc status to 12 months corrected age in preterm infants was influenced by in hospital feeding of their mother's milk together with supplemental zinc in the form of a fortifier which also contained protein, lactose, calcium and phosphorus. Secondly our goal was to determine if zinc status and growth to 12 months corrected age in premature infants fed mother's milk post-hospital discharge to six months corrected age was similar to preterm infants fed a term formula post-hospital discharge.

2. Materials and methods

Subjects

Thirty-seven premature infants with a mean gestation of 29.9±1.7 weeks and
a mean birth weight of 1311±239 grams were studied. These infants had participated in a randomized controlled trial which has been described elsewhere [11]. Briefly, premature infants fed their mother's milk in hospital were randomized to receive either a multi-nutrient fortifier (MM+MNF) or calcium and phosphorus alone (MM+CaGP). The multi-nutrient fortifier (MNF: produced, to our design specifications, by Wyeth-Ayerst, Canada) contained protein (3.7 g/L), lactose (34.7 g/L), calcium (15.2 mmol/L), phosphorus (14.1 mmol/L) and zinc (211 μmol/L) when added to breast milk. The infants randomized to MM+MNF (n=12) had a birth weight of 1.4±0.2 kg and gestational age of 29.9±1.9 wk. The infants randomized to MM+CaGP (n=13) had a birth weight of 1.3±0.2 kg and gestational age of 30.1±1.5 wk. CaGP was given to provide calcium and phosphorus at approximately 3 mmol/kg.d⁻¹. A group of premature infants fed preterm formula (PTF) (Preemie SMA, Wyeth-Ayerst Canada) served as a comparison group (n=12, birth weight: 1.2±0.2 kg, gestational age: 29.7±1.7 wk).

Upon hospital discharge at approximately 38 weeks post-menstrual age, the 25 mother's milk-fed infants who received MM+MNF or MM+CaGP in hospital were designated, based on parental feeding practices, to a post-hospital discharge formula-feeding group (Post-FF) or breast feeding group (Post-MM) (Fig 1). In the Post-FF group six infants received MM+MNF and four infants received MM+CaGP in hospital. In the Post-MM group eight infants received MM+MNF and seven infants received MM+CaGP in hospital. Breast feeding was defined as receiving over 60 % of enteral
intake as breast milk. The gender distribution was somewhat different in the Post-MM group compared to Post-FF and PTF groups with more males in the Post-MM group (Fig 1).

Methods

At approximately 35 weeks post-menstrual age zinc retention was measured in hospital by means of a five-day mass balance as described previously [11]. Following discharge from the neonatal intensive care unit the premature infants were seen for follow-up visits at the Children's Hospital of the Hamilton Health Sciences Corporation (formerly Chedoke-McMaster Hospital) in Hamilton, Canada at term, three, six and 12 months corrected age. Growth was measured by weight, length and head circumference at each visit using standardized equipment and procedures as described before [11]. Anthropometric measurements were not converted into Z-scores as it would not be appropriate to compare growth of breast-fed to formula-fed infants on which the growth centiles of the National Centre of Health Statistics standards are based. Zinc intakes were determined from food records which were kept by the parents for five consecutive days after each follow-up visit. Portions of solids and formula were estimated by the parents. For infants who were fed mother's milk, parents were provided with a scale accurate to one gram (Sartorius, Goettingen, Germany) to test-weigh their infant before and after breast feeding. The food records were analyzed with Nutrient Analysis Software (E. Warwick, PEI, Canada). At the six and 12 months follow-up visit a hair sample was obtained from the occipital portion of
the scalp. Hair samples were washed in detergent (0.5 % acationox, Monoject Scientific, MO), rinsed three times in distilled deionized water and dry ashed in a muffle furnace (Thermolyne 30400, Sybron/Thermolyne Corporation, IW) at 500 °C. The ashed samples were reconstituted in 10 % nitric acid and subsequently analyzed for zinc by flame atomic absorption spectrometry (Varian Spectra, Canada). An aliquot of reference hair was used for quality control and obtained accuracy was 2.0±4.2 %. Because of the limited amount of hair that could be obtained from the infants hair zinc could not be measured in duplicate. The coefficient of variation from reference hair samples was 4.3 %.

3. Statistics

Differences between diet groups were performed by one way analysis of variance (ANOVA) followed by a Student-Newman-Keuls multiple means test or Dunns multiple means test if data were not normally distributed. For statistical analysis of hair zinc concentrations the data were log-transformed prior to statistical analysis (Sigmastat Software, San Rafael, CA). The level of significance for all tests was p<0.05.

4. Results

*Outcomes related to pre-hospital discharge nutrition*

By design, zinc intake was significantly higher in the MM+MNF group
compared to the MM+CaGP and PTF groups. Absolute zinc retention was different amongst groups (Table 1). In hospital, calcium and phosphorus intakes were significantly greater in MM+MNF and MM+CaGP compared to PTF. Protein intake was similar between diet groups (data not shown).

After term age nutrient intakes post-hospital discharge were similar for protein, energy, energy derived from cereals, calcium or phosphorus among MM+MNF, MM+CaGP and PTF groups. Introduction of solids occurred earlier in the PTF group (2.9±1.1 months) when compared to MM+MNF (3.5±1.1 months) and MM+CaGP (3.7±1.1 months); however, this was not significant.

The addition of MNF to mother's milk and consequent higher zinc retention in hospital did not result in significantly higher hair zinc concentrations at six or 12 months corrected age (Fig. 2) compared to infants who received calcium and phosphorus supplements without zinc. Preterm infants who received mother's milk in hospital, regardless of supplementation, had significantly greater hair zinc concentrations compared to preterm infants fed a formula in hospital (Fig 2).

Outcomes related to post-hospital discharge nutrition

Zinc intake post-hospital discharge was significantly lower in Post-MM when compared to the Post-FF and PTF groups at term, three and six months corrected age (Table 2). Calcium, phosphorus and protein intake were significantly greater in PTF compared to Post-MM at term and three months corrected age. At six and 12 months corrected age, calcium and phosphorus intakes were similar between diet
groups but protein intake remained greater at these time points in PTF compared to Post-MM. Energy intake was not different between diet groups as was energy intake from cereals except at 12 months corrected age, energy derived from cereals was greater in the Post-FF compared to the Post-MM group (data not shown). Introduction of solids occurred at an earlier time in PTF (2.9±1.1 months) compared to Post-MM (3.7±1.0 months) and Post-FF (3.7±1.2 months), however this was not significant.

Hair zinc concentrations at six months corrected age were significantly greater in Post-MM infants compared to PTF infants (Fig. 3). At six months of corrected age hair zinc in Post-MM was similar to reference values derived from term born infants fed mother's milk (median: 146 μg/g; 25th-75th percentile: 106-190 μg/g [10]). Hair zinc concentrations at six months corrected age in Post-MM and Post-FF were higher than the reference values derived from term born formula-fed infants as shown in Fig. 3. At 12 months corrected age only infants in Post-MM had hair zinc concentrations above the median of reference values derived from term born formula-fed infants (Fig. 3).

Weight, length or head circumference were not significantly different between Post-MM, Post-FF and PTF at the different time points. All premature infants were within the normal range of growth curves generated from mother's milk-fed term infants [15] (Fig. 4).
Table 1
Zinc intake and retention at 35 weeks post-menstrual age in MM+MNF, MM+CaGP and PTF groups

<table>
<thead>
<tr>
<th></th>
<th>MM+MNF</th>
<th>MM+CaGP</th>
<th>PTF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc intake (μmol/kg.d⁻¹)</td>
<td>33.84±3.40⁸</td>
<td>11.64±1.75ᵇ</td>
<td>23.45±3.46ᶜ</td>
</tr>
<tr>
<td>Zinc retention (μmol/kg.d⁻¹)</td>
<td>14.74±8.58⁸</td>
<td>5.80±2.54ᵇ</td>
<td>8.47±4.43ᵃᵇ</td>
</tr>
</tbody>
</table>

Values represent mean±SD.
Values with different superscripts are significantly different with p<0.05 (ANOVA).
Recommended zinc intake is 7.7-12.3 μmol/kg.d⁻¹ [12].
Intrauterine accretion at 34 weeks post-menstrual age is 5.4 μmol/kg.d⁻¹ [13].
Table 2
Zinc intake at term, three, six and 12 months corrected age for premature infants in Post-MM, Post-FF and PTF

<table>
<thead>
<tr>
<th>Zinc intake (µmol/kg.d⁻¹)</th>
<th>Post-MM</th>
<th>Post-FF</th>
<th>PTF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Term</td>
<td>5.7±2.1ᵃ</td>
<td>14.9±1.7ᵇ</td>
<td>15.0±2.8ᵇ</td>
</tr>
<tr>
<td>3 months</td>
<td>3.7±1.4ᵃ</td>
<td>11.0±4.0ᵇ</td>
<td>11.3±1.5ᵇ</td>
</tr>
<tr>
<td>6 months</td>
<td>3.5±1.4ᵃ</td>
<td>8.9±2.8ᵇ</td>
<td>8.7±1.8ᵇ</td>
</tr>
<tr>
<td>12 months</td>
<td>10.7±2.9</td>
<td>8.1±1.7</td>
<td>9.6±1.4</td>
</tr>
</tbody>
</table>

Values represent mean±SD. Values with different superscripts are significantly different with p<0.05 (ANOVA). Recommended zinc intake for preterm infants one year following discharge from hospital is 15.0 µmol/kg (estimate) [12].
Fig 1. Overview of study design. *Breast feeding was defined as receiving over 60% of enteral intake as breast milk. *Infants in Post-MM and PTF received standard term formulas.
Fig. 2. Hair zinc concentrations (median and 25th-75th percentile) at six and 12 months corrected age for MM+MNF (●), MM+CaGP (○) and PTF (▲). The solid box represents the 25th-75th percentile with dotted line as the median derived from term born infants fed formula (n=18 [six months], n=49 [12 months]) [14]. The dotted box represent the median and the 25th-75th percentile derived from term born infants fed mother’s milk (n=13) [10]. Values with different letters are significantly different with p<0.05 (ANOVA on log-transformed hair zinc concentrations).
Fig. 3. Hair zinc concentrations (median and 25th-75th percentile) at six and 12 months corrected age in post-MM to six months (●), Post-FF (○) and PTF (●). The box represents the 25th-75th percentile with dotted line as the median derived from term born infants fed formula (n=18 [six months], n=49 [12 months]) [14]. The dotted box represents the median and 25th-75th percentile from term born infants fed mother's milk (n=13) [10]. Values with different letters are significantly different with p<0.05 (ANOVA on log-transformed hair zinc concentrations).
Fig. 4. Weight (a), length (b) and head growth (c) to one year corrected age for Post-MM (●), post-FF (○) and PTF (★). Dotted lines represent mean and 3rd to 97th percentile derived from healthy term infants fed mother's milk [15].
5. Discussion

Despite the consistently lower zinc intakes in preterm infants fed their mother's milk to six months corrected age their zinc status, as measured by hair zinc, was better compared to preterm infants fed formula after hospital discharge. This observation is consistent with findings whereby term born infants fed mother's milk had greater hair zinc concentrations when compared to term born formula-fed infants at three and six months of age [10]. A better zinc status was also demonstrated in term born infants fed mother's milk using other biochemical zinc indices such as plasma zinc [16] and erythrocyte metallothein concentrations [17] when compared to formula-fed infants.

Other studies have measured linear and/or ponderal growth as a functional indicator of zinc status and have demonstrated positive associations between zinc intake and growth in preterm infants [5,6] and term infants [4,18]. Although these observations were made predominantly in formula-fed infants, with the exception of the study of Krebs et al. [4], it has been suggested that low zinc intake from breast milk may limit growth and could therefore explain the growth "faltering" of infants fed mother's milk from the growth centiles of the National Centre of Health Statistics standards (NCHS) [4]. These findings are controversial, however, because it has recently been recognized that growth patterns of mother's milk-fed infants are different from formula-fed infants on which the growth curves of the NCHS are based [19]. Several other studies in term infants fed mother's milk have failed to demonstrate an
association between zinc intake and zinc status and/or growth [10,20]. Because zinc status in term born infants fed mother's milk appears to be better than zinc status in formula-fed infants, the slower growth observed in mother's milk-fed infants compared to formula-fed infants is not a result of inadequate zinc intake. More likely variation in growth performance may reflect differences in intakes of other nutrients (eg. protein, calcium) from mother's milk in comparison to formula as was shown in this study and which was also demonstrated in term infants [21]. Also the timing of introduction of solids has been shown to be different between breast-fed and formula-fed infants [22], which may contribute to differences in growth patterns [21].

Although it is important to investigate the role zinc plays in the growth of premature infants, to date there has existed no information on zinc status in breast-fed premature infants after discharge from hospital. In a double-blind trial in preterm infants fed standard term formula upon hospital discharge, significant increases in plasma zinc, linear growth velocity and motor development scores were observed in those receiving a zinc supplement compared to those who received a placebo supplement [6]. The findings in that study were consistent with our findings as it was implicated that zinc status was not optimal in preterm infants fed a standard term formula (containing approximately 75 μmol/L) after term age.

Supplemental zinc, given as part of a multi-nutrient fortifier, to mother's milk in the early neonatal period did not significantly improve zinc status in the first year. This was somewhat unexpected since zinc retention, and presumably zinc stores,
in MM+MNF infants was significantly greater, compared to MM+CaGP. Half of the preterm infants in these groups, however, received formula after hospital discharge and this may have obscured the effect of receiving supplemental zinc in early neonatal life on zinc status in the first year.

The differences in hair zinc concentrations between the Post-MM and the PTF group could not be attributed to differences in gender distribution. It has been shown that male infants have lower hair zinc concentrations than females [10]. Despite a proportionally greater number of males present in Post-MM, hair zinc concentrations were higher in this group at six months corrected age compared to PTF. The gender differences, however, may have obscured differences between the Post-MM and the Post-FF group.

Our findings support the suggestion that the bioavailability of zinc from breast milk is superior to that of formula, which is presumably attributed to the presence of low molecular weight zinc binding ligands in breast milk [9,23] as well as the binding of zinc to casein in cow's milk based formulas [24]. The difference in zinc status between breast-fed and formula-fed preterm infants may also relate to nutrient and/or mineral interactions in the diet. Calcium has been shown to inhibit zinc absorption in adult [25], infant-piglets [26] and preterm infant [27]. Although, energy intake from cereals was not different among diet groups, it is possible, however, that an earlier introduction of solids foods in PTF infants may have decreased zinc bioavailability. It has been demonstrated in the rat pup model that zinc
absorption was reduced when cereals were combined with milk [28]. Further, as zinc is required for growth, preterm infants fed formula may have lower zinc status as a result of a somewhat faster growth. Thus, the low zinc bioavailability from cow's milk in combination with faster growth and possible effects of mineral-and nutrient interactions in formula-fed infants may explain the lower zinc status observed in these infants in comparison to breast-fed infants.

Hair zinc was selected as an indicator of zinc status because previous studies have demonstrated that hair zinc concentrations reflect redistribution and changing zinc status in infants [18,29]. Hair zinc is easily obtained, reflects zinc intake over a longer period of time and is not susceptible to circadian variations [30] or pathological conditions [31]. It has been suggested that the interpretation of hair zinc concentrations may be obscured by reduced hair growth resulting from zinc deficiency [32]. In malnourished children with growth failure hair zinc concentrations were higher in comparison with nourished children but were accompanied by a low serum zinc concentration, which may have been associated with infection rather than low zinc status [33]. Several studies, however, in well nourished infants and children, found low hair zinc to be associated with slower linear growth [29] and supplementation with zinc improved both linear growth and hair zinc concentrations [18,34]. It appears that in the case of mild zinc deficiency, hair zinc concentrations decline, but hair growth is unaffected. In our infants, linear growth was within the normal range of growth curves generated from healthy term breast-fed infants [15],
thus the higher hair zinc concentrations found in Post-MM was not a result of reduced growth secondary to the presence of zinc deficiency.

We realize that caution is necessary in drawing conclusions from our findings. The number of premature infants described in our study is small. The number of infants needed to detect a mean difference in hair zinc of 40 µg/g [10] with α=0.05 and β=0.8 would be 16 infants per diet group. Also, the preterm infants in Post-MM received on average 70 % of total milk intake as breast milk with the rest as formula. Thus, our results do not necessarily represent preterm infants who are solely fed mother's milk.

Feeding mother's milk for premature infants has many benefits and hence it is increasingly encouraged for premature infants. Therefore, further investigations will be required to confirm our findings and to generate normal reference values for zinc status in the first year in term born infants fed mother's milk.

Acknowledgements

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6. List of references


Chapter Seven

Discussion and Future Directions
Chapter Seven

Discussion and Future Directions

A number of randomized controlled trials have investigated whether multi-nutrient supplementation to mother's milk for preterm infants in comparison to mother's milk alone resulted in better growth, mineral status and bone mineral mass (Ehrenkranz et al. 1984, Ehrenkranz et al. 1985, Modanlou et al. 1986, Carey et al. 1987, Gross et al. 1987, Venkataraman et al. 1987, Greer & McCormick 1988, Pettifor et al. 1988, Kashyap et al. 1990, Moyer-Mileur et al. 1992, Schanler & Abrams 1995, Lucas et al. 1996). The results of these studies have been inconclusive as these investigations have been confounded by their failure to consider dietary mineral bioavailability, lack of accuracy and precision of methods used to determine mineral absorption and retention, bone mineral mass and quality of growth, as well as the inappropriate use of reference standards. In order to address the question stated and to circumvent the shortcomings of previous studies, a randomized controlled trial was designed. The main objectives were to determine: dietary mineral bioavailability using stable isotope tracers; alternative indices of mineral status; bone mineral, lean and fat mass applying new and more accurate and precise methods and; outcomes of growth, bone mineral, lean and fat mass to one year corrected age in preterm infants fed supplemented mother's milk.
Dietary mineral bioavailability

Prior to the research described in chapter two, no other investigations had measured dietary mineral bioavailability from preterm infants' diets using stable isotope tracers in a randomized controlled trial. Dietary calcium, magnesium and zinc bioavailability was not reduced by the addition of a multi-nutrient supplement to mother's milk for preterm infants in comparison to supplementation with calcium and phosphorus alone. This information was of great importance for the interpretation of other results (chapter four) in this randomized controlled trial. Based on our results it can be concluded that the lack of difference in whole body BMC at term corrected age between the two mother's milk-fed diet groups cannot be attributed to poor dietary mineral bioavailability from the multi-nutrient fortifier.

It may be argued that because of the relatively large variability in fractional mineral absorption in the preterm infants described in chapter two, this study may have lacked the power to determine significant differences between diet groups. Despite this, differences in fractional mineral absorption for calcium (2 %), magnesium (7%) and zinc (1 %) between the mother's milk-fed diet groups were small. Therefore, it is not likely that a larger sample size would have changed the findings in chapter two.

In support of the use of stable isotope tracers rather than mass balance techniques to determine dietary mineral bioavailability, another important finding emerged in chapter two. Mass balance techniques, which have been traditionally used
to measure dietary mineral bioavailability (Schanler et al. 1988, Schanler & Rifka 1994, Schanler & Abrams 1995), significantly underestimated dietary mineral bioavailability. This finding may have important implications for the nutritional management of the preterm infant. Recommendations for mineral intakes of preterm infants have been based on observed mineral retention in response to variable mineral intakes as determined by mass balance techniques (AAP 1985). As a result of the underestimation of dietary mineral bioavailability by mass balance technique, the most recent recommendations (Committee on Nutrition, Canadian Pediatric Society [CPS] 1995) may be suggesting a higher mineral intake than is needed to achieve intrauterine accretion rates. High mineral intakes may impose unnecessary stress on developing digestive, metabolic and secretory systems (Greer 1989) as well as result in mineral: mineral interactions (Atkinson & Shah 1990).

The issue of dietary mineral: mineral interactions in preterm infants had not been extensively addressed prior to the development of the research described in this thesis. The impact on dietary mineral bioavailability by the addition of a multi-nutrient supplement or calcium and phosphorus alone to mother's milk was also investigated in chapter two. Fractional zinc absorption from unsupplemented mother's milk was approximately 20 % higher when compared to supplemented mother's milk (chapter 2). The dietary zinc bioavailability appeared to be compromised by the addition of calcium and phosphorus either in the form of a multi-nutrient supplement or added to mother's milk alone. Thus, when mother's milk for preterm infants is
supplemented with calcium and phosphorus, it is important to investigate whether their zinc status is being compromised.

The findings regarding dietary mineral bioavailability in the preterm infants described in chapter two cannot simply be extrapolated to smaller and more immature preterm infants. Absorption processes may be compromised in smaller and sicker preterm infants (Higashi et al. 1988, Steichen & Tsang 1992) and therefore even a small reduction in dietary mineral bioavailability may have a greater impact on mineral homeostasis in such infants.

Further, caution needs to be employed when interpreting results of dietary mineral bioavailability when using a single stable isotope as an extrinsic tracer (chapter two, addendum to chapter two). Findings in chapter two indicated that a single isotope tracer is a suitable approach to measure dietary mineral bioavailability in preterm infants. In-vitro, an extrinsic tracer, however, added to diets for preterm infants did not distribute among the different milk fractions in an equal pattern in comparison to native minerals as was demonstrated for calcium and zinc in the addendum to chapter two. Whether this would have influenced the results of mineral absorption in-vivo from the diets described in chapter two remains speculative. In-vivo studies in infants fed similar diets have demonstrated that extrinsic and intrinsic tracers for calcium, magnesium and zinc were absorbed with similar efficiency (Liu et al. 1989, Serfass et al. 1989).

*Future Directions* - The addition of a single mineral salt or a multi-nutrient
supplement to mother's milk may alter its unique composition as well as the ratios of minerals in mother's milk. Future investigations should address other mineral interactions that may occur by the addition of a multi-nutrient supplement or mineral salts to mother's milk. Interactions such as calcium:copper (Snedeker et al. 1982), zinc:copper (Festa et al. 1985) and zinc:iron (Solomons 1988) have been previously observed and need to be further explored as these minerals serve important functions for growth and development in preterm infants. In preterm infants, such studies should be performed using stable isotope tracers, with caution being employed to ensure that the extrinsic tracer is valid for the determination of dietary mineral bioavailability. Further, dietary mineral bioavailability in diets of smaller and sicker preterm infants should be explored.

In-vitro studies may provide additional information regarding the mechanisms of mineral uptake at absorption sites as well as the mechanisms responsible for mineral interactions.

**Calcium:iron interactions**

Unlike the potential mineral interactions of calcium:magnesium or calcium:zinc, the inhibitory effect of calcium on iron absorption has received ample attention in the literature (Barton et al. 1983, Dawson-Hughes et al. 1986, Cook et al. 1991). The results from these studies have been extrapolated to be relevant to infant nutrition (Hallberg et al. 1992). These studies, however, were not performed in an
infant population nor did they allow for adaptations to a diet high in calcium. The research described in chapter three was the first investigation to date which addressed the issue of calcium:iron interactions in a suitable infant-animal model. Using the infant-piglet model, a detailed investigation of the mechanisms of calcium:iron interactions at absorption sites and possible adaptation mechanisms could be performed. The result of this investigation demonstrated that calcium did inhibit iron uptake in brush border membrane vesicles, but the inhibition was less in brush border membrane vesicles derived from piglets adapted to a high calcium diet.

The nature of the calcium:iron interaction was investigated in-vitro in brush border membrane vesicles prepared from a 22 day old sow-fed control piglet (Appendix IIIa). $V_{\text{max}}$, the maximal rate of transport by the iron transporter, was decreased in the presence of calcium. However, the Michaelis constant ($K_m$), was similar in the presence or absence of calcium. Therefore, calcium appeared to alter iron absorption by a non-competitive mechanism of inhibition. In this type of inhibition, calcium would bind to a site on the iron transporter other than the iron binding site, in such way that it alters the rate of iron transport (Neame & Richards 1972). Kinetic analysis further demonstrated that the $V_{\text{max}}$ for iron uptake in brush border membrane vesicles prepared from piglets fed a regular diet was approximately 40% of the $V_{\text{max}}$ observed in brush border membrane vesicles prepared from piglets fed a diet high in calcium, while $K_m$ was similar in both groups (Appendix IIIb). Although these kinetic experiments were only performed in a small sample size, the
findings suggested that the adaptation to a high calcium diet to counteract calcium:iron interactions may be a result of an increased number of iron carriers for transport across the brush border membrane in order to maintain iron status. Future research is required to confirm this speculation. The concept that has risen from these findings is the possibility that the preterm infant, like the infant-piglet, has the capacity to adjust iron balance to fulfil its increased demands.

In order for these results from chapter three to be relevant to the preterm infant fed mother's milk, the assumption has to be made that similar results would have been found had the piglets been fed sow's milk. Whether this assumption can be made is questionable. Iron-ligand binding is known to be different between human milk and cow's milk based formulas (Davidson & Lonnerdal 1988, Lonnerdal 1991), thus iron uptake processes may be different from human milk as could be mechanisms of calcium:iron interaction.

**Future directions** - Since it remains to be determined whether the response observed in piglets would be similar in preterm infants fed mother's milk, future investigations should measure iron balance in preterm infants fed mother's milk, especially those who receive calcium supplements after active erythropoiesis has commenced. Such studies could be performed by measuring iron absorption using stable isotope tracers and monitoring the appearance of the tracer in red blood cells (Ehrenkranz et al. 1992). It was demonstrated that the extrinsic tracer method was not valid for studies of iron absorption in piglets fed sow's milk (Gislason et al. 1992).
Thus, stable isotope tracers should be preferably used as an intrinsic tracer. Iron status in preterm infants could further be measured by assessment of more sensitive biochemical indicators of iron status, such as serum ferritin or serum transferrin receptors (Jacobs & Worwood 1982, Baynes et al. 1994).

**Functional indices of mineral bioavailability in relation to nutrient supplementation of mother's milk in hospital**

As the measurement of dietary mineral bioavailability does not necessarily reflect the amount of mineral that is ultimately presented to the body, mineral retention also needs to be evaluated in order to determine mineral bioavailability.

A mineral retention similar to intrauterine values for calcium, phosphorus and zinc was not obtained in all preterm infants (chapter two, Appendix II). For these minerals, absolute mineral retention was a function of total mineral intake (chapter two). Although current nutrient recommendations for preterm infants strive to provide nutrients in order to achieve mineral accretion and growth rates similar to those of the fetus of the same gestational age (Ziegler 1981), others consider the preterm infant as a new biological entity, characterized by immaturity of many organs and functions and thus the optimal diet is the one that imposes the least stress on the developing digestive, metabolic and secretory systems even if recommendations are not met (Raiha et al. 1976). Whether the quantity and quality of minerals provided in the diets for preterm infants described in this thesis were appropriate was ascertained by the
assessment of functional outcomes related to mineral nutrition as was described in chapters four, five and six.

**Short-term bone mineral mass**

The measurement of whole body BMC in preterm infants fed different diets as described in chapter four was the first randomized controlled trial of its kind. The measurement of BMC by DXA provided information on the whole body rather than in a single bone as has traditionally been measured in preterm infants (Gross 1987, Greer & McCormick 1988, Moyer-Mileur 1992). The rationale of the investigation described in chapter four was that the addition of protein, as well as minerals, in a multi-nutrient supplement for mother's milk may improve whole body bone mass in comparison with supplementation of calcium and phosphorus alone. Minerals such as zinc, manganese, copper and magnesium function as co-factors for enzymes involved in skeletal development (Ernst & Neal, 1992). No differences in whole body BMC were observed between diet groups, however, for all preterm infants, whole body BMC was in the low normal range compared to term born infants (Atkinson et al. 1994). This latter observation supported the notion that lower intakes of calcium and phosphorus than current recommendations for preterm infants resulted in a satisfactory whole body BMC. Whether this was a result of the use of the more soluble calcium/phosphorus salt CaGP in the diets or was a result of the use of a more precise and accurate method (DXA technology) to measure BMC remains to be determined.
A shortcoming of this study design was that whole body BMC could not be measured during hospitalization in order to detect a change in whole body BMC during the nutritional intervention. Furthermore, as whole body BMC was measured approximately two to three weeks after discontinuation of the intervention, it could not be ruled out that possible differences between diet groups had been transient. This speculation, however, was contradicted by our findings and those of others (Appendix IVb, Pittard et al. 1990). Bone mass accretion in all preterm infants appeared to proceed at a slower rate than their intrauterine counterpart. Bone width at the one third distal radius followed the intrauterine bone width increments at approximately the third percentile, while radial BMC did not follow the intrauterine bone mineral accretion pattern during the early neonatal period. These observations suggest that preterm infants develop bone matrix at a constant rate while bone mineral deposition lags behind. There exists no proof, however, that preterm infants lack the metabolic maturity to mineralize bone (Tseichen & Tsang 1992). It has also been suggested that during periods of rapid growth, the high mineral intake required for the preterm infant in order to deposit minerals in the skeletal osteoid at a similar rate as the fetus of similar post-menstrual age, cannot be supplied through enteral feeding to the same extent as would have been supplied via the placenta (Steichen & Tsang 1992).

**Future Directions** - Further development of the DXA technique is required in order to determine bone mineral mass in smaller hospitalized infants so that a change in whole body BMC can be measured during a nutritional intervention. Newer,
available biochemical markers of bone mineral turnover such as hydroxyproline and serum type I procollagen peptides (Calvo et al. 1996) should be validated in infant populations as, together with measurements of bone mineral mass, they can provide valuable information on bone development in preterm infants.

The investigation described in chapter four was not designed to study the association between specific nutrients that contribute to bone mineral mass. Future studies, therefore, in animals or infants should address the role of specific nutrients such as protein, zinc and other minerals in bone mineral mass accretion to aid in gaining a better understanding of the role of nutrition for appropriate bone mineralization in preterm infants.

**Short-term growth, lean and fat mass**

Protein intake is known to be a determinant of growth because it is related to lean mass gain (Micheli & Schutz 1993). The multi-nutrient fortifier was designed to contain less protein than current commercially available fortifiers in order to reduce metabolic stress. Despite a moderate protein intake, intrauterine retention rates of nitrogen as well as intrauterine weight and length gain were attained in all preterm infants fed mother's milk supplemented with the multi-nutrient fortifier (chapter four).

Although nitrogen intake was not significantly different between preterm infants fed mother's milk supplemented with calcium and phosphorus alone and preterm infants fed preterm formula, weight and length gain in preterm infants fed a
preterm formula were significantly greater compared to those infants receiving mother's milk supplemented with calcium and phosphorus alone (chapter four). A higher protein turnover in preterm infants fed mother's milk may account for this (Pencharz et al. 1983, Boehm et al. 1990). This was consistent with findings of greater nitrogen losses in urine which were observed in preterm infants fed mother's milk compared to those fed formula (Appendix IVa). Lower nitrogen accretion in preterm infants fed mother's milk without supplemental protein compared to preterm infants fed preterm formula or mother's milk with a multi-nutrient fortifier can explain in part why intrauterine length gain was not achieved in the former group.

Growth may, however, be affected by many nutrients. As zinc has been indicated to be important for somatic growth in preterm infants (Friel et al. 1985), one could speculate that the slower growth observed in preterm infants fed mother's milk with only supplemental calcium and phosphorus may be related to suboptimal zinc status. Intrauterine zinc retention was not achieved in this group of preterm infants (chapter two). This speculation, however, could not be supported by findings described in chapter six and Appendix V. Plasma zinc concentrations in preterm infants fed mother's milk with either the multi-nutrient fortifier or calcium and phosphorus alone and preterm formula were comparable (Appendix V) and similar to values observed in term infants at a similar postnatal age (Rajaram et al. 1995).

It is recognized that plasma zinc is not a specific indicator of zinc status, however, findings of zinc status, as measured by hair zinc concentrations in the first
year (chapter six), also suggested that zinc status was not compromised in preterm infants fed mother's milk with only supplemental calcium and phosphorus. It can be postulated that the preterm infants may, to some extent, have the capacity to regulate zinc balance to meet their increased demands. Lower endogenous zinc losses in preterm infants fed unsupplemented mother's milk (0.92±0.46 μmol/kg.d⁻¹) in comparison with preterm infants fed fortified mother's milk (9.14±6.51 μmol/kg.d⁻¹) were observed in one study (Ehrenkranz et al. 1989b). This was also demonstrated in term infants fed a low zinc diet who maintained zinc balance through increased efficiency of absorption and decreased excretion of endogenous zinc (Ziegler et al. 1989).

Because the study described in chapter four involved supplementing mother's milk with multi-nutrients, it is difficult to attribute one specific nutrient to growth and further research will be required to investigate the role of specific minerals and nutrients in somatic growth.

Growth has been traditionally used as an indicator of nutritional status in preterm infants (Pereira & Georgieff 1992), whereas the quality of growth and its relationship to the diet of preterm infants has not received much attention because of the lack of accurate methodology to determine lean and fat mass. One of the objectives of the research in chapter four was to describe whole body lean and fat mass, determined by DXA, in response to different feeding practices. No studies to date have determined lean and fat mass in the whole body in relation to nutritional
intervention post-hospital. It is not known whether the quality of growth of preterm infants in early neonatal life is of importance. It was demonstrated that adiposity at birth is a predictive factor for adiposity at six years of age (Agras et al. 1990). On the other hand, since the extrauterine environment of the preterm infant is different from the reference fetus, a greater fat mass may be desirable to provide energy stores and maintain body temperature (Brook 1978). The optimal lean and fat mass composition of preterm infants has not been defined and should receive further attention.

**Long-term growth and body composition**

The short-term outcomes of growth, bone, lean and fat mass in the randomized controlled trial described in this thesis were followed by the question whether or not nutritional intervention in early neonatal life would influence outcomes of growth and body composition in the first year of life. Prior to the development of this research, none of the studies presented in the literature had performed long term follow-up of growth and whole body composition as determined by DXA. Recently, one study has measured long term outcomes of growth in preterm infants fed a multi-nutrient supplement for mother's milk in early neonatal life (Lucas et al. 1996). That study found no differences in weight and length in the first 18 months between preterm infants fed mother's milk supplemented with multi-nutrients or phosphorus and vitamins alone. In the research described in chapter five, nutritional intervention in early neonatal life did not appear to influence long-term growth, bone, lean and fat
mass.

For this long-term follow-up, however, no sample size calculations were performed because it was a descriptive analysis. The compliance in this study was good and the drop-out rate for the long-term follow-up was only 5.4%. But it was not always possible to obtain DXA scans of all the infants. Nevertheless findings in chapter five were novel. Further, the findings in chapter five also demonstrated that the variability in feeding practices (breast feeding or formula feeding) among study groups after hospital discharge may have influenced long term outcomes of growth and body composition and led us to investigate the influence of post-hospital discharge nutrition on growth and body composition.

**Future Directions** - In order to answer the question of whether early nutritional intervention has any impact on long term growth and body composition, a large clinical trial would be required in which nutrient intakes after hospital discharge can be well controlled and monitored. Further long-term follow-up past one year corrected age would also provide additional valuable information. Catch-up growth and bone mass accretion may occur past one year corrected age and early neonatal nutrition may potentially play a role in bone mass accretion up to five years of age (Schanler et al. 1992, Bishop et al. 1996).
Functional indices of mineral bioavailability in relation to breast feeding post-hospital discharge

A post-hoc analysis was performed to describe long-term growth, bone, lean and fat mass and zinc status in preterm infants fed mother's milk in hospital and mother's milk or formula post-hospital discharge (chapters five and six). This allowed an investigation into the influence of post-hospital discharge nutrition only on long term outcomes of growth, body composition and zinc status. No studies to date have described these outcomes in preterm infants fed their mother's milk post-hospital discharge.

Recently, it has been recognized that growth patterns of term infants fed mother's milk are different from those fed formula (Dewey et al. 1995). This was also observed in the preterm infants described in chapter five, although no significant differences in growth between formula-fed and breast-fed infants were found. In preterm infants fed breast-milk it was demonstrated that body composition was different from formula-fed preterm infants with a lower bone mass and greater fat mass in breast-fed preterm infants (chapter five). Further, it was observed that zinc status was better in breast-fed compared to formula-fed infants (chapter six).

It has to be kept in mind that these investigations, of the effect of post-hospital discharge nutrition on growth, body composition and zinc status were performed as a post-hoc analysis in a small number of infants and, therefore, the possibility of type I and II statistical errors may exist. Nevertheless the findings in
chapter five imply that differences in nutrient intakes from breast milk or formula accounted in part for the observed differences in body composition between breast-fed and formula-fed preterm infants.

The importance of the apparent differences in body composition and zinc status of preterm infants fed mother's milk compared to those fed formula on long-term growth and development remains unknown. No studies to date have described long-term outcomes of bone, lean and fat mass or zinc status in term infants in relation to breast feeding during the first year of life. The WHO recommends exclusive breast-feeding from birth to four to six months of age, with continued breast feeding, while receiving appropriate and adequate complementary foods, up to two years or beyond (WHO 1995). Therefore, reference data from breast-fed term born infants should represent optimal growth and development for the preterm infant fed mother's milk.

**Future Directions** - As breast feeding for preterm infants is gaining popularity, it is of great importance to determine its benefits as well its nutritional adequacy for growth and development. Long term follow-up studies measuring growth, bone, lean and fat mass in an appropriate number of breast-fed and formula-fed premature infants should be performed. Further reference data from breast-fed term born infants are needed. Only when such data are available can long-term outcomes of whole body bone, lean and fat mass be evaluated in preterm infants.
In summary this thesis investigated the mineral bioavailability from supplemented mother's milk for preterm infants. Mineral bioavailability was determined directly by measurements of fractional mineral absorption. Different and new approaches to measure functional outcomes of mineral nutrition were applied. The results of the infant and animal studies have provided new concepts and insights for the nutritional management preterm infants. The research described in this thesis has also identified areas which require further investigation. Outcomes of future studies, as suggested in the discussion, will advance the development of guidelines for the optimal nutritional management of preterm infants fed mother's milk.
REFERENCES


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Heine W. Is mother's milk the most suitable food for very low birth weight infants ?. Early Hum Dev 1992;29:345-50.


Minton SD, Steichen JJ, Tsang RC. Bone mineral content in term and preterm appropriate-for-gestational-age infants. J Pediatr 1979;95:1037


Overview of calcium metabolism

(Modified from: Linder 1990, Halbert & Tsang 1992)
Overview of magnesium metabolism

Overview of zinc metabolism

(Modified from: Linder 1990, Hunt et al. 1990)
Overview of iron metabolism

(Modified from: Linder 1990, Hunt et al. 1990)
Appendix II

Phosphorus balance in preterm infants fed mother's milk supplemented with multi-nutrients (MNF), calcium and phosphorus alone (CaGP) or preterm formula (PTF) as determined by mass balance technique

<table>
<thead>
<tr>
<th>Phosphorus balance</th>
<th>MM+MNF</th>
<th>MM+CaGP</th>
<th>PTF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intake (mmol/kg.d⁻¹)</td>
<td>3.27±0.27⁺</td>
<td>3.37±0.41⁺</td>
<td>2.18±0.35⁻</td>
</tr>
<tr>
<td>Urine (mmol/kg.d⁻¹)</td>
<td>0.66±13⁺</td>
<td>0.79±0.38⁺</td>
<td>0.26±12⁻</td>
</tr>
<tr>
<td>Stools (mmol/kg.d⁻¹)</td>
<td>0.09±0.04</td>
<td>0.10±0.08</td>
<td>0.15±0.08</td>
</tr>
<tr>
<td>Absorption (%)</td>
<td>97±1⁺</td>
<td>97±2⁺</td>
<td>93±4⁻</td>
</tr>
<tr>
<td>Retention (%)</td>
<td>77±4⁺</td>
<td>74±10⁺</td>
<td>81±10⁻ ²⁺</td>
</tr>
<tr>
<td>Net Retention (mmol/kg.d⁻¹)</td>
<td>2.52±26⁺</td>
<td>2.48±0.37⁺</td>
<td>1.78±0.35⁻</td>
</tr>
</tbody>
</table>

Values represent mean±2SD.
Different superscripts indicate p<0.05 (ANOVA).
Appendix IIIa

Analysis of the kinetics of the inhibition of iron uptake by calcium in brush border membrane vesicles prepared from one control piglet

Double reciprocal plot showing the effect of a fixed concentration of calcium (4.0 mM) on the rate of uptake of iron present at various concentrations.

[Ca] = 0 mM (○): Vmax = 9.71 (nmol/mg protein/min), Km = 2.08 (mM).
[Ca] = 4 mM (○): Vmax = 2.90 (nmol/mg protein/min), Km = 2.45 (mM).

Values for Vmax and Km are derived from Lineweaver-Burke plot (Neame & Richards 1972)
Appendix IIIb

Kinetic analysis of iron uptake in brush border membrane vesicles prepared from piglets fed a regular diet (N-Ca) or a high calcium diet (Hi-Ca)

<table>
<thead>
<tr>
<th>Iron uptake kinetics</th>
<th>N-Ca (n=4)</th>
<th>Hi-Ca (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$ (nmol/mg protein/min)</td>
<td>8.45±0.55</td>
<td>19.19±4.87</td>
</tr>
<tr>
<td>$K_m$ (mM)</td>
<td>3.60±0.22</td>
<td>3.71±0.45</td>
</tr>
</tbody>
</table>

Values are mean±SEM
Values for $V_{max}$ and $K_m$ are derived from Lineweaver-Burke plots (Neame & Richards 1972)
Appendix IVa

Nitrogen balance in preterm infants fed mother's milk supplemented with multi-nutrients (MNF), calcium and phosphorus alone (CaGP) or preterm formula (PTF) as determined by mass balance technique

<table>
<thead>
<tr>
<th>Nitrogen balance</th>
<th>MM+MNF</th>
<th>MM+CaGP</th>
<th>PTF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intake (mmol/kg.d⁻¹)</td>
<td>37.0±3.7</td>
<td>33.5±2.2</td>
<td>35.1±5.1</td>
</tr>
<tr>
<td>Urine (mmol/kg.d⁻¹)</td>
<td>8.9±4.0ᵃ</td>
<td>9.9±2.3ᵃ</td>
<td>6.1±2.7ᵇ</td>
</tr>
<tr>
<td>Stools (mmol/kg.d⁻¹)</td>
<td>2.5±1.2</td>
<td>1.7±0.9</td>
<td>1.9±0.9</td>
</tr>
<tr>
<td>Net Retention (mmol/kg.d⁻¹)</td>
<td>25.5±4.3ᵃᵇ</td>
<td>22.0±3.3ᵃ</td>
<td>27.1±5.5ᵇ</td>
</tr>
</tbody>
</table>

Values represent mean±2SD.
Different superscripts indicate p<0.05 (ANOVA).
Intrauterine accretion rate at similar post-menstrual age 20 mmol/kg.d⁻¹ (Ziegler et al. 1976).
Radial BMC and bone width in preterm infants fed mother's milk supplemented with multi-nutrients (MNF), calcium and phosphorus alone (CaGP) or preterm formula (PTF) in comparison with intrauterine reference curves.

Radial bone mineral content (BMC) (a) and bone width (b) at 33 weeks post-menstrual and at term age for preterm infants receiving MM+MNF (●), MM+CaGP (○) and PTF (■). Dotted lines represent the mean and 95% confidence interval of intrauterine reference values (Greer et al. 1983).
Appendix V

Plasma zinc in preterm infants fed mother's milk supplemented with multi-nutrients (MNF), calcium and phosphorus alone (CaGP) or preterm formula (PTF) at approximately 35 weeks post-menstrual age

<table>
<thead>
<tr>
<th></th>
<th>MM+MNF</th>
<th>MM+CaGP</th>
<th>PTF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma zinc</td>
<td>13.91±4.31</td>
<td>15.09±3.55</td>
<td>12.84±4.48</td>
</tr>
<tr>
<td>(nmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Reference values: Preterm infants at 40 weeks post menstrual age: 12.27±2.91 nmol/L. Term infants of similar postnatal age: 14.29±2.85 nmol/L (Rajaram et al. 1995).