A Physiological Study to Determine the Enteral Threonine Requirements in Infants 1 to 6 Months of age: Preliminary Results

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Nutritional Sciences
University of Toronto

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2017

Abstract

Threonine is an indispensable amino acid that is greatly used by the gut and both deficient and excessive intakes of threonine are known to negatively impact the mucosal environment. Current infant threonine requirements are based on estimates of breast milk composition, however these estimates have not been validated using newer methods. The main objective of this thesis was therefore to determine the enteral threonine requirement in 1 to 6 month olds using the indicator amino acid oxidation method. Multiple methodological and ethical aspects were considered and provide the groundwork for the current and future enteral amino acid studies in infants. Thus far, preliminary results are encouraging and appear to demonstrate that study methods are working as anticipated. As enrolment ensues beyond the completion of this thesis, a threonine requirement for this 1 to 6 month old population will be defined.
Acknowledgments

To Pasquale; we share this accomplishment together. Thank you for supporting me and sticking by my side throughout my many difficult-to-be-around moments. To my parents and sister who may have been far away but kept me focused and motivated throughout these years.

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>amino acid</td>
</tr>
<tr>
<td>AI</td>
<td>Adequate Intake</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>DAAO</td>
<td>direct amino acid oxidation</td>
</tr>
<tr>
<td>DRI</td>
<td>Dietary Reference Intakes</td>
</tr>
<tr>
<td>G-tube</td>
<td>gastrostomy tube</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>GI</td>
<td>gastrointestinal</td>
</tr>
<tr>
<td>GLT</td>
<td>glycyl-L-tyrosine</td>
</tr>
<tr>
<td>IAA</td>
<td>indispensible amino acid</td>
</tr>
<tr>
<td>IAAO</td>
<td>indicator amino acid oxidation</td>
</tr>
<tr>
<td>IOM</td>
<td>Institute of Medicine</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>sodium bicarbonate</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>NG</td>
<td>nasogastric tube</td>
</tr>
<tr>
<td>RDA</td>
<td>Recommended Daily Allowance</td>
</tr>
<tr>
<td>REB</td>
<td>research ethics board</td>
</tr>
<tr>
<td>TDG</td>
<td>threonine dehydrogenase</td>
</tr>
<tr>
<td>TDH</td>
<td>threonine dehydratase</td>
</tr>
<tr>
<td>TEF</td>
<td>tracheoesophageal fistula</td>
</tr>
<tr>
<td>UPLC</td>
<td>Ultra Performance Liquid Chromatography</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
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1 Introduction

Amino acids are the building blocks of proteins and comprise of a variety of types that differ by charge, polarity, and molecular mass. Amino acids consist of a carboxyl group, an amino group, and a unique side group, which dictate each amino acid’s specific characteristics. More than 300 different amino acids are known to exist, however only 20 are considered proteinogenic and are genetically coded into proteins\(^1\). These 20 alpha-amino acids make up all protein with nine classified as indispensable (essential), six conditionally indispensable, and five classified as dispensable (non-essential) in humans (Table 1). If an amino acid cannot be endogenously produced in sufficient amounts, it is deemed indispensable and required to be consumed through the diet\(^2\). The dispensable amino acids are a means of providing non-specific nitrogen. Lastly, certain life cycle stages and some disease states will require that other amino acids be exogenously provided, which are deemed conditionally indispensable. In addition to their use for protein synthesis, amino acids like tryptophan and glutamate act as neurotransmitters, arginine and phenylalanine regulate enzymatic activity, and leucine as a translational regulator\(^3\).

Defining optimal amino acid requirements in humans is and has been an evolving science. With respect to infants, amino acid requirements were initially defined by providing graded intakes of an amino acid and measuring resulting biological parameters such as growth and nitrogen balance\(^4\text{–}^6\). In 1985, the FAO/WHO/UNU expert consultation produced a joint report, establishing that infant amino acid requirements should be based on the pattern of amino acids in breast milk\(^7\). The recommendation to use mature breast milk composition as the standard for amino acid requirements in infants continued in the 2007 report\(^8\). This provides us with the latest recommendations for infants 0 to 6 months old.

However, it is known that breast milk composition is variable\(^9\text{–}^{11}\), and current requirements are based on multiple factors including amino acid composition of breast milk and average intakes of protein from breast milk\(^8,^{12}\). Therefore, in an effort to validate whether breast milk amino acid composition is an appropriate reference for infant amino acid requirements, researchers have begun to conduct studies to define amino acid requirements using newer methods, namely the non-invasive indicator amino acid oxidation method (IAAO)\(^13\text{–}^{15}\). This method had not been
previously used to determine enteral amino acid requirements in infants at the time of the establishment of current recommendations. Thus far, all IAAO-derived estimates for indispensable amino acids have been conducted in the less than 1 month old infant, mainly between 2011 and 2015\textsuperscript{14,16–20}. These newer requirements appear to reflect previously established breast milk-derived estimates for most amino acids studied\textsuperscript{8}. For threonine, Hogewind-Schoonenboom et al. derived the requirement in infants under 1 month old to be 68mg/kg/d\textsuperscript{14}, whereas the established threonine intakes are 76mg/kg/d for the 1 month old exclusively breast fed infant\textsuperscript{8}.

**Table 1. The Three Groups of Amino Acids\textsuperscript{2,21}**

<table>
<thead>
<tr>
<th>Dispensable</th>
<th>Indispensable</th>
<th>Conditionally Indispensable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>Histidine</td>
<td>Arginine</td>
</tr>
<tr>
<td>Asparagine</td>
<td>Isoleucine</td>
<td>Cysteine</td>
</tr>
<tr>
<td>Aspartate</td>
<td>Leucine</td>
<td>Glutamine</td>
</tr>
<tr>
<td>Glutamate</td>
<td>Lysine</td>
<td>Glycine</td>
</tr>
<tr>
<td>Serine</td>
<td>Methionine</td>
<td>Proline</td>
</tr>
<tr>
<td><strong>Threonine</strong></td>
<td>Phenylalanine</td>
<td>Tyrosine</td>
</tr>
<tr>
<td></td>
<td>Tryptophan</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Valine</td>
<td></td>
</tr>
</tbody>
</table>

In order to determine whether the current guidelines are appropriate for all infants, the next step would be the determination of amino acid requirements in the 1 to 6 month old infant, once growth slows after the neonatal period. This thesis will therefore focus on the indispensable amino acid: threonine, and defining its requirements in the 1 to 6 month old population using the IAAO method.

The piglet has become the most appropriate animal model for the study of human infant amino acid metabolism due to marked similarities between gastrointestinal development and amino acid metabolism\textsuperscript{22,23}. The piglet model has allowed for the study of protein and amino acid metabolism and effects of route of feeding on amino acid requirements\textsuperscript{22,24,25}. We know from such studies that approximately 60% of enterally-administered threonine is utilized by the gut itself, specifically for the production of threonine-rich mucins\textsuperscript{26,27}. This was later confirmed by a
study conducted in pre-term infants, demonstrating 70% first pass metabolism of threonine\textsuperscript{28}. Accordingly, it has been shown that both deficient and excessive threonine intakes can negatively impact gastrointestinal morphology\textsuperscript{29–31}. At dietary threonine intakes of 50% or 150% of appropriate threonine requirement, piglets displayed duodenal and ileal villous atrophy\textsuperscript{29}. In addition, it has been shown that either a deficient or excess threonine intake leads to reduced mucin synthesis and even reduced muscle protein synthesis in piglets\textsuperscript{32}. Moreover, insufficient dietary threonine has been associated with altered epithelial tight junction structures\textsuperscript{29}, potentially affecting gut permeability. Since studies investigating deficient or excessive intakes of amino acids in infants for prolonged periods of time are not ethically acceptable, animal studies provide evidence to support the importance of providing appropriate threonine intakes. This may be especially important in infancy, with the vulnerability of the immature and developing gastrointestinal tract\textsuperscript{33,34}.

In addition, there appear to be measurable differences in threonine metabolism when infants are fed with either formula or breast milk. Darling et al. demonstrated differences in threonine and phenylalanine metabolism of infants fed formula versus breast milk\textsuperscript{35,36}. Preterm infants fed with a whey-dominant formula oxidized less threonine as a percentage of threonine intake and demonstrated high plasma and urinary threonine concentrations compared to breast milk fed infants\textsuperscript{36}. In addition to these observations, infant formulas themselves can contain higher than recommended levels of threonine\textsuperscript{37}. Neocate and Puramino, which are standard elemental (amino acid-based) infant formulas, contain between 60-70mg threonine/g protein, whereas breast milk composition is 44mg/g\textsuperscript{8}. This translates to approximately 110mg/kg/d of threonine, compared to the current Adequate Intakes of 73mg/kg/d\textsuperscript{12}. Therefore, not only do formula fed infants metabolize threonine less efficiently than breast milk fed infants, but infant formulas themselves can provide more threonine than currently recommended.

Nitrogen balance is the traditional method used to determine amino acid and protein requirements, however multiple disadvantages, including mandatory lengthy adaptation periods, make this method unsuitable for use in vulnerable populations. Compared to older methods, it is now possible to define indispensable amino acid requirements in infant populations using the non-invasive IAAO method\textsuperscript{38,39}. Therefore, this thesis will introduce a study that aims to determine the enteral threonine requirements in infants between 1 to 6 months of age. As this
was the first study of its kind performed in our lab, this thesis will also describe the necessary methodological and research ethics requirements necessary to implement this study. The pre-study and optimization procedures will also be applicable for future paediatric enteral amino acid requirements studies.
2 Literature Review

2.1 Threonine

Threonine is an indispensable amino acid; therefore it is required in the diet and can be found in protein-rich foods. It is one of three amino acids containing a hydroxyl side group (-OH), with the others being serine and tyrosine. Threonine is therefore polar and net uncharged. Threonine can exist as 2 stereoisomers: the L and D configuration (Figure 1). L-threonine is the most abundant, and is the only form used biologically by mammalian cells, therefore proteins are made up exclusively of L amino acids. All 20 protein-forming amino acids exist as two stereoisomers, except for glycine, as it is achiral. From now on, all mentions of threonine will be referring to L-threonine unless otherwise specified.

Figure 1. Structure of L and D-Threonine (C₄H₉NO₃)

2.1.1 Metabolism

Threonine has a relatively complex metabolic pathway, and one that differs across species and changes through stages of life. Figure 2 provides a diagram of the two major threonine degradation pathways in humans. In the mammalian liver, threonine can be broken down by threonine dehydratase (TDH), yielding 2-ketobutyric acid, then CO₂ and propionic acid. Threonine can also be metabolized via threonine dehydrogenase (TDG) to form 2-amino-3-ketobutyrate, which is then cleaved to form glycine, a conditionally indispensable amino acid, and acetyl CoA.\textsuperscript{40,41}
Figure 2. Metabolic Pathway of Threonine Degradation in Humans\textsuperscript{36,42}

\textbf{L-threonine} \\
\textbf{Threonine Dehydratase} \\
(TDH) \\
\textbf{Threonine Dehydrogenase} \\
(TDG) \\

\textbf{2-amino-3-ketobutyric acid} \\
\textbf{2-ketobutyric acid} \\
\textbf{Propionic acid} \\
\textbf{Glycine} + \textbf{Acetyl-CoA} \\

\textbf{2-aminobutyric acid}
2.1.1.1 In Animals

Although mixed evidence suggests that the enzymes responsible for threonine’s metabolic pathways may or may not be regulated by factors such as protein intake, threonine, or glycine intake, animal research has provided some insight into this issue. In rat and pig data, TDG has been shown to be responsible for approximately 80% of threonine degradation in vivo\(^{41,43-45}\). Conversely, House et al. have demonstrated in-vitro that 65% of threonine degradation occurs via the TDH and not via the TDG pathway in rat hepatocytes\(^{42}\). Although TDG appears to be the major pathway, TDH activity is highly sensitive and is influenced by elevated protein intakes and hormonal factors\(^{42,45,46}\). Interestingly, in the rat foetus liver, TDH levels appear to be very low but increase rapidly after birth, and are stimulated by glucagon\(^{47}\). It therefore appears that the activity of TDG and TDG is species specific, but also may change from birth onward.

2.1.1.2 In Adults

Compared to animal data, different metabolic fates of threonine degradation occur in human adults. In men fed graded threonine intakes from 3 to 100 mg/kg/d, glycine derived from the catabolism of threonine was apparently undetectable in a multi-tracer study, and researchers concluded that the conversion of threonine to glycine in humans is insignificant\(^{48}\). Subsequently, Darling et al. then suggested that the previous study used gas chromatography (GC)-quadrupole mass spectrometry (MS), which may not have been sensitive enough to detect the glycine converted from threonine\(^{49}\). Using GC-combustion isotope ratio MS, a more sensitive instrument, Darling et al. determined that the TDG pathway was in fact minimal but not insignificant in adults, demonstrating that threonine to glycine conversion occurs at 7-10% of total threonine degradation when fed a diet containing 65mg/kg/d of threonine (this study’s control diet)\(^{49}\). These rates did not differ between test protein intakes (at 1.6 versus 2.9g/kg/d). Threonine oxidation has been shown to increase with increasing dietary threonine or protein bound threonine intakes\(^{48,49}\). However this oxidation also appears to plateau at plasma threonine levels above 200μmol/L in adult males\(^{48}\). This demonstrates a potential inability to effectively oxidize higher intakes of threonine in the body.
2.1.1.3 In Infants

In the preterm infant, threonine degradation via the TDG pathway has been estimated to be 44%\textsuperscript{36}, which is higher than the above mentioned adult TDG activity of 7-10%. Interestingly, in term newborns less than 48 hours old, Parimi et al. observed that the majority of threonine degradation occurs via the TDH pathway, with the conversion of threonine to glycine via the TDG pathway nearly undetectable\textsuperscript{50}. Since preterm infants apparently use the TDG pathway for approximately 44% threonine degradation\textsuperscript{36}, this may reflect the increasing levels of TDH from birth onward, as previously demonstrated in rat hepatocytes\textsuperscript{47}. TDH would then appear as the predominant pathway in adulthood\textsuperscript{49}.

Interestingly, formula versus breast milk fed infants degrade threonine at different capacities. Studies have shown that compared with breast milk fed infants, formula fed preterm infants demonstrate markedly elevated plasma threonine levels\textsuperscript{36,51}. Although threonine intakes varied by less than 10% between groups, Darling et al. observed that intakes of whey-dominant formula lead to a two-fold increase in plasma threonine\textsuperscript{36} with similar findings being shown in term infants as well\textsuperscript{52}. Regardless of type of formula, threonine oxidation as a percentage of threonine intake appears capped at 17% in formula fed infants, regardless of the ratio in formula of whey to casein. Breast milk fed infants however oxidized threonine at 24% of their threonine intake\textsuperscript{36}. It therefore appears that threonine oxidation occurs at an elevated and maximal rate in formula fed infants, demonstrating an inability to dispose of the slightly higher levels of threonine in the formula used in this study (63.2umol/kg/h threonine (whey dominant formula) versus 58umol/kg/h threonine (breast milk)). Similar findings have been shown in term infants, where plasma threonine is higher in formula fed infants, regardless of similar threonine intakes with breast milk fed infants\textsuperscript{53}. Although it is unclear why this happens, these finding may be explained by alternate routes of degradation other than to glycine (via TDG), or perhaps the immunological properties and growth factors present in breast milk which may trigger increased activity in the TDH or TDG pathways\textsuperscript{36}.

In addition to the altered oxidative capacities between formula or breast milk fed infants, whey dominant infant formulas can provide high amounts of threonine. Cow’s milk protein has a whey to casein ratio of $\approx 20:80$ whereas the ratio in human milk protein is $\approx 60:40$\textsuperscript{54}. In order to
replicate protein ratios in human milk, bovine proteins will be manipulated to achieve this ideal 60:40 ratio. However, bovine whey happens to be particularly rich in threonine\textsuperscript{55}. It has been shown that threonine appears to be the only amino acid that is present in significantly higher concentrations in both plasma and urine of infants fed with 60:40 formula compared to a casein dominant 20:80 formula\textsuperscript{56}. It therefore appears that by not consuming breast milk as well as consuming a whey predominant infant formula, this will contribute to elevated plasma and urinary threonine levels in infants.

### 2.1.2 Threonine Functions

As an amino acid, threonine contributes mainly to body protein via protein synthesis. The threonine composition of body protein is 42mg/g of protein\textsuperscript{12,57}. This is very similar to both the amount of threonine in breast milk and egg protein, being 44mg/g\textsuperscript{8} and 47mg/g\textsuperscript{58}, respectively.

#### 2.1.2.1 Splanchnic Metabolism of Threonine

A substantial amount of research focused on amino acid metabolism, digestion and utilization has been conducted in the pig model. The piglet model has long been an acceptable animal model for the infant, due to the comparability between amino acid metabolism and gastrointestinal development\textsuperscript{22,23,25}. Specifically, members from our lab have validated a neonatal piglet model with a primary goal to investigate amino acid requirements in parenteral nutrition\textsuperscript{24}. This then enabled the study of the effect of route of feeding on amino acid requirements.

Stoll et al. observed in 2 week old piglets that portal blood samples only represent 64\% of the amount of amino acids consumed, indicating that a third of dietary amino acids were utilized on first pass metabolism\textsuperscript{27}. First-pass metabolism is described as the direct absorption and utilization of nutrients by the splanchnic region, comprised mainly of the liver and gastrointestinal tract\textsuperscript{59}. First-pass metabolism plays a significant role in the uptake of nutrients,
and in fact, results in varying rates of systemic availability for different amino acids. It was noted early on in human neonates fed either enterally or parenterally, that protein turnover was 40% higher in the enteral fed infants, an apparent result of the energy demand of their developing gastro-intestinal tract, which is bypassed, and also atrophies, when parenterally fed. Threonine happens to be one of the amino acids that is most utilized by the gut when enterally consumed. Stoll et al. examined piglets fitted with multiple catheters in order to measure portal appearance of enterally delivered isotopically labelled protein. This study demonstrated that approximately 60% of enteral threonine is utilized in first-pass metabolism, being the highest of all indispensable amino acids. This has been confirmed by both piglet and human neonate studies observing threonine first pass metabolism between 60% and 90%. More recently, a study by Munasinghe et al. demonstrated that dietary threonine is preferentially used for the protein synthesis in the GI tract, regardless of threonine intakes. In this study, 20 piglets were fed varied threonine intakes (at 20 to 220% of threonine requirement) and tissue specific protein synthesis rates were measured by the use of $[^3]$H phenylalanine. By determining tissue-specific protein synthesis rates compared to threonine intakes, this study provides novel insight into the obligate need for threonine by mucin-producing tissues, mainly the intestines. Previously confirmed by Schaart et al, this demonstrates that at lower threonine intakes, the threonine requirement of the gut will be fulfilled at the expense of other tissues, like skeletal muscle, therefore compromising other tissue functions and growth in the piglet. These results provide insight on the importance of providing adequate dietary threonine intakes to fulfill the requirements of all organs and tissues for optimal growth and maintenance.

Based on the above-mentioned research, route of feeding impacts threonine metabolism as well as requirements. Data from Bertolo et al. demonstrated that the parenteral threonine requirement is 45% that of the enteral requirement. In adult humans, it has been confirmed that splanchnic utilization is similar between threonine and lysine, at approximately 18% of intakes. However, in the human neonate, first pass metabolism of threonine is remarkably higher, at 43% to 70%, where as lysine first pass metabolism more closely resembles the adult data, at 12%. These differences highlight a particular splanchnic need for enteral threonine in the developing infant, as the splanchnic extraction of other amino acids ranges from 12% to 24% across the lifespan.
It is well recognized that threonine’s main function within the gut is for glycoprotein and mucin production. Mucins, or mucous glycoproteins, are secreted in the intestinal lumen by epithelial cells. Threonine residues play a key role by making up a significant portion of the glycoprotein backbone. Being threonine-rich, it is therefore understandable why mucins directly utilize such an elevated proportion of threonine. MUC2 is the major secretory mucin in the gut and it is rich in threonine residues, at up to 30%. The main functions of these mucins are to create a protective insoluble barrier, which is size selective and inhibits penetrative damaging proteins. As mentioned, the gut’s affinity for threonine is so strong that threonine is utilized to maintain the mucosal layer at the expense of threonine needs of other tissues. Interestingly, Van der Schoor et al., using a multi-isotope tracer technique, were unable to detect any plasma threonine recycled from mucin origin, suggesting that either threonine degraded from mucins are immediately re-utilized within the GI tract or that they are excreted.

Lastly, animal models have demonstrated that both a low and high threonine intake can negatively impact gut morphology. In piglets, it appears as though both an insufficient or excess intake of threonine will cause villous atrophy and negatively impact the mucosal barrier by altering tight junction. In this study, when fed dietary threonine levels of 0.37% or 1.11% (whereas 0.74% is at requirement level), weanling piglets displayed duodenal and ileal villous atrophy. In addition, it has been shown that both deficient or excess threonine intake can also cause reduced mucin synthesis and even reduced muscle protein synthesis in piglets.

Such findings raise important considerations for the developing gut in infants as mucins play a key role in protecting the intestinal epithelium from bacterial, chemical and physical forces and maintaining epithelial tight junction structure is important for appropriate intestinal permeability.

2.1.2.2 Other Functions of Threonine

Wang et al. demonstrated that when challenged with *Escherichia coli*, increasing dietary threonine intake lead to increased serum antibody IgG in pigs. Also, low IgG concentrations in the plasma of sows increases in response to a diet supplemented with threonine. Threonine
appears to also play a role in collagen synthesis as ligands containing Thr-221 residue are considered important for collagen binding within the body. Lastly, threonine intake also appears to have an influence on phenylalanine metabolism. In patients with phenylketonuria (PKU), a disease characterised by an insufficient ability to metabolize phenylalanine, plasma threonine levels were negatively correlated with phenylalanine levels, with no correlation to other amino acids. Sanujirro et al. were also able to demonstrate that oral threonine supplementation reduces plasma phenylalanine concentrations in infants with either PKU or mild hyperphenylalanemia, confirming threonine’s potential for supplementing phenylalanine-restricted diets in these patients.

### 2.1.3 Threonine Deficiency

The major consequences of threonine deficiency relate to the gastro-intestinal tract. Hamard et al. has demonstrated that inadequate dietary threonine (390 mg/kg/d whereas control was 520mg/kg/d) resulted in reduced villous height and atrophy in the ileum of early-weaned piglets within 2 weeks. In a later study by the same group, to further elucidate how these changes may affect the function of the gut, demonstrated that this decrease in threonine intake results in increased gut permeability within 2 weeks, including a reduction in aminopeptidase activity. In line with these effects, multiple studies have demonstrated that reducing dietary threonine impairs mucin synthesis. Law et al. showed that deficient dietary threonine resulted in decreased mucin synthesis but also structurally affected the mucin, suggesting these piglets’ gut function could be compromised by the threonine deficient diet. These results are similar to those demonstrated in rats, where dietary threonine restriction specifically impairs mucin synthesis.

Clinically, the most relevant sign of threonine deficiency that has been observed is diarrhoea in piglets. In terms of growth however, some studies suggest no significant weight impairment when piglets are provided with a moderately deficient threonine intake (30% less than requirement) for 2 weeks. However, other piglet studies have demonstrated an effect of threonine intake on weight, with deficient intakes resulting in significantly lower weight gain. Although we do not know the fate of infant growth in response to threonine intakes,
from studies using the growing piglet model, we can interpret that a threonine-deficient diet may reduce the availability of threonine for tissues other than the GI tract\textsuperscript{62,79}. This may potentially compromise growth of extra-intestinal organs.

Overall, threonine deficiency directly impacts villi, mucin production and gut barrier function. Human studies investigating the effects of threonine deficiency are non-existent, given the highly unethical nature of such experiments, however, many relevant findings can be extrapolated from these animal models.

### 2.1.4 Threonine Toxicity

As previously mentioned, villous atrophy can occur as a result of not only deficient threonine intakes, but with excessive intakes as well\textsuperscript{29}. Elevated intakes can also lead to increased apoptosis and decreased mucin concentration in weaning piglets\textsuperscript{29}. Early research on the toxicity of threonine points towards the potential for neurological and behavioural effects. In early rat studies, excessive threonine intakes were accompanied by skin lesions, roughly 40\% growth retardation, and suppression of food intake when compared to other amino acids tested in growing rats\textsuperscript{80}. Threonine intakes that were 15 fold higher than requirement have also been associated with increased concentrations of threonine and glycine in the brain as well as in plasma, thus affecting neurotransmitter balance\textsuperscript{81,82}. However, in a study involving more than 350 weanling pigs, threonine intakes as high as 4\% of the diet appeared to contribute to the least amount of growth suppression when compared to similar excessive intakes of methionine, arginine, tryptophan or lysine\textsuperscript{83}.

In humans, experiments using excessive threonine intakes have been conducted in adults for the potential treatment of spasticity in multiple sclerosis\textsuperscript{84,85}. There were no reported toxic effects when at doses of 6-7.5g of threonine per day (approximately 100mg/kg/d) for up to 8 weeks of treatment. This is compared to the current Recommended Daily Allowance for adults at 20mg/kg/d\textsuperscript{12}. However, in healthy adults, a 22.5g dose of threonine administered parenterally produced headaches and backaches as predominant complaints\textsuperscript{86}. 
Although, most of these experiments have been performed in pig and rat models, with only few conducted in humans, it appears that threonine can be provided in relatively high intakes without major outwardly effects, in the short term at least. However, there is no research on chronically elevated threonine intakes, and as previously summarized, multiple gut-related effects of both low and high threonine intakes have been documented. It is therefore important that a requirement be defined empirically in infants and in growing children given the vulnerability of this group.

2.2  Amino Acid Requirements

The methods of determining amino acid requirements have changed with technological advances. These techniques have made advances in precision, feasibility, and reduced invasiveness.

2.2.1 Methods of Determining Amino Acid Requirements

Methods of determining amino acid requirements are based on measuring biological responses to graded intakes of amino acids. Such responses have included growth, nitrogen balance, plasma amino acids, and amino acid oxidation as indices of amino acid adequacy\(^8\). Each biological parameter should in theory provide a similar estimate of amino acid requirements, although displaying different response curves. Descriptions of the various methods are provided below and a summary of their response curves is depicted in \textbf{Figure 3}.

2.2.1.1  Growth and Nitrogen Balance

The first reported findings of threonine requirements in infants was in the 1950s by Pratt et al.\(^5\). By supplying amino acid-based diets deficient in one amino acid, it was possible to determine
the optimal requirement of threonine via a graded intake approach by measuring growth curves, nitrogen retention and plasma protein levels. By examining the nitrogen input from the diet and analyzing nitrogen output in feces and urine, researchers can determine whether the subject was in positive or negative nitrogen balance, indicative of adequate or inadequate indispensable amino acid intake. Although the nitrogen balance method has been widely used to determine amino acid and protein requirements, there are well known drawbacks. There is often an underestimation of true nitrogen excretion as researchers would omit miscellaneous losses of nitrogen (e.g. from skin or secretions), therefore underestimating the amino acid requirement to maintain nitrogen balance. In addition, the urea pool takes approximately 7 days to stabilize to test protein intakes, which is a necessary adaptive component for studies using nitrogen balance. This alone makes nitrogen balance studies difficult to perform. In terms of feasibility, today’s standards would deem it ethically unacceptable to enrol vulnerable populations like infants into a nitrogen balance study, primarily due to exposure to either deficient or elevated amino acid intakes for at least 7 days.

Plasma amino acid response analysis has also been used an indicator of amino acid sufficiency. However, tryptophan is the only amino acid that has been appropriately examined this way. Plasma amino acid levels are not recognized as a sensitive reflection of amino acid requirements since they can be affected by multiple factors including type of diet and by intakes of other amino acids.

2.2.1.2 Amino Acid Oxidation

From here, more direct metabolic measurements of amino acid requirements gained in popularity to try to overcome issues with nitrogen balance and growth. This method is based on the measurement of amino acid oxidation (and protein synthesis) using isotopic-labeled amino acids and offers an estimate of amino acid requirements needed for optimal protein synthesis. Methods of amino acid oxidation work on the premise that amino acids are not stored within the body but are transported into and out of the amino acid pool (Figure 4). Brookes et al. confirmed that once metabolized, amino acids are either broken down and subsequently
oxidized, which can be measured via breath carbon dioxide (CO₂), or they can be incorporated into protein. If one of these indispensable amino acids is limiting in the diet, all others will be in excess in comparison. Excess indispensable amino acids will therefore be oxidized. This oxidation will vary according to how limiting or in excess the test amino acid is in the diet. Altogether, this allows oxidation to be measured via an isotopically labeled indispensable amino acid. An amino acid requirement estimate is therefore established once oxidation levels begin to rise, indicating amino acid intake above requirement due to the attainment of optimal protein synthesis (Figure 3). One of the first amino acid oxidation studies was performed in rats in 1972. The researchers traced the amino acid lysine with a radioactive lysine isotope and measured tracer oxidation in breath.
Figure 3. Biological Response Versus Indispensable Amino Acid Intake

Figure 4. The Body Amino Acid Pool (adapted from)

(A) Indispensable amino acid are not endogenously synthesized
(B) Not all amino acids metabolize via non-protein pathways (e.g. phenylalanine)
* Denotes labeled $^{13}$CO$_2$ recoverable in breath from L-$[1^{-13}C]$phenylalanine oxidation
The two main types of amino acid oxidation are direct amino acid oxidation (DAAO) and indicator amino acid oxidation (IAAO). Young and colleagues used DAAO which uses the same amino acid being tested as the isotopic tracer\(^9^6\)\(^\text{--}^9^8\). For example, a phenylalanine isotope (tracer) used to trace phenylalanine (tracee) oxidation. This produces a response curve where oxidation increases but then plateaus once a requirement value is reached (Figure 3).

This differs from the IAAO which uses an indicator amino acid that is different from the test amino acid in order to trace oxidation levels relative to test amino acid intake\(^3^9\). IAAO arose as a solution to the issues with direct oxidation methods for amino acids with more complex degradation pathways, since many indispensable amino acid do not partition neatly between oxidation and protein synthesis\(^9^9\)\(^\text{--}^1^0^1\). Therefore, using the DAAO method with amino acids such threonine or methionine is inappropriate given that their carboxyl groups do not directly oxidize and appear in breath CO\(_2\)\(^3^8\). It is also impossible to study very small amino acid intakes with DAAO since the isotope provided will always contribute some amount of amino acid. On final drawback of the DAAO method is that the amino acid pool size will change with varying test amino acid intakes, affecting relative oxidation levels (since both the test and isotopically labeled amino acid are the same amino acid)\(^8^7\).

Since all indispensable amino acids are oxidized or incorporated into protein based on the adequate intakes of only one indispensable amino acid, it is acceptable to use another amino acid (indicator) to trace the test amino acid and provide accurate oxidation analysis. In IAAO method studies, the isotopically labeled indicator amino acid (i.e. tracer), must therefore satisfy the following criteria\(^3^8\):

1. Be an indispensable amino acid
2. Have a simple metabolism, where the degradation pathway leads to either CO\(_2\) production or protein incorporation
3. Carboxyl group must degrade to CO\(_2\) irreversibly
The isotopic labeling of an amino acid can occur at numerous elements on the molecule but must be done strategically to be able to observe the traced element in the amino acid pool and metabolism (i.e. CO\textsubscript{2} in breath). It is therefore imperative to know which metabolites stem from which carbons from the original amino acid. L-[\textsuperscript{13}C]phenylalanine is now used most commonly in IAAO studies\textsuperscript{16,17,102,103} and has been shown to be inversely related to protein synthesis, making it ideal for use in the IAAO model\textsuperscript{104}.

Another adaptation of amino acid oxidation methodology is the 24h amino acid oxidation balance method, validated in 1994\textsuperscript{105}. This method arose due to questions regarding the use of the short, 4-hour fed-state method to assess daily amino acid requirements. However, since results are considered similar between methods, and due to the labour intensive and time consuming nature of 24-hour amino acid oxidation studies, the 4-hour IAAO method is acceptable\textsuperscript{38}.

The first IAAO studies were performed in piglets\textsuperscript{99–101} and then in human adults\textsuperscript{48,106}. This method has been refined over the years, abiding to modern ethical standards and becoming suitable for use in infants, including premature infants. The isotopic tracer can now be given orally versus parenterally and still produce identical amino acid requirements, as demonstrated with lysine in adult men\textsuperscript{107}. It is also acceptable to use urine as a substitute for plasma measurements of isotopic enrichment for amino acid flux analysis\textsuperscript{108}, which is ideal for infant populations. To date, the IAAO has been used in preterm and term infants\textsuperscript{16,17,109,110}, school-aged children\textsuperscript{111}, pregnant women\textsuperscript{112}, and the elderly\textsuperscript{113}, and studies can now take less than 24 hours to complete.

One limitation of the IAAO method may be related to its short-duration, as requirements derived in school-aged children do not appear to reflect amino acid needs for growth\textsuperscript{111,114,115}. Since the IAAO method is not a 24h protocol, it may omit the detection of amino acid needs for growth, which in children, primarily takes place at night, as growth hormones follow a circadian pattern of secretion and peak during sleep\textsuperscript{116}. However, this limitation is largely taken care of by adding a component of amino acid deposition for growth to the IAAO derived requirement in children\textsuperscript{111,114,115}.

In 2002, the IAAO method was adapted to incorporate a 24-hour protocol, deemed the 24-hour indicator amino acid oxidation balance, in order to evaluate both fed and fasted states\textsuperscript{117,118}. This
method yields amino acid requirement estimates in adults that are comparable to those derived from IAAO119. Therefore, due to the convenient short-duration and minimal participant invasiveness, the IAAO method provides multiple advantages over the above-mentioned methods, which becomes especially important when dealing with infant populations.

A summary of the main methods used to determine amino acid requirements is provided in Table 2.
<table>
<thead>
<tr>
<th>Method</th>
<th>Strengths</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth</td>
<td>• Easy to collect</td>
<td>• Useful only in active growth states</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Unethical to expose infants to deficient or excessive intakes for long period of time</td>
</tr>
<tr>
<td>Nitrogen Balance</td>
<td>• Traditional method</td>
<td>• Requires roughly 7 days of diet adaptation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Time and resource intensive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Difficult to study same participant multiple times</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Imprecisions in determining all routes of nitrogen loss</td>
</tr>
<tr>
<td>Direct amino acid oxidation</td>
<td>• Non-invasive, short duration</td>
<td>• Oxidized amino acid pool will change size with test amino acid intakes (since they are the same amino acid)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Can only be used to study amino acids with simple metabolism (i.e. that partition between protein synthesis and oxidation)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Cannot study very low intakes of test amino acid, since some amount is supplied as the isotope tracer</td>
</tr>
<tr>
<td>Indicator amino acid oxidation</td>
<td>• Non-invasive, short duration</td>
<td>• May not account for small growth component (i.e. in school-aged children) due to short duration of study\cite{114,115}</td>
</tr>
<tr>
<td></td>
<td>• Can study all indispensable and conditionally indispensable amino acids by use of the same indicator (usually phenylalanine or lysine)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Indicator amino acid pool does not change size with test amino acid intakes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Can study wide range of intakes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Can study multiple intakes in each subject with minimal adaptation</td>
<td></td>
</tr>
<tr>
<td>24h IAAO balance</td>
<td>• Similar advantages as IAAO</td>
<td>• Lengthy study periods, increased invasiveness</td>
</tr>
<tr>
<td></td>
<td>• Captures both fed and fasted states</td>
<td>• Routine adaptation periods of 5-7 days</td>
</tr>
</tbody>
</table>
2.2.1.3 Stable Isotopes

There are no known risks of ingesting or infusing stable isotopes in humans, as they are non-radioactive, and differ only from their most abundant element by their number of neutrons. Stable isotopes differ from their respective most abundant elemental form by one neutron; producing a slight increase or decrease in molecular weight. This difference in weight is what makes stable isotopes ideal for metabolic tracing, as they act and behave exactly like their most abundant isotope but allows researchers to measure parameters like flux by using mass spectrometry methodology. A non-stable nucleus, or one that contains a disruptive excess number of neutrons will emit radioactivity as the nucleus decays with time. There are naturally occurring isotope forms however, which contain a slight excess in neutrons and will remain stable. These stable isotopic forms will therefore not emit any radioactivity. From a research perspective, stable isotopes have been used in humans for over 50 years and have consistently been shown to be safe.

The more common stable isotopes used in human research include hydrogen, carbon, nitrogen, sulfur, and oxygen. They have also been effectively used in IAAO studies, with the first studies in 1993. Carbon-13 (\(^{13}\text{C}\)), which is referred to as an environmental or natural isotope, is most commonly used in the IAAO method and is present in nature at an abundance of 1.11%. This translates to 1.11% of all the carbon in our world being composed of the slightly heavier carbon with an atomic mass of 13, with the other 98.89% made up of carbon-12. To date, the carbon-13 isotopes used in amino acid oxidation research, including paediatric research, present no risk to participants and continues to be a safe and effective tool in mapping out metabolic processes.
2.3 Threonine Requirements

2.3.1 Current Threonine Requirements

Both the current DRIs and FAO/WHO/UNU recommend that amino acid requirements for infants up to 6 months of age be based on intakes from human milk. This is based on an established assumption that for healthy infants, mother’s own milk will provide adequate nutrition as there is no evidence to suggest amino acid or protein deficiencies in healthy, term infants who are exclusively breast-milk fed\textsuperscript{125}. An Adequate Intake (AI) of 73 mg/kg/d, or 436mg/d (6 kg reference weight) of threonine has been set for infants 0-6 months of age\textsuperscript{12}. This AI is based on the average amount of milk consumption in this age group, which is 0.78 L/d, providing an average 11.9g/L protein, and multiplied by the threonine composition in average, mixed breast-milk protein. According to the WHO/FAO/UNU consultation report of 2007, the average threonine concentration in human milk protein is 44 mg/g of protein\textsuperscript{8}. However, it is well known that breast milk composition varies\textsuperscript{9–11}, and current recommendations for amino acid requirements in 0 to 6 month old infants are based on multiple assumptions in lieu of the absence of experimental data. There is therefore a need to define amino acid requirements in infants using alternative methods.

2.3.2 Threonine Requirement Studies in Infants

To date, four studies have determined threonine requirements in term infants and these are outlined in Table 3.
Table 3. Summary of Threonine Requirement Studies in Infants

<table>
<thead>
<tr>
<th>Authors</th>
<th>Study design</th>
<th>Results (mean requirement) (mg/kg/d)</th>
<th>Route</th>
<th>Samples size and infant characteristics</th>
<th>Range of Thr intakes and grades</th>
<th>Study formula used</th>
<th>Isotope used</th>
<th>Protein Intakes (g/kg/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pratt et al. (1954)⁵</td>
<td>Nitrogen Balance &amp; Growth</td>
<td>60</td>
<td>Oral</td>
<td>n=8 1-6 months old</td>
<td>0-180 5 grades</td>
<td>Crystalline amino acids</td>
<td>N/A</td>
<td>3</td>
</tr>
<tr>
<td>Fomon et al. (1973)⁶</td>
<td>Nitrogen Balance &amp; Growth</td>
<td>59 (mg/100kcal)</td>
<td>Oral</td>
<td>n=13 Full term &gt;9 days of age</td>
<td>58-77 (Observed range)</td>
<td>Soy-isolate</td>
<td>N/A</td>
<td>1.68 -1.86</td>
</tr>
<tr>
<td>Chapman et al. (2009)¹⁶</td>
<td>IAAO</td>
<td>32.8</td>
<td>IV/TPN</td>
<td>9 (16 studies) &gt;37.6 wks &lt;28 days old</td>
<td>10-100 16 grades</td>
<td>IV solution</td>
<td>¹³C phenylalanine</td>
<td>2.9</td>
</tr>
<tr>
<td>Hogewind-Schoonenboom et al. (2015)¹⁴</td>
<td>IAAO</td>
<td>68</td>
<td>Enteral</td>
<td>32 infants ≥ 37 wks &lt;1 month old</td>
<td>5-182 32 grades</td>
<td>Crystalline amino acids</td>
<td>¹³C bicarbonate &amp; ¹³C phenylalanine</td>
<td>2.96</td>
</tr>
</tbody>
</table>
Using the nitrogen balance method, Pratt et al. found that 60mg/kg/d of threonine was adequate for growth and nitrogen balance. In eight infants, these researchers provided a formula containing adequate threonine to two infants, and once they established adequate growth and health, they removed or supplemented the threonine in a graded fashion, replacing it by glycine. Subjects that had threonine completely removed from their formula, developed glossitis and redness of their buccal mucosa, which resolved once threonine was added back to their diets. Compared to today’s standards, Pratt et al.’s results should be taken with caution, given the small population size and methodological issues with the nitrogen balance technique\(^8\).

Years later, Fomon et al. examined indispensable amino acid adequacy with whole proteins via a soy-isolate formula, fed to 13 infants over several months. To determine protein status, the researchers used weight, length, and plasma albumin and monitored the infants for 3 months on this ad libidum diet. They concluded that a threonine requirement of 59mg per 100 kcals was sufficient for appropriate growth and albumin status. Authors apparently reported their findings as mg per 100 kcal as this is considerate of both infant growth rate and size. Based solely on an indirect measurement of amino acid need, it is difficult to draw definitive requirements from this study. It became imperative to discover new and more precise ways of conducting such studies. Therefore, the next two studies performed utilized the IAAO method.

In 2009, Chapman et al. set out to determine the threonine requirement in the parenterally-fed neonate. By collecting breath and urine samples in 16 infants, a requirement of 32.8 mg/kg/d (95%CI: 29.9 - 45.2 mg/kg/d) was determined via breakpoint analysis of \([1-^{13}C]\) phenylalanine oxidation. Since it would be erroneous to apply this value to an enteral requirement, it is possible to extrapolate a potential enteral requirement using the findings from a threonine requirement study conducted in the neonatal piglet model. Bertolo et al. demonstrated that the parenteral requirement is 45% of the enteral threonine requirement in neonatal piglets\(^6\), which can be used to propose a calculated threonine requirement for the enterally fed neonate, which would be approximately 72mg/kg/d. This estimate is almost identical to the current DRIs of 73mg/kg/d for the 0-6 month age group\(^1\).

In the last of the threonine requirement studies, Hogewind-Schoonenboom et al. utilized the IAAO approach to determine the optimal enteral threonine requirement in infants less than 1 month of age. 32 neonates were fed a standard amino acid based formula varying threonine from
5 to 182 mg/kg/d and by means of breath collection, determined a threonine requirement of 68mg/kg/d (95% CI: 32 – 104 mg/kg/d). Although they used almost double the population size compared to Chapman et al., their breakpoint confidence and linear fit was much wider ($r^2=0.37$ versus $r^2=0.93$), indicative of a higher variation within the population. This may also be related to the route of feeding, with more variation present when the gut is used during enteral feeding. A number of infants in this population were given I.V. antibiotics for various reasons, therefore potentially affecting individual threonine requirements by altering gut and micro-biotic state.

Diagnoses varied from unconjugated hyperbilirubinemia to pneumonia, and therefore could have affected individual protein and thus, threonine needs. Additional variability may have been introduced by the method of enrichment analysis, the infrared isotope analysis technique, compared to the more sensitive isotope ratio mass spectrometry. However given these limitations, the derived threonine requirement of 68mg/kg/d is only slightly lower than reported intakes of threonine from exclusively breast milk fed infants, which is 76mg/kg/d in the first month of life.

These four studies provide us with the only requirement studies focused on threonine in the infant population. In general, they do provide relatively similar estimates of threonine requirements, despite different populations and methods.

In addition to these studies, threonine requirement values have also been derived from the neonatal piglet model. Bertolo et al. defined threonine requirements in both parenterally and enterally fed piglets by means of a constant infusion of L-[1-$^{14}$C]phenylalanine, and established that the enteral and parenteral requirements are 0.42g/kg/d and 0.19g/kg/d, respectively. Since it is possible to extrapolate a human infant requirement estimate from such studies, as piglets grow ≈ 5x the rate of infants, an estimated threonine requirement would be 84mg/kg/d. However, in this model, the newborn piglet resembled more closely to the preterm than the term infant. This is because in humans, a larger percentage of fetal development occurs in late gestation, and newborn piglets may have different body composition than that of a newborn infant. These neonatal piglets would most closely resemble the preterm infant. In addition, piglets and human infants are highly comparable with regards to gut physiology and digestion. In conclusion, the neonatal piglet-derived requirement would more likely reflect a higher growth rate, and therefore higher protein requirement (and higher threonine requirement), than with the 1 to 6 month old infant.
Based on the above mentioned studies, which include both human infant and piglet threonine requirements, we hypothesized that the enteral threonine requirement for the 1 to 6 month old population would be approximately 73mg/kg/d, equivalent to the current DRI recommendations\textsuperscript{12}. 
3 Rationale, Hypothesis, and Objective

3.1 Rationale

Threonine is an indispensable amino acid that is critical for mucin production, gut permeability and whole body protein synthesis. In neonates, enteral threonine first pass metabolism is as high as 70% and is predominantly used for the production of the threonine-rich glycoproteins. Both deficient and elevated intakes of threonine have been shown to cause villous atrophy and appear to reduce mucin synthesis and muscle protein synthesis in pigs. From animal data, evidence suggests that providing imbalanced threonine intakes may negatively impact the vulnerable and developing infant gut. If provided with inadequate dietary threonine, the gut will spare threonine at the expense of other tissues, therefore potentially limiting growth of extra-intestinal tissues.

In the preterm infant, we know that threonine oxidation is affected by dietary source, with formula-fed infants consistently demonstrating an inability to effectively oxidize threonine, resulting in marked elevations in plasma and urine threonine levels when compared to breast milk fed infants. In addition infant formulas themselves can contain higher concentrations of threonine than the currently recommended adequate intake of 73mg/kg/d for the 0 to 6 month age group. Current recommendations, established by both the Institute of Medicine (Dietary Reference Intakes) and World Health Organization are based on the amino acid composition of mature breast milk. Due to the assumptions involved in these recommendations, researchers have begun to define amino acid requirements using newer methods in infants, which were not available at the time of guideline publications. Thus far, all newly derived estimates for indispensable amino acids have been conducted in the 1 month old infant, using the indicator amino acid oxidation method. These requirements appear to reflect previously established breast milk-derived estimates for most amino acids studied. With threonine in particular, Hogewind-Schoonenboom et al. derived a threonine requirement in 1 month olds to be 68mg/kg/d, whereas intakes of threonine from a 1 month old breast milk fed infant are estimated to be 76mg/kg/d. However, no study to date has defined amino acid requirements using newer methods in infants older than 1 month of age.
The IAAO method has become commonly used to determine amino acid requirements across the lifespan. This method has been adapted to be used safely and efficiently in paediatric populations as the stable-isotope tracer is delivered enterally\textsuperscript{108}, urine enrichment can be used as a surrogate for plasma\textsuperscript{107}, and study time is kept minimal\textsuperscript{13}. For obvious ethical reasons, infants should not be exposed to inadequate or excessive intakes of amino acids for extended periods of time, therefore, IAAO is the method best suited to estimate an enteral threonine requirement in pediatric population.

No studies to date have attempted to determine enteral threonine requirements in infants between 1 to 6 months of age. This population encompasses infants after the neonatal period (28 days of life) as growth and thus amino acid needs decrease rapidly\textsuperscript{8}, up to the age recommended for exclusive breastfeeding, at 6 months\textsuperscript{127}. Defining threonine requirements in this infant age group would be the next step in establishing amino acid requirements for the 0-6 month range.
3.2 Objectives

The main objective of this thesis was to determine the enteral threonine requirements in infants aged 1 to 6 months using the indicator amino acid oxidation method.

3.3 Hypothesis

It is hypothesized that the mean requirement derived from breakpoint analysis will be approximately 73mg/kg/d for this 1 to 6 month infant population.

This was the first infant enteral amino acid study performed in our lab as well as at The Hospital for Sick Children. Unforeseeable obstacles related to research ethics board (REB) regulation of this study contributed to significant delays in recruitment and study progression. Chapter 4 of this thesis will therefore describe the multiple ethical steps involved both prior to and during study enrolment and will describe the various methodological procedures that were established in an effort to set the groundwork for this study as well as future paediatric enteral amino acid requirement studies. Chapter 5 will then describe the study methods approved by the REB which were utilized to fulfill the above mentioned objective and ultimately to define a threonine requirement in the 1 to 6 month old infant population.
4 Method Optimization and New Ethics Responsibilities for Infant Enteral Amino Acid Requirement Studies

4.1 Introduction

This threonine requirement study was the first enteral amino acid requirement study conducted by our lab. Neonatal parenteral amino acid requirement studies were previously conducted by members of our lab almost 10 years ago\textsuperscript{15,17,109}. However, since this time, The Hospital for Sick Children has established much more rigid ethical research obligations. Specifically, the treatment of the study formula and amino acids became highly regulated, similarly to a drug in a clinical trial. The design of this study and ultimately what allows us to determine the threonine requirement in this population involves providing infants with an amino acid based infant formula, containing varied test threonine intakes, that also includes a stable L-[\textsuperscript{1}\textsuperscript{13}C] phenylalanine isotope. To this, a standard protein-free formula powder (ProPhree, Protein-Free Energy Module, Abbott Nutrition) is added to provide a complete pediatric nutrition source. All 18 L-amino acids and the [1-\textsuperscript{13}C] phenylalanine stable isotope were controlled as “investigational products”, similarly to pharmaceuticals.

In addition to ethics-related delays, recruitment of eligible infants was far below expectations. A summary of the original inclusion and exclusion criteria for this study is provided in Table 4. Infants were recruited if born at 40 +/- 3 weeks, were between 1 to 6 months old at study entry, on commercial infant formula, clinically stable, and receiving full enteral feeds. However, as recruitment fell short of expectations, amendments were put through in an effort to broaden eligibility criteria. The following sections will outline the various ethical aspects this study encountered, and the efforts made to broaden inclusion and exclusion criteria and to allow for the study to progress as smoothly as possible. In addition, multiple method optimization projects were conducted.
Table 4. Original subject inclusion and exclusion criteria.

<table>
<thead>
<tr>
<th>Inclusion</th>
<th>Exclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>• 1-6 months old</td>
<td>• Supplemental oxygen</td>
</tr>
<tr>
<td>• Born at term +/- 3 weeks</td>
<td>• Mechanical ventilation</td>
</tr>
<tr>
<td>• Commercial infant formula</td>
<td>• Endocrine or genetic anomalies</td>
</tr>
<tr>
<td>• Clinically stable (blood and vitals)</td>
<td>• Medications that may influence protein and amino acid metabolism (ex: corticosteroids)</td>
</tr>
<tr>
<td>• Receiving feeds via enteral feeding tube (NG, G)</td>
<td></td>
</tr>
</tbody>
</table>

4.1.1 Research Ethics Board Obstacles and Responsibilities

The original protocol for this threonine requirement study was submitted and approved by the SickKids research ethics board (REB) in March of 2014 with a tentative start date of October 2014. However, due to suggested issues related to patient safety along with the amino acid formulation containing a stable isotope, the REB sought regulation from Health Canada and thus made the study a compulsory “clinical trial”. This came as a major surprise to our lab since stable isotopes have been used in paediatric research for over 30 years^{128,129}. Specifically, IAAO studies are 1) not clinical trials; 2) contain no pharmaceuticals, drugs or substances other than dietary components; and 3) have been conducted safely at every stage of the lifecycle including neonates^{16-18}, infants^{28,130}, children^{114,115,131}, pregnant women^{112}, and the elderly^{132,133}. Health Canada regulation had never previously been required for amino acid requirement studies. It was therefore required that the study team provide an “investigator’s brochure”, including detailed information about the amino acid powders to be used, as well as potential adverse reactions that may arise from their use. Therefore, the amino acids and stable L-[1-^{13}C]phenylalanine isotope were to be controlled and regulated as pharmaceuticals, similar to any drug trial. In recent years, the SickKids REB has also introduced a “study activation process” (SAP) as a new hospital wide procedure and required for all Health Canada regulated trials. Therefore, not only was REB and Health Canada approval required, but also a SAP, which involved numerous meetings and sign
offs by the Research Quality and Risk Management (RQRM) team prior to official study activation. In sum, the entire REB, RQRM, and Health Canada process lead to numerous delays (approximately nine months for three amendments). In addition, whenever the study protocol or consent forms were edited (in any way), it is mandatory that in addition to the REB, Health Canada must provide final approval, which adds a minimum one extra month to the approval process. Finally, the study received full REB and Health Canada approval in February 2016, which was two years after original REB approval.

However, as recruitment advanced, it quickly became apparent that the numbers of potential participants was lower than previously expected. It was initially anticipated that roughly two infants per month would meet the original inclusion and exclusion criteria. After multiple months of a lack of eligible recruits, a protocol amendment was made to broaden the criteria by incorporating all types of tube feedings (nasogastric, gastric, and jejunostomy tube) and removing the need of “commercial infant formula” as the infant’s feeds. One limitation of including J-tube fed infants is that their individual requirement for threonine could potentially be affected by the fact that the study formula would have less contact time with the gut than with a G-tube fed baby. It was also decided to include infants on any kind type of nutrition source, including formula and breast milk. This was done mainly since multiple infants were previously excluded because they were consuming some amount of breast milk. It was also decided to provide the isotope tracer along with the formula, instead of administering it as a separate solution. This was done to minimise any work to be done by nursing staff, and to ensure that the isotope is more likely to be given since it will be mixed with the study formula. Lastly, in an effort to reduce invasiveness and encourage study enrolment, the study diet adaptation day was removed. Study diet adaptation is a standard procedure in IAAO studies, which serves to provide a similar background enrichment of $^{13}$C amongst subjects, and in adults, adapts subjects to similar protein intakes. It was decided to remove this extra day of study to reduce the entire study participation time to 24 hours. Since different diets will result in different background enrichments of $^{13}$C, the decision to remove the adaptation period may result in higher variability amongst measurements of background and plateau isotopic enrichment. By July 2016, both the SickKids REB and Health Canada approved the amendment.

However, another dry spell of eligible participants ensued, and therefore prompted another protocol amendment to further broaden inclusion and exclusion criteria. It was decided to include
patients who were born between 32 and 43 weeks gestational age, provided they were at least at 1 month corrected age at the time of enrolment. There were also a number of potential subjects who were consuming some amount of oral feeds in addition to their tube feeds. Although the staff surgeons were comfortable halting the patients’ oral feeds for the 24h duration of the study, the infants’ parents were reluctant and believed the study would hamper their child’s oral feeding progress. In an effort to accommodate parental wishes, it was decided to allow patients who were also consuming some oral feeds in addition to their tube feeds as part of the inclusion criteria. However, before this could be done, an infant formula taste panel was conducted to verify whether the lab-manufactured study formula compared in terms of palatability to other amino acid-based formulas commonly used at SickKids. The results of this study are described further below.

4.1.2 Study Diet Calculation Development

As this was the first enteral amino acid requirement study in infants conducted in our lab, considerable thought went into the development of study formula calculations. These calculations would set the groundwork for all future enteral amino acid requirement studies. In infant nutrition, one of the most important factors after ensuring the provision of adequate amounts of calories, protein and nutrients, is guaranteeing precise total fluid intakes (TFI). Therefore the calculations were designed to ensure that the following elements were taken into account:

- Total fluid intake prescribed for each infant (milliliters per kg)
- Pro-phree powder volume displacement (0.72 milliliters per g)
- Nitrogen required by alanine to maintain each formula iso-nitrogenous
- Priming and overfilling volumes (milliliters) (Table 5)
Based on Table 5, a standard overfill volume of 250ml was decided upon, in order to take into account any losses that arise from priming the enteral pump, and miscellaneous losses. The full study diet calculations can be found in Appendix E. Before each study, calculations were first performed by hand, then confirmed by an excel spreadsheet version of the calculations.

Table 5. SickKids priming guidelines for continuous enteral feeds.

<table>
<thead>
<tr>
<th>Continuous feed volume</th>
<th>Feed type</th>
<th>Extra needs for pump priming</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 10ml/hour</td>
<td>Expressed breast milk</td>
<td>15ml/d</td>
</tr>
<tr>
<td></td>
<td>Powdered formula</td>
<td>30ml/d</td>
</tr>
<tr>
<td>11-25ml/hour</td>
<td>Expressed breast milk</td>
<td>35ml/d</td>
</tr>
<tr>
<td></td>
<td>Powdered formula</td>
<td>70ml/d</td>
</tr>
<tr>
<td>&gt; 25ml/hour</td>
<td>Expressed breast milk</td>
<td>95ml/d</td>
</tr>
<tr>
<td></td>
<td>Powdered formula</td>
<td>190ml/d</td>
</tr>
</tbody>
</table>

4.1.3 Study Diet Manufacturing Procedures

As this was a Health Canada regulated study, multiple safety measures were put into place to ensure maximal hygiene and infant food-service grade standards. Figure 5 illustrates the steps involved in manufacturing the study formula prior to each study. The formula was manufactured in two areas within the hospital: our lab’s metabolic kitchen and the milk prep room (MPR) in the patient food service department of The Hospital for Sick Children. The main reasons for this were that specific pieces of equipment were used (i.e. analytical balance in the metabolic kitchen and blender in the MPR). Therefore, approval was obtained from SickKids infection control as well as from food services in order to use the metabolic kitchen for study formula preparation. Multiple changes were put into effect in our lab’s metabolic kitchen. During formula production hours, all traffic into the room was interrupted to minimize air disruptions. Similarly to MPR
procedures, all surfaces were degreased and sanitized using a food-grade degreaser (Oasis 137 Orange Force, Ecolab Co) and quaternary ammonia (200 ppm) sanitizer (Ster-Bac, Ecolab, Co), and all related glassware was replaced by temperature resistant plastic as demanded by the SickKids manager of patient food services. All formula related equipment was washed and sanitized using the MPR high temperature dishwasher (wash at 66°C for 2 minutes, rinse at 86°C for 20 seconds*). The addition of the 0.2micron filtering was put in place to provide an extra barrier against potential pathogenic contamination subsequent to amino acid weighing and mixing (Figure 5).


Figure 5. Pre-study formula manufacturing procedures.
4.1.4 Method Optimization Procedures

In an effort to ensure that the present threonine requirement study, and all future enteral requirement studies are conducted in an efficient and rigorous manner, two method optimization procedures were conducted. These occurred both prior to and during study progress and recruitment.

4.1.4.1 Amino acid formula analysis

Due to the need of filtering the amino acid solution using a 0.2 micron filter, ultra performance liquid chromatography (UPLC) analyses were performed to 1) observe any effects of pre and post-filtration on amino acid concentrations and 2) to determine whether what was physically weighed and dissolved was in fact present and detected by UPLC. This was done to confirm that infants would be receiving appropriate amounts of amino acids throughout the study day.

In amino acid requirement studies performed in our lab, a “bulk” amino acid mixture is typically weighed out which contains all amino acids which mirror the composition of egg protein, minus the subject-specific or variable amino acids (i.e. test amino acid, phenylalanine, and tyrosine). This is done to drastically cut down on time required to prepare study formulas prior to each study. In this case, the study formula is comprised of individual crystalline amino acids and designed to mimic breast milk amino acid composition\(^8\). Since the basis of this study is the manipulation of only one of the amino acids (threonine), the concentrations and proportions of all other amino acids must remain unchanging.
4.1.4.2 Infant formula taste panel

To broaden eligibility criteria, it was decided to accommodate infants who may have begun progressing to oral feeds. It is well recognized that amino acid based formulas are unpalatable and are often described as bitter tasting\textsuperscript{134} and that general palatability decreases with increasing protein hydrolysis\textsuperscript{135}. In order to provide the study formula orally, in addition to tube feeds, it was required to compare our study formula to other formulas most commonly used at SickKids. This was done by conducting an evaluative sensory taste panel in adult volunteers.

4.2 Methods

4.2.1 Amino acid formula analysis

Sample preparation and derivitization was performed by the SPARC Biocenter at SickKids. Samples were run using a UPLC System (Waters Acquity, Milford, Massachusetts, USA). Briefly, chromatography allows for the separation of mixtures of substances by molecular size and composition. A mobile phase (solvent) flows with the mixture along a stationary (column) phase. Depending on the type of solvents and columns used, mixtures will be separated based on the affinity of the analytes that make up the mixture. UPLC differs from traditional HPLC in that it allows for better separation in much shorter periods of time, with a higher pressure capacity. Samples of both pre and post 0.2 micron filtered amino acid solutions were run with a modified PICO-TAG gradient using a Waters Acquity UPLC BEH C18 column (2.1 X 100 mm) and a tunable UV detector. Data was collected, stored and processed using Waters Empower 3 Chromatography software. According to the manufacturer (Waters), precision was 0.5% relative standard deviation (i.e. coefficient of variation).
4.2.2 Infant formula taste panel

A blinded sensory taste panel was organized to determine how the study formula compared to two commercially available elemental formulas used at SickKids: Puramino A+ (Enfamil, Mead Johnson Nutrition) and Neocate (Nutricia, Nutricia North America). To conduct a formula taste panel in infants would require the involvement of the SickKids REB, resulting in inevitable time delays. Therefore it was decided to use the adult, allowing recruitment to take place in a short period of time. Infants are capable of tolerating both Puramino and Neocate by mouth, therefore by comparing the study formula to the two formulas in terms of taste, odour, and texture, it can be determined whether it too could be tolerated by infants. Since adult participants would be comparing the infant formulas to each other, it was deemed unnecessary to conduct this study in infants. Similar sensory taste panels have also used the adult instead of the infant to observe differences amongst infant formulas\textsuperscript{134,136}.

The taste panel took place in the Peter Gilgan Center for Research and Learning at SickKids in January 2017. In total, 17 panellists were recruited, consisting of graduate students and employees. Three small 2 oz glasses were provided, each filled with one of the three formulas and marked with a blind label. A structured taste panel questionnaire was provided, encompassing mainly Linkert-style questions (Appendix D). The questionnaire included ranking formulas using a 4-point scale (1 = poor, 2 = fair, 3 = good, 4 = excellent) for texture, odour, and taste, in addition to questions about formula comparisons and general palatability.

4.3 Results

4.3.1 Amino acid formula analysis

Table 6 summarizes the changes in amino acid composition after filtering with the 0.2micron filter. Amino acid changes were less than 4% for all amino acid except glycine and tyrosine, which are both derived from the GLT di-peptide. Cysteine, glycine and tyrosine were found in higher concentrations after filtration took place. Table 7 provides a summary of the differences between the amino acids that were weighed from the amino acid bulk mixture compared to what was detected by UPLC. Multiple differences were observed. Cysteine, glutamic acid and aspartic
acid were -122.2%, -44.7%, and -30.1% less than expected, respectively. Similarly, both glycine and tyrosine derived from the glycyll-L-tyrosine were -14% and -20% less than expected. Conversely, tryptophan was 50% higher than expected. All other amino acids varied from -4.2% to 16% from expected.

Table 6. Change in amino acid composition between pre and post micron filtration

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Post-Filtration change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-aspartic acid</td>
<td>-2.16</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>-2.26</td>
</tr>
<tr>
<td>L-Serine</td>
<td>-3.43</td>
</tr>
<tr>
<td>Glycine (additional)†</td>
<td>-3.57</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>-3.72</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>-3.70</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>-3.62</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>-3.41</td>
</tr>
<tr>
<td>L-Proline</td>
<td>-2.90</td>
</tr>
<tr>
<td>L-Valine</td>
<td>-3.41</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>-3.24</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>3.21</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>-3.14</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>-2.91</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>-1.82</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>-3.05</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>-0.96</td>
</tr>
<tr>
<td>Glycine (total)‡</td>
<td>9.11</td>
</tr>
<tr>
<td>Tyrosine§</td>
<td>8.95</td>
</tr>
</tbody>
</table>

† to supplement the glycine coming from GLT  
‡ sum of glycine from GLT and added glycine  
§ coming from GLT
Table 7. Differences between weighted and UPLC detected amino acid composition.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Weighed in Diet Kitchen</th>
<th>UPLC</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g</td>
<td>g</td>
<td>%</td>
</tr>
<tr>
<td>L-Aspartic Acid</td>
<td>4.1414</td>
<td>3.0584</td>
<td>-30.08</td>
</tr>
<tr>
<td>Glycine (additional)†</td>
<td>0.0667</td>
<td>0.0784</td>
<td>16.07</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>0.7823</td>
<td>1.3042</td>
<td>50.03</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>4.4175</td>
<td>4.7523</td>
<td>7.30</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>0.9936</td>
<td>1.0150</td>
<td>2.13</td>
</tr>
<tr>
<td>L-[1-13C] Phenylalanine</td>
<td>0.8964</td>
<td>0.9200</td>
<td>2.60</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>0.7363</td>
<td>0.8009</td>
<td>8.41</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>2.5309</td>
<td>2.6921</td>
<td>6.17</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>0.9663</td>
<td>1.0519</td>
<td>8.48</td>
</tr>
<tr>
<td>L-Serine</td>
<td>2.3008</td>
<td>2.3771</td>
<td>3.26</td>
</tr>
<tr>
<td>L-Glutamic Acid</td>
<td>8.1909</td>
<td>5.1977</td>
<td>-44.71</td>
</tr>
<tr>
<td>L-Valine</td>
<td>2.5309</td>
<td>2.8584</td>
<td>12.16</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>1.9500</td>
<td>2.0836</td>
<td>6.62</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>1.0584</td>
<td>1.1266</td>
<td>6.25</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>1.9500</td>
<td>1.9860</td>
<td>1.83</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>3.9666</td>
<td>3.9618</td>
<td>-0.12</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>0.7823</td>
<td>0.1886</td>
<td>-122.28</td>
</tr>
<tr>
<td>L-Proline</td>
<td>3.6813</td>
<td>3.5282</td>
<td>-4.25</td>
</tr>
<tr>
<td>Glycyl-L-Tyrosine*</td>
<td>3.1475</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Glycine (total)‡</td>
<td>1.057</td>
<td>0.9187</td>
<td>-14.00</td>
</tr>
<tr>
<td>Tyrosine§</td>
<td>2.3930</td>
<td>1.9568</td>
<td>-20.06</td>
</tr>
</tbody>
</table>

† to supplement the glycine coming from GLT  
* did not analyze dipeptide concentrations  
‡ sum of glycine from GLT and added glycine  
§ coming from GLT
4.3.2 Infant formula taste panel

The primary questionnaire results are summarized in Table 8. Calculated as median values, participants were in general agreement that Neocate was the formula with the best odour and best taste, while the study formula and Puramino were a close tie. Taste between the study formula and Puramino were apparently similar, while the study formula texture was similar to that of Neocate. Participants were also asked to rank which of the three formulas was “Best”, “Neutral” or “Worst”. The consensus indicated that Neocate was the best, the study formula was neutral, and Puramino was worst overall. Participants were then asked, “if infants can tolerate (Puramino) and (Neocate) by mouth, could they also tolerate (study formula) by mouth?” 94% of participants selected YES. In addition, a comments section was made available and the following were the major comments included:

- (Puramino) had a metallic after taste
- (Puramino) and (Study Formula) tasted like mouse food
- (Neocate) tasted very bad
- (Puramino) was definitely the worst
- They were all very similar but perhaps (Puramino) was better than the Study Formula and (Neocate)
- (Study Formula) and (Neocate) are actually very similar

<table>
<thead>
<tr>
<th></th>
<th>Neocate</th>
<th>Study Formula</th>
<th>Puramino A+</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Odour</strong></td>
<td>Excellent</td>
<td>Fair</td>
<td>Fair</td>
</tr>
<tr>
<td><strong>Taste</strong></td>
<td>Excellent</td>
<td>Good</td>
<td>Good</td>
</tr>
<tr>
<td><strong>Texture</strong></td>
<td>Good</td>
<td>Good</td>
<td>Fair</td>
</tr>
</tbody>
</table>

†Displayed as median responses, with inter quartile ranges ≤1
4.4 Discussion

4.4.1 Amino Acid formula analysis

In order to verify amino acid composition of the lab-manufactured study formula, UPLC analyses were performed to identify any differences in amino acid concentrations caused by the process of 0.2 micron filtration, and differences between expected and actual amino acid concentrations after weighing. Upon analyzing the concentrations of amino acids present in solution both pre and post micron filtration, amino acid concentrations were consistently less than expected (Table 6). Amino acid changes were less than 4% for all amino acid except glycine and tyrosine, which are both derived from the GLT di-peptide. The overall differences in amino acid concentrations were higher than the established 0.5% precision for this UPLC method. However, precision testing was not performed by personnel at the SPARC center themselves, but provided from the UPLC manufacturer, and therefore may be different for the present analysis. Despite this, the differences between pre and post filtration appear to be consistent and would not be expected to be clinically relevant. It is unclear why both the tyrosine and glycine concentrations increased after filtering, but this may have to do with the sample preparation prior to UPLC analysis, as it is the only di-peptide in the solution. However, if this increase in glycine and tyrosine is in fact true, it is less of a concern as providing excess tyrosine is an important component in IAAO studies using phenylalanine as the indicator amino acid. Realistically, however, a 0.2 micron filter should not retain any amino acid residues, since amino acid sizes vary between 57 (glycine) and 186 (tryptophan) Da, whereas 0.2 microns is equivalent to 10,000,000 Da.

When analyzing the amino acid solution to compare what was physically weighted versus what was analyzed by UPLC, multiple disparities were observed (Table 7). L-aspartic acid was found to be 30.1% less than expected and L-glutamic acid 44.7% less than expected. This can be explained either by in-solution conversions to asparagine and glutamine, or by true errors in amino acid content sampled from the amino acid bulk mixture. On the contrary, L-tryptophan was 50% higher than expected, which again may be explained by issues with amino acid bulk homogeneity. One of the largest differences was observed with L-cysteine, at 122.3% less than expected. However, this may be a result of cysteine dimerization to form cystine (two cysteine
molecules bonded by a disulfide bridge), which can readily occur\(^1\). Both glycine and tyrosine appeared to be 14% and 20% less than expected, respectively. This discrepancy with glycine appears to be related to the glycine derived from GLT, as the additional glycine alone was 16% higher than expected. Issues do arise with tyrosine concentrations, as this is an especially important amino acid in IAAO studies and therefore providing enough of this amino acid is crucial. All other amino acids appeared to differ by roughly 1 to 16%, with many appearing to be higher than what was apparently sampled from the bulk mixture.

Based on these findings, it was therefore decided to omit making a bulk amino acid mixture since issues of amino acid homogeneity could not be ruled out. This decision was based on the results described above, but also for pragmatic considerations. Even with adequate mixing prior to weighing (our standard protocol), it appears that discrepancies still occur (Table 7). Whether this is due to true differences in amino acid content or issues with UPLC detection of the specific amino acid, the most reliable and accurate method to ensure correct amino acid content in each infant formula would be to weigh out each individual amino acid prior to every study. The importance of providing appropriate amounts of amino acids in the pattern of the reference breast milk composition are critical, and concentrations need to be kept as consistent as possible between subjects. Therefore, weighing out each amino acid prior to studies would result in the most consistent amino acid formula between subjects. As subject recruitment was not as abundant as expected, the added time required to weigh out individual amino acids prior to each study was not an issue and therefore was implemented as a new, standard procedure for this infant study. The next step would be to conduct UPLC analysis of an individually weighed amino acid mixture, however, this has yet to be performed.

### 4.4.2 Infant formula taste panel

The goal of this taste panel was to determine how the study formula compared to commercially available amino acid-based formulas most commonly used at SickKids. Overall, it appears that our study formula lies somewhere in the middle, between the apparently palatable Neocate, and the lesser appealing Puramino. The number of panellists appeared to be sufficient, given that inter quartile ranges of all categories was ≤1, indicative of general consensus amongst responses. Additional comments provided by panellists were unique and varying, and it is not possible to
decipher which comments were due to the general taste of infant formulas in relation to adult
taste preferences or due to true differences between the formulas themselves. However, one
limitation is that formal determination of an appropriate number of panellists was not conducted,
therefore hindering the confidence of these results. Although the adult has been used in studies of
infant formula palatability\textsuperscript{134,136}, there are assumptions involved in using the adult to provide
information that would then be applied to the infant, relating to differing taste perceptions.
Infants generally prefer sweeter tastes and have more of an aversion to bitter (which is a flavour
identified with amino acid based formulas)\textsuperscript{138,139}. Ideally, an infant taste panel would have
provided the most suitable and applicable information regarding the palatability and acceptance
of infant formulas. Such studies utilize validated sensory evaluation methods designed
specifically to analyze infants’ hedonic responses to various sources of nutrition\textsuperscript{140,141}. Although
a palatability study was not performed in infants, from this adult study alone it is possible to
conclude that 1) the study formula appears to compare to current commercially available
formulas and 2) infants should be able to tolerate the study formula if they are capable of
tolerating commercial amino acid-based formulas.
4.5 Conclusion

Since an enteral amino acid requirement study had never been performed in our lab, multiple methodological and ethical aspects needed to be considered. First, the research ethics process at SickKids has changed over time, becoming more rigorous in an effort to ensure patient safety, which is to be expected. However, due to the REB’s reluctance regarding the safety of providing amino acids as well as a stable isotope to hospital patients, Health Canada regulation was requested. This extra level of ethical conduct was a new experience for members of the study team, and unfortunately added to the delays experienced both in attempt to begin study enrolment and for subsequent amendment approvals as well. We will therefore be better prepared to move forward with regards to the implementation of future paediatric amino acid requirement studies.

Method optimization projects were also conducted in an effort to facilitate study progress, broaden eligibility criteria, and to resolve any methodological uncertainties. First, detailed study formula calculations were carefully derived, which can be used in future enteral amino acid requirement studies. It was then determined that the best course of action would be to individually weigh out each amino acid immediately prior to studies to ensure that infants received precise and adequate intakes similar to the reference breast milk amino acid composition. Lastly, the taste of the amino acid based study formula was comparable to commercially available amino acid formulas, which therefore provides an option to parents wishing to maintain progression of their infants’ oral intake. Altogether, this background work provides a means to best implement enteral amino acid studies in infants, which includes the current threonine requirement study, of which recruitment is still active and ongoing.
5 A Physiological Study to Determine the Enteral Threonine Requirements in Infants Aged 1 to 6 Months: Preliminary Results

The following chapter outlines the actual study conducted to meet the main objective of this thesis and to meet the requirement for a master’s degree set by the Department of Nutritional Sciences at the University of Toronto.

5.1 Study Methods

5.1.1 Subjects

Recruitment began in February 2016 and is still in progress at The Hospital for Sick Children, Toronto Canada (SickKids). To date, two infants have been enrolled and have participated fully in this study. Each participant completed one threonine intake study. The original study protocol stated that 18 graded threonine intakes would be examined, implying that at least nine infants would be recruited, assuming they each perform two different intake studies. Should this be the case, the subject’s second study would be randomized to a threonine intake on the opposite side of the range of intakes in order to eliminate any risk that an infant be consecutively randomized to two potentially low or high threonine intakes.

The Hospital for Sick Children Research Ethics Board (REB) provided full study approval, and due to the nature of the study (i.e. vulnerable population and involving isotopes), Health Canada also provided “No Objection” to this clinical trial. Written approval was obtained from the most responsible physician/surgeon and written informed consent was collected from at least one parent prior to enrolment.
5.1.2 Inclusion and Exclusion Criteria

Recruitment took place in three main wards at The Hospital for Sick Children: the Neonatal Intensive Care Unit (NICU), wards 5B (General Surgery, Urology & Gynaecology) and 8C (Burns & Plastics, Ophthalmology, Surgical Short Stay). Infants were recruited if they were 1 to 6 months old (correct gestational age), born between 32 and 43 weeks gestational age, clinically stable as per blood values and vital signs, and receiving full enteral feeds via feeding tube. In terms of their diet, infants were recruited if receiving protein intakes between 2.6-3.0 g/kg/d and energy intakes ≥80 kcal/kg/d. Infants were excluded if they were receiving supplemental oxygen or mechanical ventilation, had any endocrine or metabolic conditions affecting protein or amino acid metabolism, and if they were on any medications affecting protein and amino acid metabolism (i.e. corticosteroids) (Table 9). One final consideration in recruitment was intestinal function since threonine is highly metabolized by the gut. Therefore, potential recruits were comprised of patients with diagnoses of diaphragmatic hernia, Pierre-Robin sequence, and tracheoesophageal fistula, all of which have no apparent lower-GI involvement.

Table 9. Subject inclusion and exclusion criteria.

<table>
<thead>
<tr>
<th>Inclusion</th>
<th>Exclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-6 months old</td>
<td>Supplemental oxygen</td>
</tr>
<tr>
<td>Born between 32 and 43 weeks gestational age</td>
<td>Mechanical ventilation</td>
</tr>
<tr>
<td>Clinically stable (blood and vitals)</td>
<td>Endocrine or genetic anomalies that influence protein and amino acid metabolism</td>
</tr>
<tr>
<td>Receiving feeds via enteral feeding tube (NG, G, or J tubes)</td>
<td>Medications that may influence protein and amino acid metabolism (ex: corticosteroids)</td>
</tr>
</tbody>
</table>
5.2 Experimental Design

The study design used the minimally-invasive IAAO method\textsuperscript{38}. This method is based on the premise that amino acids are not stored within the body. When metabolised, amino acids are either broken down and oxidized or they can be incorporated into protein. Optimal protein synthesis occurs only once all indispensable amino acids are present in sufficient quantities, limiting oxidation (\textbf{Figure 4}). If threonine were limiting in the diet, all other indispensable amino acids would be in excess and would be oxidized since optimal protein synthesis is halted due to an inadequate threonine intake. As threonine intake approaches levels that are required for optimal protein synthesis, oxidation would decrease accordingly until a plateau is reached, demonstrated by no further change in oxidation as threonine intakes increase (\textbf{Figure 3}). Since the oxidation of all indispensable amino acids depends on the limiting amino acid (threonine), phenylalanine will be the indicator of how oxidation and protein synthesis will change at graded intakes of threonine intake. We know this because phenylalanine oxidation has been shown to be inversely related to protein synthesis\textsuperscript{104} and is also the most commonly used indicator in IAAO studies\textsuperscript{16,17,102,103}.

Each study took 24 hours to complete. \textbf{Figure 6} provides an overview of the study protocol. Infants were monitored for approximately 48 hours before study initiation and the study was initiated once the clinical dietitian (RD) confirmed appropriate intakes of protein and calories, and adequate weight gain. Baseline measurements were collected on the study day between 9h00 and 12h00. These included weight, length, head circumference, and baseline urine and breath samples of isotopic enrichment. The study formula and L-[\textsuperscript{1-13}C]phenylalanine isotope were administered beginning at 12h00 in a continuous feeding pattern using enteral feeding pumps (Infinity Orange, Zevex), of which the infants were already using clinically. After 12 hours of formula and isotope administration, urine sample collection began, followed by breath collection \textbf{Figure 6}.
5.3 Study Diet

Infants were placed on the study formula for 24 hours. Calories, protein, and fluid intakes were kept as prescribed by either the attending surgeon or dietitian on the ward, given that they conformed to eligibility criteria. The study formula was a crystalline amino acid based formula manufactured in our lab’s metabolic diet kitchen and in collaboration with the Milk Prep Room (MPR) of the SickKids hospital kitchen. The amino acid composition of the study formula was based on the amino acid profile of mature breast milk according to the FAO/WHO/UNU 2007 guidelines8. Table 10 lists the types and amounts of amino acids used in the study formula. Amino acid composition was kept constant amongst studies except for the following which varied with each study: threonine, alanine, phenylalanine, and glycyrl-L-tyrosine. As the test amino acid, threonine varied by a graded range of intakes between 15 to 130 mg/kg/d. The first two subjects enrolled were purposefully studied at the extremes of this range in order to observe...
whether an appropriate range was chosen. The next 10 studies were to be conducted at random using the following graded intakes:

\[25 - 35 - 45 - 55 - 65 - 75 - 85 - 95 - 105 - 115 \text{ mg/kg/d}\]

Once 12 studies were completed and the data analyzed, the last six intakes would be studied in a strategic manner, and likely placed closest to the hypothesized breakpoint of 73mg/kg/d.

In terms of other variable diet components, alanine, a dispensable amino acid, was adjusted to maintain the formula as iso-nitrogenous. The dietary phenylalanine was adjusted depending on the intake of \(^{13}\)C phenylalanine isotope, which varies by body weight, and the rest provided as \(^{12}\)C phenylalanine. Lastly glycyl-L-tyrosine (GLT) was used as a means to provide water-soluble tyrosine, since the amino acid mixture needed to be passed through a 0.2 micron filter. In addition to the amino acid component of the study formula, a pediatric protein free formula (ProPhree, Protein-Free Energy Module, Abbott Nutrition) was added as a standard carbohydrate, lipid, and micronutrient component.
<table>
<thead>
<tr>
<th>WHO UNU FAO Breast Milk Composition</th>
<th>Standard breast milk composition using crystalline AAs* in the lab</th>
<th>Study AA bulk mix (Variable AAs removed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>g/kg protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Aspartic Acid</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>(Glycine from GLT)†</td>
<td>[21.5]</td>
<td>[21.5]</td>
</tr>
<tr>
<td>L-Glycine (in addition to GLT)‡</td>
<td>1.45</td>
<td>1.45</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>96</td>
<td>96</td>
</tr>
<tr>
<td>L-Phenylalanine§</td>
<td>42</td>
<td>0</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>55</td>
<td>55</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>L-Serine</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>L-Glutamic Acid</td>
<td>178</td>
<td>178</td>
</tr>
<tr>
<td>L-Valine</td>
<td>55</td>
<td>55</td>
</tr>
<tr>
<td>L-Threonine¶</td>
<td>44</td>
<td>0</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>L-Alanine#</td>
<td>38</td>
<td>0</td>
</tr>
<tr>
<td>L-Lysine – HCL††</td>
<td>86.2</td>
<td>86.2</td>
</tr>
<tr>
<td>(Tyrosine from GLT)‡‡</td>
<td>[52]</td>
<td>[52]</td>
</tr>
<tr>
<td>Tyrosine◆</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>L-Proline</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>Glycyl-L-tyrosine</td>
<td>68.4</td>
<td>0</td>
</tr>
<tr>
<td><strong>Totals</strong></td>
<td><strong>978.05</strong></td>
<td><strong>785.65</strong></td>
</tr>
<tr>
<td></td>
<td>(100%)</td>
<td>(80.33%)</td>
</tr>
</tbody>
</table>
* L-amino acids and glycine (Pharmagrade, Ajinomoto Japan via Milipore Sigma), Glycyl-L-Tyrosine (SAFC Cleveland via Milipore Sigma)  
† Glycine was not added directly to the bulk as it comes from added glycyl-L-tyrosine  
‡ Since glycyl-L-tyrosine was added to provide tyrosine, glycine was added based on what GLT was left after GLT  
§ Phenylalanine was left out of the bulk as it was made up of dietary and L-[1-13C]phenylalanine  
¶ Threonine varied between 15 and 130 mg/kg/d  
# Alanine was adjusted to maintain the formula iso-nitrogenous  
†† Lysine-HCL will be added as the source of lysine as this form has increased solubility  
‡‡ Tyrosine will be added in the form of glycyl-L-tyrosine as tyrosine alone is highly insoluble  
◆ Tyrosine will not be added as it is provided as glycyl-L-tyrosine  
Glycyl-L-tyrosine added to provide water soluble tyrosine  

As per Health Canada regulations, L-Threonine, L-Alanine, L-[1-13C]phenylalanine, and the amino acid bulk mixture were regarded as drug products and therefore were to be treated as regulated pharmaceuticals. This required 24h temperature monitoring and specific labeling measures. Although most amino acids are stable at room temperature, according to manufacturers instructions, L-[1-13C]phenylalanine was kept away from moisture and light, and GLT was kept at -20°C. All other amino acids were kept refrigerated between 2-4°C.

5.4 Tracer Protocol

L-[1-13C]phenylalanine [99 atom percent excess (APE) and 99.9% L-isomer; Cambridge Isotope Laboratories, Andover, USA] was used as the isotope used to trace breath 13CO2 and urine 13C-phenylalanine enrichment. This isotope has been used in numerous IAAO designed studies; in the elderly132, adults102, in school-aged children131 and in infants14,16,109. The tracer was administered as a 15 µmol/kg/h (2.49mg/kg/h) dose given in a continuous fashion for 24 hours in combination with the study formula. As this was the first study of its kind performed in our lab, an optimal isotope dose had not yet been used for this population. Table 11 provides a summary of previous types and amounts of isotopes used in infant populations. None of the studies looked at infants older than 1 month, and some provided isotopes for 24 hours while others for only 7 hours. It was decided that an un-primed 15µmol/kg/h dose would be sufficient for our 1 to 6 month old population, in accordance with Darling et al.’s protocol that provided adequate
measurements of isotope enrichment in urine and breath within 24 hours\textsuperscript{35}. 24 hours of isotope administration was required to ensure adequate urine sample collection at baseline and isotopic plateau. It was also decided to provide this dose in a continuous fashion in accordance with more recent intravenous studies performed by Courtney-Martin et al. and Chapman et al.\textsuperscript{15,18} (Table \textbf{11}). These studies reported that the 24 hour isotope infusion did not cause any apparent isotope recycling, which can occur with prolonged isotope administration\textsuperscript{142}. 
Table 11. $^{13}$C Phenylalanine isotope dosing in previously performed studies

<table>
<thead>
<tr>
<th>Author</th>
<th>Population</th>
<th>Route of infusion</th>
<th>Isotope prime</th>
<th>Prime dose ($\mu$mol/kg)</th>
<th>$^{13}$C Phe Infusion ($\mu$mol/kg/h)</th>
<th>Hours infused</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wykes L. 1992</td>
<td>LBW neonates</td>
<td>Enteral/ Parenteral</td>
<td>$^{13}$C Phe</td>
<td>12</td>
<td>4.8</td>
<td>12-16</td>
</tr>
<tr>
<td>Roberts 2001</td>
<td>Neonates</td>
<td>Parenteral</td>
<td>$^{13}$C Phe</td>
<td>15.6</td>
<td>13</td>
<td>24</td>
</tr>
<tr>
<td>Zello G. 2003</td>
<td>Preterm neonates</td>
<td>Enteral</td>
<td>$^{13}$C Phe</td>
<td>29</td>
<td>29</td>
<td>16-24</td>
</tr>
<tr>
<td>Darling P. 2004</td>
<td>Preterm neonates</td>
<td>Enteral</td>
<td>$^{13}$C Phe</td>
<td>15</td>
<td>15q3*</td>
<td>18</td>
</tr>
<tr>
<td>Courtney-Martin G. 2008</td>
<td>Neonates</td>
<td>Parenteral</td>
<td>$^{13}$C Phe</td>
<td>15.6</td>
<td>13</td>
<td>24.75</td>
</tr>
<tr>
<td>Chapman K. 2010</td>
<td>Neonates</td>
<td>Parenteral</td>
<td>$^{13}$C Phe</td>
<td>15.6</td>
<td>13</td>
<td>24.75</td>
</tr>
<tr>
<td>Huang L. 2012</td>
<td>Neonates</td>
<td>Enteral</td>
<td>$^{13}$C Bicarb</td>
<td>14/9*</td>
<td>34/27‡</td>
<td>4 (+3 for bicarb)</td>
</tr>
<tr>
<td>Hogewind-Schoonenboom 2015</td>
<td>Neonates</td>
<td>Enteral</td>
<td>$^{13}$C Bicarb</td>
<td>14/9‡</td>
<td>34/27‡</td>
<td>4 (+3 for bicarb)</td>
</tr>
</tbody>
</table>

* 15 every 3 hours
† 14 prime and 9 continuous administration of $^{13}$C Bicarbonate
‡ 34 prime and 27 continuous administration of $^{13}$C Phenylalanine
The amount of L-[1-13C]phenylalanine given was subtracted from the total phenylalanine intake of 126mg/kg/d, with dietary L-phenylalanine making up the difference. This intake was based on phenylalanine content in breast milk (42mg/g) at protein intakes of 3g/kg/d. This was purposeful to ensure phenylalanine, the indicator amino acid, was never limiting in the diet. In IAAO studies, the intake of tyrosine must be in excess to prevent any phenylalanine from being converted tyrosine. Therefore based on breast milk content of tyrosine (52mg/g) at protein intakes of 3g/kg/d, 156mg/kg/d of tyrosine was provided to ensure that any tyrosine derived from phenylalanine would be immediately oxidized.

5.5 Sample Collection and Analysis

Baseline weight, length, and head circumference were obtained. Breath and urine samples were collected at baseline and then at plateau which took place between 12 and 24 hours after continuous isotopic administration (Figure 6). The timing and number of sample collections was variable and dependant on clinical care of the infants, frequency of urination, and infant cooperation.

5.5.1 Breath Samples

Breath samples were collected by means of a non-invasive method using a metabolic cart with a CO2 analyzing machine and spiral reflux condenser, coupled to a ventilated hood. This breath collection method has previously been used in term and preterm infants, and in adults. However, our lab’s metabolic cart had not been used since the previous neonatal amino acid requirement studies and had undergone software and hardware upgrades by medical engineering in March of 2015. This included a vacuum pump replacement and changes in air flow linearity. To ensure appropriate readings of VCO2, flow capacities were validated against external flow sensors, and the metabolic cart was deemed functional.

As the infants lay either in their beds or in the arms of a caregiver, they were fitted with a clear plastic hood that rested loosely upon their shoulders. This hood was connected by a long tube to
a portable CO$_2$ analyzer (Servomex, 1400 series; Westech Industrial Ltd, Mississauga, Canada) and mass flow meter (5860 series; Brooks Trillium Measurement and Control, Stouffville, Canada) which was calibrated every study day using CO$_2$ and N$_2$ reference gases. Infants rested as room air flowed at 1-3 L/min. The concentration of CO$_2$ in the hood was controlled and kept between 0.2 to 0.5% and breath samples were collected with CO$_2$ concentration in the range of 0.35-0.45%. This was done to minimize the ratio variability between $^{13}$CO$_2$ and $^{12}$CO$_2$. Although a goal of six (three in duplicate) baseline breath measurements was originally planned, two to four samples were collected. Similarly with plateau breath measurements, eight (four in duplicate) sample measurements were proposed, but six to eight were actually collected on the study day. This was due to unforeseen care interruptions or infant restlessness. Each breath sample was obtained by bubbling 10ml of 1 normal NaOH solution with the expired breath over 10 minutes. After 10 minutes, a sufficient amount of CO$_2$ becomes trapped within the NaOH, converting to NaHCO$_3$. The NaHCO$_3$ was then injected into vacutainers (Beckton, Dickson and Company, NJ, USA) and frozen at -20°C until analyzed.

In preparation for breath $^{13}$C enrichment analysis, samples were defrosted and 300μL was transferred to 12ml-evacuated tubes (Exetainer, Labco, Ceredigion, UK). To this, 300μL of H$_3$PO$_4$ was added, liberating the trapped CO$_2$. Samples were run on a continuous-flow isotope ratio mass spectrometer (CF-IRMS) (CF-IRMS isotope analyzer; Sercon Limited, Cheshire, UK). In basic terms, a mass spectrometer separates molecules by mass. Therefore, an IRMS separates isotopes by their mass. The IRMS works by releasing the sample of CO$_2$ gas into an isolation vacuum chamber, where the gas is ionized by electron removal. The ions are then accelerated along a magnet, where their path becomes curved in response to the magnetic force. This curvature varies depending on mass, with lighter masses more easily deflected, separating them from heavier ions. Finally, once separated by mass (i.e. $^{13}$C and $^{12}$C), the ions are measured by a detector. This then allows for the calculation of atom percent excess, which is the number of isotope atoms in 100 atoms of the element, represented as a percentage. This provides the $^{13}$C enrichment of the breath sample which is compared to a reference standard of compressed CO$_2$ gas.
5.5.2 Urine Samples

Urine samples were collected by placing cotton balls in the infants’ diapers. At least 5 cotton balls were placed into the infants’ diaper beginning at approximately 07h00 on the study day and then again 12 hours after isotope and formula administration began. Nurses were instructed to periodically check, collect, and replace cotton balls (nursing instructions in Appendix C). Since infants are incapable of urinating on command, two to three baseline samples and four to six plateau urine samples were collected. Once the cotton balls were wet, they were placed into sterile urine collectors and if fecal matter was present, the cotton balls that were not seemingly contaminated were collected. A syringe plunger was used to draw the urine from the cotton balls. Three aliquots were taken from each sample and frozen at -20°C until analyzed.

The urine samples were analyzed using a Linear Ion Trap Quadrupole LC/MS/MS Mass Spectrometer (model 1024945-AH, AB SCIEX Instruments, Ontario, Canada) made up of a Sciex QTrap 5500 mass spectrometer coupled to an Agilent 1200 high performance liquid chromatography system (HPLC). In preparation for analysis, urine samples were defrosted, vortexed, and 75μL of each sample was added to eppendorf tubes containing 200μL methanol. After centrifuging at 13000 RPM for 10 minutes, the clear supernatant was extracted with a Pasteur pipette and added to derivitizing tubes. After a 45 minute -80°C snap freeze, samples were freeze-dried (VirTis Sentry 2.0 Freezemobile 35EL, New York, USA) for 3 hours. The dried samples were reconstituted with a 500uL buffer made up of 0.1% formic acid in a 50:50 dilution of acetonitrile and deionized water and then transferred to auto-sampler vials and run through a Waters Xterra MS C18 3.5μm, 150mm x 2.1 mm HPLC column, using an isocratic mobile phase of A) water + 0.1% formic acid and B) acetonitrile + 0.1% formic acid. The equipment was run and results analyzed using Analyst software (version 1.6.3, SCIEX). Ion chromatograms were obtained by instructing the mass spectrometer to detect the transition of ionized $^{12}$C phenylalanine, as mass 166.1, and that of $^{13}$C Phenylalanine, as mass 167.1. These corresponded to un-enriched (M) and enriched (M+1) peaks. Isotopic enrichment was calculated from the mean area ratio between $^{13}$C and $^{12}$C at baseline and plateau and was expressed as molecule percent excess.
5.6 Estimation of Isotope Kinetics

The stochastic model described and used by Matthews et al\textsuperscript{145} was applied for isotope kinetic calculations. This model assumes that the amino acid pool encompasses both labelled and unlabelled phenylalanine, and that the size of the phenylalanine pool will not change with varying threonine intakes. At a baseline or plateau steady state, the amount of isotope entering and leaving the pool is constant, therefore allowing an estimate of $^{13}$C enrichment to be made.

Using enrichment values derived from breath CO\textsubscript{2} and urinary phenylalanine; whole-body phenylalanine flux, phenylalanine oxidation, and rate of production of $^{13}$CO\textsubscript{2} ($F^{13}$CO\textsubscript{2}) were calculated using the following calculations:

Phenylalanine pool kinetics:

$$Q = S + C = B + I$$

Where $Q$ is the rate of phenylalanine flux or turnover; $S$ is the rate of phenylalanine incorporation into protein (i.e. protein synthesis); $C$ is the rate of phenylalanine oxidation; $B$ is the rate of phenylalanine released from protein (i.e. protein breakdown); and $I$ is the rate of exogenous phenylalanine intake (i.e. total phenylalanine intake).

Phenylalanine flux ($Q$), or the movement of phenylalanine in and out of the amino acid pool, is measured from the incorporation of the $[1^{-13}$C$]$phenylalanine in urinary phenylalanine once isotopic steady state is reached:

$$Q = i \left[ \frac{E_i}{E_u} - 1 \right]$$

Where $i$ is the $[1^{-13}$C$]$phenylalanine rate of intake ($\mu$mol/kg/hour); $E_i$ is the enrichment of the infused $[1^{-13}$C$]$phenylalanine (99\% APE); $E_u$ is the $[1^{-13}$C$]$phenylalanine enrichment in urine at isotopic plateau (APE); and the (-1) removes the contribution of the tracer infusion rate from the phenylalanine flux.
The rate of phenylalanine oxidation in response to threonine intake is calculated by the following:

\[
O = F^{13}\text{CO}_2 \left[ \frac{1}{E_i} - \frac{1}{E_u} \right] \times 100
\]

Where \(O\) is the phenylalanine oxidation rate (\(\mu\text{mol/kg/h}\)); and \(F^{13}\text{CO}_2\) is the rate of \(^{13}\text{CO}_2\) released by phenylalanine tracer oxidation (\(\mu\text{mol/kg/h}\)) which is calculated:

\[
F^{13}\text{CO}_2 = \frac{F_{\text{CO}_2} \cdot E_{\text{CO}_2}}{W} \left[ \frac{60 \cdot 41.6}{100 \cdot 0.82} \right]
\]

Where \(F_{\text{CO}_2}\) is the \(\text{CO}_2\) production rate (i.e. \(\text{VCO}_2\) in \(\text{ml/min}\)); \(E_{\text{CO}_2}\) is the \(^{13}\text{CO}_2\) enrichment of expired air at isotopic steady state (i.e. APE); \(W\) is the subject’s weight (kg); the constants 60 min/h and 41.6 \(\mu\text{mol/ml}\) (at standard temperature and pressure) convert \(F_{\text{CO}_2}\) to \(\mu\text{mol/h}\); the 100 changes APE from a percent to a fraction and the 0.82 accounts for the fraction of \(^{13}\text{CO}_2\) released by \(\text{L-[1-^{13}\text{C}]phenylalanine}\), taking into account carbon retained by bicarbonate fixation\(^{146}\). Labeled \(\text{CO}_2\) will pass through the bicarbonate pool prior to being excreted in breath\(^{146}\), and an amount of carbon will inevitably be retained by this pool, therefore necessitating a retention factor.
5.7 Statistical Analysis

Traditionally with IAAO studies, the effect of threonine intake on phenylalanine flux, oxidation and F_{13}CO_2 are analyzed using analysis of variance (ANOVA) with PROC GLM program (SAS/STAT version 9.4; SAS Institute). Breakpoint analysis is then performed using a 2-phase linear regression crossover model to determine the mean threonine requirement from a two lined curve plotted as F_{13}CO_2 versus threonine intake^{147,148}. The breakpoint or mean threonine requirement is reached once the curve depicts a slope that is not significantly different from zero. Once a breakpoint is established, a population safe level, analogous to the Recommended Dietary Allowance (RDA) can be calculated as the 95% confidence interval. The breakpoint (i.e. mean requirement) itself would technically cover only 50% of this population’s needs, being equivalent to the Estimated Average Requirement (EAR).
6 Study Results

6.1 Recruitment Details and Subject Characteristics

Given the timely constraints of a master’s degree, combined with difficulties encountered with regards to REB and recruitment delays, a total of two participants were enrolled and participated in this study. Recruitment took place for approximately 16 months and a total of 13 infants met the eligibility criteria (Figure 7). In accordance with REB regulations, members of the study team cannot approach patients without having first been notified about the study through someone from their clinical circle of care. Therefore, 10 of the 13 potential subjects were approached by the RD in their circle of care. However, this procedure caused slight delays between communication and action, resulting in three infants being discharged without having been approached. Five parents refused consent due to 1) unknown reasons, 2) significant oral intake which they did not want to apparently regress, 3) no immediate benefit for their child, and 4) did not want to change their infants feeding schedule from 3h boluses to continuous feeds.

The subject characteristics of the two female study participants are displayed in Table 12. Both were in born at term, in a positive state of growth, and had diagnoses of hemifacial microsomia and Pierre Robin sequence, respectively. There was a 3-month age difference between subjects and weights were 5.74kg and 4.09kg at study day, with similar weight gains (60g, 50g), over the 24 hr study period. Both subjects were clinically stable prior to and during the study. Prior to administration of the study formula, subject 1 developed a mild fever which quickly subsided with a single dose of acetaminophen. Subject 2 dislodged their NG tube during the last hour of study, causing a delay in feed administration by approximately 20 minutes. Otherwise, the study formula and procedures were tolerated well by both participants, with excess flatulence as the only adverse reaction during the study. Infants received similar intakes of calories (113 and 111kcal/kg/d) and protein intakes were within the required range (2.6 and 3 mg/kg/d) (Table 12).
*Reasons for consent refusal: unknown reasons (n=2), significant oral intake (n=1), continuous feeding (n=1), no benefit for child (n=1). Interpretive services used (n = 2)
Table 12. Study subject characteristics.

<table>
<thead>
<tr>
<th>(n=2)</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth weight (kg)†</td>
<td>3.06</td>
<td>2.81</td>
</tr>
<tr>
<td>Birth weight/age Z-score, percentile‡</td>
<td>-0.38, 35th %ile</td>
<td>-0.96, 17th %ile</td>
</tr>
<tr>
<td>Sex (F:M)</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>&gt;37</td>
<td>&gt;37</td>
</tr>
<tr>
<td>Age on study day</td>
<td>4mo 6d</td>
<td>1mo 18d</td>
</tr>
<tr>
<td>Primary diagnosis</td>
<td>Hemifacial microsomia</td>
<td>Pierre Robin sequence</td>
</tr>
<tr>
<td>Hospital diet (formula)</td>
<td>Goodstart*</td>
<td>Puramino**</td>
</tr>
<tr>
<td>Nutrition support</td>
<td>NG tube</td>
<td>NG tube</td>
</tr>
<tr>
<td>Weight (change over 24 hr study) (g)</td>
<td>5750 (+60)</td>
<td>4090 (+50)</td>
</tr>
<tr>
<td>Weight/age Z-score, percentile‡</td>
<td>-1.03, 15th %ile</td>
<td>-1.14, 13th %ile</td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>40.5</td>
<td>37</td>
</tr>
<tr>
<td>HC/age Z-score, percentile‡</td>
<td>-0.22, 41st %ile</td>
<td>-0.51, 31st %ile</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>60</td>
<td>54</td>
</tr>
<tr>
<td>Weight/length Z-score, percentile‡</td>
<td>-0.23, 41st %ile</td>
<td>-0.52, 30th %ile</td>
</tr>
<tr>
<td>Protein intake (g/kg/d)</td>
<td>2.6</td>
<td>3.0</td>
</tr>
<tr>
<td>Calorie intake (kcal/kg/d)</td>
<td>113</td>
<td>111</td>
</tr>
<tr>
<td>Threonine Intake (mg/kg/d)</td>
<td>15</td>
<td>130</td>
</tr>
</tbody>
</table>

† mean = 2.9kg,  mean = 38.75cm,  c mean = 4.9kg
* standard infant formula
** amino acid-based infant formula
‡ Z-scores and percentiles derived using WHO Anthro (plus) software version 3.2.2
6.2 Urinary Phenylalanine and Expired CO₂ Enrichment

The difference between the baseline and plateau enrichment provides the 13C percent excess which is then be used to calculated phenylalanine oxidation, indicating the level of oxidation in relation to varying intakes of threonine. Figures 8 to 11 provide the baseline and plateau enrichments in urine and in expired breath for both subjects. Verified by phenylalanine oxidation analysis, infants reached isotopic steady state after 12 hours of formula administration. The protocol did not provide leeway in terms of commencing urine collection earlier than 12 hours therefore, infants may have reached steady state earlier. The variation in plateau urinary 13C phenylalanine and breath 13CO₂ was <11% and <6%, respectively. In order to reduce the coefficient of variation amongst enrichment values, sample points are either omitted or included in order to obtain the lowest level of variation. Isotopic steady state is defined as a steady period in which there is no change in the enrichment, identified by a plateau variation <10% amongst sample points. This is a standard procedure routinely conducted as part of IAAO studies as sample enrichment is expected to vary. An example of this is demonstrated by the greyed out data points in Figure 8.

The isotopic enrichment values and subsequent percent excess calculations are summarized in Table 13. This provides an indication of the effect of threonine on phenylalanine response. 13C percent excess is higher in both urinary phenylalanine and breath CO₂ in subject 1 which is an expected finding given the subject’s low test threonine intake of 15 mg/kg/d. Subject 2 depicts smaller percent excesses; again an expected response given the higher threonine intake (Table 13).
Figure 8. Baseline and plateau urinary $^{13}$C phenylalanine enrichment (subject 1).

Figure 9. Baseline and plateau urinary $^{13}$C phenylalanine enrichment (subject 2).

Baseline CV = 2.9%
Plateau CV = 10.9%
Figure 10. Baseline and plateau $^{13}$C enrichment in expired CO$_2$ (subject 1).

Baseline CV = 49.5%
Plateau CV = 4.6%

Figure 11. Baseline and plateau $^{13}$C enrichment in expired CO$_2$ (subject 2).

$^{13}$C phenylalanine percent excess

Breath collection and times

not included in mean APE calculation
baseline CV = 7.3%
plateau CV = 5.9%
Table 13. Subject urinary $^{13}$C phenylalanine and expired $^{13}$CO$_2$ enrichment values*

<table>
<thead>
<tr>
<th>Subject</th>
<th>Threonine intake</th>
<th>$^{13}$C Phenylalanine</th>
<th>$^{13}$CO$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Baseline</td>
<td>Plateau</td>
</tr>
<tr>
<td>1</td>
<td>15</td>
<td>0.01845</td>
<td>0.18567</td>
</tr>
<tr>
<td>2</td>
<td>130</td>
<td>0.01397</td>
<td>0.16825</td>
</tr>
</tbody>
</table>

* mean values

6.3 Phenylalanine Kinetics

Due to the limited number of study subject data, it was not possible to conduct statistical analyses on the effects of threonine, protein, or caloric intake on the rate of $^{13}$CO$_2$ production or on $^{13}$C phenylalanine oxidation. However, it was possible to infer correlation based on visual inspection of the data.

Phenylalanine flux is measured via the incorporation of the L-[1-$^{13}$C]phenylalanine tracer in urinary phenylalanine at isotopic steady state. The phenylalanine flux between subjects is summarized in Table 14. Upon visual inspection, phenylalanine flux does not appear to be affected by threonine intake. The flux appears to increase slightly at the higher threonine intake (97.1 vs. 89.6 μmol/kg/hr), however variation is to be expected amongst subjects (Table 14). This are encouraging preliminary data since the indicator pool (i.e. phenylalanine flux) should not be influenced by test amino acid (threonine) intakes, as it is crucial to the IAAO design.
Table 14. Subject threonine intake and phenylalanine flux.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Threonine intake (mg/kg/d)</th>
<th>Phenylalanine flux (μmol/kg/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>89.6</td>
</tr>
<tr>
<td>2</td>
<td>130</td>
<td>97.1</td>
</tr>
</tbody>
</table>

Phenylalanine oxidation does appear to be affected by threonine intake (Figure 12). The lower threonine intake at 15 mg/kg/d produced the higher level of phenylalanine oxidation at 4.18 μmol/kg/hr. Similarly, threonine intake also appeared to affect the rate of production of $^{13}$CO$_2$ in these infants, again with the lower threonine intake contributing a higher $F^{13}$CO$_2$ amongst infants (Figure 13).
Figure 12. The effect of threonine intake on phenylalanine oxidation in 2 subjects.

Figure 13. The effect of threonine intake on the production of $^{13}\text{CO}_2$ in 2 subjects.
Due to the obstacles involved with participant recruitment and study progression, there were not enough data points to be able to conduct breakpoint analyses, and subsequent determination of a threonine requirement during the time limited for this master’s degree. In order to calculate the break point, two lines are required. With IAAO, the first line is made of at least three data points (three different threonine intakes below the requirement) decreasing in oxidation from lower to higher threonine intakes, which produces a slope significantly different from zero. The second line required is one of at least three data points where the slope is not significantly different from zero, indicating no change in oxidation versus threonine intake. Where these two lines intersect is defined as the breakpoint. Clearly, no intersection could be determined given the limited data. In the present study, both F\textsuperscript{13}CO\textsubscript{2} and phenylalanine oxidation appear to be inversely affected by threonine intakes as expected.
7 Discussion

7.1 Preliminary data

This is the first study to attempt to determine the enteral threonine requirements in infants older than one month of age. Due to unforeseeable delays, a sub-optimal number of subjects were recruited in the allotted time, therefore limiting the conclusions that can be made based on present results. Of the anticipated 18, two subjects were enrolled and strategically studied at the lowest (15mg/kg/d) and highest (130mg/kg/d) threonine intake. This was done purposefully to determine whether an appropriate threonine intake range was established for this population. The hypothesized breakpoint or requirement value for this age group was set at 73mg/kg/d, therefore the range of intakes was equally dispersed around this point. Previously, a range of threonine intakes between 10 to 100mg/kg/d allowed for the determination of the parenteral threonine requirement, which was determined to be 32.8mg/kg/d\(^{16}\). Therefore the present range was widened at the higher level to account for where the hypothesized breakpoint would lie in comparison. Our study’s threonine intakes are within range of a similar threonine requirement study performed in 1 month olds, where the range was set at 5 to 182mg/kg/d, and defined the mean requirement at 68mg/kg/d\(^{14}\).

Although preliminary, the results of the present study do provide evidence that the correct threonine intake range has been chosen for this population. The production of \(^{13}\)CO\(_2\) was higher, indicating elevated phenylalanine oxidation, at the lowest threonine intake. Conversely, a lower level of oxidation was exhibited from the highest threonine intake (130 mg/kg/d) (Figure 13). Although it would be incorrect to confidently state that this lower level of oxidation will be one of the points producing a zero slope line (indicative of lowest oxidation once more subjects are studied), we assume that it will, based on where the hypothesized breakpoint lies in comparison.

Another encouraging observation was that the subjects’ phenylalanine flux rates were not affected by drastically different intakes of threonine (Table 14). Although based on only two subjects, the relative similarity amongst phenylalanine flux values provides affirmation that the study’s IAAO design remains valid and should be able to yield an appropriate threonine
requirement once an optimal number of subjects are recruited. This is because the model of isotope kinetics used to conceptualize IAAO assumes that the indicator pool size (i.e. phenylalanine) will remain stable regardless of varied intakes of threonine. Phenylalanine flux is calculated by considering 1) the $^{13}$C phenylalanine intake per hour, 2) the enrichment of the $^{13}$C phenylalanine intake, which is 99% APE and 3) the urinary $^{13}$C phenylalanine enrichment obtained from plateau urine sample analysis. By providing a constant 126mg/kg/d of phenylalanine to all subjects, the pool size should remain stable and therefore any partitioning of phenylalanine between oxidation and protein synthesis is strictly due to the difference in threonine intake and not due to changes in phenylalanine pool size. One of the reasons phenylalanine is an ideal indicator in IAAO is due to its small pool size, but also because it partitions neatly between two pathways: protein synthesis and oxidation. However, this is only the case if excess tyrosine, an indispensable amino acid, is provided in the diet. Excess tyrosine is required in order to enable any $^{1-13}$C tyrosine hydroxylated from L-$[1-^{13}$C]phenylalanine to be shunted immediately towards $^{13}$CO$_2$ oxidation. In the present study, 156mg/kg/d of tyrosine was provided, which is in excess of the amount consumed by breast milk fed infants (120mg/kg/d). Similar intake levels of tyrosine have also been used in previously performed infant IAAO studies, therefore the amount of tyrosine provided in this study should be sufficient for optimal tracer sensitivity.

Amino acid flux can vary depending on route of tracer administration, route of feeding, and by individual amino acid kinetics. Given the same route of feed administration, route of tracer infusion does not affect requirement estimates made by breakpoint analysis, but it can affect amino acid kinetics. One study that most closely resembles the current one was conducted by Huang et al. who sought to determine lysine requirements in enterally fed 1 month olds. Their derived phenylalanine flux was 88 μmol/kg/hr, which is similar to the mean flux value of the current study at 93 μmol/kg/hr. Since our mean flux rate was derived from only two subjects, a more representative flux value will be determined once more participants have been enrolled. For now, however, the present study’s phenylalanine flux rates do seem comparable to previously established rates.

Prior to calculating $F^{13}$CO$_2$ using VCO$_2$ values, the subjects’ APEs themselves depict an expected relationship between enrichment and threonine intake. APE is determined by the
difference in $^{13}$C enrichment from baseline to plateau, and therefore is not a product of complex
calculation, as with $^{13}$CO$_2$ and phenylalanine flux calculations. Often the first indication of an
appropriately functioning IAAO design: the subject with the lowest intake of threonine
demonstrated the highest level of both $^{13}$C phenylalanine (urine) and $^{13}$CO$_2$ (breath) percent
excess at 14.3261 and 0.009825, respectively (Table 13). Although preliminary, this provides an
indication that inadequate threonine intake corresponds to a higher level of phenylalanine
oxidation, as threonine is limiting for optimal protein synthesis to occur.

Although the $^{13}$C percent excess demonstrated expected findings between threonine intakes,
there was high within-subject variation amongst enrichment estimates. However, it is difficult to
discern whether this is simply due to the fact that only two subjects were included in the analysis.
Previous infant requirement studies have demonstrated coefficient of variations amongst plateau
phenylalanine and CO$_2$ enrichment values at 5-10%, and <0.2%, respectively.$^{16,17,109}$ Presently,
our study’s urinary and breath plateau enrichments are <11% and <6%, respectively. However,
our population is much older than the neonates used in the previous studies, and encompass a
wider age range. Our population is also presumably more active than the studies using neonates
situated in the NICU. During breath collection, infants were often fussy, and sometimes crying
while under the breath collection hood. This may have affected individual rates of expired CO$_2$,
leading to potential $^{13}$C:$^{12}$C isotope fractionation.$^{146}$ Isotope fractionation can occur via
equilibrium or kinetic effects$^{150}$ and therefore a physical effect (i.e. force of breathing) may have
altered or “fractionated” the proportion of $^{12}$C to $^{13}$C due to $^{13}$C being heavier in mass. Another
possibility for increased variability may also be due to the route of feeding. Parenteral nutrition is
a very direct mode of nutrition provision, which is not influenced by GI tract motility, digestion,
or overall function. Contrary to these previously conducted parenteral neonatal studies, our study
infants were enterally fed, which introduces a new contributing factor towards enrichment
variability. Not usually reported in infant requirements studies are variation amongst baseline $^{13}$C
enrichment values. With subject 1 in particular, only two baseline breath enrichments were
collected and variation was calculated to be 49.5% (Figure 10). It is difficult to discern which
sample is more representative of true enrichment, therefore the mean of both was used. Subject
1’s baseline urinary enrichments were much less variable at 14.9% (Figure 8) while both the
baseline urinary and breath enrichments of subject 2 were at 2.9% (Figure 9) and 7.3% (Figure
It appears that the large CV in Subject 1’s baseline CO$_2$ enrichment may be the result of an artefact.

18 studies, i.e. 18 graded threonine intakes, are hypothesized to be sufficient to define a significant and well-fitted breakpoint for this population. This is based on data from members of our lab; Chapman et al.$^{16}$ and Courtney-Martin et al.$^{18}$. These studies were conducted on parenterally fed infants at SickKids, and produced well defined breakpoints using 16-18 intake studies. However, colleagues in the Netherlands have conducted similar IAAO requirement studies with enterally fed infants, and used approximately 30 varied threonine intakes to determine requirements.$^{14,19}$ As this is the first enteral amino acid requirement study in infants performed in our lab, we will be able to determine whether 18 intake levels is sufficient to define a threonine requirement in the 1-6 month population, or if more intake levels will be required.

We used urine as a means to determine amino acid enrichment. Historically, plasma samples were used, but our lab demonstrated that urine can be used as a surrogate, therefore allowing amino acid requirement studies to become less invasive.$^{13}$ The reason urine samples are collected in such studies is primarily to verify whether there is a significant change in indicator flux at different test amino acid intakes. Colleagues in the Netherlands have begun omitting this procedure, since phenylalanine flux rates have not been affected by their subjects’ test amino acid intakes.$^{130,151}$ However, this added step is non-invasive to the infants, and provides researchers with an added measure of biological response (phenylalanine oxidation) in addition to the label oxidation response (F$^{13}$CO$_2$) (Figure 12). Whenever possible, breakpoint analyses are usually conducted with F$^{13}$CO$_2$, as phenylalanine oxidation values rely on F$^{13}$CO$_2$ in its calculation. In addition, using F$^{13}$CO$_2$ produces similar breakpoint analyses compared to apolipoprotein B-100, which is a direct measure of phenylalanine hydroxylation and provides a measurable response to protein synthesis.$^{152}$

This study has also been designed so that regardless of threonine intake, infants will be receiving adequate intakes of total nitrogen. This is an important consideration since indispensable amino acid requirements could be overestimated if inadequate nitrogen (from inadequate dispensable amino acids) is supplied in the diet.$^{153}$ The nitrogen intake of the current study has been set to be identical to nitrogen provided from total amino acid content in breast milk$^8$, with alanine being
adjusted based on the nitrogen removed or provided in excess due to varying threonine intakes. Therefore, all infants received similar and adequate intakes of total nitrogen.

It is known that threonine requirements are highly sensitive to route of feeding and subsequent splanchnic demand. The parenteral threonine requirement is almost 50% that of the enteral threonine requirement\textsuperscript{63}. Besides digestive organs, other factors are known to contribute to measurable differences in threonine requirement. In pigs randomized to a number of different test diets, Myrie et al. demonstrated that specific food components altered endogenous threonine losses\textsuperscript{154}. This study confirmed that diets high in barley and wheat bran (i.e. high fibre diets) lead to the lowest level of ileal digestibility of threonine, and in general, lower ileal digestibility of all indispensable amino acids. This implies that increased threonine intake is required to fulfill requirements when such foodstuffs make up a significant amount in the diet. Ito et al. has shown that rats fed diets containing soluble fibres such as guar gum, psyllium, and konjac mannan at 50g/kg diet, resulted in up-regulation of mucin secretion and increased goblet cell number\textsuperscript{155}. Interestingly, oral administration of aspirin can also produce increases in mucin secretion, with an increase in the threonine content of mucins as well\textsuperscript{156}. Since mucin-producing tissues extract threonine at the expense of tissues like skeletal muscle\textsuperscript{62}, it is important to provide enough threonine to satisfy all organ-specific requirements. Threonine requirements will also change in response to disease states, as do protein requirements. For example, sepsis increased the threonine requirement in rats by 2.6 times, which would be endogenously covered via muscle protein breakdown if not orally administered\textsuperscript{157}. Altogether, this re-iterates the need to determine amino acid requirements, not only within healthy populations, but also at various states of disease and with variable dietary composition.

7.2 Study limitations

One limitation of this study was that adaptation diets were not used in an effort to minimize invasiveness. Such diets are a customary component of IAAO studies, which serve the purpose of adapting subjects to similar protein intakes, but also adapting them to similar background levels of $^{13}$C. Carbon 13 abundance is approximately 1.1%\textsuperscript{121} with natural variation ranging from
1.06 to 1.12%\textsuperscript{158}. Levels of background \textsuperscript{13}C vary amongst individuals due to dietary differences, and potentially in this case, due to differences amongst infant formula. However, this effect is significantly reduced by calculating the difference (APE) in \textsuperscript{13}C enrichment at baseline and at plateau, rather than just the enrichment value at plateau. However, once the study begins, a new formula (the study formula) is introduced to the infants, and it itself will provide a new background level of \textsuperscript{13}C, which combines with the \textsuperscript{13}C being administered as the tracer. Therefore, the omission of an amino acid based adaptation diet may have introduced enrichment variability by altering \textsuperscript{13}C due to changing dietary composition.

It is also customary to control protein intakes prior to and during IAAO studies, which is another function of the adaptation diet. This becomes challenging with infant populations, since clinically prescribed dietary parameters cannot be altered for the sake of the study. Thorpe et al. previously demonstrated that prior protein intake adaptation does have an effect on phenylalanine flux and oxidation, but does not affect label oxidation (F\textsuperscript{13}CO\textsubscript{2})\textsuperscript{144}. At present, it is impossible to state whether the small 0.4g/kg/d protein difference amongst subjects was enough to produce measurable effects at the level of oxidation or flux. However, once more infants have been studied, and should protein intake appear to have an effect on these parameters, the F\textsuperscript{13}CO\textsubscript{2} should still provide reliable data for breakpoint analysis\textsuperscript{144}. In a similar study conducted in our lab, infants displayed a protein range of 2.8–3.5 g/kg/d, and this range did not affect tracer oxidation\textsuperscript{17}.

Besides ethical considerations, the hospital-based infant population was chosen for this study primarily due to the ease of feeding via feeding tubes. There is also the need to deliver the formula and tracer in a consistent fashion. However, the reasons for admission to the hospital for the eligible subjects were quite variable. We purposefully selected infants who would most closely replicate the “healthy” infant. Therefore, the main aspects to consider were protein and amino acid metabolism, and gut function. Infants were excluded if they had a diagnosis known to be related to altered protein or amino acid metabolism or one that caused gut related effects such as malabsorption or suboptimal digestion in general. However, there will always be the fact that these are indeed hospitalized patients that for a number of reasons may vary from the general healthy, infant population.
In an effort to broaden inclusion and exclusion criteria, it was decided to allow infants between 32 and 43 weeks gestational age to participate in the study, provided they were at least 1 month corrected gestational age. This therefore requires the assumption that, without prior major health events, growth and therefore amino acid requirements would be similar amongst the late-preterm and term infant once corrected for gestational age. It was also decided to include all forms of tube feeding, including jejunostomy-tube placements. Since a majority of the enteral requirement is composed of the threonine required for splanchnic metabolism, delivering the study formula to the jejunum rather than the stomach may in fact alter individual threonine requirements. Although neither of the subjects was born premature, nor fed via a J-tube, these criteria will need to be monitored in future subjects, and should be kept in mind when analyzing the breakpoint requirement, as these conditions may contribute to variable threonine requirements.

7.3 Recruitment Obstacles

In the 16 months following study activation, necessary protocol amendments caused delays in the recruitment of eligible subjects by ≥3 months at a time. In addition to the multiple adjustments made to broaden the eligibility criteria, there still remains a small window of opportunity. The diagnoses of potential recruits are restricted to those that have no gut related manifestations, which can be common for infants receiving enteral nutrition. Of the infants who met inclusion criteria, consumed appropriate protein intakes, and had a non-gut related need for enteral feeds, selection becomes limited. As outlined, 13 infants were eligible for study participation in these 16 months, which was much lower than the originally predicted 32. Prior to recruitment, approval was obtained from staff surgeons in regards to recruiting infants who had variable feeding patterns, whereas for the study this would be changed to a continuous feeding pattern. Although the study protocol was eventually altered to allow infants transitioning to oral feeds to participate, no infants have been enrolled since. Clinically, this 24h switch to continuous feeds should not affect formula tolerance or progression of oral feeds. However, parents previously displayed worry about having their infant switched from bolus feeding schedules (i.e. feeding every 3 hours) to continuous feeds, even though the patients’ surgeons provided full approval. In other instances, it was unknown why parents refused consent since there was no
opportunity to further inquire, as this is not allowed within the consent process. On two occasions, interpretive services were required for translation with parents who did not speak sufficient English to demonstrate full comprehension of the study details. Surprisingly, this in itself was a recruitment barrier since the REB did not immediately know how to proceed. Although the REB is now actively working on making this process easier for researchers, it demonstrates that clinical research has largely focused on English speaking populations; excluding an important subset of potential recruits; a form of prejudice we assumed would be a non-issue.

With one parent in particular, an Arabic translation was involved. The consenting process was quickly terminated once the translator indicated that the parent had become aggressive and unreasonable. It is unclear whether this was due to translational issues or to personal comprehension, but this parent appeared to be disturbed by the fact that no immediate benefit would be provided to their child and felt as though their child was not being properly looked after. Although a relatively extreme case, this illustrates an apparent issue with subject recruitment in this paediatric hospital setting. Since all enrolment took place with in-patients, we were unable to provide any form of compensation to children or families for research participation, as they are already receiving clinical care by the hospital. This is an REB directive. The combination of no personal incentive along with the fact that their child is being hospitalized for an unrelated diagnosis, appeared to hamper subject recruitment.

In all recruitment opportunities, the notion of “no apparent risk” was reiterated multiple times. This is also clearly stated in the parental consent form (Appendix A). More recently, similar IAAO studies have been successfully performed in the enterally fed 1-month old population\textsuperscript{14,19,151}. However, the main difference between the present setting and the setting in these studies is location. Such studies have taken place in China, where ethical regulations may not be as rigorous as in Canada\textsuperscript{159}. Therefore, procedures could be put into place which make parents and children more aware that they may be approached for research participation. Such a document could include a statement about why research (as a concept) is important and can only be accomplished and kept relevant by recruiting people of all ages. It is important to foster an attitude of promoting research throughout all areas and staff in the hospital.
7.4 Implications

Approximately 1/3 of enterally delivered amino acids are utilized on first pass metabolism, however in the infant, splanchnic metabolism of threonine has been shown to be as high as 70%, with no other differences in amino acid first pass metabolism across the life cycle. This may be due to the developing gut’s affinity for threonine as it is mainly used for the incorporation into mucous glycoproteins. In the piglet, multiple studies demonstrate gut related consequences to both threonine deficient and excessive diets. When insufficient dietary threonine is provided, extra-intestinal tissues may suffer at the expense of the gut. Although these effects have never been studied in the infant, the pig model provides us with the most relevant and applicable data. There have been observations of increased plasma and urine levels of threonine in response to being formula fed, given the decreased threonine oxidation in formula fed infants. Although elevated plasma threonine levels have not been associated with any measurable consequences in infants, these observations warrant further investigation. In addition, infant formulas can contain high levels of threonine in an effort to accommodate the entire 0 to 6 age range. Since formulas need to contain amino acid levels to satisfy the first month of life, older infants may be consuming more than necessary as protein requirements decrease. Amino acid-based formulas may also contain elevated amounts of threonine. For example Neocate® (Nutricia) provides 70mg of threonine per gram of protein, which is in excess of the 44mg of threonine per gram protein from average breast milk composition. This would lead to threonine intakes at approximately 110mg/kg/d, compared to the current DRIs of 73mg/kg/d. There are therefore multiple reasons to determine an experimentally-derived threonine requirement in infants. Estimates based on breast milk composition have been in place since the 1980s, and although provide the best recommendations we have to date, are based on multiple factors and have not been validated using newer methodology. Therefore, deriving physiological estimated of amino acid requirements using IAAO will provide novel standards, which may be most useful for the formula-fed infant, or for the breast milk fed infant who may not remain exclusively breast-fed for the recommended first 6 months of life.
8 Conclusion

The primary goal of this thesis was to determine a threonine requirement in 1 to 6 month old infants. This study has been “on the go” for roughly 4 years, but was continuously interrupted by numerous REB issues. Although as researchers, we understand the relative risk of performing such studies on infants, it can become difficult to reiterate the message to regulators who themselves have expectations for “clinical drug trials”. Unlike with our lab’s previous amino acid studies, this study’s amino acids and phenylalanine tracer were regulated as pharmaceuticals and therefore required the highest levels of ethical compliance possible at our hospital. This was something no one had dealt with previously and required many new processes. In the meantime, optimization projects were performed and enabled the study to progress and be able to attain the goal of identifying the threonine needs in this infant population. This is an important goal, as no one yet has provided estimates of indispensable amino acids in infants older than 1 month of age, using IAAO methodology.

Preliminary results so far allude to the IAAO method working as expected. Phenylalanine flux values demonstrated that the changes in phenylalanine oxidation are likely due to threonine intakes alone, and not due to changes in phenylalanine turnover. The higher intake of threonine lead to the lowest oxidation level, verifying that with higher intakes, oxidation slows as threonine becomes less limiting for protein synthesis. Given the various roadblocks and years of perseverance, this study has come a long way. Although a minimal number of participants were recruited in the allotted time, eligibility criteria has expanded to encompass a larger pool of potential participants and has resulted in increased potential recruits. However, it will be necessary to weigh all aspects of conducting this study, like valuable resources, in an effort to determine the best way to investigate this as well as the other eight indispensable amino acid requirements in this population.
8.1 Future Directions

For future considerations, a dialogue will need to be had amongst the present study team in order to determine the most feasible approach to performing IAAO studies in infants under the current hospital setting. This is especially so, considering that there are 9 indispensable amino acids in all, which would each require their own study to identify a requirement value in this age group. One option would be to conduct multiple amino acid studies simultaneously, however all studies would be enrolling infants from the same eligibility pool. As only 13 infants were suitable for enrolment in the present study over 16 months, these studies may take multiple years to complete. Another option would be to conduct these studies as “multi-site” projects. However, this would require considerable organization and resources to implement.

In an effort to make the study protocol even less invasive than it currently is, which could double as a potential incentive for study participation, one consideration could be to remove urine sample collection altogether, which has been done by colleagues in the Netherlands. This was justified by the fact that previous studies demonstrated no change in flux relative to test amino acid intakes. This could significantly reduce the time required for administering the amino acid formula and stable isotope, since the current protocol is one of 24 hours, which is mainly due to the unavoidable wait time involved in infant urine collection.
9 References


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Appendix A. Consent form and consent discussion form

Research Consent Form
(Without $^{13}$C Bicarbonate Isotope)

Title of Research Project:
A physiological study to determine the enteral threonine requirement of infants aged 1 to 6 months

Principal/Qualified Investigator:
Dr. Paul Pencharz  MD, PhD, Research Institute  416-813-7454

Co/Sub-Investigators:
Dr. Christopher Tomlinson  MD, PhD, Neonatology  416-813-7340
Ms. Beth Haliburton  RD  416-813-8580
Dr. Glenda Courtney-Martin  RD, PhD  416-813-5744

Purpose of the Research:
Amino acids (AA) are the building blocks of protein and there are 20 AA that we take in through our diets. One of the AAs, threonine, is critical in the production of mucin (a key protein in the mucous membrane that protects the lining of the intestine) in the gut and contributes significantly to collagen, elastin and tooth enamel formation. Methodological advances have made it possible to determine AA requirements in humans with a more precise technique. However, no studies of threonine requirement in infants (1 – 6 months of age) using the more precise technique of Indicator Amino Acid Oxidation (IAAO) where a small amount of a stable
isotope is given in the diet and is measured in breath and urine samples have been reported. Since we do not know how much threonine should be in the diet of infants, this study will determine a threonine dietary requirement.

**Description of the Research:**

We will be administering 18 different threonine intakes to between 9 to 18 infants; each infant will receive at least one intake level and some infants may receive 2 different intakes of threonine as their feeding schedules and their parents permit.

If you choose to enrol your baby in this study, there will be an additional Registered Nurse (RN) and Dietician involved in the care of your baby for 24-48 hours. Prior to your baby’s participation in the study, we will be speaking with his/her doctor and dietician to get their permission for your baby to participate in the study and we will review your baby’s hospital chart to ensure that he/she is clinically stable and eligible to participate.

There will be one study day of 18-24 hours after ensuring that the clinical formula your infant is on provides sufficient protein. Just before we start the study diet, we will measure your baby’s length, weight, head circumference and take 3 breath and 3 urine samples. Breath samples will be collected using a Carbon Dioxide Analyzer Cart, which is similar in design and use as a clinical test device called a Calorimeter. Both devices use a vented hood system under which the infants sleep or rest where the volume of CO₂ breathed out is measured. Each breath sample will be collected over a 10-minute period by bubbling the breath effluent into a reflux condenser and trapping the breath in a test tube. To collect urine we place cotton balls in your baby’s diapers. Neither of these procedures are painful for the baby.

On the study day, we will give your baby an infant formula, which contains a specific amount of threonine. Your infant will receive the same amount of protein as he/she gets in standard formula; it is simply the mix of the AAs in the formula will be different. The delivery of the study day formula will be offered as a continuous or semi-continuous feed, either by tube feeding or by a combination of tube feeding and oral feeds, which may differ from your baby’s current feeding schedule but it has been approved by your baby’s surgeon and dietician. The study feeds will only be given for 18-24 hours (example: beginning at 12pm and finishing the next day before 12pm. Your baby’s standard feeds will then be resumed.
To measure your baby’s response to the different amount of threonine he/she is receiving, we will give your baby a stable isotope called L-\([1-^{13}C]\) Phenylalanine. Stable isotopes are present in all human bodies and we all ingest them daily through the food we eat. We are giving your baby a dietary substance that is known not to be harmful or is anything that he/she cannot digest. We have done several studies where we gave babies in the Neonatal Intensive Care Unit (NICU) the same isotopes by vein (through their IV), with no observed side effects, either short or long term. Stable isotopes are used internationally in research and several investigators here at The Hospital for Sick Children, including our group, have been using stable isotopes on all age groups for over 20 years.

During the study day, the Phenylalanine stable isotope is also given together with the study formula for the 18 – 24 hours. It is the research community’s experience that the stable isotope is processed by the human body, including infants and neonates is gone within 24 hours of being added to the diet. All of the AA’s used in the study are prepared in small quantities for research purposes only and are not used in commercial products. We have taken steps to ensure adequate safety for use with neonates, infants, children and adults.

Four plateau breath and urine samples will be collected between 12h and 24h after the introduction of the stable isotope. The study day is complete once the breath and urine samples have been collected. Your baby will resume his/her diet once it has been delivered to the ward.

**Potential Benefits:**

**To individual subjects:**

Your baby will not benefit directly from participating in this study. We can provide you with our published results from previous studies and can provide you with a copy of the results of this study once they are published.
To society:

Once the requirements for the 9 indispensable AA have been determined for infant 1 to 6 months of age, companies that manufacture infant formulas may revise their formulations to our recommendations in order to provide the optimum AA balance within the protein delivered to infants.

Confidentiality:

We will respect your baby’s privacy. No information about who you or your baby are will be given to anyone or be published without your permission, unless required by law. For example, the law requires us to give information about your baby if a child has been abused, if your baby has an illness that could spread to others, if you or someone else talks about suicide (killing themselves), or if the court orders us to give them the study papers.

SickKids Clinical Research Monitors, employees of the funder [CIHR] or sponsor of the study or the regulator of the study may see your child’s health record to check on the study. For example, people from Health Canada Health Products and Food Branch, if necessary, may look at your baby’s records.

By signing this consent form, you agree to let these people look at your baby’s records. We will put a copy of this research consent form in your baby’s patient health records. We will give you a copy for your files.

The data produced from this study will be stored in a secure, locked location. Only members of the research team (and maybe those individuals described above) will have access to the data. This could include external research team members. Following completion of the research study the data will be kept as long as required then destroyed as required by Sick Kids policy. Published study results will not reveal your baby’s identity.

A description of this study will be available on http://www.clinicaltrials.gov/. This website will not include information that can identify your baby. At most, the website will include a summary of the results. You can search this website at any time.
Potential Harms:
We know of no harm that taking part in this study could cause your baby. The interventions that differ from your baby’s clinical care are the introduction of a different intake of threonine while the amount of protein your child receives is the same as in his/her regular diet as are the amounts of fat, carbohydrate, minerals, trace elements and vitamins. There is no known risk to altering the amount of threonine in the child’s diet for 24 hours. Stable isotopes have been used in research around the world on all age groups from premature infants to senior citizens with no reported adverse events. Risk to feeding infants by tube in a research study is similar to the risk of feeding infants by tube in a clinical situation. All clinical controls to minimize this risk will be utilized in the study (i.e. Checking position of feeding tube, RN will set up and monitor the feeding, etc).

Potential Discomforts or Inconvenience:
There are no known discomforts or inconvenience to you or your infant. All study interventions will be done around your infant’s clinical care and tests. Risk to feeding infants by tube in a research study is similar to the risk of feeding infants by tube in a clinical situation. All clinical controls to minimize this risk will be utilized in the study (i.e. checking position of feeding tube, RN will set up and monitor the feeding, etc…).

Reimbursement:
Unfortunately, we are unable to offer any reimbursement for participating in this study as the study occurs during the hospitalization and clinical care of your baby.

Participation:
It is your choice to let your child take part in this study. The care your child receives at SickKids will not be affected in any way by whether your child takes part in this study. As this is a basic physiological study where we are simply trying to determine how much threonine is required in infants’ diets, there is no alternative to not participating.
During this study we may create new tests, new medicines, or other things that may be worth some money. Although we may make money from these findings, we cannot give you or your child any of this money now or in the future because your child took part in this study.

In some situations, the study doctor or the agency paying for the study may decide to stop the study. If this happens, the study doctor will talk to you about what will happen next.

If your child becomes ill or are harmed because of study participation, we will treat your child for free. Your signing this consent form does not interfere with your or your child’s legal rights in any way. The study staff, any people who gave money for the study, or the hospital are still responsible, legally and professionally, for what they do.

New information that we get while we are doing this study may affect your decision to let your child take part in this study. If this happens, we will tell you about this new information. And we will ask you again if you still want your child to be in the study.

**Sponsorship:**
This research study is being funded by The Canadian Institute of Health Research (CIHR). The sponsors of this study are Dr. Pencharz and the Hospital for Sick Children.

**Conflict of Interest:**
Dr. Pencharz and the other research team members have no conflict of interest to declare.
**Consent:**

By signing this form, I agree that:

1) You have explained this study to me. You have answered all my questions.

2) You have explained the possible harms and benefits (if any) of this study.

3) I know what I could do instead of having my child take part in this study. I understand that I have the right to refuse to let my child take part in the study. I also have the right to take my child out of the study at any time. My decision about my child taking part in the study will not affect my child’s health care at SickKids.

4) I am free now, and in the future, to ask questions about the study.

5) I have been told that my child’s medical records will be kept private except as described to me.

6) I understand that no information about my child will be given to anyone or be published without first asking my permission.

7) I have read and understood pages 1 to 5 of this consent form. I agree, or consent, that my child______________________________ may take part in this study.

*(Print Child’s name)*

_____________________________________      __________________________________
Printed Name of Parent/Legal Guardian        Parent/Legal Guardian’s signature & date

_____________________________________      __________________________________
Printed Name of person who explained consent  Signature of Person who explained consent & date

_____________________________________      ___________________________
Printed Witness’ name (if the Parent/Legal guardian does not read English)    Witness’ signature & date
☐ I agree for my child to participate in this study a second time if he/she is eligible
(should time permit, and with approval/clearance from attending surgeon)

☐ I do not agree for my child to participate in this study a second time if he/she is eligible

_______________________  ______________________
Initials                                   Date

If you have any questions about this study, please call Dr. Glenda Courtney-Martin at 416-813-5744

If you have questions about your rights as a subject in a study or injuries during a study, please call the Research Ethics Manager at 416-813-5718
INFORMED CONSENT DISCUSSION

Study Title: A Physiological Study to Determine the Enteral Threonine Requirements of Infants aged 1 to 6 Months

| Qualified Investigator (QI): Dr. Paul Pencharz | REB File #: 1000048977 |

Date (yyyy/mmm/dd): _____________________________

Approached: _______________________________________,

(Indicate parents/legal guardians and/or patient’s name) for participation in the above mentioned study.

Permission to approach regarding the study was obtained by _______________________________ or □ Not Applicable for this study

The consent was reviewed with the participant and/or parent/legal guardian. The purpose, procedures and alternatives of the study were explained as well as the risks and benefits of participating in this study. Issues of confidentiality and the voluntary nature of participating in this study were also explained.

Time alone to review the consent document was provided? □ Yes □ No, if no why not?

_____________________________________________________________________________

Adequate time was given for all questions to be answered to the satisfaction of the participant/parent/legal guardian? □ Yes □ No, if no why not?

_____________________________________________________________________________

If the response is NO to the two questions above do not proceed with consent.
*Capacity Assessment:* Should include justification as to how the QI (or designate) determined the consent decision for the participant.

A copy of the signed consent and assent (if applicable) form was given to the participant and /or parent/legal guardian prior to study procedures?  □ Yes  □ No

Comments:

___________________________________________________________________

___________________________________________________________________

Informed Consent Discussion Completed By:

________________________________________________  ______________
Printed Name                                          Signature            Date (yyyy/mmm/dd)
Appendix B. Pre-study checklist

Threonine pre-study checklist for each subject

<table>
<thead>
<tr>
<th>Pre consenting/recruiting</th>
<th>Check off</th>
<th>Date</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Talk with RD about protein/cal intake &amp; wt gain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Confirm with RD (confirmation email about appropriate Intakes 24-48hrs previously)**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Confirm with surgeon (confirmation email) about permission to participate in study**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inclusion/Exclusion criteria – signed by Chris</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Chris) note that meets inclusion/exclusion if not stated in chart</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Talk with parents – leave consent form 24hrs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Get consent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Immediately after baby consented:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Let Susan Know about baby and diet – prep MPR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coordinate with HSC kitchen about new formula x 1 day – does priming volume make sense?</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>compare to logarithm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Get study folder and paperwork ready</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do formula calculations – paper and excel</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Let nurse manager on floor know about study participation</td>
<td></td>
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<td></td>
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<tr>
<td>Diet techs – label generator?</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KidCare – miscellaneous – get Glenda to write RESEARCH *Indicate: HOLD FEEDS during study</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Coordinate: How and what diet will be ready once study done?</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prepare VCO2 cart – label breath tubes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Get urine collector bags &amp; ice bucket</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prepare and print nursing instructions, give night before</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pre-study Day – early AM in diet kitchen/formula room</strong></td>
<td></td>
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</tr>
<tr>
<td>Gather stirring plate, 1 L beaker (Susan’s plastic one), parafilm, micron filter, squeeze bottles – MAKE SURE ALL DISHWASHED and PLASTIC*</td>
<td></td>
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<tr>
<td>---</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Day before study – measure 13C and 12C Phe, Thr, Ala, GLT &amp; AAs into large plastic beaker</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sterile water up to about 80% of required volume</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cover with parafilm and stir plate until visibly dissolved – approximately 5 hours</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transfer to measuring grad. cylinder and top off with sterile water, rinsing the beaker well**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Filter AA solution using 0.2u filter, cover and refrigerate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Measure pro phree and transport downstairs*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bring large plastic grad. Cylinder just in case</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7am: Transfer to milk prep room in kitchen, bring pro phree</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-7:30am – mix pro phree with AA solution and pour into bottles, label them and bring up to ward fridge</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Study day – after making formula (7:30am)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bring formula to ward</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Begin study</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>End study – complete study completion form</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Will they be doing a second study?</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix C. Nursing bedside instructions

Your Patient: ___________________________ HSC #_________________

Is participating in the Dr. Paul Pencharz’s Enteral Threonine Requirement study

For 24 hours: ___________________________2017

His/Her scheduled activities are:

<table>
<thead>
<tr>
<th>Time</th>
<th>RN</th>
<th>Veronik Connan, RD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(Study contact)</td>
</tr>
<tr>
<td>0001 – 1200h</td>
<td>Length and Weight</td>
<td></td>
</tr>
<tr>
<td>Date:</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| 0600 – 1200h     | • With each diaper change, add > 5 cotton balls to diapers; when diapers are changed, put saturated cotton balls in urine collectors in biohazard bag and then into ice bucket (label time of collection)  
| Date:            | • Please change diapers at least 3 times in this time putting new cotton balls into fresh diaper  
|                  | Study Staff to collect baseline breath samples between 0900 to 1200: 30 minutes under the hood X 2 tests.  |
| 1200 h           | • Begin feed administration (continuous feeds) of study Threonine diet (brightly coloured labelled bottles).  
| Date:            | *SHAKE WELL before q 2 feed change*  
| To 1200h         | • Infant will remain on this formula for up to 24 hrs and then return to previously prescribed feeds.  
<p>| Date:            | • Please note any reflux or vomiting, estimated amount and time.  |</p>
<table>
<thead>
<tr>
<th>Time</th>
<th>Instructions</th>
</tr>
</thead>
</table>
| 0300-1200h | • With each diaper change, add >5 cotton balls to diapers; when diapers are changed, put saturated cotton balls in urine collectors in biohazard bag and then into ice bucket. (label time of collection)  
• Please change diapers at least 3 times in this time putting new cotton balls into fresh diaper |
| 0700-1200h | • Maintain feeding tube administration (continuous feeds) of study Threonine diet (brightly coloured labelled bottles).  
  *SHAKE WELL before q 2 feed change*  
• Infant will remain on this formula until study end (approx. 12 pm) and then return to previously prescribed feeds.  
• Please note any reflux or vomiting, estimated amount and time.  
  Study Staff to collect plateau breath samples between 0700 to 1200: 30 minutes under the hood X 3 tests. |
| 1200h      | Study end – baby returned to prescribed feeds                                                                                                                                                      |
Appendix D. Taste panel questionnaire

Please taste samples and score as follows:

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Odour</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Taste</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Texture</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Excellent:** 4
**Good:** 3
**Fair:** 2
**Poor:** 1

Please compare samples 1 with 3

Which formula smells the best? __________

Which formula tasted the best? __________

Which had the best texture? _________

Please compare samples 3 with 4

Which one smells the best? __________

Which one tasted the best? __________

Which had the best texture? _________
In your opinion:

If infants can tolerate formulas 1 and 4 by mouth, could they also tolerate 3 by mouth?

_______

Rank formulas in terms of palatability

<table>
<thead>
<tr>
<th></th>
<th>Best</th>
<th>OK</th>
<th>Worst</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Any general comments about any of the formulas?

______________________________________________________________________________
______________________________________________________________________________
______________________________________________________________________________
Appendix E. Study diet calculations

**Formula Calculations For Study Day**

Study Date (yyyy/mm/dd): ________________  Subject ID: ________________

Age: __________ months

Length: _________ cm  Weight: ____________ kg  Head Circumference: _____ cm

---

**Threonine Test Level** = __________ mg/kg/d [X]

---

**Abbreviations:**

TFI = Total fluid intake  
VTBI = Volume to be Infused  
GLT = Glycyl-L-Tyrosine  
BM = Breast Milk  
ptn = Protein  
CHO = Carbohydrates  
AA = Amino Acids  
N = Nitrogen  
Thr = Threonine  
Ala = Alanine
**Nutritional Requirements as Prescribed by RD**: 

- Protein** = ________g/kg/d (required: 2.6-3g/kg/d) [A]

- Total protein intake/day = _____[A] x _____ kg = ______ g PTN required per day [A1]

- Total Fluid Intake (TFI) aka volume to be infused (VTBI) = __________ml/kg/d x _____ kg = ____________ mls of fluid/d [VTBI]

- Calories = ____________ kcals/d [C]

*The infant will have been well tolerating these feeds as previously prescribed by the dietitian.

The infant will also have gained and maintained adequate weight status and growth while on these feeds, to ensure an anabolic state.

**As close to 3 g/kg/d as possible
<table>
<thead>
<tr>
<th>WHO UNU FAO Breast Milk</th>
<th>MW</th>
<th>Nitrogen</th>
<th>AA Breast Milk [Standard]</th>
<th>AA Breast milk using our AAs</th>
<th>1 kg bulk (test AAs removed)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gm/Mol</td>
<td>gm N/AA</td>
<td>mg AA/g total ptn</td>
<td>g/kg ptn (AA)</td>
<td>g/kg ptn (AA)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Aspartic Acid</td>
<td>133.1</td>
<td>14</td>
<td>90</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>(Glycine from GLT)</td>
<td></td>
<td></td>
<td></td>
<td>[21.5]</td>
<td>[21.5]</td>
<td>Amount of Glyc from GLT (not added directly into bulk)</td>
</tr>
<tr>
<td>2</td>
<td>Gly (additional)</td>
<td>75.1</td>
<td>14</td>
<td>23</td>
<td>1.45</td>
<td>1.45</td>
</tr>
<tr>
<td>3</td>
<td>Tryp</td>
<td>204.23</td>
<td>28</td>
<td>17</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>4</td>
<td>Leu</td>
<td>131.17</td>
<td>14</td>
<td>96</td>
<td>96</td>
<td>96</td>
</tr>
<tr>
<td>5</td>
<td>Phe</td>
<td>165.19</td>
<td>14</td>
<td>42</td>
<td>42</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>Meth</td>
<td>149.21</td>
<td>14</td>
<td>16</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>7</td>
<td>Isoleu</td>
<td>131.17</td>
<td>14</td>
<td>55</td>
<td>55</td>
<td>55</td>
</tr>
<tr>
<td>8</td>
<td>His</td>
<td>155.15</td>
<td>42</td>
<td>21</td>
<td>21</td>
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<td>(Tyr from GLT)</td>
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<tr>
<td>16</td>
<td>Tyr</td>
<td>181.19</td>
<td>28</td>
<td>52</td>
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<tr>
<td>17</td>
<td>Cys</td>
<td>121.16</td>
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<td>17</td>
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<tr>
<td>18</td>
<td>Pro</td>
<td>115.13</td>
<td>14</td>
<td>80</td>
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<td>19</td>
<td>GLT</td>
<td>238.24</td>
<td></td>
<td>68.4</td>
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<td>will be added based on Tyr content of BM; not added in the calculations of the total grams of AA in the Bulk #1. Only g’s of tyrosine and glycine supplied by GT added in the calc.</td>
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<tr>
<td><strong>Totals</strong></td>
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<td></td>
<td>322</td>
<td>966</td>
<td>978.05</td>
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Calculating the Nitrogen (N) Content of the 2 Amino Acids Being Varied in the Study: Using Breast Milk (BM) Protein Pattern and Baby’s protein requirement

Threonine:

\[\text{[Thr] in BM ptn} = 44\text{g/kg of ptn} \rightarrow 44\text{mg/g} \times \text{______ [A1]} = \text{_______ mg of Thr baby would be getting if consumed BM standard [D]}\]

Total N from Thr = \(14000 \times \text{______ [D]} \div 119119 = \text{________________mg N from Thr in amount of BM that would be consumed by this baby [E]}\)

Alanine:

\[\text{[Ala] in BM ptn} = 38\text{ g/kg of ptn} \rightarrow 38\text{mg/g} \times \text{______ [A1]} = \text{_______ mg of Ala baby would be getting if consumed BM standard [F]}\]

Total N from Ala = \(14000 \times \text{______ [F]} \div 89090 = \text{________________mg N from Ala in amount of BM that would be consumed by this baby [G]}\)

Total N from Thr & Ala in BM ptn = \______ [E] + \______ [G] = \_______mg of N [H]

Volume of formula to be made (which includes overfill for priming, waste, spillage etc.)

Total formula volume to be made = \______\(\text{(VTBI)}\) + 250 (standard overfill) = Total Volume = \______________ml [HAPPY]
**Amino Acid Mix (for study day) – Enough for 24 hours worth of continuous tube feeding**

AA Bulk Mix represents 80.32% of the total AA reqt = ______ [A1] x 0.8032 = _________g AA required from bulk mixture [HAPPY1]

The total amount of AAs to be added (Thr, Ala, GLT, 12C-phe, 13C-phe) = 19.68% of reqt = ______ [A1] x 0.1968 = _________g added to bulk [HAPPY2]

Bulk AA mix per ml of formula = _________ [HAPPY 1] ÷ ______ [VTBI] = _________

g/ml [AAMIX]

__________ [AAMIX] X __________ [HAPPY] = __________ g of AA bulk require for total volume of formula to be prepared [HAPPY3]

**Phe Intake (Diet + Isotope) – not contributing to changes in Nitrogen**

Phe intake/day = 126 mg/kg/d x _____ kg body wt = ______ mg Phe intake required in 24 hrs [I]

**Isotope Phe:**

\[^{13}\text{C}}\text{-Phe intake = 2.4927 mg/kg/h x _____ kg x 24 hours = }\]

\[\text{__________ mg }^{13}\text{C}}\text{-Phe per/24hrs [J]}\]

\[^{13}\text{C}}\text{-Phe per ml formula = _________[J] ÷ _________[VTBI] = _________mg/ml [K]}\]

\[\text{__________[K] x __________ [HAPPY] ÷ 1000 = __________ g of }^{13}\text{C}}\text{-Phe required for TFI + overfill (total formula) [K1]}\]

**Dietary Phe** = __________ [I] – __________ [J] = __________mg [M]

Dietary Phe per ml of VTBI = __________ [M] ÷ _________ [VTBI] = __________

g/ml formula of dietary Phe [N]

\[\text{__________ [N] x _________ [HAPPY] ÷ 1000 = __________ g of Phe required for TFI + overfill (total formula) [N1]}\]
Double Check Phe Calculation:

Total Phe intake = __________ mg requirement/day of Phenylalanine

$_{[I]}$ / $[VTBI]$ = __________ mg/ml Phe concentration X

$_{[HAPPY]}$ / 1000 = __________ g of Phe in total formula to be made, then

subtract 13C Phe intake _______ [K1]= should give amount of 12C Phe intake in diet

_________ g [N1]

Threonine Intake (test AA)

Thr intake (test level) = __________mg/kg/d [X]

Thr intake/day = __________ [X] x _________kg body wt = _________mg [O]

Thr amount per ml of VTBI = __________[O] / _________ [VTBI] = __________

mg/ml [P]

_________ [P] x _________ [HAPPY] / 1000 = _________ g of Thr required for TFI +

Overfill or HAPPY [P1]

Nitrogen from test Thr intake = 14000 x _________ [O] / 119119 =

___________mg N from Thr required intake [Q]

Balance Alanine to be added (to keep formula iso-nitrogenous)

Alanine to be added to diet = Nitrogen difference between standard BM & test Thr

intake

Nitrogen left to balance = _________ [H] – _________ [Q] = __________mg N from

Ala to balance [R]

Ala required = _________ [R] x 89090 / 14000 = __________mg Ala in

VTBI [S]

Ala concentration in VTBI = _____________ [S] / _________ [VTBI] = __________

mg/ml [T]

_________ [T] x _________ [HAPPY] / 1000 = __________ g of Ala required for

TFI + Overfill (Total Study Formula) [T1]

Amount of fluids used to make total AA solution = __________ mls (approx 200-500ml)

[X1]
Amount of GLT to be added (to provide 52mg/g of tyrosine as in BM composition)

52mg/g of tyrosine = 68.4mg/g of glycyl-L-tyrosine

Amount of GLT required = 68.4 mg x ________ [A1] = __________ mg of GLT intake required [O1]

Amount of GLT to be measured = (__________ [O1] ÷ __________ [VTBI]) x ________ [HAPPY] ÷ 1000 = ________ g of GLT to be added to entire formula [OO]

Double-check AA Component of Formula

Total amount of AAs to be added (Thr, Ala, GLT, 12C-phe, 13C-phe) = ___________ g [HAPPY2]*

Thr: ___________ mg [O]
Ala: ___________ mg [S]
GLT: ___________ mg [O1]
13C Phe: ___________ mg [J]
12C Phe: ___________ mg [M]

Total = ([O] + [S] + [O] + [J] + [M]) / 1000 = _____________ g of AA to be consumed by baby *

*Should match with [HAPPY2] → some variation OK since alanine added based on nitrogen and not requirement…

Energy Intakes as prescribed by attending dietitian

___________ kcal/day [C]

Total Diet Constituents = Amino Acids + Formula (Pro-Phree)

Calories from AA intake = ________[A1] x 4 kcals/g = ____________ kcals from dietary AA [U]

Caloric difference to be made up by ptn-free formula Pro-Phree = ___________ [C] – ___________[U] = _____________ kcals required from Pro-Phree [V]
Study Formula Preparation*

*Need to calculate displacement for TFI (VTBI), then calculate displacement for overfill/prime – This is then subtracted from the Total volume minus the volume from the AA mix to yield total free water that we are able mix with pro-phree

Formula (Pro-Phree) required = ________ [V] ÷ 5.1 kcal/g (caloric concentration of Pro-Phree) = ______________ g of Pro-Phree powder required [V1]

Formula Displacement = __________[V1] x 0.72 ml/g displacement= __________mls displaced [W]

Available water to make ________ [VTBI] – _________ [W] – _______ [X1] = __________ max amount (ml) of water that can be added [maxH20]

_________ [V] ÷ _________[VTBI] = _________ kcal/ml X volume required for prime/overfill 250ml = __________ kcals pro-Phree in priming volume → then ÷ 5.1 kcal/g = __________ g of Pro-phree required for priming volume [PP] + ________ [V1] = __________ g total Pro-Phree required for total volume of formula to be made [TP]

Displacement of pro-phree required for prime = __________[PP] x 0.72 ml/g = __________ mls displaced [W1]

250ml – _________ [W1] = __________ mls of free water to add in priming volume [W2]

## Amino Acid Recipe on Study Day

In diet kitchen:

<table>
<thead>
<tr>
<th></th>
<th>Amount (g) required in total volume [HAPPY]</th>
<th>Instructions for formula production</th>
</tr>
</thead>
<tbody>
<tr>
<td>13C Phe</td>
<td>[K1] =</td>
<td>To be mixed together and dissolved in sterile water using a stirring plate and a beaker covered with parafilm**</td>
</tr>
<tr>
<td>12C Phe</td>
<td>[N1] =</td>
<td></td>
</tr>
<tr>
<td>Thr</td>
<td>[P1] =</td>
<td></td>
</tr>
<tr>
<td>GLT</td>
<td>[O0] =</td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>[T1] =</td>
<td></td>
</tr>
<tr>
<td>Bulk</td>
<td>[HAPPY3] =</td>
<td></td>
</tr>
<tr>
<td>Sterile Water</td>
<td>[X1]=</td>
<td></td>
</tr>
</tbody>
</table>

**Note:** Amino acid formula ➔ Dissolved in approx. 150 ml distilled water. Once dissolved - top up in flask to 200 ml or to 400ml (for 6kg baby on 3g/kg ptn needs) – depending on protein requirement of the infant.

All contents of table are to be added to bulk, mixed on stirrer until dissolved and filtered with 0.2u filter together then added to non-protein formula (pro-phree) (See below)

Then filter through 0.2u filter ➔ transport filtrate downstairs to Formula Preparation Room (FPR)
In formula/ milk prep room:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Study day amount (g or ml) to be measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro-phree powder [TP]</td>
<td></td>
</tr>
<tr>
<td>Total free sterile water to be added [FW]</td>
<td></td>
</tr>
</tbody>
</table>

Formula Requisition (Study day) 100% of baby’s needs to be made in 1 formula and divided into multiple bottles as per clinical practice (multiple feeds to go into each bottle → nurses to measure proper amount q 2hrs)  
Each bottle can hold up to 210ml
## Appendix F: Data collection form

A Physiological Study to determine Enteral Threonine Requirements in Infants aged 1-6 Months

### Study Data Collection Form: PreStudy

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<thead>
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<th>Times</th>
<th>Comments</th>
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<tr>
<td>1 2 3 4 5 6 months</td>
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<tr>
<td>Clinically Stable Y  N</td>
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<td>in-Pt Y N</td>
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<td><strong>Excl Criteria:</strong></td>
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<td><strong>Vital Signs</strong></td>
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<td>BP:</td>
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<td><strong>Baseline Breath 1 Urine 1</strong></td>
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<td><strong>Baseline Breath 1 Urine 1</strong></td>
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A Physiological Study to determine Enteral Threonine Requirements in Infants aged 1-6 Months

**Study Data Collection Form:** Study day #1

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<td>L-1-13(^{13})Phe Isotope Admin 0600 - 1000h</td>
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Qualified Investigator Signature & Date