INDIVIDUAL GROWTH TRAJECTORIES FOR PRETERM INFANTS

INDIVIDUAL REFERENCE GROWTH TRAJECTORIES FOR PRETERM INFANTS WITH POSTNATAL WEIGHT LOSS AND CONVERGENCE WITH TERM TRAJECTORIES OPTIMIZED TO MINIMIZE DISEASE RISK (DOHAD) - IMPLICATIONS FOR CALCULATION OF POSTNATAL GROWTH RATES IN CLINICAL PRACTICE

By:

ERIN LANDAU-CRANGLE (Honours BSc.)

A thesis submitted in partial fulfillment of the requirements for the degree
MASTER OF SCIENCE

MCMASTER UNIVERSITY

May 2016

INSTITUTION: McMaster University, Hamilton, Ontario

DEGREE: Master of Science (M.Sc.) Medical Sciences: Metabolism and Nutrition (2016)

TITLE: Individual Growth Trajectories for Preterm Infants

AUTHOR: Erin Landau-Crangle, Honours BSc.

SUPERVISOR: Dr. Christoph Fusch

NUMBER OF PAGES: x,75

LAY ABSTRACT:

It has been well established that growth of preterm infants has a long-term impact on health in adulthood. Since the survivability of preterm infants has drastically improved in the last decades, there has been a shift in focus to improving quality of life, including improved growth. Infants that grow too quickly or too slowly may develop inappropriate body compositions, with either too much or too little fat. A sub-optimal body composition can put infants at an increased risk for developing cardiovascular, metabolic or neurodevelopmental diseases later in life. In order to prevent these diseases and optimize growth, it is necessary to have a better understanding of how preterm infants should grow. This thesis aims to improve the characterization of growth for preterm infants by providing individual reference growth trajectories for preterm infants that take into consideration postnatal adaptation and aim to minimize later disease risk.

ABSTRACT:

Background: The DoHAD hypothesis suggests that preterm infants should achieve similar growth and body composition to healthy term-born infants in order to minimize disease risk. Postnatal growth of preterm infants is not fully understood and requires additional characterization, particularly in terms of differences to and transition from intrauterine growth. The period of postnatal adaptation to extrauterine life has been described in preterm infants by Rochow et al., 2016 and was seen to last 21 days. During these first 21 days of life, preterm infants experience a physiological, one-time, permanent contraction of extracellular water spaces (water loss), which causes a downward shift in the growth trajectories. This period of adaptation/water loss and the transition to extrauterine growth rates to achieve WHOGS target trajectories need to be incorporated into individual reference curves for preterm infants.

Objectives: To develop and evaluate approaches to establish individualized growth trajectories for preterm infants to achieve growth similar to the WHO growth standards (WHOGS) for healthy infants at term, using recently published data about the physiological postnatal adaptation.

Methods: Two approaches were compared: 1) Postnatal-Percentile Approach: growth following the percentile at day of life (DOL) 21 until term; 2) Growth-Velocity Approach: using day-specific Fenton median growth velocities between DOL 21 and term. The impact of these approaches were compared using body compositions of 57 healthy preterm infants obtained before discharge (36+0/7 to 42+6/7 weeks PMA).

The main outcome was the weight difference between the predicted trajectory and WHOGS target at 42+0/7 weeks PMA for the infants' birth weight percentile.

Results: Postnatal-Percentile Approach: Trajectories deviated by up to 930g and did not match with WHOGS. Growth-Velocity Approach: Trajectories converged with term WHOGS after adjusting growth velocities with a factor of 1.0017 (approximately 10% increase in daily growth velocities). The validation of the Growth-Velocity Approach in preterm infants with minimal medical interventions revealed little deviation between predicted and actual weights. Infants were symmetrically distributed around zero deviation with a mean deviation of -10±370g and an average of 20% fat mass. In contrast, the Postnatal-Percentile Approach showed large deviations between predicted and actual weights and a skewed distribution around zero deviation with a mean deviation of -310±380g or 70±350g, following the birth or DOL 21 percentile, respectively.

Conclusions: Individualized growth trajectories for preterm infants converged with the WHOGS when Fenton daily median growth velocities were applied and optimized with a single factor. The simplicity of the model and its ability to predict target weights that correspond to an appropriate fat mass suggests a biological principle. These results provide a superior understanding of preterm infant's growth including the physiological postnatal adaptation and new trajectories to achieve WHOGS target trajectories. Results can be used to develop a bedside tool to aid clinicians in monitoring growth, guiding nutrition and preventing chronic adult diseases as a consequence of unguided, inappropriate growth.

ACKNOWLEGEMENTS:

Foremost, I would like to thank my graduate supervisor Dr. Christoph Fusch for his continuous mentorship and guidance, helping me to transform from an inexperienced third year undergraduate student to a more experienced graduate student who will always aim to think critically. I am appreciative of the scientific, research and clinical expertise Dr. Fusch has contributed to my learning and of his passion to improve clinical care through research.

I would also like to thank Dr. Gerhard Fusch, our lab manager, who has continuously provided an alternative perspective and guidance throughout this project.

Thank you to Dr. Niels Rochow, who has been a major collaborator on this project. I am extremely appreciative of the patience Dr. Rochow has shown and the time he has taken to teach me and help me with this project.

Thank you to Dr. Joseph Macri, who provided a tremendous amount of knowledge, and expertise to the wet lab (gel electrophoresis/mass spectrometry) portion of my master's work and continues to me an incredible mentor.

Thank you to Dr. Tarnopolsky for his scientific contributions, discussions and expertise through committee meetings and Dr. Morrison for her valuable feedback as the external examiner.

Thank you to Dr. Michael Marrin, Dr. Tanis Fenton, Anaam Ali and Kai Liu for their collaboration and contributions to the manuscript that is the basis of this project. Dr. Marrin kindly provided his expertise as a neonatologist and Dr. Fenton her expertise on growth.

Thank you to Preeya Raja, for without her Master's thesis, there would be no basis for my project.

As well, thank you to Laura Bagnulo, for her tireless administrative and moral support. Lastly, I would like to sincerely thank all of the Fusch Lab members for both their scientific contributions and discussions and for making the lab a fun and inviting place.

TABLE OF CONTENTS:

LIST OF TABLES:

LIST OF FIGURES:

ABBREVIATIONS:

VLBW = very low birth weight
EUGR = extrauterine growth restriction
WHOGS = World Health Organization Growth Standards
GA = gestational age
PMA = postmenstrual age
DOL = Day of Life
DOHaD = developmental origins of health and disease
NICU = neonatal intensive care unit
SGA = small for gestational age
LGA = large for gestational age
AGA = appropriate for gestational age
IUGR = intrauterine growth restriction
BIA= bioelectrical impedence
IFO = individualized fortification of breast milk
AAP = American Academy of Pediatrics
PreCES = Preterm Contraction of Extracellular Spaces
TeCES = Term Contraction of Extracellular Spaces

DECLARATION OF ACADEMIC ACHIEVEMENT:

This thesis is an expansion of a manuscript prepared for submission:

> **Landau-Crangle, E**., Rochow, N., Fenton, TR., Liu, K., Ali, A., Fusch, G., Marrin, M. Fusch, C. [n.d.]. A new concept and practical tool to predict preterm infant's individual growth trajectories. In preparation for submission.

Erin Landau-Crangle, Niels Rochow, Christoph Fusch: Developed study design, analyzed and interpreted the data, wrote the manuscript

Erin Landau-Crangle performed the literature review and wrote the thesis

Michael Marrin and Tanis Fenton: Helped to interpret the data and wrote the manuscript

Kai Liu: Employed the predictive model

Anaam Ali: Collected body composition data and was involved in interpretation of data.

Gerhard Fusch: Was involved in interpretation of the data.

INTRODUCTION

Survival rates in preterm infants, particularly VLBW infants (<1500g) have drastically improved in the last decade, shifting the focus of clinical care towards quality of life for preterm infants (Horbar et al., 2015; St. John & Carlo, 2003). According to the developmental origins of health and disease (DOHaD) hypothesis, nutrition and growth in early life are related to later disease risk. Thus, in order to improve quality of life and minimize the risk of adult diseases, it is important to consider growth and nutrition (Uauy & Koletzko, 2014). Suboptimal early growth can cause infants to develop an inappropriate body composition and expose the infants to an increased risk for chronic diseases in adulthood including metabolic, cardiovascular and neurological disease (Euser et al., 2005; Gluckman, P.D., Hanson, M.A., Beedle, 2007; Metcalfe & Monaghan, 2001; Wells, Chomtho, & Fewtrell, 2007). During the 2^{nd}-3^{rd} trimester of pregnancy, fetuses experience a very high growth rate of approximately 17-20 g/kg/day (Johnson, Wootton, Leaf, & Jackson, 2012; A Lucas et al., 1984; Ee Ziegler, 2011). Infants may be born preterm due to the disturbance of the feto-placental maternal unit, which places the fetus' well being at risk. Outside the womb, preterm infants are exposed to a distinct set of conditions compared to their counterparts that remain *in utero* until term age. Additionally, preterm infants cared for in the NICU are at risk for diseases of prematurity and sepsis (Fusch & Samiee-Zafarghandy, 2014). These conditions can lead to postnatal growth restriction of preterm infants (commonly defined as growth less than the 10^{th} percentile), which has consequences for later disease risk (Clark, Thomas, & Peabody, 2003; Marks, Reichman, Lusky, & Zmora, 2006; E. E. Ziegler & Carlson, 2009).

DOHAD

The Developmental Origins of Health and Disease (DOHaD) hypothesis was proposed by David Barker, a British Epidemiologist, in 1990 after he observed a correlation between infants with lower birth weights and a higher risk for cardiovascular disease in adulthood. Barker's work provided the foundation for many further research studies that tested whether foetuses and infants in early life can undergo programming effects based on their environmental conditions (Barker & Osmond, 1986; Barker, 1992). For example, evidence suggests that infants that were developing *in utero* during the Dutch famine of World War II, were born smaller, and experienced programming effects leading to impaired glucose tolerance, higher body mass index (BMI), and increased cardiovascular disease risk. These negative outcomes may be a result of the mismatch between adaptation to famine conditions in prenatal life and instead experiencing non-famine conditions postnatally (Ravelli et al., 1998). The evolutionary adaptive advantage that would have been provided by being prepared for a famished environment ended up putting these infants at greater risk in adult life due to a mismatch between early and late environment. This study was repeated by Yudkina et al in a Finnish population where there was also a famine. They found no differences in insulin resistance, likely due to the fact that the famine in Finland lasted much longer (28 months vs. 6 months in the Netherlands) and so the mismatch in environments (pre- vs. postnatal) was not present (Yudkina & Stannera, 1998). Another possible trade-off that is important to consider is that with smaller body size, in particular less fat mass, infants may also be at a greater risk for impaired neurodevelopment. Many studies since, have observed the relationship between early life conditions and later disease risk.

De Jong and colleagues performed a systematic review of observational studies looking at adult, adolescent and children's resting blood pressure for individuals born at term age (>37 weeks) or born preterm (<37 weeks). They found that preterm-born infants had higher resting blood pressure and higher risk of hypertension (Femke De Jong, Monuteaux, Van Elburg, Gillman, & Belfort, 2012). Catch-up growth is commonly defined as periods of very high growth rates after periods of growth restriction (Hack et al., 2003). This study also found that infants who experienced more rapid catch-up growth between preterm birth and term age had a higher risk of increased blood pressure at school-age (Femke De Jong et al., 2012). Another systematic review was performed by Parkinson et al. in 2013, examining metabolic disease in adults born preterm. They found that these individuals had higher low-density lipoprotein (LDL) cholesterol, higher blood pressure and greater risks for cardiovascular disease and atherosclerosis (Parkinson, Hyde, Gale, Santhakumaran, & Modi, 2013). In 2005, Johansson et al. performed a cohort study on adult men that were born preterm in Sweden and measured their blood pressure as adults. They found that men born preterm had a higher risk of high blood pressure and there was a dose-response effect, where the higher the gestational age the individual was born at, the lower the risk of high blood pressure (Johansson et al., 2005).

Keijzer-Veen et al, 2010, did a retrospective follow-up study of preterm infants (born <32 weeks gestational age) that were born small for gestational age (SGA) due to intrauterine growth restriction (IUGR), term infants born appropriate for gestational age (AGA) and preterm infants born AGA. The follow-up was done at age 20 and measured blood pressure and renin concentration (marker of kidney function). This study found that

premature infants had higher blood pressure but no effect of IUGR was seen on blood pressure (Keijzer-Veen, Dülger, Dekker, Nauta, & van der Heijden, 2010). Another study found that AGA and SGA preterm infants had lower insulin sensitivity than term infants and higher systolic and diasoltic blood presure (Rotteveel, van Weissenbruch, Twisk, & Delemarre-Van de Waal, 2008). In 2013, Lewandowski and colleagues published a prospective follow-up study of 25-year-old adults born preterm (3 groups varying by degree of prematurity) or born at term. They did a lifestyle questionnaire, blood sample, blood pressure and cardiac MRI. This study found that more premature infants had smaller right ventricles and smaller right ventricle end-diastolic volume; this was the most extreme in the most premature infants. They also found preterm infants had higher ratios between right ventricle mass and end-diastolic volume, lower stroke volume, higher heart rates, lower cardiac output, lower ejection fraction and overall poorer cardiovascular health as adults (Lewandowski et al., 2013). In 2015, this group published another prospective follow-up study of 25-year-old adults born preterm vs. at term. They measured blood pressure, circulating angiogenesis markers (soluble endoglin, soluble fms-like tyrosine kinase-1), as well as assessments of cardiovascular function including aortic stiffness and carotid-femoral pulse. This study found preterm-born adults had increased soluble endoglin (sENG), which is a marker of cardiovascular disease and this was related to degree of prematurity (Lewandowski et al., 2015).

In 2002, a prospective study of preterm infants followed them until 13-16 years of age. They looked at nutrient intake (randomized to nutrient rich preterm formula, standard formula or donor breast milk) and measured serum leptin, fat mass (using bioelectrical impedance and skinfold thickness) at 13-16 years. This study found that the

ratio of leptin to fat mass was greater in the preterm formula group compared to the standard formula or donor breast milk group. This study suggested that leptin levels could be the result of early programming effects from the nutrient-rich early diet and could be linked to a potential mechanism of later obesity (Singhal et al., 2002).

In 1994, Lucas and Morley executed a randomized control trial where preterm infants were randomized to different diets (preterm formula, standard formula or breast milk) and measured blood pressure at 7.5-8 years of age. The group found major differences in the nutrient intake between diets but saw no difference in blood pressure. Although this does not support the idea that blood pressure may be programmed by early diet, this data may support the idea that early nutrition to overcome growth restriction could prevent programming effects on blood pressure (A Lucas & Morley, 1994). 4 years later, this group studied preterm infants less than 1850g and performed a randomized, blinded, longitudinal study where infants received either 4 weeks of only standard formula, only nutrient rich preterm formula, or preterm formula as a supplement to maternal milk. They measured IQ at 7-8 years with the Weschler scale and found a significant sex difference. Males fed standard formula had a significantly lower IQ, especially verbal IQ; however this could be due to males being sicker by chance. Both sexes had lower verbal IQ on standard formula but and no cognitive disadvantages when on preterm formula. Standard-formula fed infants had higher incidences of cerebral palsy. The preterm formula group gained more weight and had a higher head circumference, a marker of neurodevelopment. The best outcomes were seen in the breast milk supplemented with preterm formula group, which supports current recommendations that

breast milk with appropriate supplementation is the best source of nutrition for preterm infants (A Lucas, Morley, & Cole, 1998).

Belfort and colleagues did a longitudinal prospective follow-up study of preterm infants and found that higher growth rates during the first year provided a modest neurodevelopmental advantage and a small increase in blood pressure (M. B. Belfort, Martin, Smith, Gillman, & McCormick, 2014). In a prospective long-term follow-up study Ehrenkranz et al. found that growth velocity during hospital stay was significantly correlated with neurodevelopment and growth outcomes in later life (Richard A Ehrenkranz et al., 2006). This adds significant justification to studying preterm infant's growth during NICU hospitalization.

A study examining BMI and weight patterns in infants, children and adolescents in the New Delhi Birth Cohort measured waist circumference, blood pressure, glucose, insulin, lipid concentrations, and prevalence of metabolic syndrome in 26-32 year olds who had been followed for weight and height during infancy, childhood and adolescence (every 6 months). They found more rapid weight gain or BMI increase (earlier adiposity) associated with increased risk of metabolic syndrome and insulin resistance. Glucose intolerance was associated with rapid BMI gain in childhood and adolescence but lower BMI in infancy (catch-up effect) (Fall et al., 2008).

Taking many of these studies into consideration, Lucas has suggested that in fact it may be the rapid postnatal growth experienced in the post-term period, aptly named the "postnatal growth acceleration hypothesis", that puts infants at risk for adult chronic diseases rather than characteristics such as birth weight alone. As mentioned above, he found that the relationship between prematurity and later blood pressure disappeared

when nutrition in the preterm period was controlled (Alan Lucas, 2010). This provides strong evidence for the importance of studying nutrition, growth and body composition in infancy in order to guide growth and prevent negative developmental programming effects.

NUTRITION & GROWTH RATES

In term infants, nutritional intake needed for growth is controlled via neuro-endocrinal appetite regulation (Ross & Desai, 2014). Hungry term infants cry to be fed. Preterm infants are not able to communicate their hunger. In comparison to foetuses remaining *in utero* until term age, preterm infants lack a placenta to provide nutrients and nutrient clearance (Fusch & Samiee-Zafarghandy, 2014). As a result, preterm infants are fully under the nutritional control of the clinical team who therefore also control their postnatal growth. According to the DOHaD hypothesis, growth is related to risk of developing chronic diseases in adulthood. Therefore is important that the nutrition given and the growth and body composition achieved are optimized for each individual infant in order to ensure the infant's disease risk is minimized (A Lucas et al., 1998; A Lucas & Morley, 1994).

The American Academy of Pediatrics (AAP) suggests that preterm infants achieve growth similar to that of a fetus *in utero*, 17-20 g/kg/day (Nutrition, 1985). This can be difficult to emulate outside the womb, particularly during the first weeks after birth (Clark et al., 2003; R A Ehrenkranz et al., 1999; Marks et al., 2006; E. E. Ziegler & Carlson, 2009). After preterm birth, the premature infant is cut off from the placental supply of nutrients (Fusch & Samiee-Zafarghandy, 2014). *In utero*, the placenta provides the growing fetus with all necessary nutrients. The nutrition is characterized mainly by

high amounts of amino acids and glucose and the necessary essential fatty acids (Nutrition, 1985; C. H. . van den Akker et al., 2008; C. H. P. Van Den Akker et al., 2011; C. H. Van den Akker & Van Goudoever, 2010; C. Van Den van den Akker et al., 2009; E. E. Ziegler, O'Donnell, Nelson, & Fomon, 1976). After birth, postnatal nutrition for preterm infants is provided by parenteral and enteral nutrition. Due to the gut immaturity, enteral feeding is slowly advanced during the first weeks of life. Preterm infants are at a high risk for developing feeding intolerance; therefore enteral feeding advancement is prolonged on average for extremely low birth weight (ELBW) infants approximately 14-21 days. Some NICUs reported requiring 30 or more days to achieve full enteral feeding (Agostoni et al., 2010). During enteral feeding advancement most nutrition is provided by parenteral nutrition. Parenteral nutrition is also increased slowly until the end of the first week of life. Full parenteral nutrition provides a lower calorie intake (90 to 100 kcal/kg/day) than what is recommended and provided by full enteral feeding (130 to 140 kcal/kg/day) (Koletzko, Goulet, Hunt, Krohn, & Shamir, 2005a, 2005b, 2005c, 2005d). As a result, preterm infants are exposed to a cumulative nutritional deficiency leading to extrauterine growth restriction (EUGR) (R A Ehrenkranz et al., 1999; E. E. Ziegler & Carlson, 2009).

It is important to consider that preterm infants actually have a higher energy requirement than foetuses because their organs are functioning without the help of the mother and placental support, they are also more physically active, have to breath, and require energy for thermogenesis. Therefore, preterm infants are at a high risk for extrauterine growth restriction. Extrauterine growth restriction is continually reported to be a major issue for preterm infants (Clark et al., 2003; R A Ehrenkranz et al., 1999;

Marks et al., 2006; E. E. Ziegler & Carlson, 2009). Despite these challenges, postnatal growth rates can be optimized by nutritional regime. In an attempt to achieve optimal extrauterine growth rates and body composition similar to healthy fetuses that stayed *in utero* until term, different strategies exist to feed preterm infants (Arslanoglu, Moro, & Ziegler, 2006, 2009; A Lucas et al., 1984; Polberger, 2009; Rochow et al., 2013).

These strategies all aim to optimize nutrient intake. This includes optimizing the energy, amino acids, lipids and carbohydrates provided via parenteral and enteral nutrition. Preterm infants, had they stayed *in utero* until term age would have been exposed to mostly glucose and amino acids by the placenta while postnatal nutrition provides glucose, amino acids and high amounts of fat to facilitate the higher postnatal energy demands. Recent studies have shown that growth rates of preterm infants can be adjusted with nutritional regimes (Adamkin & Radmacher, 2014; R. a Ehrenkranz, 2014; Rochow et al., 2012; Senterre & Rigo, 2011; EE Ziegler, 2014).

It has been shown by our group and others that early and higher nutritional intake via parenteral nutrition, faster enteral feeding advancement and daily adjustment of nutrition according to body weight gained, increases growth rates (Rochow et al., 2012; Senterre & Rigo, 2011). Breast milk is well known to be the gold standard nutrition for preterm infants; however, it needs to be fortified in order to meet the nutritional requirements of rapidly growing preterm infants. Previous studies have shown that breast milk is highly variable, particularly in mothers of preterm infants. It has been reported that 25-50% of breast milk samples were deficient, which means that even under common clinical practice, where a standard fortifier would be added to a fixed volume of breast milk, the nutrient supply for the preterm infants would be insufficient, contributing to growth

restriction (Henriksen et al., 2009). One strategy to improve breast milk composition for preterm infants is Adjustable Fortification. Adjustable fortification measures Blood Urea Nitrogen (BUN) values as an indicator of protein intake in order to determine how much protein to add to breast milk. This approach particularly focuses on protein because of its importance in growth, particularly for lean mass growth (Arslanoglu et al., 2006, 2009). However, studies by others have shown that not only is protein important, but also energy and carbohydrate to fat ratios. In particular, the higher the total energy provided, the higher the growth rate. The non-protein energy described as carbohydrate to fat ratios impact the ability to utilize protein. Higher carbohyrdate to fat ratios were observed to positively impact protein metabolism and growth, particularly increasing head circumference and length, which are markers of neurodevelopment and lean mass, respectively (Embleton, 2007; S Kashyap et al., 1988; Sudha Kashyap, 2001; Sudha Kashyap et al., 1985, 2001; van Goudoever, Vlaardingerbroek, van den Akker, de Groof, & van der Schoor, 2014). Glucose is an easily accessible energy source. In the case that glucose intake is limited, energy will be provided by fat and protein. Under this circumstance, protein will be oxidized and accretion for growth is limited.

Another approach to improve nutrition using breast milk is target fortification. Bedside breast milk analyzers have been developed which measure breast milk composition in less than one minute, using only 1mL of sample volume. This allows the clinical team to target fortify breast milk according to the recommendations by adding extra protein, fat and/or carbohydrates if any of these macronutrients would still be deficient after the addition of a standard fortifier (Rochow et al., 2013, 2015).

However, evidence for physiologically appropriate postnatal growth trajectories is scarce for preterm infants who are developing under different physiological conditions. These conditions are specifically without maternal and placental support, and requiring fully self-regulated metabolic and respiratory functioning (Fusch & Samiee-Zafarghandy, 2014). As a result, the most appropriate individual growth trajectory for each preterm infant is not known. As well, due to differences in local clinical guidelines, there is a high variation in achieved weight gain values between hospitals despite caring for infants with similar characteristics (Blackwell et al., 2005).

As mentioned previously, according to the DOHaD hypothesis infants that grow too fast or too slow are likely to achieve an inappropriate body composition (Wells et al., 2007). If an infant is healthy, within a certain range there is a linear relationship between nutritional intake and growth. Infants fed diets very rich in nutrition have higher growth rates (A Lucas et al., 1984). Nutrient rich diets may also lead to a body composition with a high fat mass index (Singhal et al., 2002). Preterm infants at term age were seen to have a higher percentage of fat mass, despite reasonable weights. Johnson and colleauges did a systematic review of 8 articles comparing body composition at term equivalent age of preterm (<37 weeks) and term-born infants (>37 weeks). The study reported mixed results, depending on method used to measure body composition. They found a higher percentage of body fat and significantly less lean mass (fat free mass) in preterm infants than in term infants at term equivalent age (Johnson et al., 2012).

More fat mass and a higher head circumference are surrogate markers of good brain development (Richard A Ehrenkranz et al., 2006) (this effect plateaus after a normal fat mass is achieved) however, a high proportion of fat mass to lean mass puts the infant at

risk for developing hypertension, cardiovascular disease, atherosclerosis, coronary heart disease, leptin resistance, insulin resistance, obesity and type 2 diabetes in later life (F. de Jong, Monuteaux, van Elburg, Gillman, & Belfort, 2011; Eriksson, 2011; Johansson et al., 2005; Johnson et al., 2012; Lewandowski et al., 2015; Singhal et al., 2002; Vohr, Allan, Katz, Schneider, & Ment, 2010). On the other hand, infants fed nutrient-deficient diets will grow more slowly and have lower body fat levels (A Lucas et al., 1984). Higher nutrient intake is associated with higher IQ in childhood and adulthood (M B Belfort, Gillman, & McCormick, 2012; A Lucas et al., 1998).

Growth restriction is still a significant problem in preterm infants and nutrition is controlled and can be optimized by the clinical team. This can result in major effects on growth and have long-lasting effects on chronic disease risk in adulthood. Thus, it is critical that we develop individual reference growth curves to help clinicians guide nutrition and growth.

CURRENT GROWTH MONITORING

Currently, growth is monitored retrospectively by measuring weight, length, head circumference and using growth charts to plot the growth (R. A. Ehrenkranz, 2014). Common charts include those by Fenton and Kramer (Fenton & Kim, 2013; M S Kramer et al., 2001). The WHO growth standards provide a gold standard postnatal growth chart for term-born infants, which were created with data from healthy, term-born, breast fed infants (de Onis et al., 2004). No such chart exists for preterm infants, because there are several idiosyncrasies that preterm infants provide. Mainly, the common Fenton charts smooth the intrauterine data (up to 36 weeks) into the post-term data from the WHOGS at

50 weeks (Fenton & Kim, 2013). These charts do not consider the period of postnatal adaptation and weight loss in preterm infants.

There are multiple types of reference growth curves currently available, all with different drawbacks for preterm infants. There are three types of reference curves to characterize growth during the postnatal period for preterm infants. The first type of reference curves are those created from cross sectional birth weight data from neonatal surveys, including several million infants of known gestational ages (Alexander, Himes, Kaufman, Mor, & Kogan, 1996; Bertino et al., 2010; Bonellie et al., 2008; Dollberg, Haklai, Mimouni, Gorfein, & Gordon, 2005; Niklasson & Albertsson-Wikland, 2008; Oken, Kleinman, Rich-Edwards, & Gillman, 2003; Olsen, Groveman, Lawson, Clark, & Zemel, 2010; Roberts & Lancaster, 2006; Voigt et al., 2010). These curves are confounded because the data about preterm babies inherently are established from pregnancies that came to an end prematurely and thus implies a pathology during pregnancy, of which a high proportion have abnormal intrauterine growth (Burkhardt, Schäffer, Zimmermann, & Kurmanavicius, 2008; Lausman, McCarthy, Walker, & Kingdom, 2012). A recent study optimized these charts by using data from a meta-analysis combined with longitudinal, postnatal data from healthy term infants (Fenton & Kim, 2013). The second type of reference curves are growth charts developed from postnatal longitudinal weight data from preterm infants that were cared for in different NICUs (Cole, Williams, & Wright, 2011; Richard A Ehrenkranz et al., 2006). These charts reflect what is being achieved by center-specific practice rather than the true physiological growth potential of these infants. Newer publications show a trend towards higher weight gain, which can be explained by improved medical standards in NICUs.

This observation further suggests that these curves may not provide physiological growth standards. Theoretically, there is a new third type of growth reference data. These data are established from longitudinal fetal ultrasound measurements from pregnancies that led to term birth and may reflect the most physiological growth. The drawback of this method is that the applied fetal ultrasound technique measures length, circumferences and diameters instead of weight and an algorithm is applied which has an error rate of up to 25% and was recently criticized by Ehrenkranz (Richard A. Ehrenkranz et al., 2006; Liotto et al., 2010).

The common Fenton curves are an example of a postnatal growth chart created using cross-sectional data from preterm infants born at different gestational ages and these curves are smoothed into the WHO Growth Standard curves from 36 to 50 weeks postmenstrual age to show the transition from intrauterine to postnatal growth (Fenton & Kim, 2013). However, these smoothed charts do not fully apply to individual preterm infants because they do not show the physiological postnatal adaptation. This is currently the best tool clinicians have to assess and monitor growth of their preterm patients. Clinicians usually check whether preterm infants are growing in parallel to the percentiles on these growth charts. However, these charts do not provide a target weight trajectory and it is unclear on which percentile a preterm infant should grow after postnatal adaptation. Inappropriate growth is usually interpreted by downwards crossing of percentiles on a growth chart (Clark et al., 2003). In such cases the nutritional regime will be adjusted in order to change the growth trajectory (Rochow et al., 2012; Senterre & Rigo, 2011). This approach has limitations because by the time an infant starts to display falling percentiles, small head circumference, too high or low fat mass, or poor BMI, the

programming effects that may put the infant at risk for adverse outcomes in adulthood may have already occurred (Barker, 1992; M. B. Belfort et al., 2014; Parkinson et al., 2013). Thus, it is important to be able to prospectively predict growth and provide clinicians with a tool to help them monitor growth.

Additionally, growth monitoring has implications for the diagnosis of extrauterine growth restriction (EUGR). Often, EUGR is diagnosed as infants with weights and growth falling below the 10th percentile. However, this does not take into consideration the infant's individual characteristics and genetic potential. An infant could be at the 50th percentile but actually be growth restricted because their genetic potential would indicate they should be larger. This would go unrecognized with the current growth monitoring tools. An individualized trajectory would provide clinicians with a "map" tailored to each individual infant in order to assess whether the infant is growing off-course.

GROWTH PERIODS

To characterize the growth of preterm infants it is necessary to consider the physiological periods of growth. Preterm infants experience four distinct periods of growth. 1) Intrauterine growth reflected on the Fenton growth curves, prematurely interrupted (before 37 weeks gestational age) by preterm birth; 2) a period of postnatal adaptation and weight loss due to a one-time, permanent, physiological loss in extracellular fluid, from birth until day of life 21; 3) a period of stable growth from day of life 21 until 42 weeks postmenstrual age; 4) continuation of stable growth on the WHO growth standard curves after term age.

POSTNATAL ADAPTATION

A recent study by Rochow et al., studied a cohort of almost 1000 'healthy' preterm infants, without major clinical interventions in order to better understand how preterm infants should grow during the first weeks of life. The study modeled how preterm infants adjust to a new percentile after completed postnatal adaptation from birth until day of life 21 (period 3 above) (Rochow et al., 2016). After birth, all term and preterm newborns with minimal medical interventions experience a physiological period of adaptation to extrauterine life. During this adaptation there is a characteristic weight loss, which offsets the postnatal growth trajectories. As illustrated in Figure 1, this weight loss is constituted in part by a temporary decrease in growth due to a caloric deficit until feeding is established. However, the main contributor to this weight loss is the physiological, irreversible, one-time, "term contraction of extracellular spaces" (TeCES) in term infants and "preterm contraction of extracellular spaces" (PreCES) in preterm infants (Rochow et al., 2016).

This weight loss mainly from water leads to a shift in growth trajectories and sets the starting point for a "new" growth trajectory. In preterm infants the water loss contributes to approximately 10% loss of body water and body weight. The loss is smaller in term-born infants who have a higher relative amount of fat mass. As well, preterm infants adaptation was found by Rochow et al. to last approximately 21 days, vs. approximately 14 days in term-born infants (Rochow et al., 2016).

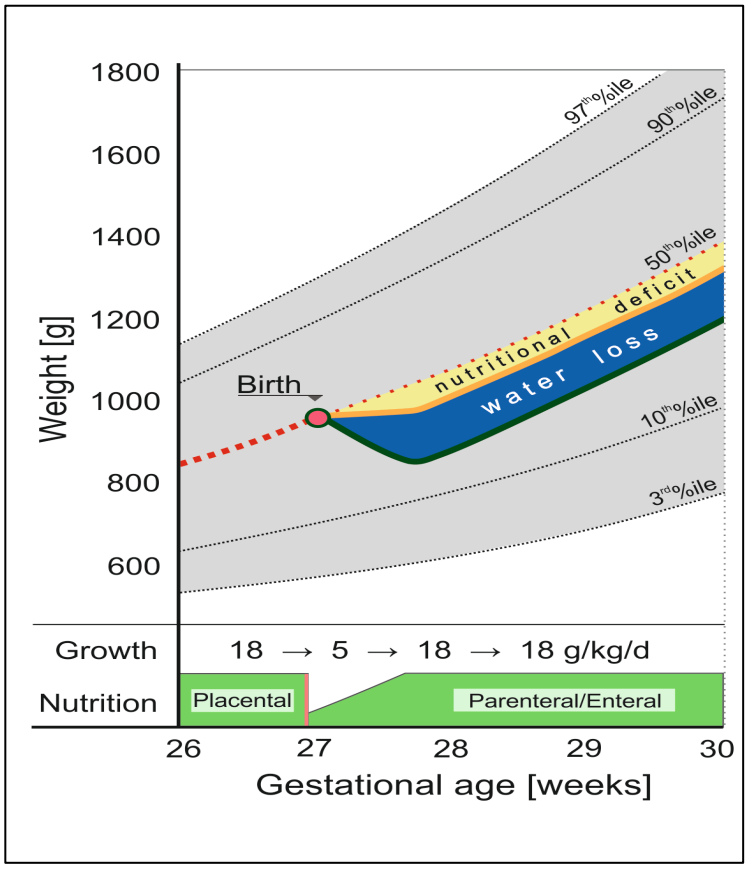

Figure 1: Physiological weight loss during postnatal adaptation: Minor contribution from temporarily decreased nutrient intake (yellow area), major contribution from contraction of extracellular space with water loss (blue), "new" growth trajectory (yellow + blue = weight difference from fetus to preterm infant after postnatal adaptation)(Fusch & Samiee-Zafarghandy, 2014).

On the WHOGS curves, the postnatal adaptation of term infants is reflected by the dip at the beginning of the curves (40-42 weeks PMA) (de Onis et al., 2004). However, this period of postnatal adaptation is not reflected in intrauterine growth curves and postnatal growth curves for preterm infants (24-34 weeks). A unique set of curves, which reflects the postnatal weight loss is required for infants born with different gestational ages (24 + 0/7 to 34 + 6/7) and individual birth weights. After postnatal adaptation, preterm infants are shifted off of the percentile they were born at due to this one-time, irreversible water loss (Rochow et al., 2016). It would not be physiological to

immediately replace this loss of water with fat or lean mass. This implies that a preterm infant should not catch up to the birth weight percentiles on the intrauterine chart.

Current growth charts do not provide a target weight or trajectory to guide preterm infants' growth after postnatal adaptation. It is unclear, where a preterm should grow on the growth curves (Figure 2). The position on the growth curves, as a result of slightly different growth rates, impacts the body composition of the infant (Figure 3). The aim is to achieve optimal growth and minimize disease risks.

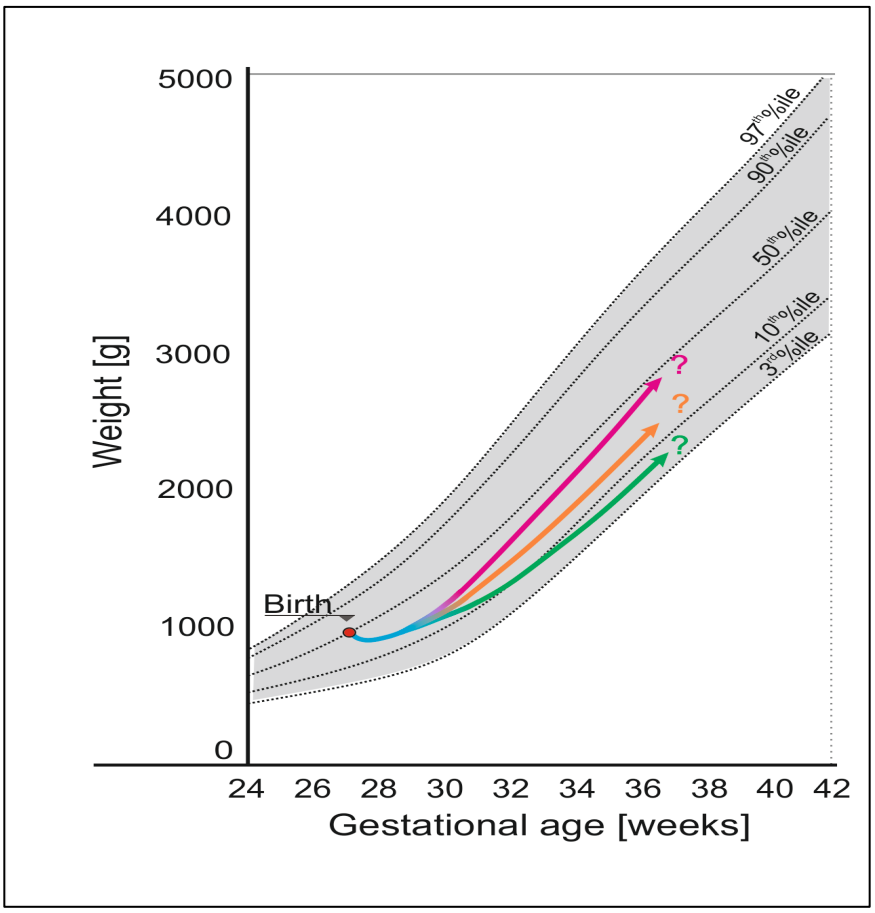

Figure 2: Possible Growth Trajectories following American Academy of Pediatrics recommendation for growth rates similar to *in utero* (17-20g/kg/day).

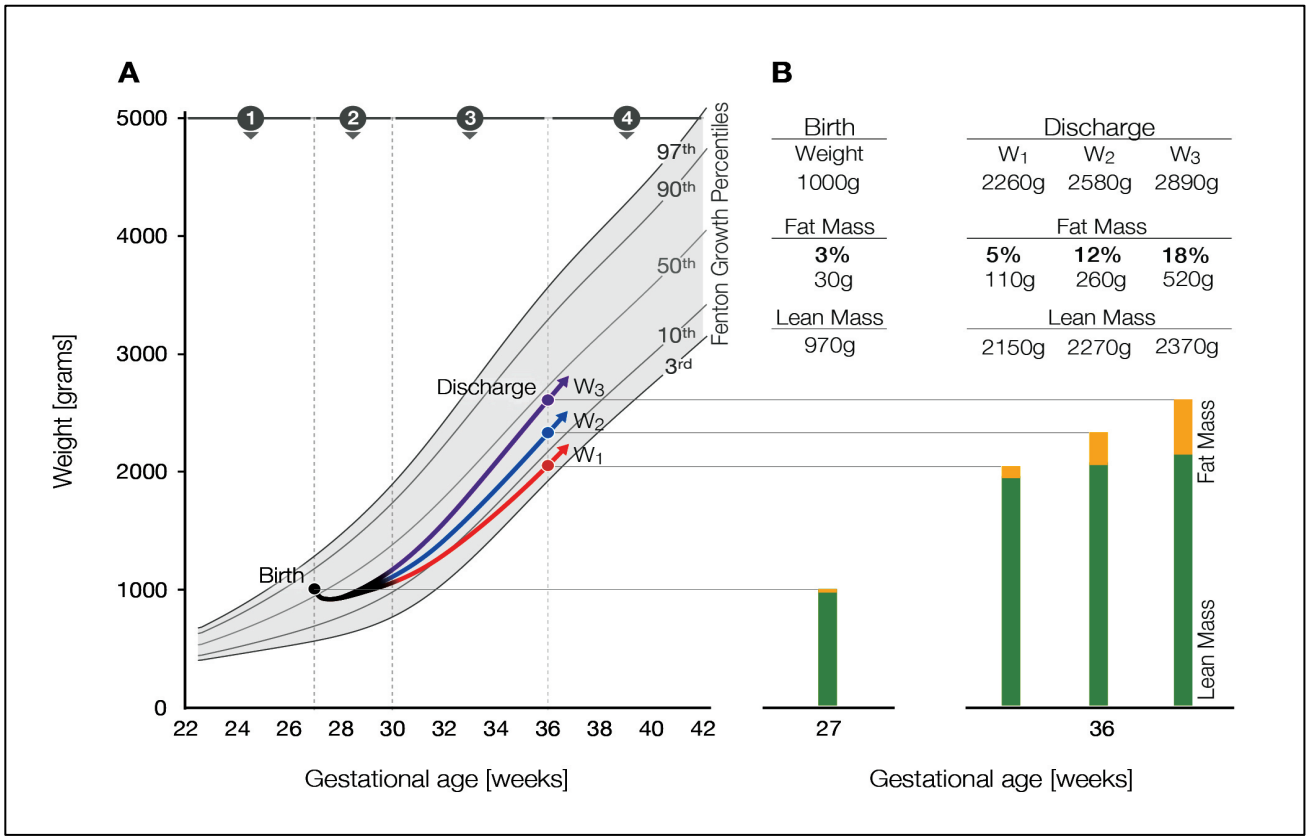

Figure 3: Position on the growth curves due to slightly different growth rates, results in different body compositions and disease risk profiles(Rochow et al., 2016)**.**

In order to guide the preterm infants growth after postnatal adaptation, following the AAP growth rate recommendations, it is important to consider the growth goal. We suspect that preterm infants should achieve the growth and body composition that they would have achieved had they stayed *in utero*, in a healthy, undisturbed pregnancy, until term age. Preterm infants experience Preterm Contraction of Extracellular Spaces (PreCES) with weight loss. Term infants experience Term Contraction of Extracellular Spaces (TeCES) and weight loss. These periods of weight loss shift the infant to a lower trajectory, earlier in preterm infants. The implication of this phenomenon can be illustrated with the hypothetical case of a twin pregnancy, where one twin is born preterm. This twin experiences weight loss due to postnatal adaptation and should

continue to grow with the same rate as their twin counterpart that remains *in utero*. At term, the twin that remained *in utero* would be born and experience postnatal adaptation and weight loss, leading to a downshift in the growth trajectory. Thus, preterm infants growth trajectories should aim to merge with gestational age matched term infants' growth trajectories *after* the term infants' postnatal adaptation and TeCES is completed (42 weeks PMA) (Figure 4).

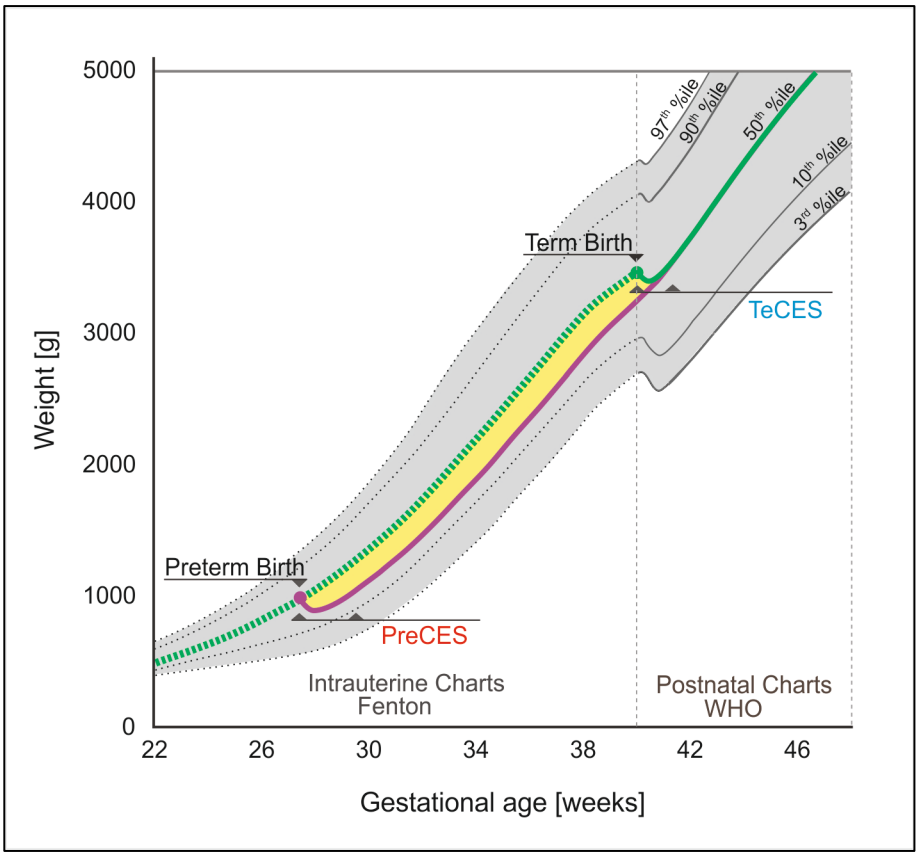

Figure 4: Postnatal offset of growth trajectories of preterm infants and convergence of preterm with term trajectories at 42 weeks; dotted curves-intrauterine trajectories(Fenton et al. 2013), solid line postnatal trajectories - preterm (purple) merging with term (green) (de Onis et al. 2004).

GROWTH RATES

In reference to the AAP's recommendation that preterm infants grow like a fetus *in utero* (with growth rates of approximately 17-20g/kg/day), it is important to consider that the loss of water described in the period of postnatal adaptation, affects the calculation of growth rates between pre- and post- adaptation. Preterm infants lose around 8 to 12% of their weight and term infants lose about 5 to 7% of their weight from water loss during postnatal adaptation (K Bauer & Versmold, 1989; Karl Bauer et al., 1991; Crossland, Richmond, Hudson, Smith, & Abu-Harb, 2008; Schaefer et al., 2015).

Further analysis of postnatal growth trajectories is necessary to improve the understanding of how preterm infants should grow and what their target growth trajectory should be. As well, there is a need for an improved method to assess, monitor and predict the growth of preterm infants.

GROWTH AFTER POSTNATAL ADAPTATION UNTIL TERM

Using common growth charts in accordance with the AAP recommendation allows, in theory, three possible preterm growth trajectories. These growth trajectories are illustrated in Figure 5: 1) Following the birth weight percentile from birth to the same birth weight percentile at term (yellow); 2) Catching up from the postnatal weight loss to the birth weight percentile trajectory, continuing to the corresponding birth weight percentile at term (dark blue); or 3) Growing along the "new" trajectory achieved after postnatal adaptation until term age (purple).

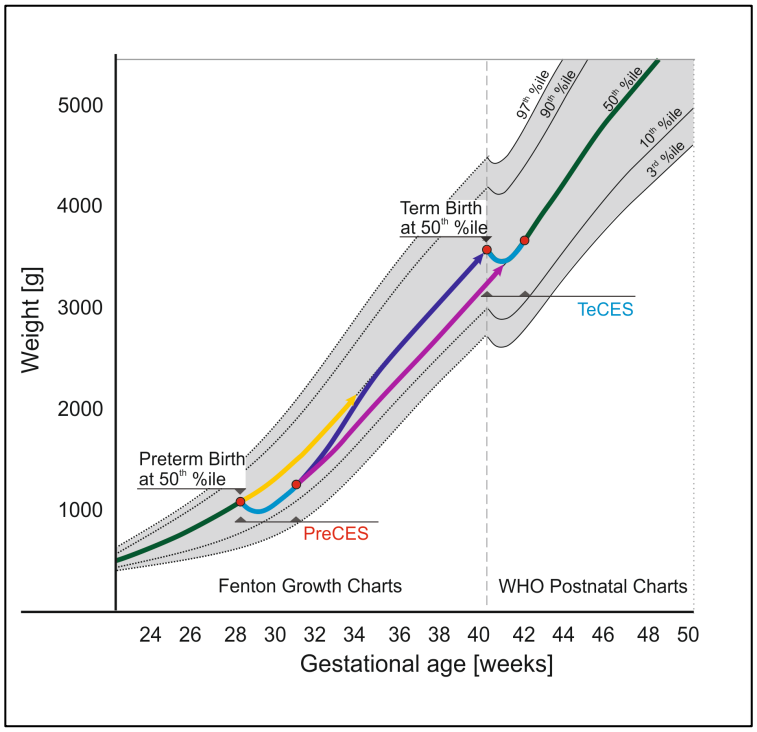

Figure 5: Filling the gap between end of postnatal adaptation in preterm and term infants - possible growth trajectories.

Scenario 1 has the limitation that it does not include the physiological postnatal adaptation and weight loss. Scenario 2 implies that the water, which is lost during PreCES should be replaced by fat or lean mass in order to catch up to the birth weight percentile. These first two scenarios do not incorporate the postnatal weight loss due to TeCES that will occur in term infants at birth and is reflected in the dip at the beginning of the WHO growth standards (WHOGS) for term infants. Therefore, to merge with term infants' trajectories, the preterm infant would need to lose body water or body mass at around term age, which is not physiological. Scenario 3 incorporates the physiological postnatal adaption of preterm infants and aims to merge with the post-adaptation trajectory of term infants born at the corresponding percentile. Scenario 3 may be the most physiologically appropriate, taking into consideration the prediction model for

postnatal adaptation, the Fenton data, and WHOGS data (de Onis et al., 2004; Fenton & Kim, 2013; Rochow et al., 2016).

As described earlier, there are four important chronological periods of growth to consider in defining individual growth trajectories. Three of them are well characterized by evidence. First, the intrauterine period, represented by intrauterine growth curves like the one by Fenton (Fenton & Kim, 2013). Second, is the period of postnatal adaptation during the first three weeks of life, as recently described by our group (Rochow et al., 2016). In this study we observed that healthy preterm infants grew parallel to the Fenton growth chart after PreCES. These healthy infants at a variety of gestational ages maintained an average z-score of -0.8 after PreCES and weight loss was completed (Rochow et al., 2016). The fourth period is the postnatal growth of healthy, breast-fed; term infants reflected in the WHOGS chart (de Onis et al., 2004), which acts as a target for preterm infants after term age.

What is missing is a reference for the third period, to define the individual trajectory from the end of postnatal adaptation in preterm infants (DOL 21) until the end of postnatal adaptation in term infants on the WHOGS (42+0/7 weeks PMA). This is a long period where many clinical decisions may impact a child's later health (Richard A. Ehrenkranz et al., 2006). We speculate that the lack of tools to plan and to assess appropriate postnatal growth is a contributor to extrauterine growth restriction (EUGR), which is still frequently reported in NICUs. As well, the lack of understanding that it is normal for most preterm infants to be growing below their birth centile after PreCES may contribute to incorrect diagnoses of EUGR.

We have considered different approaches to describe the individual growth trajectories for the third period. The Postnatal-Percentile Approach assumes that infants follow the "new" percentiles that they have adjusted to after completing their postnatal adaptation (DOL 21). In contrast, the Growth-Velocity Approach applies the day-specific median growth velocities from the Fenton curves for the period of DOL 21 until 42+0/7 weeks PMA.

The aim of the current study is to compare these two approaches and to develop individualized, reference growth trajectories for preterm infants, as well as to perform a preliminary validation of the concept using healthy preterm infants' weight and body composition data.

METHOD

GROWTH MODEL

The objective of this study is to develop postnatal growth trajectories for individual preterm infants from birth until 42+0/7 weeks PMA. The model incorporates the physiological postnatal adaptation with weight loss, which leads to a shift in the postnatal growth trajectory to a new percentile between 14 and 21 days of life (Rochow et al., 2016). We considered this new percentile to be the new reference point for further extrauterine growth. Subsequently, two approaches were applied to describe individual growth trajectories. Both approaches applied commonly used clinical growth chart data from Fenton (Fenton & Kim, 2013), one following percentiles and the other using the median growth velocities.

Figure 6 provides an illustration of how the growth trajectories were predicted with the two approaches, including the periods, time points, anchor points and reference data that make up the model. The growth trajectory for any infant begins in the intrauterine period. We use the Fenton intrauterine growth charts as a reference for the growth trajectory before birth. The beginning of the postnatal growth trajectory and the first significant time point for the model is preterm birth. This time point serves as the anchor for the predicted postnatal growth trajectory. After preterm birth, the first 21 days of life are considered to be a period of physiological postnatal adaptation and can be modelled using a published prediction model (Rochow et al., 2016). DOL 21 is the end of postnatal adaptation and is considered to be the second significant time point. DOL 21 is the starting anchor point for the period of stable growth until 42+0/7 weeks PMA. During

this period of stable growth, one of the two previously described approaches is used to define the growth trajectory.

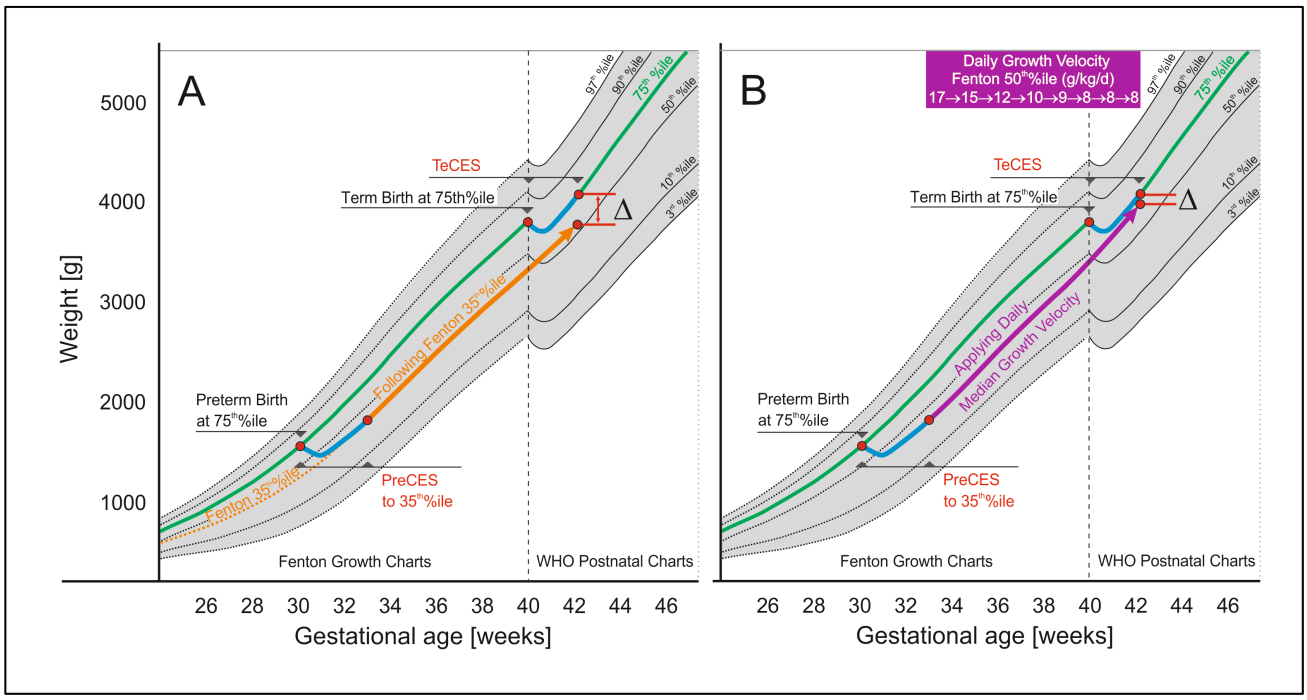

Figure 6: Individualized trajectories for preterm infants: (A) Postnatal-Percentile Approach: after premature contraction of extracellular spaces (PreCES- blue) infants follow the percentile achieved (orange) on Fenton chart at DOL 21 until 42+0/7 weeks. (B) Growth-Velocity Approach: application of Fenton daily median growth velocities from DOL 21 until 42+0/7 weeks. Δ = difference between target WHOGS weight at 42+0/7 weeks PMA and predicted individual growth trajectory weight using either Postnatal-Percentile or Growth-Velocity Approach

The Postnatal-Percentile Approach (figure 6A) assumes that infants follow the "new" percentiles that they have adjusted to after completing their postnatal adaptation (DOL 21). In contrast, the Growth-Velocity Approach (figure 6B) applies the day-specific median growth velocities from the Fenton curves for the period of DOL 21 until 42+0/7 weeks PMA.

For post-term postnatal growth starting at 42+0/7 weeks, the commonly used WHOGS data are applied (de Onis et al., 2004). The difference between predicted weight and the target weight on the WHOGS for the corresponding birth percentile at 42+0/7 weeks PMA serves as the outcome parameter. To assess the appropriateness of the model across gestational ages, the trajectories were calculated in 1-week increments for all gestational ages between 24 and 34 weeks. The calculation was repeated for infants whose birth weights represented the 3rd, 10th, 25th, 50th, 75th, 90th and 97th percentiles. This resulted in 77 individual growth trajectory combinations (11 gestational weeks x 7 percentiles) that were compared to the WHOGS target weights corresponding to the birth weight percentiles at 42+0/7 weeks PMA.

The growth trajectory models were validated using body composition data. The postnatal-percentile approach was executed in a way that either the birth weight percentile or the percentile achieved at DOL 21 was followed until 42+0/7 weeks on Fenton charts. Weight and body composition was measured with a Cosmed USA Peapod device. Deviation of achieved weights from the target weights was compared with percentage of body fat (36+0/7 to 42+6/7 weeks PMA).

PATIENT DEMOGRAPHICS:

We included 57 patients, 35 male, 22 female, with mean gestational age of 27.1 +/- 1.4 weeks and mean birth weight of 930 +/- 210g. These infants were selected from a randomized control trial “Individualized Fortification of Breast Milk” at McMaster Children’s Hospital. The inclusion criteria for the study were: gestational age <30 weeks; tolerating enteral intake of ≥100 ml/kg/d for ≥ 24h. Exclusion criteria included: gastrointestinal malformation, major congenital anomalies, chromosomal abnormalities;

infants with enterostoma or short gut syndrome; infants fed more than 25% of mean caloric intake for a consecutive week with formula milk; fluid restriction <140mL/kg/d for ≥ 3 consecutive days; infants with gram-negative sepsis, necrotizing enterocolitis, defined by feeding intolerance associated with positive x-ray findings (pneumatosisintestinalis – Bell Stage 2; air in the biliary tract or free air in the peritoneum – Bell Stage 3; Renal disease, defined by symptoms (oliguria, anuria, proteinuria, hematuria) associated with an increased blood urea nitrogen10 mmol/L[27] and creatinineof130mmol/L[28]; Hepatic dysfunction, defined by jaundice (direct bilirubin >1.0 mg/dl) that is associated with one or more abnormal liver function tests (AST, ALT or GGT); Participation in another clinical trial that may affect outcomes of this study; Probability of transfer to another NICU or level II nursery outside the McMaster Children's Hospital.

STATISTICS:

The data analysis was performed with Microsoft Excel. The day specific growth velocities were calculated by $weight_{(day+1)}$ / $weight_{(day)}$ for each gestational week (Patel et al. 2005, Patel et al. 2009).

Reference data provided parameters for the LMS method, which was used to calculate percentiles and weights (de Onis et al. 2004, Fenton et al. 2007, Fenton et al. 2013).

RESULTS

Table 1 and Figure 7 show weight residuals comparing the individual growth trajectories with the WHOGS for the Postnatal-Percentile Approach at 42+0/7 weeks for boys. There were large differences between the Postnatal-Percentile Approach weights at 42+0/7 weeks compared to the target WHOGS weights for the same birth weight percentile (Figure 7A). The residuals between the individual growth trajectory weights at 42+0/7 and the WHOGS target weights were smallest for birth weights at the 25th percentile, ranging from -49g to 80g. At lower percentiles the estimated individual growth trajectories achieved higher weights than the WHOGS at 42+0/7 weeks. The residuals ranged from -52g to -316g for the 3rd percentile. At higher percentiles the estimated individual growth trajectories achieved weights below the WHOGS at 42+0/7 weeks. The residuals ranged from 377g to 926g for the 97th percentile. A factor to correct for these residuals could not be identified. With the Growth-Velocity Approach there was a consistent decrease in the deviation from the target weight at 42+0/7 weeks for the 10 to 97th percentiles from 24 to 34 weeks of gestation (Table 2, Figure 7B). Due to this consistent pattern one factor (1.0017), which was multiplied with all day-specific median growth velocities was introduced to correct for this deviation. The resulting residuals were small and a close match to the WHOGS target weights at 42+0/7 weeks was achieved, except for the 3rd percentile (Table 3, Figure 7C). The analysis using reference data for girls yielded similar results for both methods. The Postnatal-Percentile Approach showed a large deviation and the optimized Growth-Velocity Approach predicted a weight and percentile at 42+0/7 weeks, which closely coincided with the WHOGS data (Tables 4, 5, 6 and Figure 8).

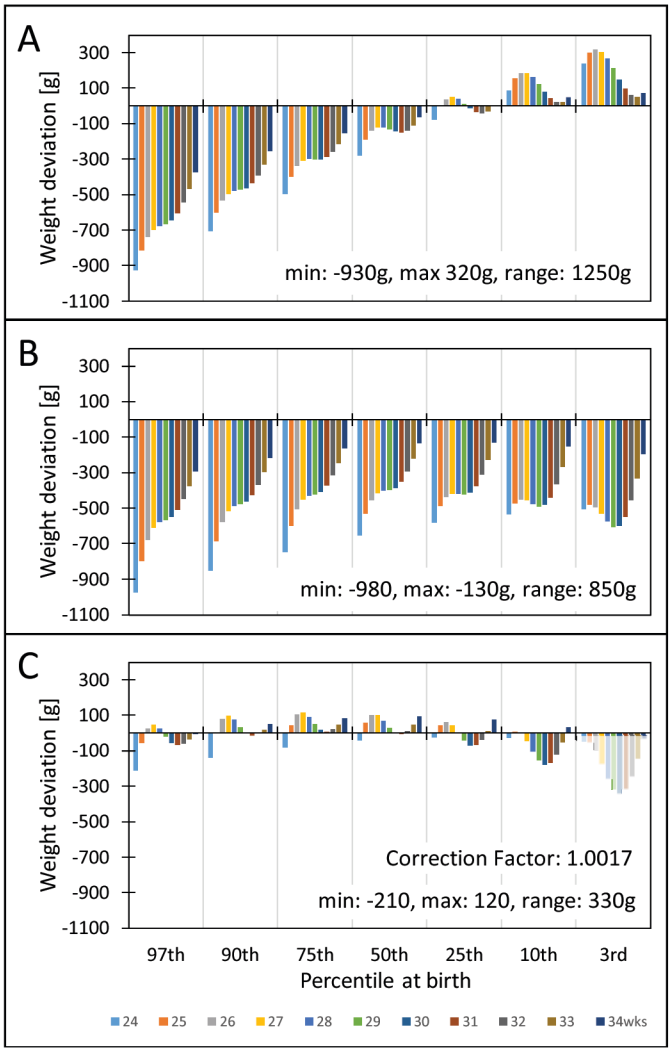

Figure 7: Deviation from target weight - Boys: Difference between weights from the WHOGS and achieved weights using the individual target trajectory at 42+0/7 weeks using (A) Postnatal-Percentile Approach: Growth during the period of stable growth was defined by following the percentile achieved at day of life 21 until 42+0/7 weeks; (B) Growth-Velocity Approach: Growth during the period of stable growth was defined by applying daily Fenton median growth velocities from the day of life 21 achieved weight, each day until 42+0/7 weeks; (C) Optimized Growth-Velocity Approach: Growth-Velocity Approach optimized with a simple, single correction factor to accommodate for errors in the reference data.

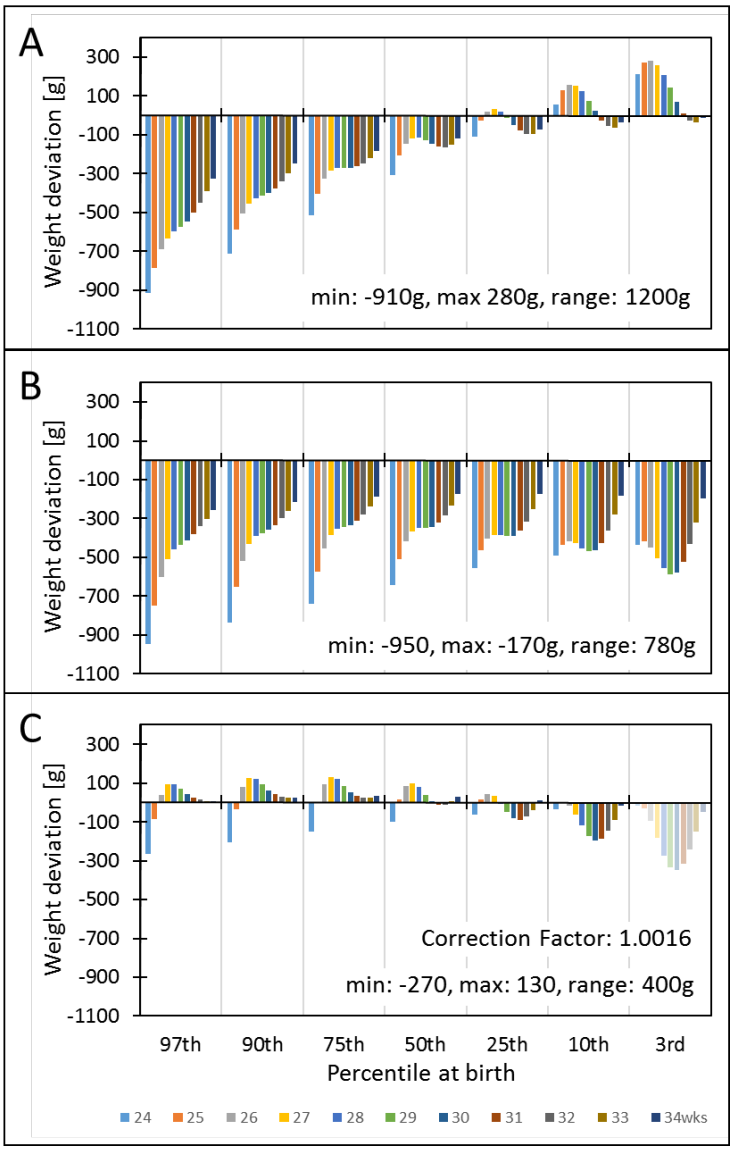

Figure 8: Deviation from target weight - girls: Difference between weights from the WHOGS and achieved weights using the individual target trajectory at 42+0/7 weeks using (A) Postnatal-Percentile Approach: Growth during the period of stable growth was defined by following the percentile achieved at day of life 21 until 42+0/7 weeks; (B) Growth-Velocity Approach: Growth during the period of stable growth was defined by applying daily Fenton median growth velocities from the day of life 21 achieved weight, each day until 42+0/7 weeks; (C) Optimized Growth-Velocity Approach: Growth-Velocity Approach optimized with a simple, single correction factor to accommodate for errors in the reference data**.**

Table 1: Postnatal Percentile Approach - Boys: Difference between the World Health Organization Growth Standards and weights and percentile (in brackets) achieved using the individual target trajectory at 42+0/7 weeks (growth during the period of stable growth was defined to follow the percentile achieved at day of life 21 until 42+0/7 weeks).

	Deviation from target weight [g]										
GA [weeks]	24	25	26	27	28	29	30	31	32	33	34
Birth %ile											
97th	-926	-814	-741	-699	-678	-666	-645	-605	-545	-468	-377
90th	-706	-601	-534	-497	-481	-474	-464	-438	-393	-332	-257
75th	-498	-400	-340	-309	-299	-302	-302	-289	-261	-216	-156
50th	-280	-191	-142	-121	-121	-132	-145	-150	-139	-111	-65
25th	-80	-2	37	49	38	12	-15	-37	-43	-31	3
10th	86	154	183	185	161	122	78	42	21	21	47
3rd	239	298	316	304	267	212	149	96	61	52	74

GA – gestational age

Table 2: Growth Velocity Approach - Boys: Difference between the World Health Organization Growth Standards and weights and percentile (in brackets) achieved using the Fenton median growth velocity from day of life 21 until 42+0/7 weeks.

	Deviation from target weight [g]										
GA [weeks]	24	25	26	27	28	29	30	31	32	33	34
Birth %ile											
97th	-978	-798	-679	-610	-580	-569	-551	-511	-449	-376	-294
90th	-853	-689	-580	-517	-490	-480	-463	-426	-368	-297	-218
75th	-748	-602	-506	-453	-430	-423	-408	-372	-316	-246	-165
50th	-653	-532	-456	-417	-404	-400	-387	-352	-295	-221	-134
25th	-582	-490	-440	-420	-420	-425	-414	-377	-313	-230	-131
10th	-535	-475	-453	-458	-477	-491	-483	-441	-366	-268	-153
3rd	-506	-481	-495	-532	-577	-607	-602	-550	-456	-334	-196

Table 3: Optimized Growth Velocity Approach - Boys: Difference between the World Health Organization Growth Standards and weights and percentile (in brackets) achieved using the Fenton median growth velocity adjusted by 1.0017

	Residuals of weight [g] (percentile of target trajectory on WHOGS)										
GA [weeks]	24	25	26	27	28	29	30	31	32	33	34
Birth %ile											
97th	-215	-57	26	47	24	-21	-58	-70	-60	-37	-8
90th	-139	4	78	97	74	32	-3	-15	-4	19	50
75th	-83	42	104	116	92	51	18	8	21	48	84
50th	-43	56	100	100	70	28	-2	-7	11	46	94
25th	-25	43	61	43	2	-43	-71	-69	-40	11	75
10th	-27	8	-4	-46	-103	-155	-181	-169	-123	-52	34
3rd	-44	-48	-97	-173	-255	-320	-344	-317	-244	-144	-29

Table 4: Postnatal Percentile Approach - Girls: Difference between the World Health Organization Growth Standards and weights and percentile (in brackets) achieved using the individual target trajectory at 42+0/7 weeks (growth during the period of stable growth was defined to follow the percentile achieved at day of life 21 until 42+0/7 weeks).

	Deviation from target weight [g]										
GA [weeks]	24	25	26	27	28	29	30	31	32	33	34
Birth %ile											
97th	-915	-786	-692	-634	-599	-575	-545	-503	-449	-390	-328
90th	-713	-591	-505	-454	-427	-414	-399	-375	-340	-299	-249
75th	-517	-404	-328	-287	-270	-269	-270	-264	-247	-221	-182
50th	-309	-209	-147	-119	-116	-129	-148	-161	-163	-151	-120
25th	-112	-26	19	32	19	-12	-48	-80	-98	-97	-72
10th	56	129	157	152	122	75	21	-25	-56	-62	-38
3rd	213	272	283	259	209	142	71	11	-28	-38	-12

Table 5: Growth Velocity Approach - Girls: Difference between the World Health Organization Growth Standards and weights and percentile (in brackets) achieved using the Fenton median growth velocity from day of life 21 until 42+0/7 weeks.

	Residuals of weight [g] (percentile of target trajectory on WHOGS)										
GA [weeks]	24	25	26	27	28	29	30	31	32	33	34
Birth %ile											
97th	-948	-750	-600	-508	-459	-435	-412	-381	-342	-301	-255
90th	-838	-654	-517	-434	-392	-374	-358	-333	-299	-260	-215
75th	-739	-576	-456	-386	-355	-344	-334	-312	-279	-238	-188
50th	-642	-509	-417	-367	-350	-348	-342	-321	-284	-234	-173
25th	-557	-461	-405	-383	-385	-392	-388	-362	-314	-250	-173
10th	-491	-434	-417	-429	-452	-470	-465	-429	-364	-279	-182
3rd	-434	-420	-450	-504	-557	-587	-577	-522	-431	-321	-199

Table 6: Optimized Growth Velocity Approach - Girls: Difference between the World Health Organization Growth Standards and weights and percentile (in brackets) achieved using the Fenton median growth velocity adjusted by 1.0016 from day of life 21 until 42+0/7 weeks.

	Residuals of weight [g] (percentile of target trajectory on WHOGS)										
GA [weeks]	24	25	26	27	28	29	30	31	32	33	34
Birth %ile											
97th	-267	-84	39	93	95	70	43	25	16	8	4
90th	-204	-34	78	125	123	94	63	42	32	25	26
75th	-150	-1	94	129	120	87	53	32	24	24	33
50th	-101	16	83	99	78	40	6	-11	-10	3	28
25th	-63	14	44	32	-6	-50	-82	-88	-71	-38	9
10th	-36	-1	-15	-61	-120	-171	-197	-187	-148	-89	-18
3rd	-15	-29	-94	-184	-273	-333	-348	-314	-243	-151	-51

VALIDATION:

57 healthy preterm infants met the inclusion criteria and had Peapod body composition measurement between 38 and 42 weeks PMA. For these infants the mean z-score at birth was 0 +/- 0.8, 3 infants were SGA, 50 AGA and 4 LGA, suggesting a normal distribution of infants. The average PMA for body composition measurement was 39.0 +/- 2.0 weeks. The mean weight at measurement of body composition was 2990 +/- 630g. The z-score at measurement was -0.7 +/-0.9. The mean deviation of the achieved weight from the target weight was -10±370g for the Growth-Velocity Approach, 70±350g for the Postnatal-Percentile Approach and -310±380g for the birth weight percentile approach.

Figure 9 shows that the Postnatal-Percentile Approaches (based upon birth weight percentile or percentile achieved at DOL 21) both lead to a skewed distribution where the bulk of the infants were observed to have achieved weights either higher or lower than the target weight. In contrast, the Growth-Velocity Approach showed a symmetrical distribution of infants around the target weights. As well, the target weights corresponded to approximately 20% body fat.

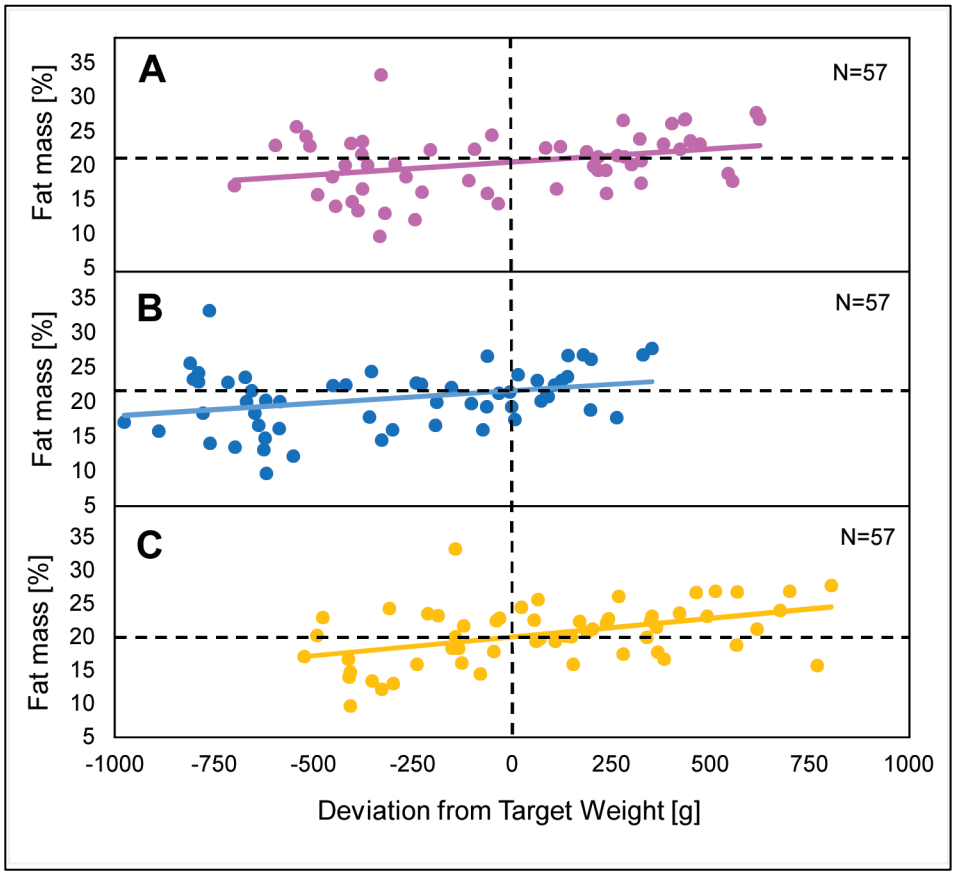

Figure 9: Validation of growth trajectory: Percentage body fat by deviation from target weight (weight at discharge measurement minus target weight) using: (A) Individualized growth trajectories (Growth-Velocity Approach); (B) Following birth weight percentile; (C) Following the percentile achieved at day of life 21.

DISCUSSION

In our study, we describe a new concept to predict individual growth trajectories for preterm infants. This innovative concept takes current evidence-based principles about growth for preterm infants and fills the gap in reference data between the end of postnatal adaptation (DOL 21) in preterm infants and the end of postnatal adaptation in term infants (42+0/7 weeks). The four evidence-based principles used to predict individual growth trajectories are: 1) The use of Fenton's growth chart data for the intrauterine period. These are robust data developed from a meta-analysis of perinatal surveys from millions of infants obtained from different countries (Germany, USA, Canada, Australia, UK and Italy) (Bertino et al., 2010; Bonellie et al., 2008; Fenton & Kim, 2013; M S Kramer et al., 2001; Olsen et al., 2010; Roberts & Lancaster, 2006; Voigt et al., 2010); 2) the use of the WHO Growth Standards (WHOGS) data for the post-term period. This data is longitudinal, comprised of healthy, term-born breast fed infants and thus is a gold standard for the post-term period for preterm infants(de Onis et al., 2004); 3) Term infants born on the 50th percentile will transition to the 50th percentile on the WHO curves after postnatal adaptation; 4) Preterm infants undergo the same postnatal adaptation but earlier(Rochow et al., 2016) and transition to their birth weight percentile on the WHO curves. Our concept aimed to use these four evidence based principles to describe individual growth trajectories for preterm infants that incorporated their physiology.

Individual growth trajectories yielded from day-specific median growth velocities resulted in the closest prediction to the WHOGS target weights at 42+0/7 weeks. The day-specific growth velocities are applied to preterm infants' individual weights after the

period of postnatal adaptation and weight loss (PreCES) at DOL 21(Rochow et al., 2016). The Fenton median growth velocities represent the natural growth pattern with a physiological decrease in weight gain across gestational ages until term. Although there was a deviation between the predicted and target weights when using the raw Fenton median growth velocities, the introduction of a single factor led to a close match with the WHOGS target weight at 42+0/7 weeks, after TeCES was completed. This factor was valid for infants born at gestational ages from 24 to 34 weeks PMA and for all major percentiles.

The necessity of the factor in order to match with WHOGS target weights at 42 weeks suggests that preterm infants need to grow approximately 10% faster after postnatal adaptation compared to *in utero* growth rates. There are two physiological reasons why preterm infants need to growth with faster rates compared to their *in utero* counterparts. First, preterm infants have a higher percentage of weight loss during postnatal adaptation compared to term infants (8 to 12% vs. 5 to 7%). This is accompanied by a reduction in extracellular water spaces of about 40 to 60%. As one compartment has shrunk, the other has the increase at a fast rate to maintain growth rates (K Bauer & Versmold, 1989; Karl Bauer et al., 1991; Crossland et al., 2008; Schaefer et al., 2015). Second, preterm infants may experience cumulative nutritional deficits leading to temporary growth restriction during the first days of life. Growth restriction during this period becomes immediately evident because expected growth rates are high(Clark et al., 2003). Thus, in order to maintain the same protein accretion rate, the ex utero growth rate may need to be 10% higher. This is in line with our finding of a correction factor

corresponding to a difference in growth rate of approximately 10% necessary to meet the WHOGS target growth trajectories at 42+0/7 weeks PMA.

The observed variation between predicted and WHOGS weights using the Growth-Velocity Approach was within the range of error in weight measurements. Common sources of measurement error include: 1) differences between measurements done before or after infants' voiding; 2) whether or not delayed cord clamping was done; 3) differences between scales 4) with or without respiratory devices or lines included in the measurements. Thus, the deviation between predicted and target weights was clinically acceptable.

Surprisingly, the common Postnatal-Percentile Approach, which follows currently published intrauterine percentiles from DOL 21 to 42+0/7 weeks PMA, provided a less appropriate target trajectory for postnatal growth in preterm infants. We found high deviations from the WHOGS target weight at 42+0/7 weeks PMA with systematic differences (Fig. 3). In particular, we observed that these deviations were highest for higher birth weight percentiles and more immature infants. These observations may be explained by the skewness inherent in the growth charts that are derived from birth weight data and thus represent a conceptual error in the use of the Postnatal-Percentile Approach.

ASSESSMENT OF GROWTH USING BIRTH WEIGHT CHARTS - SYSTEMATIC ERROR:

When following the Postnatal-Percentile Approach from DOL 21 until 42+0/7 weeks PMA, systematic errors were observed, which can be explained by the nature of the birth weight data. Figure 10 shows a sinusoidal pattern across gestational ages within each percentile.

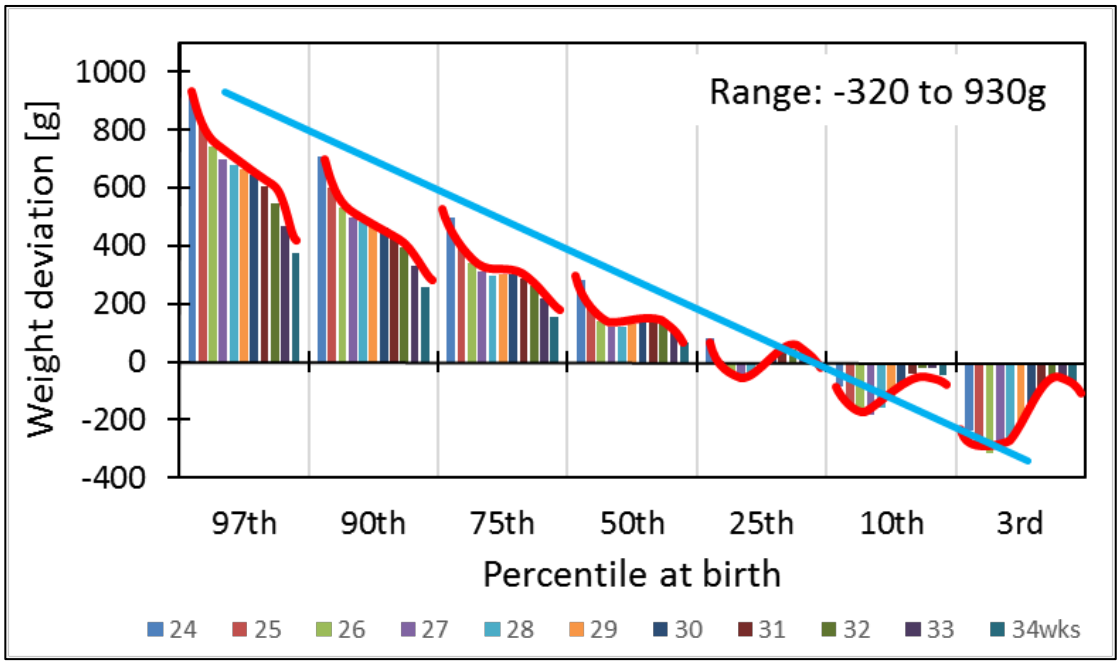

Figure 10: Deviations in achieved weight at 42+0/7 weeks PMA using the Postnatal-Percentile Approach compared to the WHOGS for term infants of the same birth weight percentile

The following principles can explain the observed patterns:

1) The weight data for the Fenton charts and corresponding WHOGS at 42+0/7 weeks PMA have an offset (Fig. 11A). This offset is because the Fenton charts provide a reference for infants that continued to grow *in utero* and therefore did not account for PreCES in preterm infants. This offset corresponds to higher percentiles on the WHOGS

at 42+0/7 weeks (Fig. 11B) when the infants transition from the Fenton chart to the WHOGS. For example, the 50th percentile on Fenton corresponds to the 58th percentile on WHOGS.

2) At lower percentiles and gestational ages the distribution of the percentiles (Fenton, 2013) are more skewed because of higher variation (Fig. 11C). Consequently, according to the reference charts, infants at lower percentiles cross less percentiles with PreCES than infants at higher percentiles (Fig. 11D). Interestingly, our analysis revealed that following the 25th Fenton birth percentile provides a close match to the WHOGS target weight at 42+0/7 weeks. This observation at the 25th percentile is because the weight difference caused by postnatal crossing of percentiles happens to counteract the offset between the Fenton and WHOGS percentiles. Above and below the 25th birth percentile the deviation is higher and does not cancel out.

3) Preterm infants born earlier (24 to 26 weeks) have a higher percentage weight loss during postnatal adaptation compared to more mature infants (33 to 34 weeks) leading to crossing of more percentiles (Fig. 11E). Following lower percentiles until term age leads to a higher deviation from the target percentile on the WHOGS at 42+0/7 weeks PMA.

4) The skewness of the percentile distribution varies over the range of gestational ages. Distances between percentiles are larger towards 28 weeks and continuously decreasing from 30 weeks onwards (Fig 11F). This leads to the sinusoidal pattern observed across the range of gestational ages for percentiles, and weight deviation for the same percentiles (Figure 10).

In summary, following percentiles from the current reference charts leads to an offset from the target weight by 42+0/7 weeks PMA.

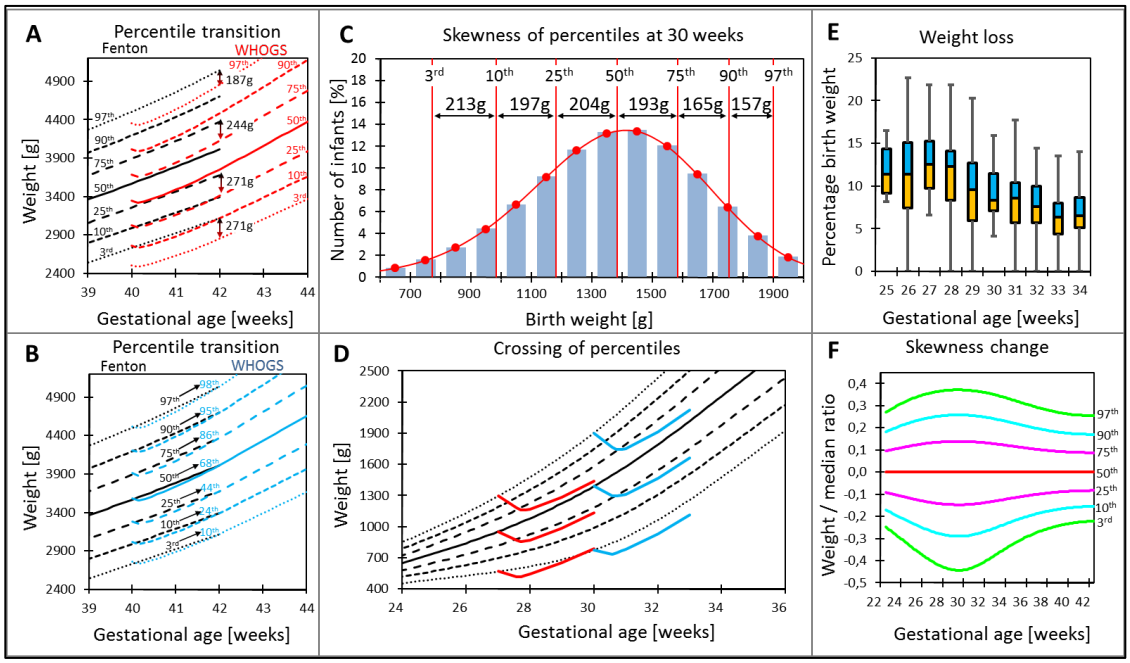

Figure 11: Characteristics of intrauterine growth chart affecting individual trajectories A) Weight offset between Fenton and WHOGS for the same percentile; B) Percentile difference between Fenton and WHOGS for the same weight; C) Skewness of Fenton percentile distribution; D) Crossing of percentiles during postnatal adaption; E)Weight loss for PMA; F) Skewness of percentile distribution for PMA.

The Postnatal-Percentile Approach may work on less-skewed reference data from healthy fetuses followed longitudinally until term, but no such data currently exist. Our findings are further supported by a recent study, which showed that cross-sectional observations within a sample or population does not reflect true longitudinal growth. Percentile growth charts present smoothed statistical distributions for a specific age within a sample or population. The interpolated percentile lines across different gestational ages do not represent how infants grow physiologically(Lampl & Thompson, 2007).

In contrast, the optimized Growth-Velocity Approach results in individual growth trajectories for preterm infants that coincided with the WHOGS target weights at 42+0/7 weeks PMA after TeCES in term infants is completed. The fundamental aspect of the Growth-Velocity Approach is that it incorporates evidence from published growth concepts for different time-periods: 1) intrauterine growth until preterm birth on the Fenton growth curves, 2) PreCES from birth to DOL 21 (Rochow et al., 2016), 3) day-specific median growth velocities from Fenton charts (Fenton & Kim, 2013), and 4) transition to WHOGS (de Onis et al., 2004). The reference data for periods 1 and 4, the Fenton and WHOGS curves, are well established as appropriate for the intrauterine period, and the postnatal growth period after term birth, respectively (de Onis et al., 2004; Fenton & Kim, 2013). For period 2, data from a recent publication of a selected healthy group of preterm infants, 24 to 34 weeks of gestational age, with little to no clinical interventions required, were used. These data describe how a healthy preterm infant would physiologically adjust its growth trajectory after birth if undisturbed by illness. This provides the new set point for day of life 21 and continuation of stable growth (Rochow et al., 2016). For period 3, the Fenton chart median growth velocities were the most robust estimate of the population. The Fenton chart median growth velocities were recently compared in a validation study with three cohorts of stable growing preterm infants and the median growth velocities and patterns were found to be almost identical for all three cohorts (Fenton & Kim, 2013).

If neonatologists were to use the individual growth trajectory concept in practice it would have implications for assessment of extrauterine growth restriction (EUGR). EUGR is usually defined as weight less than the 10^{th} percentile at the time of discharge

(Clark et al., 2003). This method of classification is not necessarily accurate because an infant growing well could easily weigh less than the 10th percentile and thus might be misclassified as EUGR. In contrast, an infant growing at the 50th percentile may actually be restricted because their genetic potential indicates that they should be larger, but remain unidentified as restricted because of their percentile. The Growth-Velocity Approach presented here allows for the assessment of deviation from target growth trajectories. Thus, small infants would not automatically be classified as EUGR irrespective of the growth velocity and an infant with a high weight and slow weight gain may be correctly identified as EUGR. A similar recommendation was recently published for intrauterine growth restriction (IUGR), which suggested using falling percentiles (e.g., from the 25th to the 10th) to classify IUGR(Royal College of Obstetricians and Gynaecologists, 2013).

One limitation of the individual growth trajectory concept is that it currently cannot be used for infants that are born small for gestational age (SGA) due to intrauterine growth restriction (IUGR). This is because the concept has the underlying assumption that birth characteristics reflect the genetic potential of the infants. These characteristics are then used to create the individual growth trajectories (birth weight, gestational age, sex). Some studies have suggested that IUGR can be identified by looking at the asymmetry between head and body growth, since a growth restricted infant can develop a peripheral insulin resistance that causes glucose to be preferentially taken up by the brain (Devaskar & Chu, 2016). As a result, such foetuses showed decreased weight gain and an attenuation of length increase. However, the head circumference would be affected last and often continues to grow normally (head sparing). This results

in head circumference growth that is classified in a higher percentile than the body growth. Another important point is the consideration that small for gestational age (SGA) infants have a higher genetic potential for their target weight, than what may appear from their birth characteristics. Two issues remain unsolved. First, the current concept may underestimate the target weight. Second, the optimal trajectory for SGA infants is unknown. Specifically, whether or not SGA infants should catch up immediately or more slowly over an extended period of time in order to achieve optimal outcomes in light of the DOHaD hypothesis (Cho & Suh, 2016; Maciejewski, Hamon, Fresson, & Hascoet, 2016; Sebastiani et al., 2015).

It will be important in future validation studies of this concept to identify and exclude infants with IUGR, and further to develop a method to predict individual growth trajectories for IUGR infants. This may include assessing the maternal and paternal height and weight data and adding those characteristics into the prediction of the individual growth trajectories.

GROWTH TRAJECTORY CALCULATOR:

A clinical tool to use at bed-side to predict individual growth trajectories and target weights from birth to 42+0/7 weeks PMA for preterm infants is proposed. This tool would be valid for gestational ages between 24 and 34 weeks PMA and all birth weights. Such a tool would provide individualized target growth trajectories by incorporating the four periods of growth (intrauterine growth, postnatal adaption, period of stable growth until 42+0/7 weeks and term growth).

This proposed tool requires personalized input of gestational age, birth weight and sex in order to plot the individual infants' target growth trajectories. The prediction

model first calculates the target weight for day of life 21. The target postnatal trajectory for the period of stable growth is calculated using the Fenton day-specific median growth velocities applied to the previous day's weight starting with the weight from day of life 21. The individual trajectory is superimposed on a corresponding set of individualized reference curves created in the same way as the individual curve, however using Fenton birth weight data from the 3rd, 10th, 50th, 90th and 97th percentile as a reference, starting at the same gestational age as the individual infant. A range of ±0.5 standard deviation at DOL 21 corresponded to an allowed weight deviation of 5% at DOL 21 and 42 0/7 weeks PMA. The 5% deviation is a result of the uncertainty in the prediction model that predicts the DOL 21 weight. A weight deviation of ±5% is provided in the calculator as the range for the postnatal trajectory where the individual could reasonably grow. 5% weight deviation also falls within a clinically reasonable range, as compared to birth weight measurement error propagation, as discussed above.

The reference charts shift dynamically with the target curve based on birth weight, gestational age and sex. These reference curves then join the WHOGS reference curves at 42±0/7 weeks PMA (Figure 12). Figure 13 shows a preview of what this growth trajectory looks like in its first stages as an online tool (currently in development).

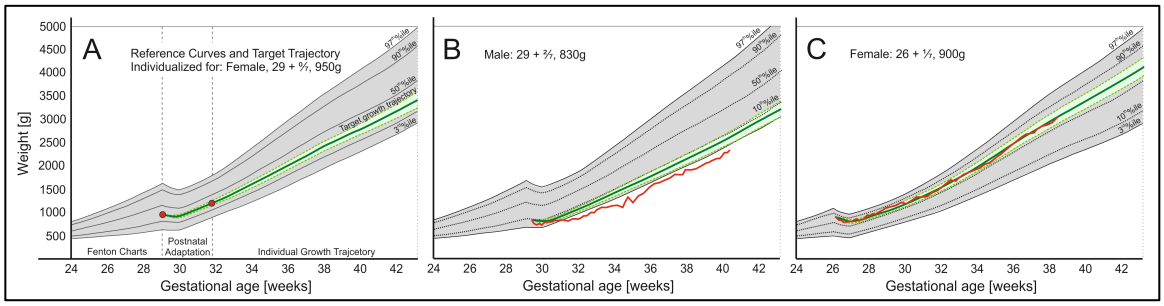

Figure 12: Growth Trajectory Calculator: Clinical tool to predict individual growth trajectory and target weight shown for three example infants. Solid green line shows

target growth trajectory ±0.5 SD (dotted green). Individualized reference curves are shown for the three example infants.

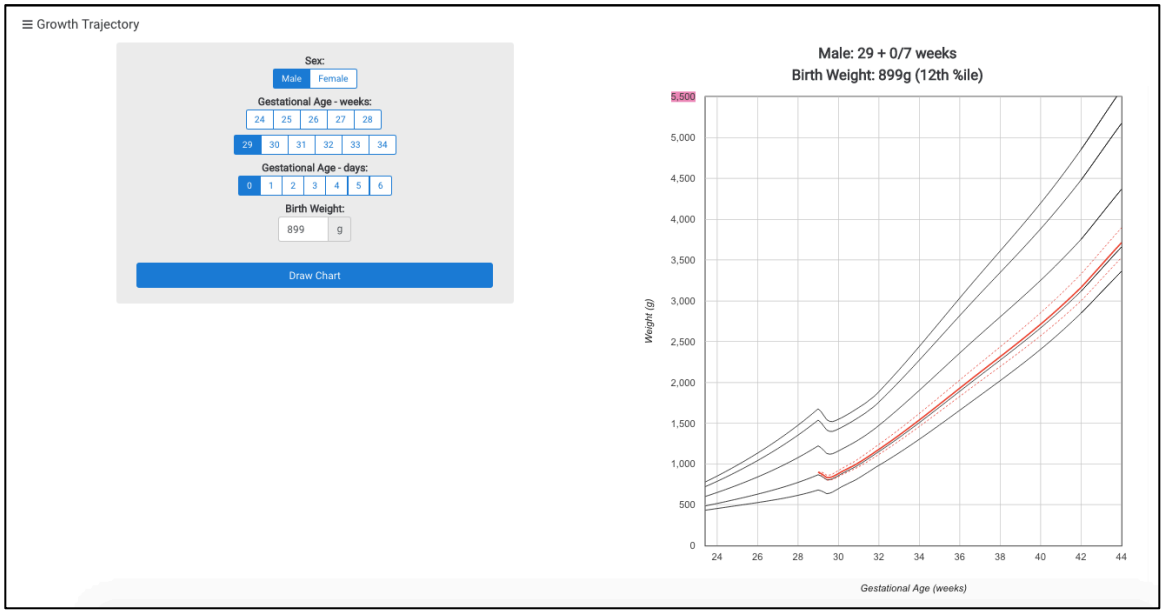

Figure 13: Preview of Growth Trajectory Calculator Online Tool: Individualized input of sex, gestational age and birth weight for a particular infant generates an individualized target trajectory (red) with customized reference curves showing postnatal adaptation.

VALIDATION:

The weights predicted with the Growth-Velocity Approach had an average deviation of 10g from actual measured weights in a selective subset of healthy, nutritionally controlled preterm infants. These infants required minimal medical support and had an enteral feeding intake of 150 to 165 mL/kg/d of fortified breast milk. At the time of measurement (term age) these infants were 0.7 z-score below their birth weight percentile, which is similar to the healthy infants as recently described by Rochow et al. 2016. The body composition of these infants was similar to recently described non-growth restricted preterm infants(Roggero et al., 2012). Of note is that percentage of body fat was symmetrically distributed around zero deviation from the target weight at measurement (term age). In contrast, the Postnatal-Percentile Approaches (following

birth weight percentile or DOL 21 achieved percentile) led to a skewed distribution where the majority of infants deviate away from the target weight in one direction more than the other. Following the birth weight percentile leads to a distribution where achieved weights are lower than target weights, whereas following the DOL 21 percentile leads to a distribution where achieved weights are higher than the target weights at discharge. In summary, the growth velocity approach is supported by this validation using weight and body composition data of infants with minimal risks for growth faltering. However, the Growth-Velocity Approach needs to be studied in a larger trial following infants' growth prospectively and observing neurodevelopment, cardiovascular and metabolic outcomes in later life.

This study has several strengths. The Growth-Velocity Approach predicts individualized growth trajectories for preterm infants based on gestational age, birth weight, and sex, and the individual growth trajectories fuse with term reference data at 40-42 weeks PMA. Physiological growth periods including postnatal adaptation and stable growth are incorporated into the approach. The reference data used in this study are based on large surveys from developed countries and the WHOGS, which is developed from longitudinal data from healthy, breast fed, term infants. The use of median growth velocities is a robust estimate of the population and can be considered as a reference for physiological intrauterine growth in order to follow the AAP's recommendation that preterm infants should grow like healthy foetuses *in utero*. The resulting predicted growth trajectories from the Growth-Velocity Approach appear to simulate physiological growth and the consistency in the results suggests that there is an underlying biological principle. With only a single, simple factor to optimize the day-

specific growth velocities our Growth-Velocity Approach is able to make an elegant, accurate prediction of preterm infant growth trajectories. Our study incorporates the four evidence-based principles of growth in preterm infants to predict physiological growth: 1) The use of Fenton's growth chart data for the intrauterine period. These are robust data from millions of infants form perinatal surveys obtained from different countries; 2) the use of the WHO Growth Standards (WHOGS) data for the post-term period. This data is longitudinal, comprised of healthy, term-born breast fed infants and thus is a gold standard for the post-term period for preterm infants; 3) Term infants born on the 50th percentile will transition to the 50th percentile on the WHO curves after postnatal adaptation; 4) Preterm infants undergo the same postnatal adaptation but earlier and transition to their birth weight percentile on the WHO curves.

Our study is an improvement from previous studies that were more descriptive than predictive in nature (Porcelli & Rosenbloom, 2014; Reeves & Bernstein, 2008; Riddle & DonLevy, 2010). Porcelli et al. created a neural network model to estimate growth trajectories. However, this model obtained weight and nutritional intake information from currently growing infants. This kind of growth trajectory prediction may be affected by clinical conditions and sickness of the infants. Thus, it does not provide a reference for physiological growth. The INTERGROWTH study (Villar et al., 2015) analyzed preterm infants born to a selected population of mothers with minimal risk factors and studied those infants with few complications and morbidities during NICU stay. This growth references does not consider postnatal adaptation and does not provide individual growth trajectories. Further, this reference may be confounded by current clinical care, rather than reflecting true physiology. In conclusion, in order to

create growth charts that reflect physiological growth and lead to optimal development it is extremely important to perform prospective follow-up studies that look at the long-term outcomes of following different growth trajectories.

Another strength of our study is that the concept can be easily integrated into a bed-side tool for physicians to use to predict growth of their preterm patients, for example as an electronic application.

This study has limitations. Reference data from different studies were combined leading to cross-sectional characteristics. The preliminary validation was performed in a small sample size of healthy, preterm infants. No effect on long-term health outcomes or biomarkers were given. As well, infants' genetic potential and infants with IUGR were not included in this initial analysis.

CONCLUSION

Four evidence based principles for preterm infants growth can be combined to create a new concept to describe the physiological postnatal growth of preterm infants. Using the Optimized Growth-Velocity Approach to describe individual growth trajectories to close the gap for the period of stable growth for preterm infants results in an accurate and precise match with the WHOGS target weight at 42+0/7 weeks PMA. The correction factor required for the optimized growth velocity approach, corresponding to a 10% increase of the daily growth velocities, and may represent a physiological pattern. On average, preterm infants lose 10% of their body water from extracellular water spaces in the first 21 days after birth (K Bauer & Versmold, 1989; Karl Bauer et al., 1991; Rochow et al., 2016). Thus, needing a 10% increase in growth rates (that are

normalized to body weight) to maintain protein accretion rates from *in utero*, suggests an underlying biological principle. The presented concept may provide neonatologists with a reference for individual growth trajectories and target weights. This concept may present metaphorical guardrails to guide growth and to target the infants' nutrition.

It is promising that the preliminary validation that has been performed, suggests that following the Optimized Growth Velocity Approach correlated with normal amounts of fat mass. As well it is encouraging that a normal population of infants, were found to be symmetrically distributed above and below the target trajectory, suggesting the predicted individual growth trajectories may not have biases towards higher or lower growth. Future clinical studies need to validate this concept in a large, multicenter study. To test the appropriateness of the individual growth trajectory concept, it is important to test whether adherence to the individual growth trajectories leads to improved body composition, cardiovascular outcomes (blood pressure, biomarkers, intima media thickness) and neurodevelopment (Bayley Scores).

REFLECTION AND FUTURE DIRECTIONS

We have already taken the initial steps to put a large, multicenter validation study into place to validate the individual growth trajectory concept. It will include thousands of infants from Canada, Australia, Germany and Sweden, including their growth data in the NICU and follow-up data (18 months, 2 years, 5 years) with body composition, neurodevelopment and cardiovascular outcomes.

The growth trajectory calculator concept that has been created from this project, is currently being developed into website and mobile application that can be used by clinicians to guide growth in clinical practice once it has been validated.

When I started this project in September of 2014, the aim was to identify a biomarker to predict healthy growth in preterm infants in serum. After a year of gel electrophoresis and mass spectrometry, it became clear that growth of preterm infants, specifically what was considered healthy growth, was very poorly characterized in literature. When I divided infants into growth groups according to growth rate, no differences in biomarkers were found between groups even when screening 200 proteins with mass spectrometry. This led us to speculate about whether or not dividing infants into groups by growth rate was sufficient, particularly since growth rate is a snapshot of specific time point and changes depending gestational age as well as the period of time that growth rate is calculated for. *See Appendix for method and results regarding Gel Electrophoresis and Mass Spectrometry experiments aiming to identify biomarkers for healthy growth*. This led to what has become the main part of my thesis - characterizing growth of preterm infants, predicting individual growth trajectories and translating this into a growth trajectory calculator tool.

The development of the individualized growth trajectories and the growth trajectory calculator will not only have a major impact on clinical management of growth (once validated and implemented) but will also allow for better characterization of growth of preterm infants for future research studies. Now, using this characterization of growth, infants can be divided into groups according to how closely they follow their predicted growth trajectories and this may allow researchers to identify a biomarker to predict healthy growth in preterm infants.

REFERENCES:

Adamkin, D. H., & Radmacher, P. G. (2014). Fortification of Human Milk in Very Low Birth Weight Infants (VLBW <1500 g Birth Weight). *Clinics in Perinatology*, *41*(2), 405–421. http://doi.org/10.1016/j.clp.2014.02.010

Agostoni, C., Buonocore, G., Carnielli, V. P., De Curtis, M., Darmaun, D., Decsi, T., … Ziegler, E. E. (2010). Enteral nutrient supply for preterm infants: commentary from the European Society of Paediatric Gastroenterology, Hepatology and Nutrition Committee on Nutrition. *Journal of Pediatric Gastroenterology and Nutrition*, *50*(1), 85–91. http://doi.org/10.1097/MPG.0b013e3181adaee0

Alexander, G. R., Himes, J., Kaufman, R. B., Mor, J., & Kogan, M. (1996). A United States National Reference for Fetal Growth. *Obstetrics & Gynecology*, *87*(2), 163–168.

Arslanoglu, S., Moro, G. E., & Ziegler, E. E. (2006). Adjustable fortification of human milk fed to preterm infants: does it make a difference? *Journal of Perinatology : Official Journal of the California Perinatal Association*, *26*(10), 614–21. http://doi.org/10.1038/sj.jp.7211571

Arslanoglu, S., Moro, G. E., & Ziegler, E. E. (2009). Preterm infants fed fortified human milk receive less protein than they need. *Journal of Perinatology : Official Journal of the California Perinatal Association*, *29*(7), 489–92. http://doi.org/10.1038/jp.2009.50

Barker, D. J. P. (1992). “ Impact of diet on critical events in development .” *Proceedings of the Nutrition Society*, *51*, 135–144.

Barker, D. J. P., & Osmond, C. (1986). Infant Mortality, Childhood Nutrition and Ischaemic Heart Disease in England and Wales. *The Lancet*, 1077–1081.

Bauer, K., Bovermann, G., Roithmaier, A., G??tz, M., Pr??lss, A., & Versmold, H. T. (1991). Body composition, nutrition, and fluid balance during the first two weeks of life in preterm neonates weighing less than 1500 grams. *The Journal of Pediatrics*, *118*(4 PART 1), 615–620. http://doi.org/10.1016/S0022-3476(05)83390-6

Bauer, K., & Versmold, H. (1989). Postnatal weight loss in preterm neonates less than 1,500 g is due to isotonic dehydration of the extracellular volume. *Acta Paediatrica Scandinavica. Supplement*, *360*(7), 37–42. http://doi.org/10.1111/j.1651-2227.1989.tb11280.x

Belfort, M. B., Gillman, M. W., & McCormick, M. C. (2012). Prenatal and perinatal predictors of blood pressure at school age in former preterm, low birth weight infants. *Journal of Perinatology : Official Journal of the California Perinatal Association*, *32*(4), 265–9. http://doi.org/10.1038/jp.2011.88

Belfort, M. B., Martin, C. R., Smith, V. C., Gillman, M. W., & McCormick, M. C. (2014). Infant Weight Gain and School-Age Blood Pressure and Cognition in Former Preterm Infants. *Pediatrics*, *125*(6), 1–17. http://doi.org/10.1542/peds.2009-2746.Infant

Bertino, E., Spada, E., Occhi, L., Coscia, A., Giuliani, F., Gagliardi, L., … Milani, S. (2010). Neonatal anthropometric charts: the Italian neonatal study compared with other European studies. *Journal of Pediatric Gastroenterology and Nutrition*, *51*(3), 353–361. http://doi.org/10.1097/MPG.0b013e3181da213e

Blackwell, M. T., Eichenwald, E. C., McAlmon, K., Petit, K., Linton, P. T., McCormick, M. C., & Richardson, D. K. (2005). Interneonatal Intensive Care Unit Variation in Growth Rates and Feeding Practices in Healthy Moderately Premature Infants. *Journal of Perinatology*, *25*(7), 478–485. http://doi.org/10.1038/sj.jp.7211302

Bonellie, S., Chalmers, J., Gray, R., Greer, I., Jarvis, S., & Williams, C. (2008). Centile charts for birthweight for gestational age for Scottish singleton births. *BMC Pregnancy and Childbirth*, *8*, 5. http://doi.org/10.1186/1471-2393-8-5

Burkhardt, T., Schäffer, L., Zimmermann, R., & Kurmanavicius, J. (2008). Newborn weight charts underestimate the incidence of low birthweight in preterm infants. *American Journal of Obstetrics and Gynecology*, *199*(2), 1–6. http://doi.org/10.1016/j.ajog.2008.01.011

Cho, W. K., & Suh, B. (2016). Catch-up growth and catch-up fat in children born small for gestational age, *59*(1), 1–7. http://doi.org/10.3345/kjp.2016.59.1.1

Clark, R. H., Thomas, P., & Peabody, J. (2003). Extrauterine growth restriction remains a serious problem in prematurely born neonates. *Pediatrics*, *111*(5 Pt 1), 986–990. http://doi.org/10.1542/peds.111.5.986

Cole, T. J., Williams, A. F., & Wright, C. M. (2011). Revised birth centiles for weight, length and head circumference in the UK-WHO growth charts. *Annals of Human Biology*, *38*(1), 7–11. http://doi.org/10.3109/03014460.2011.544139

Crossland, D. S., Richmond, S., Hudson, M., Smith, K., & Abu-Harb, M. (2008). Weight change in the term baby in the first 2 weeks of life. *Acta Paediatrica, International Journal of Paediatrics*, *97*(4), 425–429. http://doi.org/10.1111/j.1651-2227.2008.00685.x

de Jong, F., Monuteaux, M. C., van Elburg, R. M., Gillman, M. W., & Belfort, M. B. (2011). Systematic Review and Meta-Analysis of Preterm Birth and Later Systolic Blood Pressure. *Hypertension*, *59*(2), 226–234. http://doi.org/10.1161/HYPERTENSIONAHA.111.181784

De Jong, F., Monuteaux, M. C., Van Elburg, R. M., Gillman, M. W., & Belfort, M. B. (2012). Systematic review and meta-analysis of preterm birth and later systolic blood pressure. *Hypertension*, *59*(2), 226–234. http://doi.org/10.1161/HYPERTENSIONAHA.111.181784

de Onis, M., Garza, C., Victora, C., Onyango, A., Frongillo, E., & Martines, J. (2004). The WHO Multicentre Growth Reference Study: planning, study design, and methodology. *Food Nutr Bull*, *1*(15-26).

Devaskar, S. U., & Chu, A. (2016). Intrauterine Growth Restriction: Hungry for an Answer. *Physiology*, *31*(2), 131–146. http://doi.org/10.1152/physiol.00033.2015

Dollberg, S., Haklai, Z., Mimouni, F. B., Gorfein, I., & Gordon, E. S. (2005). Birthweight standards in the live-born population in Israel. *Israel Medical Association Journal*, *7*(5), 311–314.

Ehrenkranz, R. a. (2014). Nutrition, Growth and Clinical Outcomes. In *Nutritional Care of Preterm Infants: Scientific Basis and Practical Guidelines. World Review of Nutiriton and Dietetics.* (pp. 11–26).

Ehrenkranz, R. A., Dusick, A. M., Vohr, B. R., Wright, L. L., Wrage, L. a, & Poole, W. K. (2006). Growth in the neonatal intensive care unit influences neurodevelopmental and growth outcomes of extremely low birth weight infants. *Pediatrics*, *117*(4), 1253–61. http://doi.org/10.1542/peds.2005-1368

Ehrenkranz, R. A., Younes, N., Lemons, J. A., Fanarof, A. A., Donovan, E. F., Wright, L. L., … Papile, L. A. (1999). Longitudinal growth of hospitalized very low birth weight infants. *Pediatrics*, *104*(2), 280–289. http://doi.org/10.1542/peds.104.2.280

Embleton, N. D. (2007). Optimal protein and energy intakes in preterm infants. *Early Human Development*, *83*(12), 831–837. http://doi.org/10.1016/j.earlhumdev.2007.10.001

Eriksson, J. G. (2011). Early growth and coronary heart disease and type 2 diabetes : findings from the Helsinki Birth Cohort Study (HBCS) 1 – 4, *94*, 1–4. http://doi.org/10.3945/ajcn.110.000638.It

Euser, A. M., Finken, M. J. J., Keijzer-Veen, M. G., Hille, E. T. M., Wit, J. M., & Dekker, F. W. (2005). Associations between prenatal and infancy weight gain and BMI, fat mass, and fat distribution in young adulthood: A prospective cohort study

in males and females born very preterm. *American Journal of Clinical Nutrition*, *81*(2), 480–487. http://doi.org/81/2/480 [pii]

Fall, C. H. D., Sachdev, H. S., Osmond, C., Lakshmy, R., Biswas, S. D., Prabhakaran, D., … Bhargava, S. K. (2008). Adult metabolic syndrome and impaired glucose tolerance are associated with different patterns of BMI gain during infancy: Data from the New Delhi Birth Cohort. *Diabetes Care*, *31*(12), 2349–56. http://doi.org/10.2337/dc08-0911

Fenton, T. R., & Kim, J. H. (2013). A systematic review and meta-analysis to revise the Fenton growth chart for preterm infants. *BMC Pediatrics*, *13*, 59. http://doi.org/10.1186/1471-2431-13-59

Fusch, C., & Samiee-Zafarghandy, S. (2014). Promoting healthy growth and nutrition in preterm infants: A challenge for clinicians and researchers. *Clinical Biochemistry*, *47*(9), 711–713. http://doi.org/10.1016/j.clinbiochem.2014.05.021

Gluckman, P.D., Hanson, M.A., Beedle, A. S. (2007). Feature Article Early Life Events and Their Consequences for Later Disease : A Life History and Evolutionary Perspective. *American Journal of Human Biology*, *19*(August 2006), 1–19. http://doi.org/10.1002/ajhb

Hack, M., Schluchter, M., Cartar, L., Rahman, M., Cuttler, L., & Borawski, E. (2003). Growth of very low birth weight infants to age 20 years. *Pediatrics*, *112*(1 Pt 1), e30–e38. http://doi.org/10.1542/peds.112.1.e30

Henriksen, C., Westerberg, A. C., Rønnestad, A., Nakstad, B., Veierød, M. B., Drevon, C. a, & Iversen, P. O. (2009). Growth and nutrient intake among very-low-birth-weight infants fed fortified human milk during hospitalisation. *The British Journal of Nutrition*, *102*(8), 1179–86. http://doi.org/10.1017/S0007114509371755

Horbar, J. D., Ehrenkranz, R. A., Badger, G. J., Edwards, E. M., Morrow, K. A., Soll, R. F., … Bellù, R. (2015). Weight Growth Velocity and Postnatal Growth Failure in Infants 501 to 1500 Grams: 2000-2013. *Pediatrics*, *136*(1), e84–92. http://doi.org/10.1542/peds.2015-0129

Ignjatovic, V., Lai, C., Summerhayes, R., Mathesius, U., Tawfilis, S., Perugini, M. a., & Monagle, P. (2011). Age-related differences in plasma proteins: How plasma proteins change from neonates to adults. *PLoS ONE*, *6*(2), e17213. http://doi.org/10.1371/journal.pone.0017213

Johansson, S., Iliadou, A., Bergvall, N., Tuvemo, T., Norman, M., & Cnattingius, S. (2005). Risk of high blood pressure among young men increases with the degree of immaturity at birth. *Circulation*, *112*(22), 3430–3436.

http://doi.org/10.1161/CIRCULATIONAHA.105.540906

Johnson, M. J., Wootton, S. a, Leaf, A. a, & Jackson, A. a. (2012). Preterm birth and body composition at term equivalent age: a systematic review and meta-analysis. *Pediatrics*, *130*(3), e640–9. http://doi.org/10.1542/peds.2011-3379

Kashyap, S. (2001). Effects of Quality of Energy Intake on Growth and Metabolic Response of Enterally Fed Low-Birth-Weight Infants. *Pediatric Research*, *50*(3), 390–397.

Kashyap, S., Forsyth, M., Zucker, C., Ramakrishnan, R., Dell, R. B., & Heird, W. C. (1985). FETAL AND NEONATAL MEDICINE Effects of varying protein and energy intakes on growth and metabolic response in low birth weight infants. *Clinical Research*.

Kashyap, S., Schulze, K. F., Forsyth, M., Zucker, C., Dell, R. B., Ramakrishnan, R., & Heird, W. C. (1988). Growth, nutrient retention, and metabolic response in low birth weight infants fed varying intakes of protein and energy. *The Journal of Pediatrics*, *113*(4), 713–21. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3139856

Kashyap, S., Towers, H. M., Sahni, R., Ohira-kist, K., Abildskov, K., Schulze, K. F., & Nutr, A. J. C. (2001). Effects of quality of energy on substrate oxidation in enterally fed , (1), 374–380.

Keijzer-Veen, M. G., Dülger, A., Dekker, F. W., Nauta, J., & van der Heijden, B. J. (2010). Very preterm birth is a risk factor for increased systolic blood pressure at a young adult age. *Pediatric Nephrology (Berlin, Germany)*, *25*(3), 509–16. http://doi.org/10.1007/s00467-009-1373-9

Koletzko, B., Goulet, O., Hunt, J., Krohn, K., & Shamir, R. (2005a). Guidelines on Paediatric Parenteral Nutrition: Amino Acids. *Journal of Pediatric Gastroenterology and Nutrition*, *41*(12-18), 1–9.

Koletzko, B., Goulet, O., Hunt, J., Krohn, K., & Shamir, R. (2005b). Guidelines on Paediatric Parenteral Nutrition: Carbohydrates. *Journal of Pediatric Gastroenterology and Nutrition*, *41*(12-18), 28–32.

Koletzko, B., Goulet, O., Hunt, J., Krohn, K., & Shamir, R. (2005c). Guidelines on Paediatric Parenteral Nutrition: Fluid and Electrolytes (Na , Cl and K). *Journal of Pediatric Gastroenterology and Nutrition*, *41*(12-18), 1–6.

Koletzko, B., Goulet, O., Hunt, J., Krohn, K., & Shamir, R. (2005d). Guidelines on Paediatric Parenteral Nutrition: Lipids. *Journal of Pediatric Gastroenterology and Nutrition*, *41*(12-18).

Kramer, M. S., Platt, R. W., Wen, S. W., Joseph, K. S., Allen, a, Abrahamowicz, M., … Bréart, G. (2001). A new and improved population-based Canadian reference for birth weight for gestational age. *Pediatrics*, *108*(2), E35. http://doi.org/10.1542/peds.108.2.e35

Kramer, M. S., Platt, R. W., Wen, S. W., Joseph, K. S., Allen, A., & Abrahamowicz, M. (2015). Birth Weight for Gestational Age, *108*(2).

Lampl, M., & Thompson, A. (2007). Growth Chart Curves Do Not Describe Individual Growth Biology. *American Journal of Human Biology*, *19*(2), 653–653. http://doi.org/10.1002/ajhb

Lausman, A., McCarthy, F. P., Walker, M., & Kingdom, J. (2012). Screening, diagnosis, and management of intrauterine growth restriction. *Journal of Obstetrics and Gynaecology Canada : JOGC = Journal D'obstétrique et Gynécologie Du Canada : JOGC*, *34*(1), 17–28. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/22260759

Lewandowski, A. J., Bradlow, W. M., Augustine, D., Davis, E. F., Francis, J., Singhal, A., … Leeson, P. (2013). Right ventricular systolic dysfunction in young adults born preterm. *Circulation*, *128*(7), 713–20. http://doi.org/10.1161/CIRCULATIONAHA.113.002583

Lewandowski, A. J., Davis, E. F., Yu, G., Digby, J. E., Boardman, H., Whitworth, P., … Leeson, P. (2015). Elevated blood pressure in preterm-born offspring associates with a distinct antiangiogenic state and microvascular abnormalities in adult life. *Hypertension*, *65*(3), 607–14. http://doi.org/10.1161/HYPERTENSIONAHA.114.04662

Liotto, N., Radaelli, T., Orsi, A., Taricco, E., Roggero, P., Giannì, M. L., … Cetin, I. (2010). Relationship between in utero sonographic evaluation and subcutaneous plicometry after birth in infants with intrauterine growth restriction: an exploratory study. *Italian Journal of Pediatrics*, *36*(1), 70. http://doi.org/10.1186/1824-7288-36-70

Lucas, A. (2010). Growth and later health: A general perspective. *Nestle Nutrition Workshop Series: Pediatric Program*, *65*, 1–11. http://doi.org/10.1159/000281107

Lucas, A., Gore, S. M., Cole, T. J., Bamford, M. F., Dossetor, J. F., Barr, I., … Lucas, P. J. (1984). Multicentre trial on feeding low birthweight infants: effects of diet on early growth. *Archives of Disease in Childhood*, *59*(8), 722–730. http://doi.org/10.1136/adc.59.8.722

Lucas, A., & Morley, R. (1994). Does early nutrition in infants born before term

programme later blood pressure? *BMJ*, *309*(6950), 304–309. http://doi.org/10.1136/bmj.309.6950.304

Lucas, A., Morley, R., & Cole, T. J. (1998). Randomised trial of early diet in preterm babies and later intelligence quotient. *BMJ*, *317*(November).

Maciejewski, E., Hamon, I., Fresson, J., & Hascoet, J.-M. (2016). Growth and neurodevelopment outcome in symmetric versus asymmetric small for gestational age term infants. *Journal of Perinatology*, (November 2015), 1–6. http://doi.org/10.1038/jp.2016.48

Marks, K., Reichman, B., Lusky, A., & Zmora, E. (2006). Fetal growth and postnatal growth failure in very-low-birthweight infants. *Acta Paediatrica*, (May 2005), 236–243. http://doi.org/10.1080/08035250500358315

Metcalfe, N. B., & Monaghan, P. (2001). Compensation for a bad start: grow now, pay later? *Trends in Ecology & Evolution*, *16*(5), 254–260. http://doi.org/10.1016/S0169-5347(01)02124-3

Niklasson, A., & Albertsson-Wikland, K. (2008). Continuous growth reference from 24th week of gestation to 24 months by gender. *BMC Pediatrics*, *8*, 8. http://doi.org/10.1186/1471-2431-8-8

Nutrition, A. A. of P. C. on. (1985). Nutritional Needs of Low-Birth-Weight Infants. In *Pediatrics* (Vol. 75, pp. 976–986).

Oken, E., Kleinman, K. P., Rich-Edwards, J., & Gillman, M. W. (2003). A nearly continuous measure of birth weight for gestational age using a United States national reference. *BMC Pediatrics*, *3*, 6. http://doi.org/10.1186/1471-2431-3-6

Olsen, I. E., Groveman, S. a, Lawson, M. L., Clark, R. H., & Zemel, B. S. (2010). New intrauterine growth curves based on United States data. *Pediatrics*, *125*(2), e214–e224. http://doi.org/10.1542/peds.2009-0913

Parkinson, J., Hyde, M., Gale, C., Santhakumaran, S., & Modi, N. (2013). Preterm Birth and the Metabolic Syndrome in Adult Life : A Systematic Review and Meta-analysis. *Pediatrics*, *131*, e1240–e1263.

Polberger, S. (2009). Emerging societies : coexistence of childhood malnutrition and obesity. *Nestlé Nutrition Institute Workshop Series Pediatric Program, 63*(63), xiv, 273 p. http://doi.org/10.1159/000209974

Porcelli, P. J., & Rosenbloom, S. T. (2014). Comparison of New Modeling Methods for Postnatal Weight in ELBW Infants Using Prenatal and Postnatal Data. *Journal of Pediatric Gastroenterology and Nutrition*, *59*(1), e2–e8.

http://doi.org/10.1097/MPG.0000000000000342

Rabilloud, T., Chevallet, M., Luche, S., & Lelong, C. (2010). Two-dimensional gel electrophoresis in proteomics: Past, present and future. *Journal of Proteomics*, *73*(11), 2064–77. http://doi.org/10.1016/j.jprot.2010.05.016

Ravelli, a C. J., Vandermeulen, J. H. P., Michels, R. P. J., Osmond, C., Barker, D. J. P., Hales, C. N., & Bleker, O. P. (1998). Glucose-tolerance in adults after prenatal exposure to famine. *The Lancet*, *351*, 173–177. Retrieved from http://fox.novo.dk/netacgi/getref.pl?ref=C-980111330

Reeves, S., & Bernstein, I. M. (2008). Optimal Growth Modeling. *Seminars in Perinatology*, *32*(3), 148–153. http://doi.org/10.1053/j.semperi.2007.11.001

Riddle, W. R., & DonLevy, S. C. (2010). Generating expected growth curves and Z-scores for premature infants. *Journal of Perinatology : Official Journal of the California Perinatal Association*, *30*(11), 741–750. http://doi.org/10.1038/jp.2010.32

Roberts, C. L., & Lancaster, P. A. L. (2006). National birthweight percentiles by gestational age for twins born in Australia, *11*(December 1998), 278–282.

Rochow, N., Fusch, G., Choi, A., Chessell, L., Elliott, L., McDonald, K., … Fusch, C. (2013). Target fortification of breast milk with fat, protein, and carbohydrates for preterm infants. *The Journal of Pediatrics*, *163*(4), 1001–7. http://doi.org/10.1016/j.jpeds.2013.04.052

Rochow, N., Fusch, G., Muehlinghaus, A., Niesytto, C., Straube, S., Utzig, N., & Fusch, C. (2012). A nutritional program to improve outcome of very low birth weight infants. *Clinical Nutrition*, *31*(1), 124–131. http://doi.org/10.1016/j.clnu.2011.07.004

Rochow, N., Fusch, G., Zapanta, B., Ali, A., Barui, S., & Fusch, C. (2015). Target fortification of breast milk: How often should milk analysis be done? *Nutrients*, *7*(4), 2297–2310. http://doi.org/10.3390/nu7042297

Rochow, N., Raja, P., Liu, K., Fenton, T., Landau-Crangle, E., Göttler, S., … Fusch, C. (2016). Physiological adjustment to postnatal growth trajectories in healthy preterm infants. *Pediatric Research*, (August 2015), 1–10. http://doi.org/10.1038/pr.2016.15

Roggero, P., Gianni, M. L., Piemontese, P., Amato, O., Agosti, M., & Mosca, F. (2012). Effect of nutrition on growth and body composition in infants born preterm. *Journal of Maternal-Fetal & Neonatal Medicine*, *25 Suppl 3*(April), 49–52. http://doi.org/10.3109/14767058.2012.712318

Ross, M. G., & Desai, M. (2014). Developmental Programming of Appetite/Satiety. *Annals of Nutrition and Metabolism*, *64*(s1), 36–44. http://doi.org/10.1159/000360508

Rotteveel, J., van Weissenbruch, M. M., Twisk, J. W. R., & Delemarre-Van de Waal, H. a. (2008). Infant and childhood growth patterns, insulin sensitivity, and blood pressure in prematurely born young adults. *Pediatrics*, *122*(2), 313–21. http://doi.org/10.1542/peds.2007-2012

Royal College of Obstetricians and Gynaecologists. (2013). The Investigation and Management of the Small for Gestational Age Fetus. *Royal College of Obstetricians and Gynaecologists*, (31).

Schaefer, E. W., Flaherman, V. J., Kuzniewicz, M. W., Li, S. X., Walsh, E. M., & Paul, I. M. (2015). External Validation of Early Weight Loss Nomograms for Exclusively Breastfed Newborns. *Breastfeeding Medicine*, *10*(10), 458–463. http://doi.org/10.1089/bfm.2015.0126

Sebastiani, G., Díaz, M., Bassols, J., Aragonés, G., López-Bermejo, A., de Zegher, F., & Ibáñez, L. (2015). The sequence of prenatal growth restraint and post-natal catch-up growth leads to a thicker intima-media and more pre-peritoneal and hepatic fat by age 3-6 years. *Pediatric Obesity*, 1–7. http://doi.org/10.1111/ijpo.12053

Senterre, T., & Rigo, J. (2011). Optimizing early nutritional support based on recent recommendations in VLBW infants and postnatal growth restriction. *J Pediatr Gastroenterol Nutr*, *53*(5), 536–542. http://doi.org/10.1097/MPG.0b013e31822a009d

Singhal, A., Farooqi, I. S., Rahilly, S. O., Cole, T. J., Fewtrell, M., & Lucas, A. (2002). Early nutrition and leptin concentrations in later life 1 – 3.

St. John, E. B., & Carlo, W. A. (2003). Respiratory distress syndrome in VLBW infants: Changes in management and outcomes observed by the NICHD Neonatal Research Network. *Seminars in Perinatology*, *27*(4), 288–292. http://doi.org/10.1016/S0146-0005(03)00056-9

Uauy, R., & Koletzko, B. (2014). Defining the Nutritional Needs of Preterm Infants. In *Nutritional Care of Preterm Infants: Scientific Basis and Practical Guidelines.* (pp. 4–10). World Rev Nutr Diet.

van den Akker, C. H. ., Schierbeek, H., Rietveld, T., Vermes, A., Duvekot, J. J., Steegers, E. A. ., & van Goudoever, J. B. (2008). Human fetal albumin synthesis rates during different periods of gestation. *American Journal of Clinical Nutrition*, *88*(4), 997.

Van Den Akker, C. H. P., Schierbeek, H., Minderman, G., Vermes, A., Schoonderwaldt,

E. M., Duvekot, J. J., … Van Goudoever, J. B. (2011). Amino acid metabolism in the human fetus at term: Leucine, valine, and methionine kinetics. *Pediatric Research*, *70*(6), 566–571. http://doi.org/10.1203/PDR.0b013e31823214d1

Van den Akker, C. H., & Van Goudoever, J. B. (2010). Recent advances in our understanding of protein and amino acid metabolism in the human fetus. *Current Opinion in Clinical Nutrition and Metabolic Care*, *13*(1), 75–80. http://doi.org/10.1097/MCO.0b013e328333aa4f

van den Akker, C. Van Den, Schierbeek, H., Dorst, K. Y., Schoonderwaldt, E. M., Vermes, A., Duvekot, J. J., … Goudoever, J. B. Van. (2009). Human fetal amino acid metabolism at term gestation 1 – 3. *American Journal of Clinical Nutrition*. http://doi.org/10.3945/ajcn.2008.26553.1

van Goudoever, J., Vlaardingerbroek, H., van den Akker, C., de Groof, F., & van der Schoor, S. (2014). Amino acids and proteins. In *Nutritional Care of Preterm Infants: Scientific Basis and Practical Guidelines.* (pp. 49–63). World Rev Nutr Diet.

Villar, J., Giuliani, F., Bhutta, Z. A., Bertino, E., Ohuma, E. O., Ismail, L. C., … Waller, S. (2015). Postnatal growth standards for preterm infants: The Preterm Postnatal Follow-up Study of the INTERGROWTH-21st Project. *The Lancet Global Health*, *3*(11), e681–e691. http://doi.org/10.1016/S2214-109X(15)00163-1

Vohr, B. R., Allan, W., Katz, K. H., Schneider, K. C., & Ment, L. R. (2010). Early Predictors of Hypertension in Prematurely Born Adolescents. *Acta Paediatrica*, *99*(12), 1812–1818. http://doi.org/10.1111/j.1651-2227.2010.01926.x.Early

Voigt, M., Rochow, N., Straube, S., Briese, V., Olbertz, D., & Jorch, G. (2010). Birth weight percentile charts based on daily measurements for very preterm male and female infants at the age of 154-223 days. *Journal of Perinatal Medicine*, *38*(3), 289–295. http://doi.org/10.1515/JPM.2010.031

Wells, J. C. K., Chomtho, S., & Fewtrell, M. S. (2007). Programming of body composition by early growth and nutrition. *Proceedings of the Nutrition Society*, *66*(03), 423–434. http://doi.org/10.1017/S0029665107005691

Yudkina, J. S., & Stannera, S. (1998). Prenatal exposure to famine and health in later life. *Lancet*, *351*(9112), 1362.

Ziegler, E. (2011). Low protein intake from human milk fortified in a routine fashion is the main cause of postnatal growth restriction. *Ann Nutr Metab*, *58*(suppl 1), 8–18.

Ziegler, E. (2014). Human milk and human milk fortifiers. In B. Koletzko (Ed.), *Nutritional Care of Preterm Infants: Scientific Basis and Practical Guidelines.* (pp. 215–27). World Rev Nutr Diet.

Ziegler, E. E., & Carlson, S. J. (2009). Early nutrition of very low birth weight infants. *The Journal of Maternal-Fetal & Neonatal Medicine : The Official Journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstetricians*, *22*(3), 191–7. http://doi.org/10.1080/14767050802630169

Ziegler, E. E., O'Donnell, A. M., Nelson, S. E., & Fomon, S. J. (1976). Body Composition of the Reference Fetus. In *Growth* (pp. 329–341).

APPENDIX

OTHER WORK COMPLETED IN MASTER'S PROGRAM:

(**Not** part of Master's work submitted for defense)

As described briefly in the future directions, the initial year of my Master's work was dedicated to search for a biomarker to predict healthy growth in preterm infants. This is a brief summary of that work, which led to the main thesis "Predicting Individualized Growth Trajectories for Preterm Infants". The hope is that this work will inform future studies searching for biomarkers to predict healthy growth in preterm infants and that the improved characterization of growth in preterm infants will allow superior classification of growth groups for research, in addition to it's clinical utility.

The gel electrophoresis work was completed by myself in collaboration with an undergraduate student, Augustine Nguyen.

OBJECTIVES:

- To test if 2D-gel electrophoresis or mass spectrometry can be used to identify differences in protein expression patterns or protein concentration of specific biomarkers between distinct growth groups to predict healthy growth in preterm infants.

- To test if the differences in protein expression patterns or protein concentration between distinct growth groups of preterm infants reflect clinical growth differences.

METHODS:

STUDY DESIGN:

Prospective study, McMaster Children's Hospital

Study Period: September 2013-August 2016

Study population: Preterm infants enrolled in the "Individualized Fortification of Breast Milk" study at McMaster Children's Hospital, separated into high, normal and low growth groups.

SAMPLE COLLECTION & STORAGE:

GROWTH GROUPS:

Leftover plasma samples collected from the Core Lab at McMaster Children's Hospital, from all preterm infants enrolled in the ongoing Randomized Control Trial "Individualized Fortification of Breast Milk (IFO)". The blood is drawn on Mondays, sent to the Core Lab for analysis and leftover blood is stored in 4 degrees Celsius until Tuesday when it is picked up and stored in -80 degrees Celsius until further analysis. Volume of plasma collected from each sample ranges from 5ul to 300ul, averaging 50-100ul per sample. Note: no additional blood is drawn from the infants; therefore there is no additional risk to the infants participating in this sub-study of the IFO study.

CONTROL GROUPS:

The collection of plasma from healthy term infants will be done at either i) the day 3 well baby exam or ii) when infants are tested for hypoglycemia and are negative, in either case with parental consent. In these cases 200ul of additional blood will be drawn at the same time blood is routinely drawn at these visits, so that no additional risk or discomfort is posed to the infant.

SAMPLE SIZE:

We aimed to collect a minimum of 250 plasma samples for the growth groups based on feasibility and availability from patients in the ongoing clinical trial.

GROWTH AND HEALTH INFORMATION:

In accordance with the REB, information for each of the infants collected as part of the growth groups were extracted from MEDITECH, Hamilton Health Sciences' electronic patient record system. The information collected is stored without the use of patient identifiers in a secured excel document. Unique codes were assigned to each infant and are kept in a separate secured excel document. The information collected includes: birth date, sex, weight in grams for each day a weight is taken while the infant is treated in McMaster Children's Hospital's NICU, gestational age at birth, gestational age at the date(s) of sample collection, date of sample collection, discharge date, antibiotic and steroid administration dates. From this information the following parameters were calculated: growth rate, length of stay, number of days between sample collection and discharge, size for gestational age.

2D GEL ELECTROPHORESIS REPEATABILITY EXPERIMENTS:

Two-dimensional (2D) gel electrophoresis is a useful technique to separate proteins in biological fluids such as plasma in order to visualize patterns and compare patterns in protein expression between groups (Rabilloud, Chevallet, Luche, & Lelong, 2010). 2D gel electrophoresis separates gels by isoelectric point and mass, ideally giving each protein a unique position on the gel. 2D gel electrophoresis has been a popular proteomics method often used in combination with mass spectrometry. If protein spots are found to differ between two groups where differences in protein expression are expected, the gel spot can be excised, digested and run with mass spectrometry for protein identification.

Due to the small volume of infant plasma available large gels were not feasible for our study. Instead we wanted to validate the use of mini-gels to compare different groups in preterm infants.

In order to evaluate the validity and repeatability of 2D gel electrophoresis using mini-gels (Novex® 8cm × 8cm pre-cast mini-gels in the XCell SureLock™ Mini-Cell device) as a method to observe differential protein expression in neonates, 5 samples were run in quintuplicate for a total of 25 mini-gels. Two samples were healthy adults, two samples were healthy neonates and one was cord blood. The fibrinogen gamma chain was chosen as a reference protein to compare between gels because of its well-known placement, visibility and use in other papers comparing neonates and adults. The fibrinogen gamma chain was compared between all gels, including its post-translational modifications for a total of up to 10 spots per gel.

SAMPLE SELECTION CRITERIA

The healthy adult blood donors were aged 20-22, male and were not taking any medications. The neonate samples were samples from the growth groups chosen for being healthy and having a normal growth rates. The following criteria were applied to choose the healthy neonate samples: growth rate between 15 and 25 g/kg/day, SGA and LGA infants excluded, samples where the infant was on antibiotics or steroids 7 days before or after the sample was collected were excluded, samples with shorter times from sample to discharge (indicating that the infant was relatively healthy because close to discharge) were preferred as well as shorter length of stay (an indication that the infant had relatively few complications and thus a shorter stay in the NICU). The two samples were matched for sex, birth weight, GA at birth and sample collection. The cord blood sample was from a healthy, caesarian delivery.

SAMPLE PREPARATION & IMAGE ANALYSIS:

To determine the concentration of protein in each sample, a BCA protein assay from Thermo Scientific was performed in duplicate before running each sample. Each sample was loaded in a standard amount of 1500ug. Before loading onto the first dimension, samples were depleted for the two most abundant human plasma proteins albumin and IgG, which often interfere with the visualization of less abundant proteins. This was done using the Multiple Affinity HSA/IgG Removal Spin Cartridge from Agilent. After centrifugation the depleted sample is diluted because of the buffer and was then desalted and concentrated using a Microcon 10kD centrifugal filter device from Milipore. To fit the volume of unconcentrated sample into the centrifugal filter devices, the samples are mixed with a vortex and split into 2 aliquots, creating a duplicate.

Rehydration buffer including detergents (urea, thiourea, CHAPS) to denature the proteins, destreak solution to minimize streaking on the gels and bromophenol blue to track the protein's movement through the gel, is added to the sample and incubates while shaking for 1 hour.

The first dimension of the two-dimensional gel electrophoresis is isoelectric focusing, where the proteins are separated by pH and fixed at their isoelectric point. The first dimension gels used were the 7cm IPG strips in pH 3-11 nonlinear from GE Healthcare on the IPGphor Isoelectric Focusing System from Pharmacia Biotech. The isoelectric focusing starts with a slow ramping up of voltage to allow the proteins to enter the gel, reaching a maximum voltage of 5000V and finally holding at 100V until stopped and removed the next morning.

The second dimension is preceded by an equilibration step where the IPG strips containing the proteins separated by pH are incubated in equilibration solution containing DTT then equilibration solution containing iodoacetamide for 15 minutes each with washing steps in between and after. This allows for reduction and alkylation of proteins. The strips are then placed into the 8cm x 8cm precast NuPAGE 4-12% Bis-Tris SDS-PAGE gels for the second dimension. The second dimension further separates the proteins by mass. In some of the gels, an albumin standard was added into the ladder well to act as an internal standard to allow for absolute quantification of the protein spots later on. SDS-MOPS buffer is used as a running buffer starting with a 10 minute ramp-up at 100V then the voltage is increased to 200V until the dye front reaches the bottom of the 2D gel.

After the second dimension step, the gel is fixed in a solution of methanol and acetic acid then stained in SYPRO Ruby Fluorescent stain for 48 hours. The gel is then washed and scanned with the Typhoon Scanner. Gel spots are manually circled, saved and measured using ImageJ. ImageJ can measure the integrated density of a spot, which is then used to create a relative quantification by normalizing all the values to a reference spot. The reference spot was chosen because it appeared on all the gels with approximately the same spot density. Circling of spots and relative quantification was done by two researchers independently to attempt to understand the inter-observer bias inherent in this manual quantification method.

MASS SPECTROMETRY:

In an initial, exploratory, biomarker screening experiment, ten plasma samples were analyzed using mass spectrometry with the aim of seeing if differences in protein concentration were observed. Two of the ten samples were pools of five individual samples, one high growth pool and one low growth pool. The other eight samples consisted of 4 high and 4 low growth samples, different from the samples included in the pool. The experiments were done using Tandem Mass Tagging (TMT) mass spectrometry on the LC/MS/MS Q-Exactive at SickKids SPARC Biocenter. ten samples were isotopically labeled and run simultaneously through the LC/MS/MS. Each of the samples were depleted for IgG and albumin using antibody-based depletion columns, alkylated, reduced and de-salted. 6ug of each sample were digested and labeled with the isobaric TMT reagent. All 10 samples were then combined and run in one mass spectrometry run. The proteins within each sample are identified and relative abundances are quantitated. Comparing the proteins expressed and the abundances of these proteins in the samples

from different growth groups we aimed to identify proteins, which may be of interest as potential biomarkers for healthy growth in preterm infants.

SAMPLE SELECTION CRITERIA:

High and low growth groups of plasma samples are chosen based on the following criteria: High growth = 20-25 g/kg/day for the 6 days before and after sample collection. Low growth = 10-15 g/kg/day. Samples were excluded if the infant was SGA, LGA. The samples were also excluded if the infant was administered antibiotics or steroids 7 days before or after the sample was collected. Samples in the two groups are matched for gestational age at birth and time of collection. Samples are preferentially chosen for shorter lengths of stay and time between the sample and discharge of the infant. Since all the infants included in the growth groups are part of the "Individualized Fortification of Breast Milk Study", they are all fed breast milk, minimizing different nutrition regimes as a confounding factor to differential protein expression.

Growth rate was calculated using a linear regression over a period of +/- 6 days surrounding the date of sample collection. Growth (g/kg/day) $= \frac{m}{7days \cdot avgweight} \cdot \frac{1000g}{kg}$ where m is the slope of the linear regression. The growth rates were plotted for each infant to ensure that no outlying weights skewed the relationship. Growth curves following a non-linear regression also did not follow a polynomial regression and were eliminated. Size for gestational age was determined using the Health Canada/Canadian Perinatal Surveillance System charts for calculating gestational age from birth weight (in grams)(Michael S Kramer et al., 2015). There is a separate chart for males and females and the charts are based on information for singleton pregnancies based on completed weeks of gestation. These charts allowed me to classify the infants into a percentile. I

used the clinically acceptable definition of AGA between the 10^{th} and 90^{th} percentile, LGA>90^{th} percentile and SGA<10^{th} percentile(R. a Ehrenkranz, 2014).
Growth was later also calculated using an exponential and solver method and the methods were compared for precision. The exponential model was chosen as the superior model going forward with future experiments.

RESULTS:

GEL ELECTROPHORESIS REPEATABILITY:

In order to assess the usefulness of our gel electrophoresis as a method to screen differences between groups of neonates, we need to see the variation and repeatability in the gels. First we looked at the variation in relative quantification between researchers. To do this we used a Bland-Altman test. The Bland-Altman seen in Figure 1 in the appendix shows that the variation is concentrated between +/- 3 relative quantification units with no obvious outliers. After seeing this result, we made the assumption that the two independent quantifications were similar enough to average and use the one averaged quantification for the rest of the statistics.

Using the averaged data between the two quantifiers and including missing values as the lowest detectable limit (0.002), we ran a one-sided ANOVA and Tukey's Post Hoc test comparing the 5 replicate gels within each sample. A separate ANOVA and Tukey's was run for each of the 5 samples. Ideally, we wanted to see that all 5 replicates within each sample were not significantly different from one another. In reality, there were differences between replicate 1 and 4 and 1 and 5 in sample AU (an adult sample) and between replicate 7 and 1 in sample 91 (a neonate sample). There are outliers, which

when removed, eliminated any significant differences. Further statistics are needed to assess these points as true outliers. The five replicates in one representative sample can be seen in Figure 2.

If we accept that the replicates are similar enough to one another, we can use the averages of each spot between the 5 replicate gels for an analysis between samples. Using an ANOVA and Tukey's Post Hoc Test to compare each of the 5 samples with one another we expected to see the two adult samples having a non-significant difference as well as the two neonate samples. We also expected the cord-blood to be not significantly different compared to the neonate samples and significantly different from the adult sample, according to changes reported by Ignjatovic in 2011(Ignjatovic et al., 2011). In reality, 50% of the results were as expected and the other 50% were not. The statistics were all run in GraphPad Prism 6.

In order to see the variation in each of the samples, the coefficient of variation was calculated for each of the 5 samples. Any time there was a coefficient of variation greater than 70%, the values were eliminated. The average coefficient of variation for each sample (after normalization to compare all spots within the sample) was 42% with the outliers still remaining. When the outliers were removed the CV decreased to 33%. 2D-gels are reported to have an average intra-lab coefficient of variation of 20%. However, for depleted plasma the intra-lab coefficient of variation is reported to range from 18-69%.

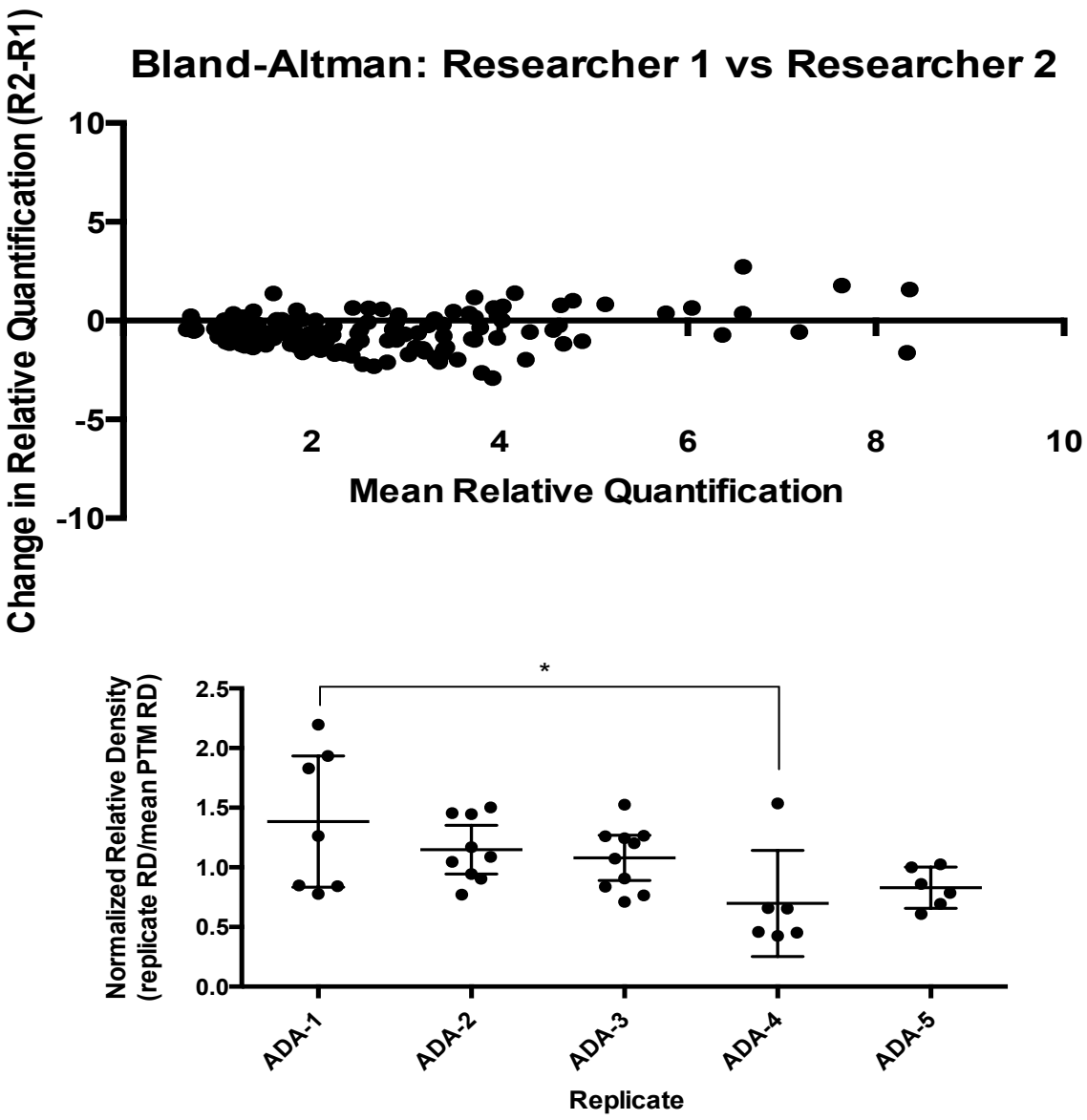

Figure 1: Intra-*ADA* sample variation (mean ± 95% CI) of normalized FIBG relative density quantification using ImageJ. ANOVA followed by Tukey *post-hoc* reveals *p<0.05, significant differences between *ADA*-1 and *ADA*-4 quantification of replicates.

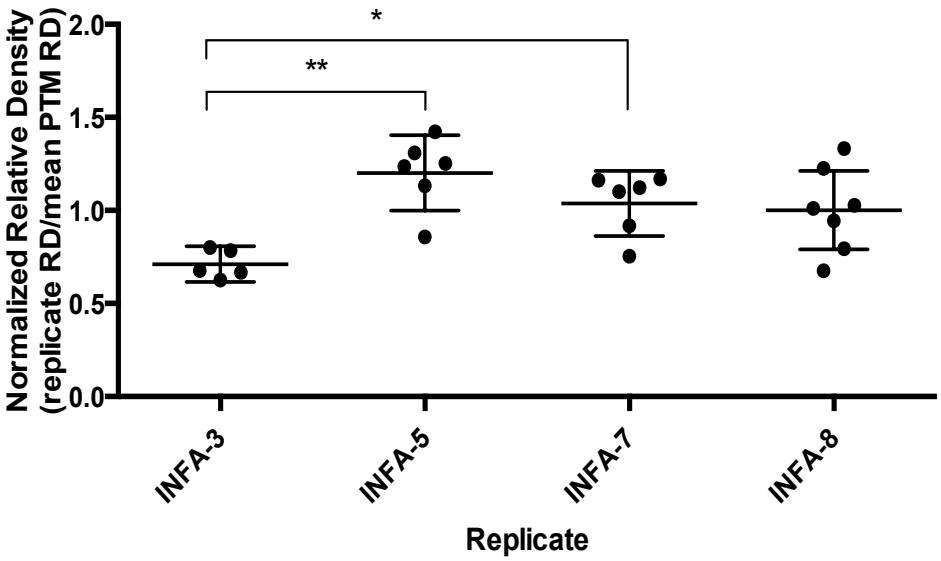

Figure 2: Intra-*INFA* sample variation (mean ± 95% CI) of normalized FIBG relative density quantification using ImageJ. ANOVA followed by Tukey *post-hoc* reveals: *p<0.05, significant difference between *INFA*-3 and *INFA*-7; and **p<0.01, significant difference between *INFA*-3 and *INFA*-5 quantification of replicates.

MASS SPECTROMETRY:

Over 200 proteins were identified with a high probability using the TMT mass spectrometry pilot experiment. Consistent, significant differences were not observed between growth groups.