Characteristics Associated with Neonatal Carnitine Levels: A Systematic Review & Clinical Database Analysis

Sarah C. Sutherland

Thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the requirements for the M. Sc. Degree in Epidemiology

Department of Epidemiology and Community Medicine

Faculty of Medicine

University of Ottawa

Abstract

Newborn screening programs measure analyte levels in neonatal blood spots to identify individuals at high risk of disease. Carnitine and acylcarnitine levels are primary markers used in the detection of fatty acid oxidation disorders. These analytes may be influenced by certain pre/perinatal or newborn screening related factors. The primary objective of this study was to explore the association between these characteristics and levels of blood carnitines and acylcarnitines in the newborn population. The study was composed of two parts: a systematic review and a clinical database analysis of existing newborn screening data. The systematic review results suggested considerable variability across studies in the presence and directionality of associations between analyte levels and birth weight, gestational age, age at time of blood spot collection, type of sample, and storage time. Sex was not significantly associated with carnitine or acylcarnitine levels in neonatal blood. We identified a need to more fully investigate a potential interaction between gestational age and birth weight in regard to analyte levels. The secondary data analyses indicated a statistically significant relationship between analyte levels and all perinatal / infant and newborn screening related factors of interest, but effect sizes were generally small. The interaction between gestational age and birth weight was significant in all models; when further explored through graphical analysis with conditional means, extremely premature neonates stood out as having distinct analyte patterns in relation to birth weight. Variation in the ratio of total acylcarnitine to free carnitine was better accounted for by the perinatal and newborn factors than was variation in any individual carnitine or acylcarnitine, indicating that proportions of carnitine and acylcarnitines may be more important in understanding an individual's metabolic functioning than individual analyte levels. A low proportion of

variation was explained in all multivariate models, supporting the use of universal algorithms in newborn screening and suggesting the need for further large scale empirical research targeted at previously unaccounted for perinatal factors such as birth stress.

Acknowledgements

I would like to take this opportunity to extend my sincere appreciation to all those individuals who have provided me with their continuous support and guidance throughout this process.

First and foremost, I would like to thank Dr. Beth Potter without whom this thesis would not have been possible. Her insight, guidance, and infinite patience over the last few years have been invaluable and I am incredibly lucky to have her as my supervisor. I would also like to thank Dr. Kumanan Wilson for his mentorship, direction, and perspective, as well as his constant efforts to keep this project on track.

Next, I would like to thank Dr. Julian Little for lending his extensive experience and knowledge of epidemiology and physiology to the planning and scientific guidance of this project. Likewise, Dr. Pranesh Chakraborty and Dr. Michael Geraghty have my deepest appreciation for their time and expertise, and for helping me to understand the intricacies of fatty acid oxidation and metabolic disease in neonates. In addition, I owe a great debt of gratitude to Steven Hawken who has been wonderful and incredibly patient in cultivating my understanding of statistical analyses and SAS programming.

I am also extremely grateful to Jennifer Milburn and the staff of Newborn Screening Ontario who have always been quick to answer my questions and provide me with whatever support is necessary. Working with these exemplary individuals has proven both mentally stimulating and highly enjoyable. Similarly, I would like to thank the University of Ottawa

and the staff of the Department of Epidemiology and Community Medicine for giving me the opportunity and tools to study in such an interesting field, as well as my classmates for making this an unforgettable and highly entertaining experience.

Lastly, I would like to extend my heartfelt thanks to the friends and family who have kept me sane throughout this process. Special thanks go out to Ania and Jelena for always being there to bounce ideas off of, my forever girls Trisha, Erin, Michelle, and Robyn, Allie and Mary-Elizabeth, and Lauren who has always been there for me despite the distance. To my brilliant family, I can never thank you enough for providing such an amazing foundation and environment in which to explore, learn, and grow as a person. Thank you so much Mom, Dad, Amanda, and Matthew, as well as the lovely non-human members of our family, for your endless encouragement, support, love, and inspiration.

Table of Contents

List of Figures

List of Tables

1.0 Introduction

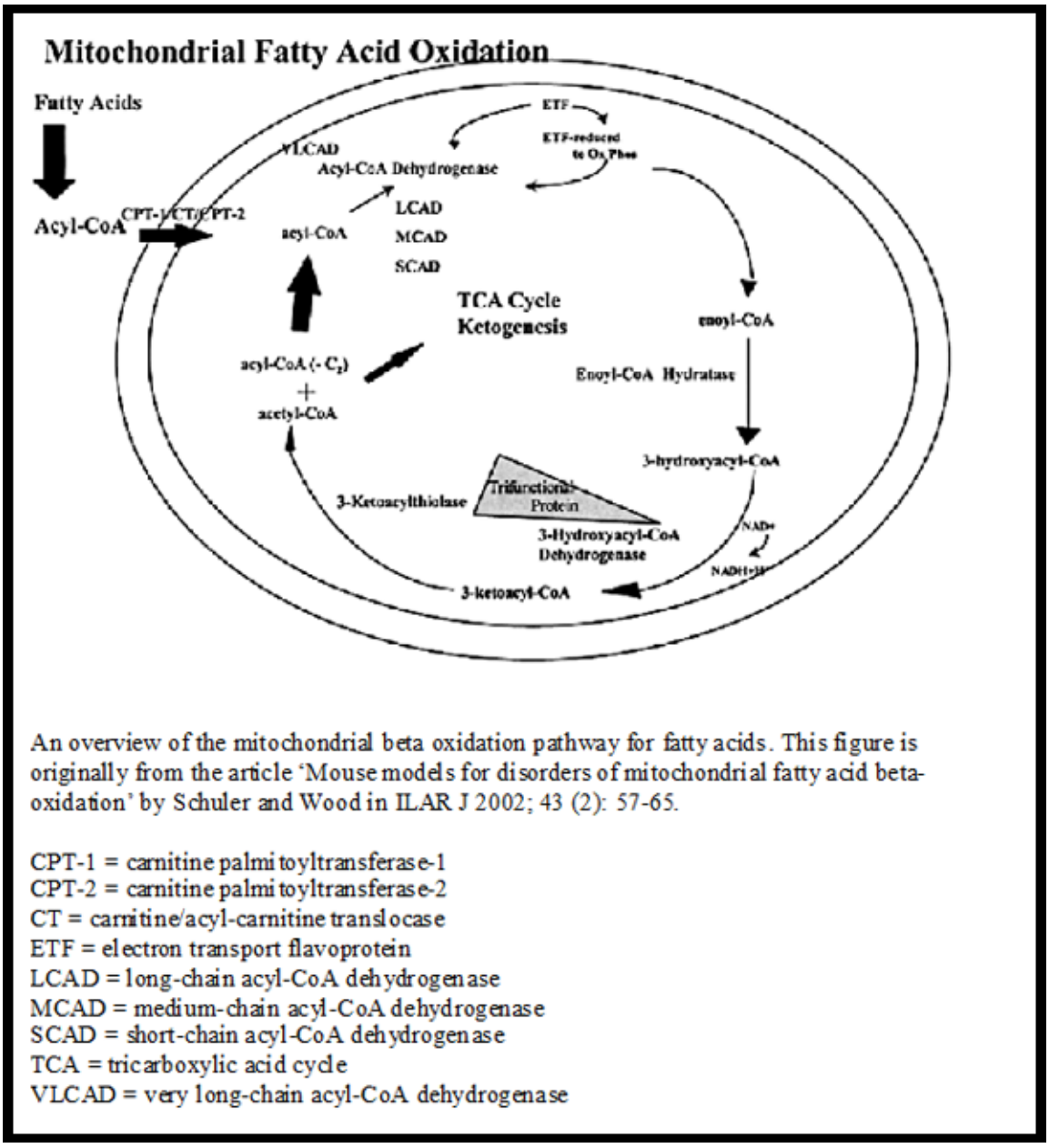
Population based newborn screening programs rely on analyte levels to identify neonates who are at high risk of disease. These analytes include carnitine and acylcarnitines, which are related largely to pathways in fatty acid metabolism. Certain pre/perinatal factors may influence carnitine and acylcarnitine levels in neonatal blood spots. It is important to understand these associations in order to better comprehend and interpret neonatal screening results.

1.1 Fatty Acid Metabolism

During periods of fasting or prolonged exercise, human energy needs are met by the breakdown of fatty acids through the carnitine cycle and mitochondrial β-oxidation, primarily in liver and muscle cells. In the liver, resulting acetyl-CoA is converted into ketone bodies which undergo further oxidation and are used for energy in tissues such as the brain. In cardiac and skeletal muscle, acetyl-CoA is oxidized completely to create energy via the tricarboxylic acid cycle [1]. This metabolic pathway is particularly important in the early neonatal period, when infants are typically in a catabolic state.

Once inside liver or muscle cells, the transport of fatty acids into the mitochondria and subsequent oxidation to create acetyl-CoA involves two processes: the **carnitine cycle** and the **beta oxidation spiral** (Figure 1). The first of these, the carnitine cycle, applies only to long-chain fatty acids. The carnitine cycle functions to transport long chain fatty acids (as fatty acid CoA) into the mitochondria and to transform them into mitochondrial fatty acyl-CoA. Medium chain fatty acids do not undergo the carnitine cycle as they are able to pass

Figure 1 – Fatty Acid Oxidation Pathway [2]



An overview of the mitochondrial beta oxidation pathway for fatty acids. This figure is originally from the article 'Mouse models for disorders of mitochondrial fatty acid beta-oxidation' by Schuler and Wood in ILAR J 2002; 43 (2): 57-65.

CPT-1 = carnitine palmitoyltransferase-1
CPT-2 = carnitine palmitoyltransferase-2
CT = carnitine/acyl-carnitine translocase
ETF = electron transport flavoprotein
LCAD = long-chain acyl-CoA dehydrogenase
MCAD = medium-chain acyl-CoA dehydrogenase
SCAD = short-chain acyl-CoA dehydrogenase
TCA = tricarboxylic acid cycle
VLCAD = very long-chain acyl-CoA dehydrogenase

directly into the mitochondria where they are activated to form acyl-CoA by acyl-CoA synthetase [1]. The carnitine cycle is composed of four steps:

1) Carnitine is transported across the plasma membrane into the cell cytosol via carnitine uptake proteins.

2) The fatty acid CoA's acyl group is transferred to carnitine via carnitine palmitoyl transferase I in the outer mitochondrial membrane, yielding acylcarnitine. Released CoA rejoins the cytosolic pool of CoA.

3) The acylcarnitine is then transferred into the mitochondrial matrix by the carnitine carrier protein carnitine/acylcarnitine translocase, which is embedded in the inner mitochondrial membrane.

4) Within the matrix of the mitochondria, carnitine palmitoyl transferase II facilitates the transfer of the acylcarnitine's acyl group to CoA resulting again in fatty acyl-CoA, now inside the mitochondria [1].

Within the mitochondria, fatty acyl-CoA compounds originating from medium and long chain fatty acids cycle through the beta oxidation spiral multiple times, creating as many two-carbon acetyl-CoA units as possible given the original chain length (Figure 1). Of particular importance is the mitochondrial trifunctional protein which houses three of the four enzymes used for the breakdown of long chain fatty acids in the beta oxidation spiral [1]. The steps involved in beta oxidation include:

1) Dehydrogenation of fatty acyl-CoA by acyl-CoA dehydrogenases creating 2-trans-enoyl-CoA. During this process FAD is reduced to FADH2 which then goes on to be reoxidized and enter the electron transport chain. The dehydrogenase used is dependent upon the chain length of the fatty acid (short [4-6 carbons or C4-C6], medium [C4-C12], long [C12-C18], or very long [C14-C20] chain acyl-CoA dehydrogenase).

2) Hydration of 2-trans-enoyl-CoA by 2-enoyl-CoA hydratase to create L-3-hydroxy-CoA.

3) Dehydrogenation of L-3-hydroxy-CoA by L-3-hydroxyacyl-CoA dehydrogenase resulting in beta-ketoacyl-CoA. NAD is also reduced to NADH+H+.

4) Thiolytic cleavage of the alpha-beta bond catalyzed by 3-ketoacyl-CoA thiolase and CoA to form a fatty acyl-CoA two carbons smaller than the original molecule that entered the beta oxidation spiral, as well as a acetyl-CoA. The acetyl-CoA units are then oxidized directly to produce energy or are converted into ketone bodies in the liver for further oxidation [1].

1.2 Inborn Errors of Metabolism

In inborn errors of fatty acid oxidation, individuals cannot complete the carnitine cycle or beta oxidation spiral, resulting in an accumulation in the blood of intermediate components, which may include acylcarnitines (fatty-acid carnitine esters). The specific acylcarnitines that accumulate depend upon the step in the metabolic pathway where the defect occurs [3].

Several of the more common fatty acid oxidation disorders (FAODs) include primary and secondary carnitine deficiency, short chain acyl-coenzyme A dehydrogenase deficiency (SCADD), medium-chain acyl-coenzyme A dehydrogenase deficiency (MCADD), long chain 3-hydroxyacyl-coenzyme A dehydrogenase deficiency (LCHADD), very long chain acyl-coenzyme A dehydrogenase deficiency (VLCADD), multiple acyl-coenzyme A dehydrogenase deficiency (MADD), carnitine palmitoyltransferase I or II (CPT-I; CPT-II) deficiency, and mitochondrial trifunctional protein deficiency (MTPD). In each case, the excess acylcarnitines in the blood correspond to the part of the metabolic process in which

the specific enzyme or transporter protein is used [1]. Eight analytes are the focus of this study (Table 1). All carnitine and acylcarnitine concentrations are measured in μM (micromolar) which is equivalent to micromoles / litre. The analytes that are used as the dependent variables throughout this study are: free carnitine (C0 or FC), octanoylcarnitine (C8), total carnitine (TC), total acylcarnitine (TA), short chain acylcarnitine (SCA), medium chain acylcarnitine (MCA), long chain acylcarnitine (LCA), and the ratio of total acylcarnitine to free carnitine.

Free carnitine (C0 or FC) is necessary for the transportation of fatty acids into the mitochondria in lipid metabolism. Free carnitine binds long chain fatty acids to create long chain acylcarnitine which is then able to move from the cytosol of a cell into the mitochondrial matrix where it can be broken down by β-oxidation [1]. It is an important marker in neonatal screening both independently and in terms of the ratio of free carnitine to acylcarnitines. Free carnitine concentration is of particular importance in screening for conditions including primary carnitine deficiency and acquired carnitine deficiency [4].

Octanoylcarnitine (C8) is a medium-chain acylcarnitine. It is used as a primary marker to identify infants at risk of medium chain acyl-CoA dehydrogenase deficiency (MCADD). In individuals with MCADD, deficiencies in the activity of medium chain acyl-CoA dehydrogenase lead to increased levels of medium chain acylcarnitines including C8. Screening for abnormally high C8 levels is a primary method of detecting MCADD, as it identifies individuals at high risk of the disorder regardless of variation in the specific underlying genetic mutation or symptoms [5].

Table 1 – Primary & Derived Analytes

Dependent Variable	Definition	Additional Information
Free carnitine (C0)	Unbound carnitine	• Necessary for transport of fatty acids in lipid metabolism (leading to β-oxidation) • Important marker in neonatal screening both independently and in combination with other acylcarnitines • Useful in detection of disorders including primary carnitine deficiency and acquired carnitine deficiency
Octanoylcarnitine (C8)	A medium chain acylcarnitine with 8 carbon saturated fatty acids linked covalently to carnitine (C8)	• Primary marker used to identify infants at risk of medium chain acyl-CoA dehydrogenase deficiency (MCADD)
Total Carnitine	Combined concentration of free carnitine & total acylcarnitine	• Total carnitine provides a baseline for the concentration of all forms of carnitine present in a whole blood sample • Useful in detection of primary carnitine deficiency
Total Acylcarnitine	Combined concentration of all short, medium, and long chain acylcarnitines	• Total acylcarnitine provides a baseline for the concentration of all esterified derivatives of carnitine in a whole blood sample • Useful in detection of disorders including primary carnitine deficiency and multiple acyl-coA dehydrogenase deficiency
Short Chain Acylcarnitine	Combined concentration of all acylcarnitines of chain lengths C2 - C5	• Short chain acylcarnitine provides a baseline for the total summed concentration of all acylcarnitines with a chain length of between 2 and 5 carbon atoms • Includes markers useful in detection of disorders including ketosis, primary or secondary carnitine deficiency, and short chain acyl-CoA dehydrogenase deficiency (SCADD)
Medium Chain Acylcarnitine	Combined concentration of all acylcarnitines of chain lengths C6 – C12	• Medium chain acylcarnitine provides a baseline for the total summed concentration of all acylcarnitines with a chain length of between 6 and 12 carbon atoms • Includes markers useful in detection of disorders including MCADD
Long Chain Acylcarnitine	Combined concentration of all acylcarnitines of chain lengths C14 – C18	• Long chain acylcarnitine provides a baseline for the total summed concentration of all acylcarnitines with a chain length of between 14 and 18 carbon atoms • Includes markers useful in detection of disorders including long chain acyl-CoA dehydrogenase deficiency (LCHADD), very-long chain acyl-CoA dehydrogenase deficiency (VLCADD), carnitine palmitoyltransferase I & II deficiency (CPT-I, CPT-II), and mitochondrial trifunctional protein deficiency (MTPD).
Ratio Total Acylcarnitine / Free Carnitine	Ratio of total acylcarnitine concentration to free carnitine concentration	• Ratios provide insight into the dynamic process of fatty acid metabolism by comparing multiple analyte markers • Useful in detection of disorders including ketosis, carnitine palmitoyltransferase II deficiency (CPT-II), and MCADD

Short, medium, and long chain acylcarnitines each represent a different step in the metabolism of long chain fatty acids. Short chain acylcarnitines refer to acylcarnitines of chain lengths C2 - C5. Abnormal levels of these analytes may indicate a range of disorders characterized by ketosis, primary or secondary carnitine deficiency, as well as other FAODs [6]. Medium chain acylcarnitines have a chain length of C6 – C12. Abnormal concentrations may indicate disorders including MCADD [6]. Long chain acylcarnitines have a chain length of C14 – C18. Abnormal levels may indicate very-long-chain acyl-CoA dehydrogenase deficiency (VLCADD), long-chain acyl-CoA dehydrogenase deficiency (LCHADD), or carnitine palmitoyltransferase II deficiency (CPT-II) among other disorders [7]. For the purpose of this study, total short, medium, and long chain acylcarnitine values were determined through the addition of concentrations of all individual acylcarnitines included in the specified category.

Total acylcarnitine level is determined through the addition of levels of all short, medium, and long chain acylcarnitines. This summed total of carnitine that has undergone esterification is a useful marker of metabolic status both alone and in comparison with other carnitine and acylcarnitine concentrations. Increases in all acylcarnitine concentrations may indicate multiple acyl-coA dehydrogenase deficiency among other disorders [6].

Total carnitine level is determined through the addition of total acylcarnitine and free carnitine levels. Total carnitine concentration is useful both alone and as a baseline for determining what proportion of total blood carnitine has been esterified and in what form (short, medium, and long chain acylcarnitines). Abnormal total carnitine levels may indicate disorders such as primary carnitine deficiency [4].

The ratio of acylcarnitine to free carnitine level is an important marker in fatty acid metabolism and neonatal screening because it provides insight into the dynamic process of fatty acid metabolism and the catabolic state by comparing multiple analyte markers. Abnormal ratios may indicate disorders including CPT-II or MCADD among others. The ratio of acylcarnitine to free carnitine is also used as a factor of interest in the diagnosis of other disorders including primary carnitine deficiency [7].

1.3 Newborn Screening for FAODs and the Role of Acylcarnitines

Newborn screening is a process in which all neonates in a specified population are screened for a number of rare but treatable diseases. Population based newborn screening programs have been in place for decades in most developed countries, including Canada. Their purpose is to identify infants at risk for diseases that may not be apparent at birth for which early diagnosis and treatment are likely to be beneficial. In Canada, newborn screening programs are organized at the provincial level [8].

Within the first few days of life and typically while mothers and newborns are still in hospital, a heel prick is used to deliver a small whole blood sample onto newborn screening-specific filter paper. To identify infants at a high risk for one of the screened diseases, these blood spot samples are checked for integrity and analyzed for the presence of disease-specific biomarkers, using enzyme activity testing, immunoassays, high performance liquid chromatography, and tandem mass spectrometry. Notably, the technology of tandem mass spectrometry (MS/MS) has been incorporated into many newborn screening programs in the

past few years. MS/MS can be used to detect newborns at risk for a range of metabolic diseases (>20) simultaneously, based on concentrations of various amino acids and acylcarnitines. This generally entails setting dichotomous cut-points for the analytes (in the case of newborn screening, these cut-points are typically within the highest 99.8th percentile) [9]. If a patient screens positive for any disorder, confirmatory testing is done in order to verify the diagnosis [8].

While the panel of disorders tested in newborn screening varies across programs both within and among countries, FAODs have recently been added as targets of many such programs around the world. FAODs are identified by characteristic acylcarnitine profiles generated using MS/MS [10]. Early detection is important for this group of diseases because a combination of increased monitoring and changes to lifestyle and diet can lead to reduced morbidity and mortality associated with FAODs [11].

Understanding sources of variation in carnitine and acylcarnitine levels in the newborn population is of particular importance because of the impact variations in these analytes may have on screening test results and associated diagnoses of specific conditions. Screening programs use algorithms that combine acylcarnitine markers and set cut-offs to yield high sensitivity for detecting FAODs while minimizing false positives [9]. High sensitivity is important because it increases the probability that an individual who is affected by a disorder will screen positive (i.e., with high sensitivity there will be fewer missed cases) [12]. Prevention of false positives, or high test specificity, is also important. False positives increase the number of tests that need to be repeated, creating an additional burden on the

health care system. Affected families are also subject to the unnecessary psychological impact of a false positive result [13].

Research has shown that newborn screening false positive rates are considerably higher in certain population subgroups including extremely premature, low birth weight, or ill neonates [14,15]. This is due in part to both biological immaturity and increased likelihood of having received medical intervention. The exact nature of these relationships, and the specific roles that various acylcarnitines play, is unclear [14,15]. In several metabolic disorders, such as the FAODs MTPD and LCHADD, the cut-off for a screen positive result is quite close to the reference interval for the healthy population. As a result, minor differences may seriously impact screening results, and possibly the resulting diagnosis of disease [16].

1.4 Rationale for Studying Sources of Variation in Metabolite Levels

From the above discussion it follows that studying the relationships between perinatal and infant characteristics (e.g., sex, gestational age, birth weight) and acylcarnitine levels in newborns is likely to be useful to newborn screening programs, for adjusting or tailoring screening algorithms and cut-offs. Beyond screening for rare metabolic diseases, the complexity of mitochondrial energy metabolism, with multiple interacting pathways, suggests that such research may also provide insights that inform the science of metabolism and have implications for the care of infants who do not have single gene disorders. For example, in 2004 a study found that infants with octanoylcarnitine (C8) levels in the high-normal range were more likely than those in the low-normal range to be heterozygous carriers for mutations in the MCAD gene or to have experienced a high degree of metabolic

stress due to prematurity, low birth weight, or other pre/perinatal factors [17]. Indeed, metabolic profiling, which includes the analysis of metabolite levels in blood and the study of their association with health outcomes, represents a new epidemiological approach to identifying the contributions of genetics and environmental factors to health and disease in general [18].

Thus, there is clear value to exploring and documenting important predictors of variation in infant metabolite levels. Several pre/perinatal characteristics (for example, sex, gestational age, feeding status) and newborn screening related factors (for example, blood sampling methods, analytic technique) have been reported to affect observed concentrations of carnitine and its esters [3,6,17-20]. However, there is inconsistency in the literature regarding which factors influence levels and the degree to which these levels are affected.

For example, several studies have found no association between sex and carnitine or acylcarnitine levels [10], while others have observed minor differences [21]. Still others have found significantly higher levels of free and total carnitine in male infants as compared to females [22,23]. Evidence of a relationship between birth weight and metabolite levels are even more diverse, with studies reporting a range of effects from inverse [24] to positive correlations [10]. Studies on gestational age have likewise shown mixed results [10,23,24]. Furthermore, little information is available regarding the relationship between carnitine and acylcarnitine levels and variables such as feeding type, socioeconomic status, or newborn screening related factors including infant age at the time of blood sample collection, time between sample collection and analysis, and the nature of the sample.

1.5 Role of Newborn Screening Ontario

Prior to April of 2006, newborn screening in Ontario was conducted by the public health lab in Toronto and consisted of testing for two disorders: PKU (phenylketonuria) and congenital hypothyroidism. In 2006, funded by the Ontario Ministry of Health and Long-term Care, the Children's Hospital of Eastern Ontario opened Newborn Screening Ontario (NSO). NSO now performs screening for the entire province at this Ottawa facility. Since re-locating to Ottawa, Ontario's newborn screening program has also undergone a major expansion, implementing a new electronic database to house both demographic and medical information and growing to include over twenty additional conditions (including FAODs) beyond the two traditionally included. This presents a unique opportunity for exploring the relationship between environmental factors and metabolite levels in the infant population. By January 2010 the expanded program at NSO had collected health information from over 611,000 samples, allowing for large scale exploration of factors associated with metabolic variation at the population level [25].

1.6 Significance

Through this thesis project, I conducted a systematic review and a clinical database analysis of the Newborn Screening Ontario database to consolidate existing evidence and to form a more comprehensive picture of the potential influence of pre/perinatal factors and newborn screening factors on observed carnitine and acylcarnitine levels in newborn infants. This research has the potential to contribute to the understanding of fatty acid metabolism and related metabolic pathways, which may in turn aid in the improvement of diagnosis and treatment of metabolic conditions.

2.0 Goal and Objectives

2.1 Overall Goal

The primary goal of this study was to explore the association of pre/perinatal factors and newborn screening factors with levels/ratios of blood carnitines and acylcarnitines in the newborn population. This project was composed of two main components: a systematic review and a clinical database analysis of an existing newborn screening dataset.

2.2 Systematic Review Objectives

The systematic review component investigated carnitine and acylcarnitine levels/ratios and their relationship with pre/perinatal and newborn screening related factors. Pre/perinatal factors of particular interest included: socioeconomic status, birth weight, gestational age, and sex. Newborn screening related factors of interest included: age of child at time of blood sample collection, nature of sample, time between sample collection and analysis, analysis technique, and conditions of storage of sample. Results of the systematic review were used in part to determine which variables to include in the clinical database analysis.

2.3 Clinical Database Analysis Objectives

The clinical database analysis component investigated carnitine and acylcarnitine levels/ratios and their relationship with pre/perinatal and newborn screening related factors in the Ontario newborn population. Pre/perinatal factors of interest included socioeconomic status, birth weight, gestational age, feeding status, and sex. Newborn screening related

factors included age of child at time of blood sample collection and time between sample collection and analysis.

3.0 Systematic Review of Sources of Population Variation in Carnitine & Acylcarnitine Levels in Neonates

3.1 Methods

3.1.1 Literature Search

We conducted a systematic search in the electronic bibliographic databases OVID Medline, Embase, Cochrane, and Scopus for articles published before January 1, 2011. Search strategies for each database included MESH terms and free text terms and were created using predetermined inclusion and exclusion criteria with the aid of a specialist librarian (Medline search strategy, Appendix A - strategies for other databases were adapted from the Medline strategy). We also searched the reference lists of included articles to identify additional relevant studies.

3.1.2 Inclusion Criteria & Screening Process

In the broad screen, two independent reviewers (SCS, BKP) screened citation titles and abstracts using the predetermined inclusion and exclusion criteria to exclude reports that clearly did not meet the inclusion criteria. Articles deemed potentially relevant by at least one reviewer proceeded to the focused screening level where full articles were considered (again independently by SCS, BKP) to determine final eligibility. Disagreements were resolved by discussion. Inter-reviewer reliability at the focused screening level was assessed using the Kappa score.

Articles included in the systematic review had to present primary research reporting on carnitine and/or acylcarnitine levels (from our list of 8 analytes, see Table 1 in Chapter 1)

or their ratios in neonatal (≤1 month of age) blood samples and their association with one or more specific perinatal factors. These were divided into two groups: factors originating from the infant (birth weight, gestational age, and/or infant sex) and factors related to newborn screening (age at bloodspot collection, nature of sample, and/or storage of sample). We excluded articles that reported these metabolites solely among children at high risk of rare metabolic diseases, since our aim was to gain insight into sources of population variation in carnitine and acylcarnitine levels.

3.1.3 Data Abstraction & Synthesis of Findings

Articles that passed the focused screen underwent data abstraction using standardized forms developed for this project (Appendix B). Information on study characteristics, design, participants, outcome measures, analyses, and results was included. Data abstraction for each article was performed by one primary reviewer (SCS) and verified by a second reviewer (BKP). Discrepancies were addressed through discussion.

No standard tool exists for conducting risk of bias assessment of observational studies. We considered the potential value of a variety of tools for this review but none fully met its needs [21-24]. We therefore drew on the domains from these previous tools and considered our study objectives to identify two study characteristics that we believed could be related to results: 1) degree to which the study represents natural carnitine variation independent of intervention in an unspecialized population; and 2) centrality of pre/perinatal or newborn screening factors to the purpose of the study. We incorporated these two characteristics into our data abstraction process.

A quantitative synthesis of the literature on each perinatal factor of interest was not possible due to the diversity of outcomes considered and methods of investigation of the studies. Instead, a narrative synthesis of the evidence focused on factors associated with carnitine and acylcarnitine levels among infants. In studies where significant differences were observed, effect sizes were expressed as the difference in means wherever possible.

3.2 Results

Using the predetermined inclusion and exclusion criteria, 1502 articles were examined at the broad screening level (Figure 2 – prepared according to PRISMA guidelines). Of these, 84 articles deemed potentially relevant by at least one reviewer advanced to the focused screening level. High levels of inter-reviewer reliability were noted at the focused screen. The two reviewers were in complete agreement on allocation status of all 84 articles. Disagreement over the primary reason for exclusion for 3 articles was resolved via discussion (kappa score of 0.85).

3.2.1 Perinatal & Infant Characteristics

Ten articles were deemed eligible for inclusion in the review of pre/perinatal factors intrinsic to the neonate (birth weight, gestational age, sex). Articles included in the review were published from 1980 to 2010 (Table 2). Three of the ten articles originated in Germany, two in the USA, and one each in Japan, Italy, Belgium, Australia, and both Australia and the UK. The five most recently published studies used tandem mass spectrometry to measure carnitine and acylcarnitine levels, while radioisotopic assays were used in the other five. Seven studies used exclusively hospital based data, two used newborn screening based data,

and one included both. Four studies were cross-sectional, while six used a longitudinal study design. Sample sizes were considered small (<50) in five studies, medium (50 - 2000) in three studies, and large (>2000) in two studies.

With respect to the predetermined study characteristics that we believed may be associated with study findings, in 6 of 10 studies, the sample population was either somewhat or very representative of the general population (Table 3) [3,25,27-30]. The other 4 reflected study samples comprised of high proportions of preterm or ill/hospitalized neonates [31-34]. Pre/perinatal factors of interest were central to 7 of 10 studies [3,25,27-31]. In the other 3, data on birth weight, gestational age, and/or sex were presented but were not the central purpose of the study [32-34].

Figure 2 – Systematic Review Flow Diagram [26]

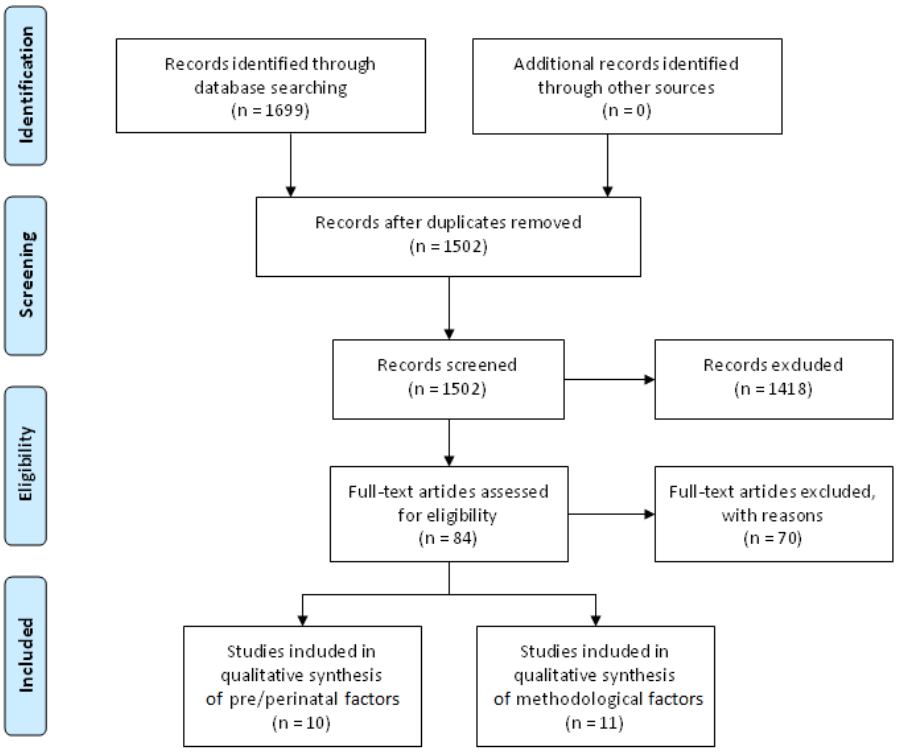


Table 2 – Characteristics of Studies Measuring Effects of Perinatal & Infant Factors on Carnitine & Acylcarnitine Levels

Citation	Study Design	Sample Size (neonates)	Assay & Sample Type	Independent Variables	Dependent Variables
Khalid JM, et al. [29] 2010 Australia & UK	Newborn screening based Cross sectional	227098	Tandem mass spectrometry Whole blood	Birth weight Gestational age Sex	C8
Cavedon CT, et al. [28] 2005 Belgium	Hospital & newborn screening based Cross sectional	117 (neonates) 67 (cord blood)	Tandem mass spectrometry Whole blood	Sex	Free carnitine Short chain acylcarnitines Medium chain acylcarnitines Long chain acylcarnitines
Meyburg J, et al. [25] 2002 Germany	Hospital based Longitudinal	120	Tandem mass spectrometry Whole blood	Gestational age	Free carnitine Total carnitine Total acylcarnitines Short chain acylcarnitines Medium chain acylcarnitines Long chain acylcarnitines
Meyburg J, et al. [3] 2001 Germany	Hospital based Longitudinal	95	Tandem mass spectrometry Whole blood	Birth weight Gestational age Sex	Free carnitine Total carnitine Total acylcarnitines Short chain acylcarnitines Medium chain acylcarnitines Long chain acylcarnitines
Wilcken B, et al. [30] 2001 Australia	Newborn screening based Cross sectional	149527	Tandem mass spectrometry Whole blood	Birth weight	Free carnitine
Christensen ML, et al. [31] 1989 USA	Hospital based Longitudinal	40	Radioisotopic assay Plasma	Gestational age Sex	Total carnitine
Yeh YY, et al. [34] 1985 USA	Hospital based Longitudinal	21	Radioisotopic assay Plasma	Gestational age	Free carnitine Total carnitine Total acylcarnitines
Takahashi M, et al. [33] 1983 Japan	Hospital based Longitudinal	29	Radioisotopic assay Plasma	Gestational age	Free carnitine Total carnitine
Schmidt-Sommerfeld E, et al. [32] 1983 Germany	Hospital based Longitudinal	14 (non-supplemented)	Radioisotopic assay Plasma	Gestational age	Total acylcarnitines Total carnitine
Battistella PA, et al. [27] 1980 Italy	Hospital based Cross sectional	44	Radioisotopic assay Plasma	Birth weight Gestational age Sex	Free carnitine

Table 3 - Quality Assessment Tool Results

Systematic Review	Total Studies #	Criteria Met	
		Study population representative of general population (unspecialized)	Pre/perinatal or newborn screening related factors are a central component of the study
Perinatal & Infant Characteristics	10	6	7
Newborn Screening Related Factors	11	10	11

3.2.1.1 Birth Weight

Four studies examined the association between birth weight and carnitine or acylcarnitine levels in neonatal blood (Table 2). In two studies, birth weight was positively correlated with either selected or all carnitine and acylcarnitine levels [3,27]. In one of these, free carnitine levels increased significantly with birth weight in premature neonates weighing <1.8 kg at birth but was not associated with birth weight in term neonates or those above 1.8 kg (Table 4) [27]. Meyburg et al. investigated birth weight in association with total carnitine, total acylcarnitine, and short, medium, and long chain acylcarnitine levels (free carnitine was also investigated) (Tables 4-7) [3]. A positive correlation was found between birth weight and all of these analytes among full term neonates with birth weight appropriate for gestational age (except for the medium chain acylcarnitines where no data were available). However, no significant association with the analytes existed when comparing full term neonates of appropriate birth weight with those small for gestational age [3]. A third study found an inverse association between free carnitine and birth weight although the statistical significance of this association was not reported (Table 4): free carnitine was higher in neonates weighing <2000g compared with those >2000g [30]. Finally, a fourth study found no clinically significant association between birth weight (>1.5 kg versus ≤1.5 kg) and a single medium-chain acylcarnitine, octanoylcarnitine (C8) (Table 7) [29].

3.2.1.2 Gestational Age

Eight studies examined the association between gestational age and carnitine or acylcarnitine levels in neonatal blood (Table 2). Results were mixed. With respect to free carnitine, 3 of 5 studies found at least one positive correlation with gestational age [3,33,34], while 2 of 5 identified at least one inverse association [25,27] (Table 4). For total carnitine, 3 of 6 studies found at least one positive association with gestational age [3,33,34] (Table 5), while Meyburg and colleagues (2002) found an inverse association. In terms of total acylcarnitines, 2 of 4 studies identified a positive correlation with gestational age at birth [3,32] (Table 6), while again Meyburg and colleagues (2002) found an inverse association, this time in one subgroup of very premature as compared to full term neonates. Only two studies, both from the same group of investigators [3,25], examined the association of gestational age with acylcarnitines across chain length (Table 7). In one of these studies, Meyburg and colleagues found either an inverse association with gestational age or no significant association for short and medium chain acylcarnitines [25], while in a second study these investigators found a positive association with gestational age in one short and one medium chain acylcarnitine [3]. Results for long chain acylcarnitines were mixed, with both studies finding positive associations in certain acylcarnitines and no significant association with gestational age in others. In one of these studies [25], certain long chain acylcarnitines were also inversely associated with gestational age. In a further study, Khalid and colleagues investigated only the medium chain acylcarnitine C8 and found no clinically meaningful difference (statistical significance not reported) when comparing infants born at ≥ 37 weeks gestational age with those born at < 37 weeks gestational age (Table 7) [29].

Table 4 – Associations between Perinatal & Infant Factors and Free Carnitine Levels

Factor	Study	Findings	Effect Size (% difference in means)	Significant Association ($p < 0.05$)
Birth Weight (BW)	Meyburg (2001) [3]	FC increased with BW in cord blood in full term AGA neonates FC increased with BW at postnatal day 5 in full term AGA neonates NS in cord blood in full term AGA vs. full term SGA neonates	Not presented Not presented	Yes
	Wilcken (2001) [30]	FC decreased normal BW vs. <2000g BW (significance not tested)	Not presented	Significance not reported
	Battistella (1980) [27]	FC increased with BW in premature neonates weighing 1.15 – 1.80 kg NS in full term neonates (any BW) NS in neonates weighing >1.80 kg (any GA) NS in premature neonates <1.80 kg vs. premature neonates >1.80 kg	Not presented	Yes
Gestational Age (GA)	Meyburg (2002) [25]	Compared with term neonates (37-41 weeks GA) on postnatal day 5: FC higher in 32-36 weeks GA FC higher in 28-31 weeks GA FC higher in 22- 27 weeks GA	 32 21 46	Yes
	Meyburg (2001) [3]	FC lower in preterm neonates vs. full term AGA in cord blood NS on postnatal day 5 in full term AGA neonates	Not presented	Yes
	Yeh (1985) [34]	FC lower in premature vs. full term neonates (prior to lipid infusion)	-56	Yes
	Takahashi (1983) [33]	Compared with full term neonates: FC lower in premature (during TPN) NS in premature (before TPN)	 -55 (day 5)	Yes
	Battistella (1980) [27]	Compared with full term neonates: FC higher in 30-33 weeks GA NS in 33-36 weeks GA NS in all premature combined (30-36 weeks GA) NS in preterm neonates (30-36 weeks GA)	 38	Yes
Sex	Cavedon (2005) [28]	NS		No
	Meyburg (2001) [3]	NS in full term AGA neonates		No
	Battistella (1980) [27]	NS (full sample & any BW)		No

Table 5 – Associations between Perinatal & Infant Factors and Total Carnitine Levels

Factor	Study	Findings	Effect Size (% difference in means)	Significant Association ($p < 0.05$)
Birth Weight (BW)	Meyburg (2001) [3]	TC increased with BW in cord blood in full term AGA neonates NS in cord blood in full term AGA vs. full term SGA neonates	Not presented	Yes
Gestational Age (GA)	Meyburg (2002) [25]	Compared with term neonates (37-41 weeks GA) on postnatal day 5: TC higher in 32-36 weeks GA NS in 28-31 weeks GA TC higher in 22- 27 weeks GA	 Not presented Not presented	Yes
	Meyburg (2001) [3]	TC lower in preterm vs. full term AGA neonates in cord blood NS on postnatal day 5 in full term AGA neonates	Not presented	Yes
	Christensen (1989) [31]	No significant difference was found in gestational age among neonates with TC $\leq$ 13 nmol/ml vs. those with TC > 13 nmol/ml		No
	Yeh (1985) [34]	TC lower in premature vs. full term neonates (prior to lipid infusion)	-50	Yes
	Schmidt-Sommerfeld (1983) [32]	NS in 29-33 vs. 34-37 weeks GA (during TPN)		No
	Takahashi (1983) [33]	Compared with full term neonates: TC lower in prem ature (during TPN) NS in premature (before TPN)	 -34 (day 5)	Yes
Sex	Christensen (1989) [31]	NS (in ratio males to females among higher & lower TC groups)		No

Table 6 – Associations between Perinatal & Infant Factors and Total Acylcarnitine Levels

Factor	Study	Findings	Effect Size (% difference in means)	Significant Association ($p < 0.05$)
Birth Weight (BW)	Meyburg (2001) [3]	TA increased with BW in cord blood in full term AGA neonates NS in cord blood in full term AGA vs. full term SGA neonates	Not presented	Yes
Gestational Age (GA)	Meyburg (2002) [25]	Compared with term neonates (37-41 weeks GA) on postnatal day 5: NS in 32-36 weeks GA NS in 28-31 weeks GA TA higher in 22- 27 weeks GA	 Not presented	Yes
	Meyburg (2001) [3]	TA lower in preterm neonates vs. full term AGA in cord blood NS on postnatal day 5 in full term AGA neonates	Not presented	Yes
	Yeh (1985) [34]	NS in premature vs. full term		No
	Schmidt-Sommerfeld (1983) [32]	TA lower in 29-33 weeks vs. 34-37 weeks GA (TPN after fat infusion)	-40	Yes
Sex	Meyburg (2001) [3]	NS in full term AGA neonates		No

Table 7 – Associations between Perinatal & Infant Factors and Chain Length Specific Acylcarnitine Levels

Factor	Study	Findings	Effect Size (% difference in means)	Significant Association ($p < 0.05$)
Short Chain Acylcarnitines (SCA), Medium Chain Acylcarnitines (MCA), Long Chain Acylcarnitines (LCA)				
Birth Weight (BW)	Khalid (2010) [29]	No clinically important difference in >1.5 kg BW vs. ≤1.5 kg BW in C8 (MCA)		Not reported
	Meyburg (2001) [3]	In full term AGA neonates (cord blood):		Yes
		SCA increased with BW in ≥4 of 5 [b]	Not presented	
		Total SCA increased with BW	Not presented	
		LCA increased with BW in 2 of 10 [b]	Not presented	
		Total LCA increased with BW	Not presented	
		Association between MCA & BW studied but results not reported		
		In full term AGA neonates (postnatal day 5):		
		SCA increased with BW in 2 of 5 [b]	Not presented	
		Total SCA increased with BW	Not presented	
		Association between MCA & BW studied but results not reported		
		Association between LCA & BW studied but results not reported		
		Compared with full term AGA neonates (cord blood):		
		SCA NS in full term SGA neonates		
		MCA NS in full term SGA neonates		
		LCA NS in full term SGA neonates		
Gestational Age (GA)	Khalid (2010) [29]	No clinically important difference in ≥ 37 weeks GA vs. < 37 weeks GA in C8 (MCA)		Not reported
	Meyburg (2002) [25]	Compared with term neonates (37-41 weeks GA) on postnatal day 5:		Yes
		SCA higher in 32-36 weeks GA in 2 of 4 [b]	28 to 46 [a]	
		SCA higher in 28-31 weeks GA in 1 of 4 [b]	42	
		SCA higher in 22-27 weeks GA in 3 of 4 [b]	80 to 143 [a]	
		Total SCA NS in any premature GA category		
		Compared with term neonates (37-41 weeks GA) on postnatal day 5:		
		MCA higher in 32-36 weeks GA in 3 of 6 [b]	27 to 100 [a]	
		MCA higher in 28-31 weeks GA in 1 of 6 [b]	67	
		MCA higher in 22-27 weeks GA in 5 of 6 [b]	53 to 100 [a]	
		Total MCA higher in 32-36 weeks GA	Not presented	
		Total MCA NS in 28-31 weeks GA		
		Total MCA higher in 22-27 weeks GA	Not presented	
		Compared with term neonates (37-41 weeks GA) on postnatal day 5:		
		LCA higher in 32-36 weeks GA in 1 of 10 [b]	71	
		LCA higher in 28-31 weeks GA in 2 of 10 [b]	32 to 40 [a]	
		LCA higher in 22-27 weeks GA in 6 of 10 [b]	14 to 194 [a]	
		LCA lower in 22-27 weeks GA in 1 of 10 [b]	-9	
		Total LCA NS in 32-36 weeks GA, 28-31 weeks GA		
		Total LCA higher in 22-27 weeks GA	Not presented	
	Meyburg (2001) [3]	Compared with full term AGA neonates (cord blood):		Yes
		SCA lower in preterm neonates in 1 of 5 [b]	Not presented	
		Total SCA NS in preterm neonates		
		MCA lower in preterm neonates in 1 of 6 [b]	Not presented	
		Total MCA NS in preterm neonates		
		LCA lower in preterm neonates in 7 of 10 [b]	Not presented	
		Total LCA lower in preterm neonates	Not presented	
Sex	Khalid (2010) [29]	No clinically important difference in C8 (MCA)		Not reported
	Cavedon (2005) [28]	Sex related differences observed (not explained) in C14, C16, and C18:1 (LCA) NS in 9 of 9 SCA NS in 7 of 7 MCA NS in 7 of 10 LCA	Not presented	Yes

[a] Range (smallest significant % difference of means to largest significant % difference of means for included acylcarnitines)
[b] Only results with significant associations were reported

3.2.1.3 Sex

Five studies examined the relationship between sex and carnitine or acylcarnitine levels in neonatal blood (Table 2). Sex was not significantly associated with any carnitine or acylcarnitine group level in any study (Tables 4-7). In one study, Cavedon and colleagues stated that there was a difference in males versus females for three of ten individual long chain acylcarnitines (C14, C16, C18:1) but the nature of the association was not specified (Table 7) [28].

3.2.2 Newborn Screening Related Factors

Eleven articles were deemed eligible for inclusion in the review of newborn screening related factors (age at blood spot collection, nature of sample, storage of sample). Articles included in the review were published from 1980 to 2010 (Table 8). Four of the 11 studies took place in Germany, 2 in Australia, and 1 each in Belgium, the Czech Republic, Italy, the USA, and both Australia and the UK. Tandem mass spectrometry was used by the 7 most recently published studies, while the other 4 used radioisotopic assays. Newborn screening based data were used exclusively in 5 of 12 studies. Hospital based data were used exclusively in 5 studies with 1 study using a combination of newborn screening and hospital based data. Seven studies used a cross sectional study design, while 4 used a longitudinal approach. Sample sizes were considered small (<50) in 2 studies, medium in 6 studies (50 – 2000), and large (>2000) in 3 studies.

In regard to the predetermined study characteristics that we believed may be associated with study findings, in 10 of 11 studies the sample population was either somewhat or very representative of the general population (Table 3) [3,25,27-30,35-38]. The

remaining sample populations were comprised entirely of hospitalized neonates. Newborn screening related factors of interest were central to all 11 studies.

Table 8 – Characteristics of Studies Measuring Effects of Newborn Screening Related Factors on Carnitine & Acylcarnitine Levels

Citation	Study Design	Sample Size (neonates)	Assay & Sample Type	Independent Variables	Dependent Variables
Khalid JM, et al. [29] 2010 Australia & UK	Newborn screening based Cross sectional	227,098	Tandem mass spectrometry Whole blood	Age of collection	C8
Maier EM, et al. [35] 2009 Germany	Newborn screening based Cross sectional	376,657	Tandem mass spectrometry Whole blood	Age of collection	C8
Strnadova KA, et al. [36] 2007 Czech Republic	Newborn screening based Cross sectional	660	Tandem mass spectrometry Whole blood	Storage of sample	Free carnitine
Cavedon CT, et al. [28] 2005 Belgium	Hospital & newborn screening based Cross sectional	117 67 (cord blood)	Tandem mass spectrometry Whole blood	Age of collection	Free carnitine Short chain acylcarnitines Medium chain acylcarnitines Long chain acylcarnitines
Meyburg J, et al. [25] 2002 Germany	Hospital based Longitudinal	120	Tandem mass spectrometry Whole blood	Age of collection	Free carnitine Total carnitine Total acylcarnitines
Meyburg J, et al. [3] 2001 Germany	Hospital based Longitudinal	95	Tandem mass spectrometry Plasma & whole blood	Age of collection Nature of sample	Free carnitine Total carnitine Total acylcarnitines Short chain acylcarnitines Medium chain acylcarnitines Long chain acylcarnitines
Wilcken B, et al. [30] 2001 Australia	Newborn screening based Cross sectional	149,707	Tandem mass spectrometry Whole blood	Age of collection	Free carnitine
Barns RJ, et al. [37] 1991 Australia	Newborn screening based Cross sectional	1843	Radioisotopic assay Plasma & whole blood	Nature of sample Storage of sample	Free carnitine Total carnitine
Christensen ML, et al. [31] 1989 USA	Hospital based Longitudinal	40	Radioisotopic assay Plasma	Age at collection	Total carnitine
Schmidt-Sommerfeld E, et al. [38] 1988 Germany	Hospital based Longitudinal	74	Radioisotopic assay Plasma	Age at collection	Free carnitine Total carnitine Total acylcarnitines
Battistella PA, et al. [27] 1980 Italy	Hospital based Cross sectional	44	Radioisotopic assay Plasma	Age at collection	Free carnitine

3.2.2.1 Age of the Newborn at the Time of the Blood Sample Collection

Nine studies examined the association between the age of the newborn at the time of the blood sample collection and carnitine or acylcarnitine levels in neonatal blood (Table 8). Results were mixed in the six studies that examined this association for free carnitine levels (Table 9). In two such studies, free carnitine levels were not significantly different when cord blood was compared to samples taken at several postnatal days of age [3,28]. In another study, free carnitine levels were seen to increase significantly in the postnatal period consistently throughout the first 14 days of life [27] and in a different study levels appeared higher postnatal days 5-8 as compared to day 2 [30], though significance was not tested. Schmidt-Sommerfeld and associates demonstrated a pattern wherein free carnitine levels increased for the first 8 hours of life, then decreased through days 2-7, increasing again through days 8-28 [38]. A similar pattern was found by Meyburg and colleagues (though significance was not reported) in neonates born at 28-31 weeks gestation. In these infants, free carnitine increased postnatally to day 3, decreased days 3-7, then increased to day 28. No significant difference in free carnitine level was found in this group between postnatal days 1 and 28. In the same study, neonates born at less than 27 weeks experienced a consistent postnatal decrease in free carnitine from days 1-14, with levels remaining steady from days 14-28. Free carnitine levels declined approximately 50% between days 1 and 28. Infants born at 37-42 weeks gestational age saw an increase in free carnitine of approximately one third between days 5 and 28 [25].

Four studies examined the relationship between age at blood collection and total carnitine level (Table 10). One found no significant difference in age of collection between neonates with total carnitine levels of $\leq$ 13 nmol/ml vs. those with > 13 nmol/ml [31]. Another study found that total carnitine level was increased significantly in blood drawn on postnatal

Table 9 – Associations between Newborn Screening Related Factors and Free Carnitine Levels

Factor	Study	Findings	Effect Size (% difference in means)	Significant Association ($p < 0.05$)
Age at Blood Collection (AoC)	Cavedon (2005) [28]	No significant difference was found in cord blood vs. 3-6 postnatal days		No
	Meyburg (2002) [25]	FC increased postnatal days 8-14 (28-31 weeks GA) FC increased postnatal day 28 vs. 5 (approximately 1/3 higher) (37-42 weeks GA) FC decreased postnatal days 1-14 (<27 weeks GA) FC decreased postnatal day 1 vs. 28 by 50% (<27 weeks GA) FC decreased postnatal days 1-7 (28-31 weeks GA) No significant difference was found postnatal day 1 vs. 28 (28-31 weeks GA)	Not presented Not presented Not presented Not presented Not presented	Yes
	Meyburg (2001) [3]	No significant difference was found in cord blood vs. postnatal day 5		No
	Schmidt-Sommerfeld (1988) [38]	FC increased 8 postnatal hours vs. cord blood FC increased 8-28 vs. 2-7 postnatal days FC decreased 2-7 vs. 1 postnatal days	19 46 -35	Yes
	Wilcken (2001) [30]	FC increased 5-8 vs. 2 postnatal days	Not presented	No (not reported)
	Battistella (1980) [27]	FC increased with AoC from 0-14 days of life in full term	Not presented	Yes
Nature of Sample	Barns (1991) [37]	Significance of comparison between FC in whole blood vs. plasma not reported (whole blood: median = 44 µM (95% CI 26-76); plasma: median = 33 µM (95% CI 12-60)		No (not reported)
Storage of Sample	Strnadova (2007) [36]	FC increased during years 1-5 of storage (largest increase in year 1) FC decreased during years 6-15 of storage	7.6 per year -1.4 per year	Yes

Table 10 – Associations between Newborn Screening Related Factors and Total Carnitine Levels

Factor	Study	Findings	Effect Size (% difference in means)	Significant Association ($p < 0.05$)
Age at Blood Collection (AoC)	Meyburg (2002) [25]	TC increased postnatal days 8-14 (28-31 weeks GA) TC increased postnatal day 28 vs. 5 (approximately 1/3 higher) (37-42 weeks GA) TC decreased postnatal days 1-14 (<27 weeks GA) TC decreased postnatal day 1 vs. 28 by 50% (<27 weeks GA) TC decreased postnatal days 1-7 (28-31 weeks GA) No significant difference was found postnatal day 1 vs. 28 (28-31 weeks GA)	Not presented Not presented Not presented Not presented Not presented	
	Meyburg (2001) [3]	TC increased postnatal day 5 vs. cord blood	8	Yes
	Christensen (1989) [31]	No significant difference was found in TC ≤ 13 nmol/ml vs. TC > 13 nmol/ml		No
	Schmidt-Sommerfeld (1988) [38]	TC increased 8 postnatal hours vs. cord blood TC decreased 2-7 vs. 1 postnatal days TC increased 8-28 vs. 2-7 postnatal days	26 -44 31	Yes
Nature of Sample	Meyburg (2001) [3]	TC is increased in whole blood vs. plasma	21	Yes
	Barns (1991) [37]	Significance of comparison between FC in whole blood vs. plasma not reported (whole blood: median = 60 µM (95% CI 35-102); plasma: median = 51 µM (95% CI 23-84)		No (not reported)
Storage of Sample	Barns (1991) [37]	TC extraction reliability decreased with time stored (significantly compromised by 6 years)	Not presented	Yes

Table 11 – Associations between Newborn Screening Related Factors and Total Acylcarnitine Levels

Factor	Study	Findings	Effect Size (% difference in means)	Significant Association ($p < 0.05$)
Age at Blood Collection (AoC)	Meyburg (2002) [25]	TA increased postnatal days 8-28 (28-31 weeks GA) TA increased postnatal day 28 vs. 5 (approximately 1/3 higher) (37-42 weeks GA) TA decreased postnatal days 1-14 (<27 weeks GA) TA decreased postnatal day 1 vs. 28 by 50% (<27 weeks GA) TA decreased postnatal days 1-7 (28-31 weeks GA) No significant difference was found postnatal day 1 vs. 28 (28-31 weeks GA)	Not presented Not presented Not presented Not presented Not presented	Yes
	Meyburg (2001) [3]	TA increased postnatal day 5 vs. cord blood	21	Yes
	Schmidt-Sommerfeld (1988) [38]	TA increased 8 postnatal hours vs. cord blood TA decreased 2-7 vs. 1 postnatal days No significant difference was found 8-28 vs. 2-7 postnatal days	43 -58	Yes

Table 12 – Associations between Newborn Screening Related Factors and Chain Length Specific Acylcarnitine Levels

Factor	Study	Findings	Effect Size (% difference in means)	Significant Association ($p < 0.05$)
Short Chain Acylcarnitines (SCA), Medium Chain Acylcarnitines (MCA), Long Chain Acylcarnitines (LCA)				
Age at Blood Collection (AoC)	Khalid (2010) [29]	No significant difference in first 14 days (MCA)		No
	Maier (2009) [35]	No significant difference in 95th percentile C8 from 1-7 postnatal days (MCA)		No
	Cavedon (2005) [28]	SCA increased 3-6 postnatal days vs. cord blood in 5 of 9** MCA increased 3-6 postnatal days vs. cord blood in 6 of 7** LCA increased 3-6 postnatal days vs. cord blood in 6 of 10** SCA decreased 3-6 postnatal days vs. cord blood in 1 of 9** LCA decreased 3-6 postnatal days vs. cord blood in 1 of 10**	16 to 43* 21 to 47* 33 to 51* -30 -25	Yes
	Meyburg (2001) [3]	SCA increased postnatal day 5 vs. cord blood (combined SCA) MCA increased postnatal day 5 vs. cord blood (combined MCA) LCA increased postnatal day 5 vs. cord blood (combined LCA) SCA increased postnatal day 5 vs. cord blood in 3 of 5** MCA increased postnatal day 5 vs. cord blood in 4 of 6** LCA increased postnatal day 5 vs. cord blood in 5 of 10**	15 11 30 9 to 18* 14 to 24* 19 to 42*	Yes
Nature of Sample	Meyburg (2001) [3]	LCA increased in whole blood vs. plasma	77	Yes
Storage of Sample	Strnadova (2007) [36]	C2 decreased years 1-15 of storage C3 decreased years 1-15 of storage	-18.5 per year (1-5) -7.5 per year (6-15) -27.4 per year (1-5) -7.8 per year (6-15)	Yes

* range (smallest % difference of means to largest % difference of means for included acylcarnitines)
** Only results with significant associations were reported

day 5 as compared with cord blood [3]. Both Schmidt-Sommerfeld and associates as well as Meyburg and colleagues found the same patterns in total carnitine level as were present in free carnitine [25,38].

Three studies addressed the relationship between total acylcarnitines and age at blood collection (Table 11). In one, total levels of acylcarnitine were seen to increase significantly in blood drawn on postnatal day 5 as compared with cord blood [3]. Schmidt-Sommerfeld and colleagues presented findings in which total acylcarnitine level increased for the first 8 hours of life, then decreased through days 2-7, but underwent no significant difference between days 2-7 and 8-28 [38]. Though significance was not tested, Meyburg and associates further grouped the study population by gestational age and found that acylcarnitine levels remained steady days 1-3 in neonates born at less than 27 weeks, and then experienced a consistent decrease from days 4-7, with levels remaining steady from days 7-28. Total acylcarnitine level declined approximately 50% between days 1 and 28 for these neonates. In the same study, acylcarnitine levels remained steady days 1-5 in neonates born at a gestational age of 28-31 weeks, decreased days 5-7, and remained steady through day 28. In this group, no significant difference in total acylcarnitine level was found between postnatal days 1 and 28. Infants born at 37-42 weeks gestational age saw an increase in free and total carnitine and total acylcarnitine of approximately one third between days 5 and 28 [25].

Short, medium, and long chain acylcarnitines all displayed mixed results in regard to association with age of collection (Table 12). In one study, postnatal increases in acylcarnitine level were seen in 5 of 9 short, 6 of 7 medium, and 6 of 10 long chain acylcarnitines. Postnatal decreases were present in 1 of 5 short and 1 of 10 long chain

acylcarnitines [28]. In another, postnatal increases were seen in 3 of 5 short, 4 of 6 medium, and 5 of 10 long chain acylcarnitines. Combined totals of short, medium, and long chain acylcarnitines were each shown to independently increase between the collection of cord blood and that at several postnatal days [3]. Two additional studies examining only specific medium chain acylcarnitines presented with no significant difference associated with age of collection [29,35].

3.2.2.2 Nature of Sample

Two studies examined the relationship between the nature of a blood sample (whole blood versus plasma) and carnitine or acylcarnitine levels in neonatal blood (Table 8). Barns and associates compared median levels of free carnitine and total carnitine in whole blood (free carnitine: 44 μM (95% CI 26-76); total carnitine: 60 μM (95% CI 35-102)) and plasma (free carnitine: 33 μM (95% CI 12-60); total carnitine: 51 μM (95% CI 23-84)) samples but did not report the significance of their findings (Tables 9 & 10) [37]. Meyburg et al. compared whole blood with plasma for total carnitines and long chain acylcarnitines (Tables 10 & 12), finding levels of both these analytes to be significantly higher in whole blood vs. plasma only samples [3]. No study investigated the other analytes we considered with respect to differences in whole blood versus plasma.

3.2.2.3 Length of Storage of Sample

Two studies examined the relationship between the length of storage of the blood sample and carnitine or acylcarnitine levels in neonatal blood (Table 8). Strnadova and associates found that free carnitine increased from years 1-5 of storage at a rate of 7.6% per year, then decreased in years 6-15 at a rate of 1.4% per year (Table 9) [36]. In the same study,

C2 and C3 levels were also shown to decrease with years of storage at rates ranging from 7.5% (C2 years 6-15) to 27.4% (C3 years 1-5) per year of storage (Table 12). Barns and colleagues found total carnitine extraction reliability to be significantly compromised by 6 years of storage (Table 10) [37].

3.3 Discussion

3.3.1 Perinatal & Infant Characteristics

Birth weight and gestational age were significantly associated with carnitine and acylcarnitine levels in the majority of studies. The presence and directionality of these associations varied between and within studies for both variables. With respect to birth weight, when a significant association with carnitine and/or acylcarnitines was identified, it was typically positive. The only exception was a single comparison in one study of free carnitine [30]. The relationship with gestational age was more varied. Gestational age was observed to have both positive and inverse associations with every carnitine and acylcarnitine type investigated, with effect sizes ranging from -56 to +194 (% difference in means). In contrast to the findings for birth weight and gestational age, sex was not associated with carnitine or acylcarnitine levels with the exception of three individual long chain acylcarnitines which were investigated in only one study [28]. The direction and strength of association was not reported in that study. Quantitatively, gestational age was the most important perinatal factor associated with carnitine and acylcarnitine levels in neonates.

The associations of carnitine or acylcarnitines with gestational age and birth weight are further complicated by the relationship between these two perinatal factors. A strong positive correlation exists between gestational age and birth weight, such that it is difficult to

separate the effect of one from the other [39]. In particular, low birth weight can result from prematurity and/or from fetal growth restriction; these conditions may have different underlying causes [40]. While some studies of birth weight in relation to carnitine/acylcarnitines examined this association independent of gestational age (e.g., by defining low birth weight in terms of "small for gestational age" or by restricting the analysis to full-term infants) [3,27], others have mixed these effects [33,34]. Furthermore, fetal growth and gestational age may interact in their association with carnitine/acylcarnitines, as well as having potentially independent relationships. The three studies in this review to include both birth weight and gestational age approached these challenges in different ways. Battistella and colleagues separated participants into groups based upon gestational age and examined birth weight as a continuous variable. Furthermore, preterm neonates were divided into 1.15-1.80 and >1.80 kg birth weight for analysis [27]. Meyburg et al. used categories that grouped full term appropriate weight for gestational age (term AGA), full term small for gestational age (term SGA), and preterm neonates [3]. Khalid et al. grouped participants into birth weight categories of ≤ 1.5kg versus > 1.5 and into gestational age categories of preterm vs. full term [29]. Studies directly examining only birth weight or gestational age also encountered this challenge, as not acknowledging the other variable does not discount its impact. In these studies, participants of all gestational ages or birth weights were examined together. These divergent approaches yielded results that were difficult to compare directly and this may in part explain the inconsistencies in their findings.

3.3.2 Newborn Screening Related Factors

The age of the newborn at the time of blood sample collection was significantly associated with carnitine and acylcarnitine levels in the majority of studies, though the presence and nature of the relationship varied across studies. Where a significant association was identified, free and total carnitine levels typically increased in the days following birth [25,27,30,38]. Where a pattern existed, free and total carnitine as well as acylcarnitine increased to 8 hours of age then decreased over the first week of life [25,38]. Free and total carnitine then increased over the first month while total acylcarnitine levels remained constant.

The potential interaction between a neonate's gestational age and age at collection of a blood sample is an important factor to consider in relation to carnitine and acylcarnitine levels. Carnitine and acylcarnitine levels change naturally over time, with samples obtained from the same infant over the course of the first month potentially yielding substantially different carnitine and acylcarnitine levels [25]. In premature neonates, these changes in the carnitine and acylcarnitine profile may be delayed compared to those of term neonates. Consequently, age of collection becomes particularly important, as a cord blood sample from a term neonate may have a similar profile to one taken from a premature neonate at an older age [25]. This may explain some of the variation found in this research where levels of free carnitine, total carnitine, and total acylcarnitine were found to decrease over the first month of life in highly premature infants while increasing in term neonates. In this study, significant differences were observed over the first month of life depending upon level of prematurity. In the most premature group, free
and total carnitine as well as total acylcarnitine levels decreased by 50% in the first two weeks of life and remained constant for the rest of the month. In neonates of 28-31 weeks

gestational age, free and total carnitine levels varied, increasing then decreasing over the first week; while total acylcarnitine levels stayed steady then decreased. From birth to day 28 of life, average levels of free carnitine, total carnitine, and total acylcarnitine remained unchanged in this group. In term infants, free and total carnitine as well as acylcarnitine levels increased by a third over the first month of life. The net increase in free carnitine, total carnitine, and total acylcarnitines in the most premature category; their unchanged nature in less premature infants; and their increase among term neonates suggests that the interaction between the age of the baby at the time of sample collection and gestational age may be an important consideration in understanding carnitine and acylcarnitine levels in neonates. Further supporting this assertion, while combined totals of short, medium, and long chain acylcarnitines each increased significantly between birth and several postnatal days, the presence and directionality of associations between individual short, medium, and long chain acylcarnitines and age of collection varied within each category.

Carnitine and acylcarnitine profiles may also vary based on the type of blood sample used. Newborn screening programs use whole blood spot analysis to screen for disease, but clinical research may rely on either whole blood or plasma [3,27,29]. Studies included in this review used primarily one of two types: plasma only [27,31-34] or whole blood [3,25,28-30]. Whole blood levels of carnitine and acylcarnitines have been shown to be consistently higher than plasma levels [3], however the degree to which carnitine and acylcarnitine levels vary between whole blood and plasma analysis may differ for different analytes [3] and little research exists detailing these potential differences. Of the studies examined, where significant, whole blood had higher levels of total carnitine and long chain acylcarnitines than plasma samples [3]. This

association was also seen in free and total carnitine in another study, but statistical significance was not reported [37].

The effect of storage on levels of carnitine and acylcarnitines in neonatal blood spots varied, but changes were generally larger in years 1-5 of storage than in years 6-15 [36]. Similarly, total carnitine extraction reliability was found to be significantly compromised by year 6 of storage [37]. Modern newborn screening programs' relatively uniform use of whole blood spots and short storage times mean that differences in analyte levels over longer term storage are of less concern. The effect of longer storage on dried blood spots is important, however, when considering their use for secondary purposes such as research or later clinical use [41].

3.3.3 Supplementation

Though data directly comparing neonates receiving carnitine supplementation versus non-supplemented individuals were excluded from this review, it is possible that infants in some of the studies may have received a form of supplementation. This may confound the association between perinatal characteristics and analyte levels. Understanding the impact of neonatal illness and interventions to manage or mitigate illness on carnitine or acylcarnitine levels in neonatal blood, and the relationship between these factors and the perinatal characteristics we studied, is an important direction for future research.

3.3.4 Statistical vs. Clinical Significance

Heterogeneity in the findings of the included studies also highlights the importance of distinguishing between statistically significant and clinically significant differences in the

context of neonatal bloodspot analyte levels. Statistical significance [3,25,27,28,30-34] provides evidence that an association exists but does not necessarily denote a clinically important difference. Clinical significance [29] implies practical relevance and is of greatest interest to decision-making in the areas of newborn screening and clinical management. There are no standards in this field for determining effect sizes that are likely to be clinically significant, and clinical significance is likely to differ depending on the purpose of measuring analytes (e.g., comparing values with cut-offs denoting positive newborn screening results, monitoring the impact of interventions, investigating the cause of symptoms). For these reasons, our study relied mainly on statistical significance ($p < 0.05$) for summarizing findings across studies. However, we also reported the observed effect sizes (e.g., difference between means) whenever possible. Even relatively small differences may be important when applied across an entire population, and may lead to hypotheses that further our understanding of the physiology of neonatal metabolism and related questions regarding the impact of prematurity, growth restriction, illness, and intervention. Ultimately, these insights may contribute to the management of both well and ill newborns.

Statistical significance is dependent upon the power of a study which is affected by sample size. Sample size was not related to any discernable patterns in the relationship between analyte levels and perinatal and infant characteristics or newborn screening related factors, though comparisons were challenging due to the disparate methods of measuring the exposure variables.

3.3.5 Risk of Bias

While every study in this review passed broad and detailed level screening, their methods were heterogeneous, suggesting that it may be useful to assess the potential risk of bias for each study individually. We researched published tools for evaluating the risk of bias / methodological quality in observational studies [21,22,24], but found that none had been definitively established as a standard and none seemed ideally applicable to our study. For these reasons, we identified two study characteristics that may be related to results, informed by existing instruments. These addressed the representativeness of the study sample in terms of the general population of newborns, and the centrality of the associations of interest in terms of the stated purpose of the study. These two characteristics reflect themes that are common across generic quality assessment tools: namely, generalizability (representativeness) and adequacy of reporting of methods (centrality of the associations of interest). We found that 10 of 14 studies included a somewhat or very representative sample; and that pre/perinatal factors were of central importance in 11 of 14 studies. However, if the 4 studies without representative samples and/or where pre/perinatal factors were not of central importance were removed from this review, no considerable differences would exist in the results with the exception that we would have reviewed no information on the association between sex and total carnitine.

3.3.6 Limitations

Despite the systematic nature of this review, several limitations exist. Notably, considerable variation across studies in terms of their design, measurement, and subgroup definitions precluded a quantitative summary of findings. In addition, sample sizes were generally small (with three notable exceptions) (Tables 2 & 8), limiting representativeness of the studies to the general population of neonates.

3.4 Conclusion

Our review results indicate a significant association between carnitine and acylcarnitine levels in neonates and birth weight, gestational age, age at time of blood spot collection, type of sample (plasma vs. whole blood), and storage time, though the presence and directionality of these associations varied. Sex was not significantly associated with carnitine and acylcarnitine levels in neonatal blood. Complex interactions may exist between gestational age and birth weight in regard to analyte levels, though this relationship is not fully investigated in the literature.

The use of unstandardized methods, generally small sample sizes, and heterogeneity of results highlight the importance of further research directed at examining the association between perinatal factors and carnitine and acylcarnitine concentrations in neonatal blood. In particular, attention should be paid to the association of gestational age with these analyte levels, incorporating a thoughtful consideration of its potential complex interactions with birth weight / fetal growth, and age at the time of sample collection. Empirical, large scale cross-sectional studies in representative samples would be most informative, as would longitudinal studies for assessing the trajectories of analyte levels over time.

4.0 Clinical Database Analysis

4.1 Methods

4.1.1 Data Source

To further investigate the relationship between perinatal factors and levels of carnitine and acylcarnitines in neonatal blood, we conducted a clinical database analysis of the Newborn Screening Ontario (NSO) database. This database houses information on the approximately 140,000 babies born in Ontario every year since its inception in 2006. Hospitals, physicians, midwives, and other neonatal health care provides from across Ontario collect newborn screening bloodspots and the associated health information from their patients, usually within the first few days after birth. These bloodspots and information cards are mailed to NSO where they are analyzed. Demographic and other health information from the bloodspot screening card is entered into the NSO database and critical fields are reentered for confirmation by a second clerk. Where vital fields are missing, data entry clerks contact the submitter to obtain this information [8].

Data were obtained from Newborn Screening Ontario with ethics approval from the Children's Hospital of Eastern Ontario (Appendix C). The data sharing agreement encompassed individual levels of carnitine and acylcarnitine markers, as well as information on pre/perinatal factors including gestational age, birth weight, sex, feeding status, transfusion status, postal code (six characters to link with census data and create a proxy for socioeconomic status), screen positive & true positive status, transit time (the time between collection of a blood sample and receipt of the sample by Newborn Screening Ontario), age of collection, and date of birth among others.

4.1.2 Data Cleaning

Data included in this analysis were screened using predetermined inclusion criteria. Samples included in the analysis had to belong to patients born between the beginning of electronic data collection by Newborn Screening Ontario (January 1, 2007) and December 31, 2009. Only the data for the first satisfactory sample for each unique patient were included. Samples belonging to patients found to be true positives for metabolic disorders were excluded.

Satisfactory samples are defined as those collected from an infant >24 hours of age and received by NSO within 14 days of collection. All important information including the identification of the child, and date and time of birth as well as date and time of blood spot collection have to be recorded. In addition, a sample must be of appropriate quality for screening in the lab. Data that met these inclusion criteria were further cleaned to remove corrupted entries and address issues such as data entry field misalignment.

4.1.3 Variable Definitions

Variables in the final dataset can be subdivided into independent variables of interest, dependent (carnitine or acylcarnitine) variables of interest, and covariates. Independent variables of interest included: age of the baby at the time of the collection of the bloodspot sample, the baby's birth weight, gestational age, and sex, an area-based proxy indicator of socioeconomic status (derived from the six character postal code), and the transit time from the date of blood sample collection until analysis at Newborn Screening Ontario. The dependent variables included the following carnitines, acylcarnitines, and their ratios and sums: C0 (free carnitine), C8 (octanoylcarnitine), the total of all short chain acylcarnitines,

the total of all medium chain acylcarnitines, the total of all long chain acylcarnitines, the total of all measured acylcarnitines, total carnitine, and the ratio of total acylcarnitines to free carnitine (refer to Chapter 1, Table 1). Covariates were included to assess and/or address potential selection bias, confounding, and effect modification of the main associations of interest. These variables included: infant feeding, transfusion status, screen positive status, true positive status, first acceptable sample status (first sample received from an infant that is of acceptable quality for newborn screening), and date of birth.

4.1.3.1 Independent Variables

Age of collection measures the number of hours between birth and the collection of an infant's blood spot sample for newborn screening. Protocols dictate that samples are not to be taken until 24 hours post-birth, as the neonatal metabolic profile changes drastically within the first day of life. As a result, test results from samples taken prior to the 24 hour cutoff are not valid for some of the conditions screened by NSO. In cases where a child is transfused, transferred, or released from the hospital prior to 24 hours of age, a bloodspot sample may be taken earlier than the 24 hour minimum. In these cases, a second sample is required and is usually collected within 5 days [42].

The vast majority of samples are collected from infants between 24 and 72 hours of age, however, for a variety of reasons including unsatisfactory original samples, sampling may occur at a later date. For the purposes of this study, we included data only for those infants with age of collection between 1 and 14 days of age in order to eliminate outliers that might have a significantly different metabolic profile as a result of age. The age of the baby at the time of the blood sample collection is recorded on the bloodspot card sent to NSO.

Age of collection was further divided into four meaningful categories corresponding to the age of the infant in days and reflecting that the majority of valid samples are taken in the first twelve hour period of eligibility for screening: 24-35 hours (the majority of samples are taken early on the second day of life), 36-47 hours (i.e., by the end of the second day of life), 48-71 hours (on the third day of life), and 72+ (beyond day 3 of life). Categories were used in order to mitigate the effect of the right skewed nature of the age of collection variable.

Infant sex refers to the biological sex of the patient (female, male, or not recorded). The sex of the infant is also recorded on the bloodspot card.

Gestational age refers to the length of time between conception and birth. Gestational age was divided into five categories based on standard definitions: extremely premature (< 28 weeks), very premature (28 - 31 weeks), medium premature (32 – 33 weeks), late premature (34 – 36 weeks), and term (≥ 37 weeks) [43]. These categories were chosen in order to comply with previous studies and current definitions as used by the medical community [43,44]. The gestational age of the baby at birth is recorded on the bloodspot card. However, it is common practice for submitting centers to record the gestational age only when the baby is not full term. Therefore, for our study, when no gestational age was recorded it was assumed that the child was full term.

Birth weight measures the weight of a child in grams at birth and was also recorded on the bloodspot card. For analysis, birth weight was divided into quintiles within categories

of sex and gestational age. Birth weight is highly correlated to gestational age and connected to sex. Correcting for these two variables allowed the impact of birth weight to be examined independently, without confounding by sex and gestational age [45].

Socioeconomic status quintile was determined by linking infants' reported postal codes (recorded on the bloodspot card) with data from the 2006 Canadian Census, using the Postal Code Conversion File (PCCF) [46]. We used the income quintile variable that is included in the PCCF, which calculates quintiles within small geographic regions.

The method of infant feeding prior to blood spot collection is recorded on the bloodspot card. Infant feeding was divided into four categories: breast milk only, formula only, a combination of breast milk and formula only, and other (including total parenteral nutrition and combinations of feeding strategies other than the exclusive use of breast milk and formula).

Transit time measures the amount of time in days between collection of a blood spot sample and its receipt by Newborn Screening Ontario. Transit time can vary between 0 and 14 days. Samples with a transit time of greater than 14 days are considered to be unsatisfactory by Newborn Screening Ontario.

4.1.3.2 Dependent Variables

The dependent variables were described thoroughly in the introduction and in Table 1. This chapter will refer back to the following eight variables: free carnitine, C8, total carnitine, total acylcarnitine, short chain acylcarnitine, medium chain acylcarnitine, long chain acylcarnitine, and the ratio of total acylcarnitine to free carnitine.

4.1.3.3 Covariates

Date of birth (recorded on the bloodspot card) was used to restrict the sample population to children born between January 1, 2007 and December 31, 2009.

First satisfactory sample status (derived from the NSO database) was used to restrict the sample population to the first satisfactory sample received for each unique, eligible patient within the specified time. First satisfactory sample status was divided into two categories: yes, and no.

Screen positive status refers to whether a blood spot sample met the criteria to be considered positive for a disorder by Newborn Screening Ontario. In cases where a screen positive sample is obtained, a repeat sample is requested in order to determine whether the patient the original sample was a true or false positive. Screen positive status was divided into two categories: yes, and no.

True positive status refers to whether a patient was confirmed positive for a disorder by Newborn Screening Ontario. When an infant receives a positive screening result for any one of the disorders on the Ontario newborn screening panel, that positive result is reported

by NSO to a treatment centre (one of five tertiary children's hospitals in Ontario) nearest the residence of the infant. The treatment centre is responsible for contacting the infant's family and arranging for confirmatory diagnostic testing as well as any necessary ongoing care. Once confirmatory testing is complete, the treatment centre reports the final result (true positive, false positive, or uncertain) back to NSO for the purposes of evaluating the screening program. For our study, true positive status was used to restrict the sample population to blood spot samples received from individuals who were not confirmed to be affected by disorders. True positive status was divided into three categories: yes, no, and unknown.

The transfusion status variable refers to whether a patient received a transfusion prior to blood spot collection. Since blood transfusions may affect the analyte levels for some of the newborn screening tests, receipt of a blood transfusion prior to the bloodspot sample collection is recorded on the bloodspot card. Transfusion status was divided into two categories: yes, and no. Samples with no recorded transfusion status or transfusion date were assumed to be untransfused.

Table 13 – Variable Characteristics

Variable	Type	Description
Independent Variables		
Age of Collection	Categorical	24-35 hours 36-47 hours 48-71 hours 72+ hours
Infant Sex	Categorical	Male Female Unknown
Gestational Age	Categorical	Extremely premature Very premature Medium premature Late premature Term
Birth Weight Quintile	Categorical	1-5 (quintile within gestational age & sex category)
Socioeconomic Quintile	Categorical	1-5 (quintile)
Infant Feeding	Categorical	Breast milk only Formula only Breast milk & formula Other
Transit Time	Continuous	0-14 days
Dependent Variables		
C0	Numeric	n/a
C8	Numeric	n/a
Short chain acylcarnitine	Numeric	n/a
Medium chain acylcarnitine	Numeric	n/a
Long chain acylcarnitine	Numeric	n/a
Total acylcarnitine	Numeric	n/a
Total carnitine	Numeric	n/a
Ratio acylcarnitines / free carnitine	Numeric	n/a
Covariates		
Date of Birth	Date	(YYYY/MM/DD)
First Satisfactory Sample	Categorical	Yes No
Screen Positive Status	Categorical	Yes No Unknown
True Positive Status	Categorical	Yes No
Transfusion Status	Categorical	Yes No

4.1.4 Analysis

All analyses were conducted using SAS version 9.1. The distribution and properties of each variable were explored, reporting frequencies for categorical variables and means with standard deviations as well as medians for continuous variables. We addressed the skewed nature of the analytes by re-defining the outcome variables into percentiles, with each participant in a percentile being assigned the median analyte concentration of that percentile. Based upon the distribution and variance within the dependent variables, no further transformations were necessary.

Bivariate analyses were used to examine the association between each independent variable of interest and each dependent variable. Since all dependent variables were continuous, bivariate analysis relied on simple linear regression. Correlations between individual dependent variables were assessed using Pearson correlations. To identify the unique associations of each independent variable with each outcome, multiple linear regression was used to model the relationship between carnitine and acylcarnitine levels and ratios and all independent variables of interest. One multivariable model was developed for each dependent variable. Multivariable models included all of the independent variables of interest that were prespecified (Table 13); no statistical variable selection methods (e.g., stepwise regression) were used.

The potential interaction between birth weight and gestational age in regard to carnitine and acylcarnitine levels and ratios in neonatal blood was also examined in each multiple linear regression model. The birth weight and gestational age interaction term is a combination of birth weight quintile (within sex and gestational age) and gestational age

category. Because a significant interaction was present between these two highly interconnected variables in the analyses for every dependent variable, we used graphical analysis to examine each of the gestational age and birth weight variables while taking into account the influence of the other variable and the other independent variables. Specifically, graphs displayed conditional mean values of the dependent variable for each unique combination of gestational age and birth weight category, adjusted for all other independent variables of interest.

Several sensitivity analyses were conducted. Specifically, we were concerned about the potential impact of transfusion status and feeding status. Newborns who are ill are more likely to be transfused and to receive nutrition by means other than breast milk or formula (e.g., total parental nutrition). Transfused blood is known to affect some newborn screening analyte levels and special nutrition formulas including TPN may contain nutrients (e.g., carnitine) that also affect levels. To examine the potential impact of these effects, we restricted the sample population. Specifically, in one sensitivity analysis, all patients who had received a transfusion prior to their blood sample collection were excluded. In the other sensitivity analysis, all patients receiving nutrition through means other than breast milk or formula were excluded from the sample population. These were of particular interest since we anticipated that our findings would have implications for newborn screening, where the potential effects of variation in feeding methods and medical interventions may be important due to the large population size.

All sensitivity analyses were run with both C0 and C8 as outcome variables. In addition, to further examine the data in a way that would be highly relevant to newborn

screening, we also examined the probability of a false positive newborn screening result by category of birth weight or gestational age.

In all analyses, P-values < 0.05 were used to denote significant relationships. Due to the large sample size, it was expected that most associations would be statistically significant. This held true even after applying the Bonferroni correction accounting for all eight independent variables (including the interaction term) for each of the eight dependent variables. Therefore, we chose to examine trends and make judgments based on effect size, using regression coefficients and r-squared values as indicators of strength of association, rather than make interpretations based mainly on statistical significance.

Regression diagnostics were performed to assess the sample for outliers, linearity, constant variance, and multicollinearity. Carnitine and acylcarnitine concentrations in neonatal blood samples are measured through tandem mass spectrometry and results are directly recorded into a computerized database. Outliers were examined though most were removed when individuals who were true positives for the disorders were eliminated from the sample. After the dependent variables were re-defined into percentiles, the skewed nature of the analyte distribution was minimized. Linearity and constant variance were visually assessed through plotting the analyte levels and their residuals. Multicollinearity was assessed both numerically and graphically in independent variables.

4.2 Results

4.2.1 Main Dataset

Newborn Screening Ontario collected 425,822 samples from neonates born between January 1, 2007 and December 31, 2009 that met the inclusion criteria for this study (Table 14). After reviewing the preliminary results from the clinical database analysis, the decision was made to remove all samples with an age of collection of ≥ 72 hours from our population, due to the considerably different metabolic profile demonstrated by this group. In particular, there was evidence of an important interaction between age of collection and gestational age for some analytes (notably short-chain and medium-chain acylcarnitines) whereby the relationship between gestational age and the metabolite level was quite different among infants at least 72 hours old at the time of the blood sample collection (Figures 3 & 4). One of the primary goals of the clinical database analysis was to examine the interaction between gestational age and birth weight (as suggested by the results of the systematic review). Inclusion of samples taken at an age of ≥ 72 hours would have necessitated consideration of the potential three way interaction between gestational age, birth weight, and age of collection, making it challenging to interpret the findings. Considering the small proportion of the total sample population in this minority group (8.5%), we decided that it made sense to remove these samples. The remainder of the results focus only on those newborns whose screening sample was collected at < 72 hours of age. After this removal, 389,714 samples remained.

Female infants made up 49.0% of the population (Table 15). Only 0.5% of samples screened positive (false positives) once true positives were eliminated. Term children

comprised 95.7% of the sample, with 3.6% late premature, 0.4% medium premature, and 0.1% of each very and extremely premature as defined in the methods section. Birth weight

Table 14 – Clinical Database Analysis Flow Diagram

Dataset	Sample size (#)
Original dataset (all samples in timeframe)	448355
Data cleaning (remove samples that are): *- NSO unsatisfactory (6019)* *- Repeats (12226)* *- Feeding status = TPN (6782)* *- True positives (2420)* *- Corrupted or missing critical data (371)*	
Cleaned dataset (all samples meeting inclusion criteria)	425822
*Remove all samples with age of collection = 72+ hours (36108)**	
Main dataset	**389714**

Remove transfused = yes		Remove feeding status = 'other'	
Sensitivity analysis: No transfused	**389472**	**Sensitivity analysis: Feeding**	**387671**

* refer to text for more details (Section 4.2: Clinical Database Analysis; Results)

Figure 3 – Short Chain Acylcarnitine Level Predicted by Gestational Age & Age of Collection

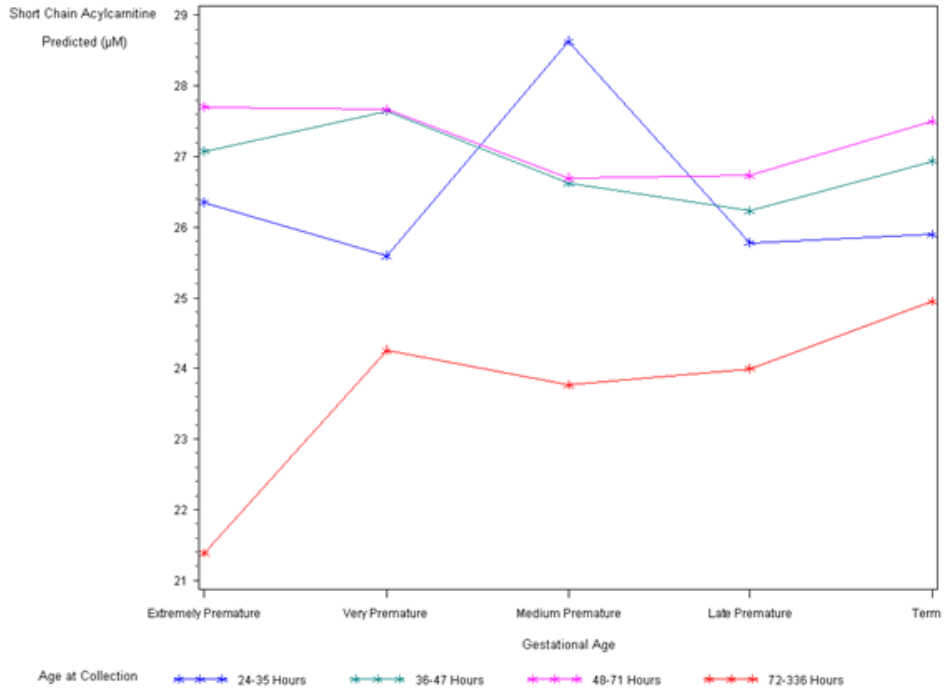
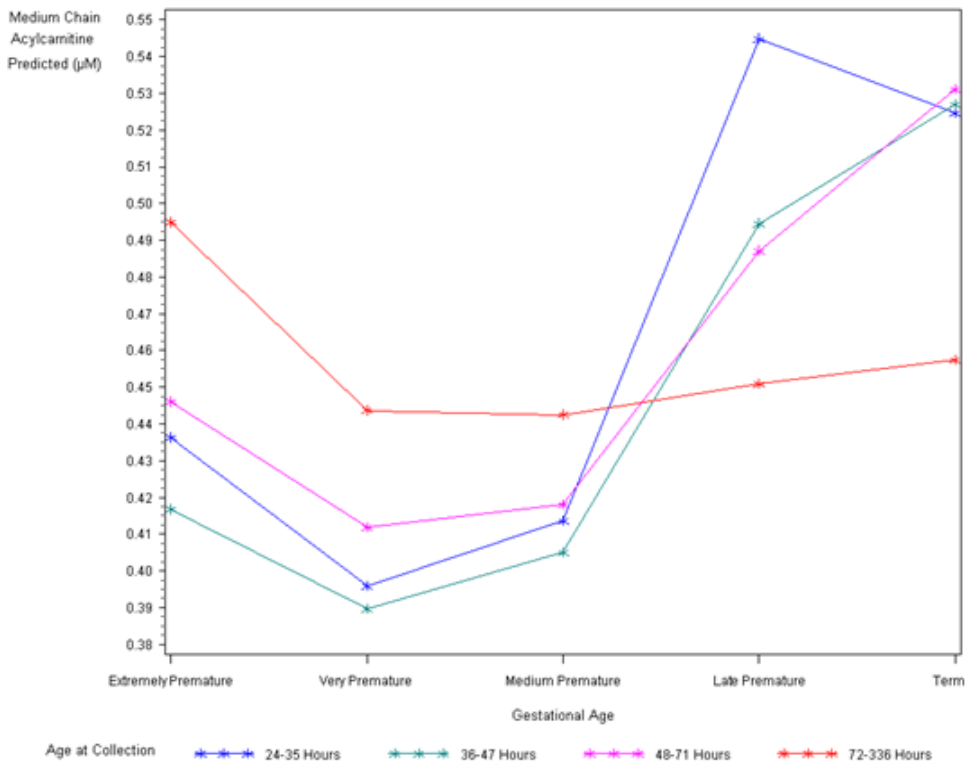


Figure 4 – Medium Chain Acylcarnitine Level Predicted by Gestational Age & Age of Collection



ranged from 501 g to 9947 g with a mean weight of 3393 g and a median of 3396 g (Table 15). For the purposes of analysis, birth weight was divided into quintiles within categories of gestational age and sex. The majority of children were reported to be fed exclusively with breast milk at the time of the blood sample collection (73.8%), with 14.0% receiving a combination of breast milk & formula, 11.7% on formula exclusively, and 0.6% on another form of nutrition (Table 15). Less than 0.1% of children were reported to have had a blood transfusion before their sample was taken. The age of the baby at the time of the blood sample collection ranged from 24 to 71 hours (samples taken ≥ 72 hours were removed from the sample population as explained in section 4.2). Most samples were collected when the neonate was between 24 and 35 hours of age (60.4%), followed by 36-47 hours (21.3%), and

48-71 hours (18.3%). The average age of the baby at the time of sample collection was 35.6 hours and the median was 31.0 hours (Table 15). The transit time (time between the sample collection and receipt by newborn screening) ranged from 0 to 14 days with a mean of 4.3 days and a median of 4.0 days.

Table 15 – Description of the Sample

Categorical Variable	**Categories**			**%**	#
Infant Feeding	Breast Milk & Formula			14.0	50389
	Breast Milk Only			73.8	264964
	Formula Only			11.7	41871
	Other			0.6	2043
Sex	Female			49.0	188893
	Male			51.0	196920
Gestational Age	Extremely Premature			0.1	345
	Very Premature			0.1	601
	Medium Premature			0.4	1478
	Late Premature			3.6	14204
	Term			95.7	373086
Age of Collection	24-35 Hours			60.4	235501
	36-47 Hours			21.3	83057
	48-71 Hours			18.3	71156
Screen Positive	No			99.5	387773
	Yes			0.5	1941
Transfusion	No			99.9	389472
	Yes			0.1	242
Continuous Variable	**Mean**	**Standard Deviation**	**Median**	**25th Percentile**	**75th Percentile**
Age of Collection (hours)	35.6	12.2	31.0	25.0	43.0
Birth Weight (g)	3392.7	525.6	3396.0	3070.0	3725.0
Transit Time (days)	4.3	1.8	4.0	3.0	5.0

Mean levels of the analytes we considered as dependent variables (after redefining into percentiles) were as follows (Table 16): C0 (mean 33.437 μM), C8 (mean 0.085 μM), total carnitine (mean 66.324 μM), total acylcarnitine (mean 32.884 μM), short chain acylcarnitine (mean 26.376 μM), medium chain acylcarnitine (mean 0.524 μM), long chain acylcarnitine (mean 5.982 μM), ratio of total acylcarnitine to free carnitine (mean 1.026). Information on the median and 25^{th} and 75^{th} percentiles of each analyte level is provided in Table 16.

Correlations between levels of each combination of carnitine and acylcarnitine (free carnitine, C8, total carnitine, total acylcarnitine, short chain acylcarnitine, medium chain acylcarnitine, long chain acylcarnitine, ratio total acylcarnitine to free carnitine) were all significant with P-values of <0.0001 (Table 17). The highest positive correlations were detected between total acylcarnitine and short chain acylcarnitine (0.991); and total carnitine with free carnitine (0.953), total acylcarnitine (0.928), and short chain acylcarnitine (0.917). The highest inverse correlation existed between the ratio of acylcarnitine to free carnitine and free carnitine (-0.510).

Table 16 – Dependent Variable Distribution (all in units of uM except the ratio of total acylcarnitine / free carnitine, which is unitless)

	Mean	Standard Deviation	Median	25th Percentile	75th Percentile
C0	33.437	12.169	31.000	25.000	39.300
C8	0.085	0.042	0.080	0.060	0.110
Total Carnitine	66.324	20.769	63.000	51.730	77.310
Total Acylcarnitine	32.884	9.858	31.420	25.800	38.300
Short Chain Acylcarnitine	26.376	8.543	25.040	20.230	30.970
Medium Chain Acylcarnitine	0.524	0.160	0.500	0.410	0.610
Long Chain Acylcarnitine	5.982	1.695	5.780	4.770	6.990
Ratio Total Acylcarnitine / Free Carnitine	1.026	0.228	1.007	0.864	1.165

Table 17 – Dependent Variable Correlations

	C0	C8	Total Carnitine	Total Acylcarnitine	Short Chain Acylcarnitine	Medium Chain Acylcarnitine	Long Chain Acylcarnitine	Ratio Total Acylcarnitine / Free Carnitine
C0	1.000	-----	-----	-----	-----	-----	-----	-----
C8	0.147	1.000	-----	-----	-----	-----	-----	-----
Total Carnitine	0.953	0.178	1.000	-----	-----	-----	-----	-----
Total Acylcarnitine	0.775	0.193	0.928	1.000	-----	-----	-----	-----
Short Chain Acylcarnitine	0.764	0.169	0.917	0.991	1.000	-----	-----	-----
Medium Chain Acylcarnitine	0.141	0.637	0.219	0.286	0.246	1.000	-----	-----
Long Chain Acylcarnitine	0.641	0.208	0.748	0.786	0.703	0.328	1.000	-----
Ratio Total Acylcarnitine / Free Carnitine	-0.510	0.016	-0.256	0.091	0.098	0.149	0.021	1.000

Next, a detailed analysis of predictors of analyte levels is presented. We begin each section with a text box summarizing a few key findings for each analyte. We then present tables and figures for each analysis. Finally, we include the detailed description of the results. Please refer back to Table 1 for the definition of each analyte.

Linearity and variance were considered acceptable. Birth weight and gestational age are highly correlated. In our analysis, birth weight quintiles were defined within categories of gestational age and sex, minimizing confounding effects. In addition to confounding, birth weight and gestational age each act as effect modifiers of the other. These issues were addressed through an interaction term in the multivariate analyses.

4.2.1.1 Associations of Independent Variables with Free Carnitine (C0)

Multivariate R-Square: 0.042 ($P < 0.0001$)

Most important predictors: sex (1.4%) and infant feeding status (1.3%)

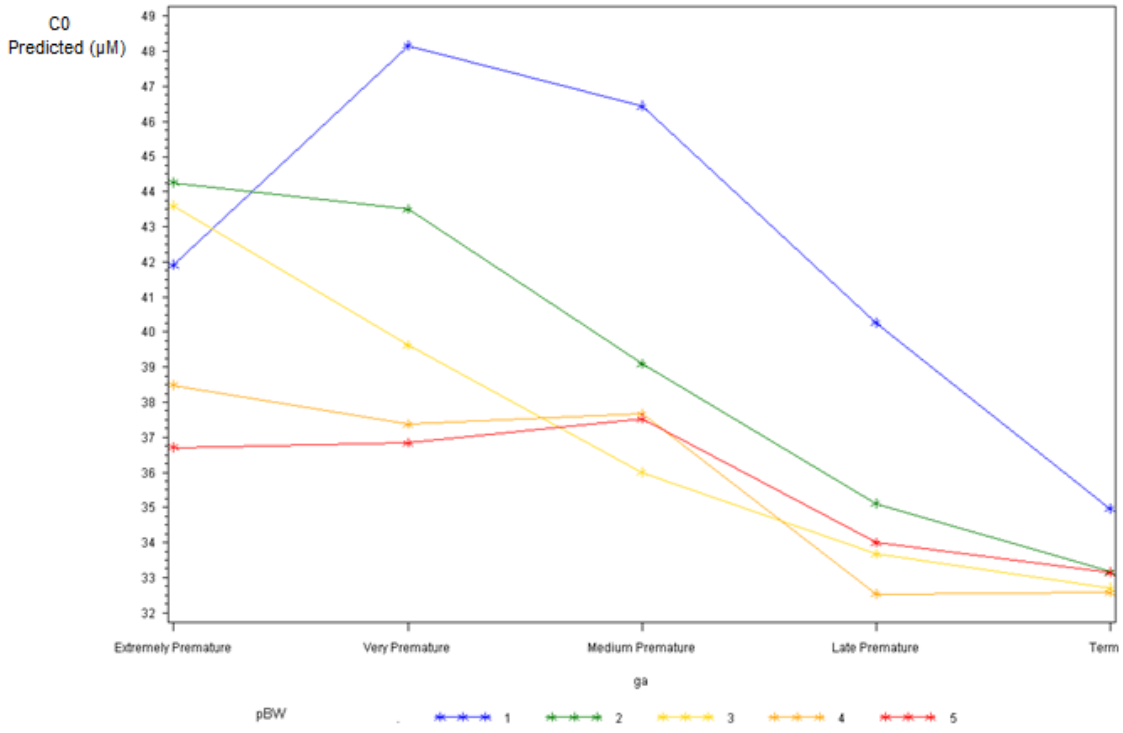
Interaction between birth weight & gestational age (trends):

- Free carnitine level varied with birth weight quintile (level of variation decreased with increasing gestational age)
- Inverse association between C0 level and birth weight quintile
- Inverse association between C0 level and gestational age

Table 18 – Factors Associated with Free Carnitine in Newborn Infants in Ontario (2007-2009)

C0	R-Square	Intercept (µM)	Regression Coefficient (µM)		
Multivariate Model	0.042 (p < 0.0001)	32.273	**Categories**	**Bivariate Model**	**Multivariate Model**
Infant Feeding	0.013 (p < 0.0001)	32.545	Breast Milk & Formula	3.405	2.942
			Breast Milk Only	0.000	0.000
			Formula Only	2.745	2.272
			Other	4.276	1.527
Sex	0.014 (p < 0.0001)	34.844	Female	-2.887	-2.856
			Male	0.000	0.000
Gestational Age	0.003 (p < 0.0001)	33.329	Extremely Premature	7.757	-----
			Very Premature	7.724	-----
			Medium Premature	6.149	-----
			Late Premature	1.807	-----
			Term	0.000	-----
Age of Collection	0.002 (p < 0.0001)	33.830	24-35 Hours	0.000	0.000
			36-47 Hours	-1.241	-1.278
			48-71 Hours	-0.704	-1.094
Birth Weight Quintile	0.006 (p < 0.0001)	33.205	Q1	2.021	-----
			Q2	0.087	-----
			Q3	-0.433	-----
			Q4	-0.593	-----
			Q5	0.000	-----
Interaction Term (BW*GA)	0.010 (p < 0.0001)	-----	(See Appendix D for conditional means)	-----	-----
Socio Economic Status Quintile	0.007 (p < 0.0001)	31.882	Q1	2.930	2.306
			Q2	2.102	1.577
			Q3	1.425	1.075
			Q4	0.764	0.598
			Q5	0.000	0.000
Transit Time	0.002 (p < 0.0001)	32.326	Transit Time	0.260	0.224

Figure 5 – C0 Level Predicted by Gestational Age & Birth Weight Quintile



There was little difference in the bivariate and multivariate results (Table 18). Bivariate analyses of the association between free carnitine and each independent variable were statistically significant at $P < 0.0001$. These associations were individually able to account for between 0.2% and 1.4% of variation in C0 levels. In the bivariate analysis, free carnitine level was explained most strongly by sex (1.4%) and infant feeding status (1.3%). Gestational age, transit time, and the age of the baby at the time of the blood sample collection each explained less than 0.4% of the variance in C0 (0.3%, 0.2%, and 0.2%, respectively). The multivariate analysis involving all independent variables (including the interaction term between gestational age and birth weight) was statistically significant at $P < 0.0001$ and accounted for 4.2% of variation in free carnitine.

In the multivariate analysis, female infants had on average levels of C0 that were close to 2.86 μM lower than their male counterparts. Infants receiving formula alone, a combination of breast milk and formula, or TPN / other undisclosed forms of nutrition all had generally higher levels of C0 than those reported to be exclusively breastfed (coefficients 2.27, 2.94, and 1.53, respectively). In the multivariable results, newborns receiving a combination of formula and breast milk had the highest concentrations of C0, since the regression coefficient for the "TPN/Other" group (which had the highest levels of C0 in the bivariate analysis) was lower after adjusting for potential confounding factors. Samples collected from newborns of ages 36-47 and 48-71 hours had generally lower levels of C0 than those collected from infants 24-35 hours of age (coefficients -1.28, and -1.09, respectively), though the effect sizes were small. Free carnitine level decreased as socioeconomic quintile increased. Compared with newborns in the highest income quintile, those in lower quintiles had higher levels of C0 (from lower to higher income quintiles, compared with the highest quintile, coefficients were 2.31, 1.58, 1.08, and 0.60, respectively). C0 level also increased by approximately 0.22 μM for every unit increase in transit time (days).

In the bivariate analysis, in comparison with term infants, all categories of premature neonates had higher levels of C0 (from extremely premature through late premature categories related to term infants, coefficients 7.76, 7.72, 6.15, and 1.81, respectively). Infants in birth weight quintiles 1 and 2 had on average higher levels of C0 than those in birth weight quintile 5 (coefficients 2.02 and 0.09, respectively). Infants in birth weight quintiles 3 and 4 had generally lower levels, though the effect sizes were small (coefficients -0.43 and -0.59, respectively).

In the multivariate analysis, the interaction between birth weight and gestational age was significant ($P < 0.0001$). We explored this interaction by graphing the conditional mean levels of free carnitine (y axis) by gestational age (x axis) stratified by birth weight (Figure 5 & Appendix D) and adjusted for all other variables in the multivariable model. Free carnitine level varied considerably with birth weight quintile in extremely, very, and medium premature infants. This level of variation generally decreased with increasing gestational age. In term infants, the level of variation with birth weight was considerably lower than in any category of preterm neonates. Figure 5 also shows that in general, newborns in a lower birth weight quintile tended on average to have higher levels of C0 than those in higher quintiles, with the largest difference between quintile 1 and quintile 2. Generally, C0 levels decreased with increased gestational age, the exception being in neonates in the lowest quintile of birth weight, where C0 levels in very premature infants were higher than those in the extremely premature (Figure 5).

4.2.1.2 Associations of Independent Variables with C8

Multivariate R-Square: 0.017 ($P < 0.0001$)

Most important predictors: infant feeding status (0.7%) and sex (0.5%)

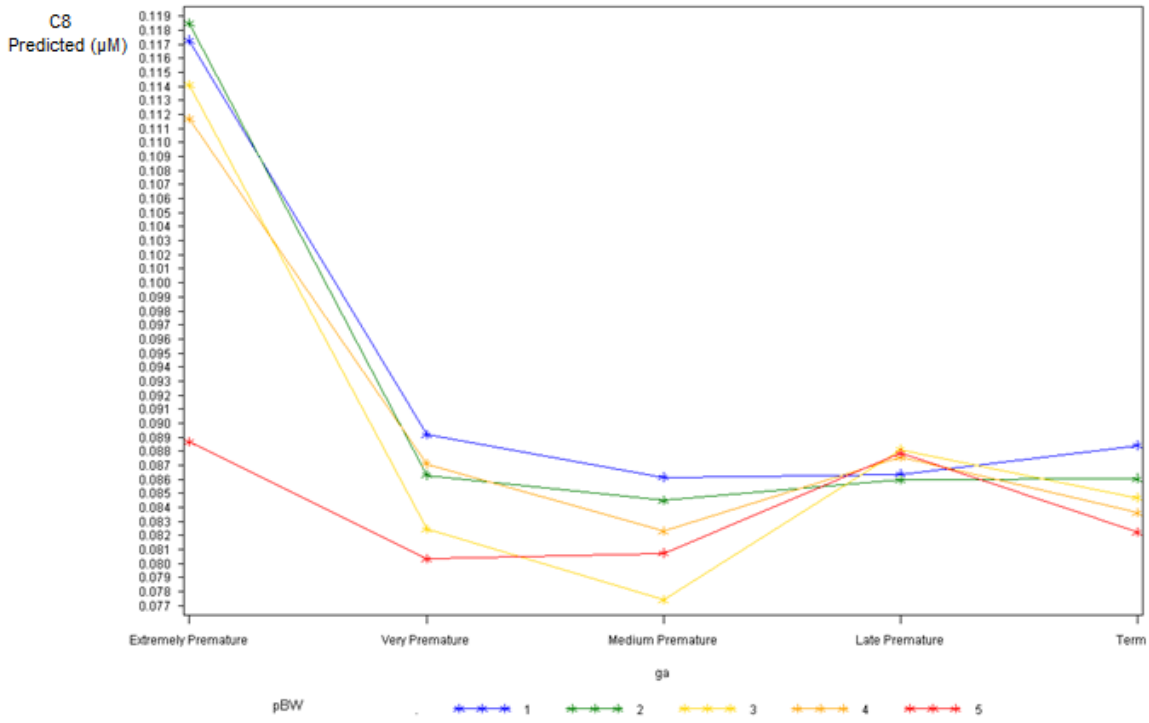
Interaction between birth weight & gestational age (trends):

- C8 level was most strongly associated with birth weight quintile in extremely premature (highest birth weight quintile had lowest C8 levels by 20% - 25%)
- Level of variation with birth weight decreased with increasing gestational age
- Inverse association between C8 level and birth weight quintile
- C8 stable across gestational age (with exception of extremely premature)

Table 19 – Factors Associated with C8 in Newborn Infants in Ontario (2007-2009)

C8	R-Square	Intercept (µM)	Regression Coefficient (µM)		
Multivariate Model	0.017 (p < 0.0001)	0.087	**Categories**	**Bivariate Model**	**Multivariate Model**
Infant Feeding	0.007 (p < 0.0001)	0.087	Breast Milk & Formula	-0.004	-0.005
			Breast Milk Only	0.000	0.000
			Formula Only	-0.011	-0.012
			Other	-0.003	-0.009
Sex	0.005 (p < 0.0001)	0.088	Female	-0.006	-0.006
			Male	0.000	0.000
Gestational Age	0.000 (p < 0.0001)	0.085	Extremely Premature	0.025	-----
			Very Premature	0.000	-----
			Medium Premature	-0.003	-----
			Late Premature	0.002	-----
			Term	0.000	-----
Age of Collection	0.000 (p < 0.0001)	0.086	24-35 Hours	0.000	0.000
			36-47 Hours	-0.002	-0.002
			48-71 Hours	<0.001	<0.001
Birth Weight Quintile	0.002 (p < 0.0001)	0.082	Q1	0.006	-----
			Q2	0.004	-----
			Q3	0.002	-----
			Q4	0.001	-----
			Q5	0.000	-----
Interaction Term (BW*GA)	0.003 (p < 0.0001)	-----	(See Appendix D for conditional means)	-----	-----
Socio Economic Status Quintile	0.000 (p = 0.0392)	0.085	Q1	<0.001	0.001
			Q2	<0.001	<0.001
			Q3	<0.001	<0.001
			Q4	<0.001	<0.001
			Q5	0.000	0.000
Transit Time	0.000 (p = 0.0019)	0.085	Transit Time	<0.001	<0.001

Figure 6 – C8 Level Predicted by Gestational Age & Birth Weight Quintile



There was little difference in the bivariate and multivariate results (Table 19). Bivariate analyses of the association between C8 level and each independent variable were statistically significant at $P < 0.0001$ with the exception of the relationship between C8 level and socioeconomic quintile ($P = 0.0392$), and C8 level and transit time ($P = 0.0019$). These associations were individually able to account for between 0.0% - 0.7% of variation in C8. In the bivariate analysis, C8 level was explained most strongly by infant feeding status (0.7%) and sex (0.5%). The multivariate analysis involving all independent variables (including the interaction term between gestational age and birth weight) was statistically significant at $P < 0.0001$ and accounted for 1.7% of variation in C8.

In the multivariable analysis, female neonates had on average levels of C8 that were 0.006 µM lower than their male counterparts. Infants receiving formula alone, a combination

of breast milk and formula, or TPN / other undisclosed forms of nutrition all had generally lower levels of C8 than those reported to be exclusively breastfed (coefficients -0.012, -0.005, and -0.009, respectively), with newborns receiving formula alone demonstrating the lowest concentrations of C8. As with free carnitine, the category of "TPN/Other" experienced the greatest change (from -0.003 to -0.009) after adjusting for potential confounding factors. Samples collected from newborns of ages 36-47 and 48-71 hours had generally lower levels of C8 than those collected from 24-35 hours of age (coefficients -0.002, and <0.001, respectively), though the effect sizes were small. In the multivariable model, C8 level decreased on average by very small amounts with increasing socioeconomic quintile (coefficients 0.001, <0.001, <0.001, and <0.001, respectively), whereas in the bivariate analysis C8 level generally increased with socioeconomic quintile, though the effect size was also very small. With a similarly small effect size, C8 level increased by less than 0.001 μM for every unit increase in transit time (days).

In the bivariate analysis, in comparison with term infants, extremely premature, very premature, and late premature neonates had generally higher levels of C8 (coefficients 0.025, <0.001, and 0.002, respectively), while medium premature neonates had generally lower levels (coefficient -0.003). C8 level decreased with increasing birth weight quintile (coefficient 0.006, 0,004, 0.002, and 0.001, respectively).

In the multivariable model, the interaction between birth weight and gestational age was again statistically significant ($P < 0.0001$). The mean C8 level conditional on all other independent variables in the analysis appeared to be strongly associated with birth weight quintile in extremely premature infants (Figure 6 & Appendix D); specifically, newborns in

the highest quintile of birth weight had 20% - 25% lower levels of C8 compared with those in the other birth weight quintiles. This level of variation generally decreased with increasing gestational age. In term and late premature infants, C8 levels were similar across quintiles of birth weight. Where differences were observed, neonates in lower birth weight quintiles had on average higher levels of C8 than those in higher quintiles.

4.2.1.3 Associations of Independent Variables with Total Carnitine

Multivariate R-Square: 0.026 ($P < 0.0001$)

Most important predictors: sex (1.4%) and socioeconomic status (0.5%)

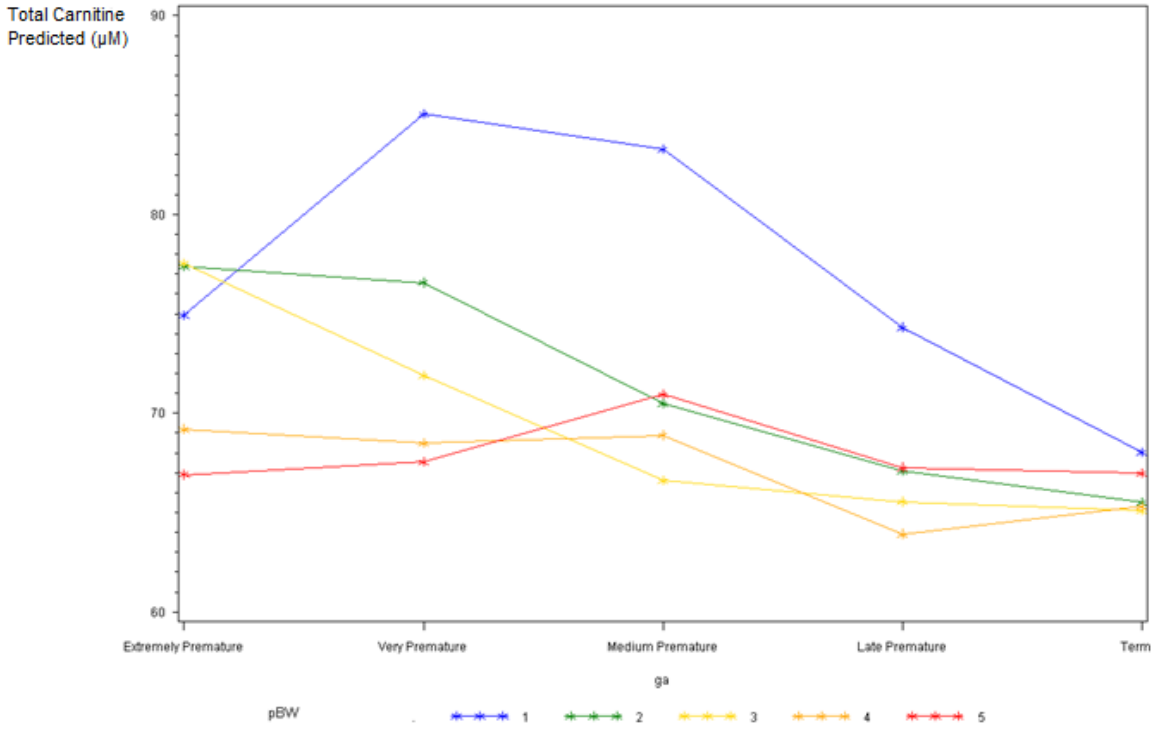
Interaction between birth weight & gestational age (trends):

- Total carnitine level varied with birth weight quintile (level of variation decreased with increasing gestational age)
- Inverse association between total carnitine level and birth weight quintile
- Inverse association between total carnitine level and gestational age (with the exception of the highest birth weight quintile)

Table 20 – Factors Associated with Total Carnitine in Newborn Infants in Ontario (2007-2009)

Total Carnitine	R-Square	Intercept (µM)	Regression Coefficient (µM)		
Multivariate Model	0.026 (p < 0.0001)	67.324	**Categories**	**Bivariate Model**	**Multivariate Model**
Infant Feeding	0.004 (p <0.0001)	65.477	Breast Milk & Formula	3.799	3.157
			Breast Milk Only	0.000	0.000
			Formula Only	1.768	1.156
			Other	2.242	-1.598
Sex	0.014 (p <0.0001)	68.664	Female	-4.852	-4.798
			Male	0.000	0.000
Gestational Age	0.001 (p <0.0001)	66.232	Extremely Premature	7.146	-----
			Very Premature	7.597	-----
			Medium Premature	5.968	-----
			Late Premature	1.416	-----
			Term	0.000	-----
Age of Collection	0.000 (p <0.0001)	66.160	24-35 Hours	0.000	0.000
			36-47 Hours	-0.140	-0.183
			48-71 Hours	1.060	0.650
Birth Weight Quintile	0.004 (p <0.0001)	66.992	Q1	1.355	-----
			Q2	-1.380	-----
			Q3	-1.862	-----
			Q4	-1.721	-----
			Q5	0.000	-----
Interaction Term (BW*GA)	0.005 (p < 0.0001)	-----	(See Appendix D for conditional means)	-----	-----
Socio Economic Status Quintile	0.005 (p <0.0001)	64.200	Q1	4.191	3.691
			Q2	2.794	2.397
			Q3	1.788	1.568
			Q4	0.911	0.818
			Q5	0.000	0.000
Transit Time	0.000 (p = 0.0004)	66.598	Transit Time	-0.064	-0.104

Figure 7 – Total Carnitine Level Predicted by Gestational Age & Birth Weight Quintile



There was very little difference in the bivariate and multivariate results (Table 20). Bivariate analyses of the association between total carnitine level and each independent variable were statistically significant at $P < 0.0001$ with the exception of the relationship with transit time ($P = 0.0004$). These associations were individually able to account for between 0.0% - 1.4% of variation in total carnitine. Total carnitine level was explained most strongly by sex (1.4%) and socioeconomic status (0.5%) in the bivariate analyses. The multivariate analysis involving all independent variables (including the interaction term between gestational age and birth weight) was statistically significant at $P < 0.0001$ and accounted for 2.6% of variation in total carnitine.

In the multivariable analysis, female infants had on average levels of total carnitine that were approximately 4.80 µM lower than their male counterparts. Infants receiving

formula alone or a combination of breast milk and formula had generally higher levels of total carnitine than those reported to be exclusively breastfed (coefficients 1.16 and 3.16, respectively), while neonates receiving TPN / other undisclosed forms of nutrition had generally lower levels (coefficient -1.60); this latter finding differed from the bivariate results. Infants receiving a combination of breast milk and formula had the highest levels of total carnitine. Similarly, total carnitine concentration was highest in samples collected from newborns of ages 48-71 hours (coefficient 0.65), although the effect was stronger in the bivariate analysis; and was lowest in those collected between 36-47 hours of age (coefficient -0.18). Total carnitine level decreased with increasing socioeconomic quintile. Compared with newborns in the highest income quintile, those in lower quintiles had higher levels of total carnitine (relative to the highest quintile, coefficients for the lowest to second highest quintiles were 3.69, 2.40, 1.57, and 0.82, respectively). Total carnitine level decreased by approximately 0.10 μM for every unit increase in transit time (days).

In the bivariate analysis, in comparison with term infants all categories of premature neonates had higher levels of total carnitine. With the exception of very premature neonates (who had on average the highest total carnitine levels), total carnitine decreased as gestational age increased (coefficients 7.15, 7.60, 6.00, and 1.42, respectively). Infants in birth weight quintile 1 had on average higher levels of total carnitine than those in birth weight quintile 5 (coefficient 1.36), while infants in quintiles 2, 3, and 4 had generally lower levels (coefficients 1.38, -1.86, and -1.72, respectively).

In the multivariable model, the interaction of gestational age and birth weight in relation to total carnitine concentration was statistically significant ($P < 0.0001$). Total

carnitine level varied considerably with birth weight quintile in extremely, very, and medium premature infants (Figure 7 & Appendix D). This level of variation generally decreased with increasing gestational age. In term infants, the concentration of total carnitine varied little across birth weight quintiles. Infants in lower birth weight quintiles had on average higher levels of total carnitine than those in higher quintiles. In these lower birth weight individuals, total carnitine concentrations generally declined with increased gestational age, with the exception of an increase in very premature relative to extremely premature newborns. Total carnitine levels in higher birth weight individuals remained fairly constant across gestational age (Figure 7).

4.2.1.4 Associations of Independent Variables with Total Acylcarnitine

Multivariate R-Square: 0.026 ($P < 0.0001$)

Most important predictors: sex (1.0%), age of collection (0.5%), and transit time (0.4%)

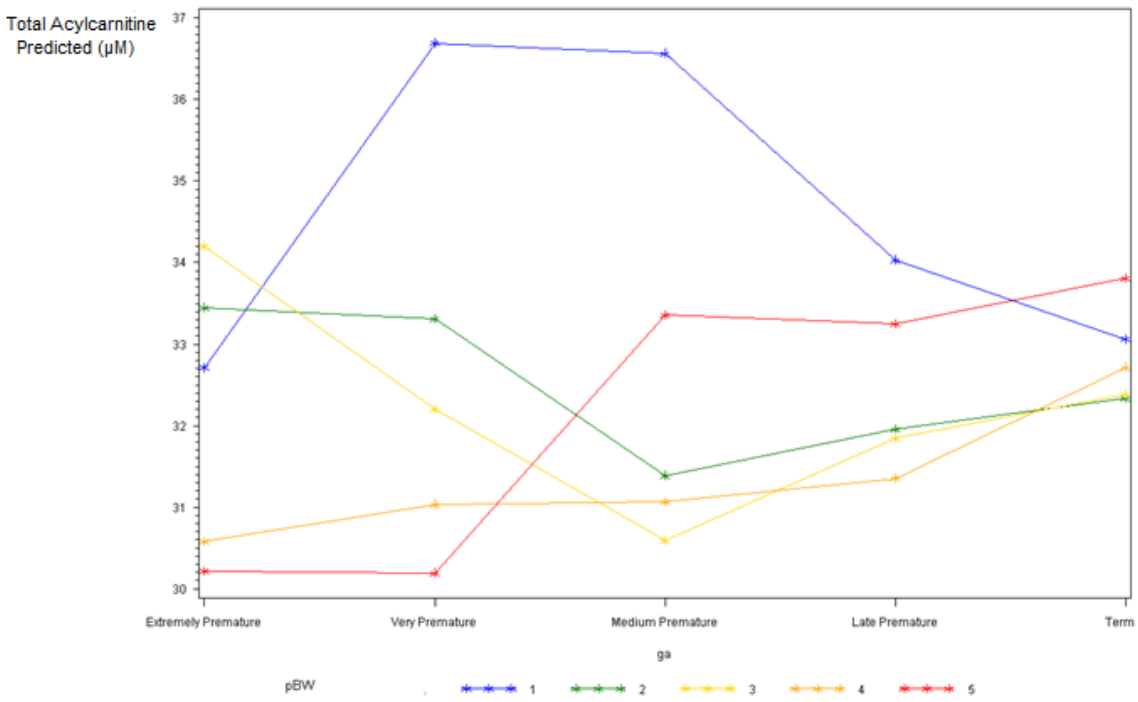
Interaction between birth weight & gestational age (trends):

- Total acylcarnitine level varied with birth weight quintile (level of variation decreased with increasing gestational age)
- Inverse association between total acylcarnitine level and birth weight quintile in extremely, very premature; no consistent association in later gestational age categories
- Total acylcarnitine level steady across gestational age with exception of late premature and term, where levels were higher than in earlier gestational ages

Table 21 – Factors Associated with Total Acylcarnitine in Newborn Infants in Ontario (2007-2009)

Total Acylcarnitine	R-Square	Intercept (µM)	Regression Coefficient (µM)		
Multivariate Model	0.026 (p <0.0001)	35.037	**Categories**	**Bivariate Model**	**Multivariate Model**
Infant Feeding	0.002 (p <0.0001)	32.929	Breast Milk & Formula	0.391	0.212
			Breast Milk Only	0.000	0.000
			Formula Only	-0.968	-1.105
			Other	-2.017	-3.082
Sex	0.010 (p <0.0001)	33.817	Female	-1.965	-1.944
			Male	0.000	0.000
Gestational Age	0.000 (p <0.0001)	32.900	Extremely Premature	-0.574	-----
			Very Premature	-0.238	-----
			Medium Premature	-0.258	-----
			Late Premature	-0.399	-----
			Term	0.000	-----
Age of Collection	0.005 (p <0.0001)	32.328	24-35 Hours	0.000	0.000
			36-47 Hours	1.095	1.092
			48-71 Hours	1.767	1.749
Birth Weight Quintile	0.003 (p <0.0001)	33.779	Q1	-0.664	-----
			Q2	-1.460	-----
			Q3	-1.419	-----
			Q4	-1.125	-----
			Q5	0.000	-----
Interaction Term (BW*GA)	0.004 (p <0.0001)	-----	(See Appendix D for conditional means)	-----	-----
Socio Economic Status Quintile	0.002 (p <0.0001)	32.312	Q1	1.261	1.385
			Q2	0.694	0.822
			Q3	0.367	0.500
			Q4	0.153	0.227
			Q5	0.000	0.000
Transit Time	0.004 (p <0.0001)	34.267	Transit Time	-0.324	-0.327

Figure 8 – Total Acylcarnitine Level Predicted by Gestational Age & Birth Weight Quintile



There was very little difference in the bivariate and multivariate results (Table 21). Bivariate analyses of the association between total acylcarnitine level and each independent variable were statistically significant at $P < 0.0001$. These associations were individually able to account for between 0.0% - 1.0% of variation in total acylcarnitine. Total acylcarnitine level was explained most strongly by sex (1.0%), age of collection (0.5%), and transit time (0.4%) in the bivariate analyses. The multivariate analysis involving all independent variables (including the interaction term between gestational age and birth weight) was statistically significant at $P < 0.0001$ and accounted for 2.6% of variation in total acylcarnitine.

In the multivariable analysis, female infants had levels of total acylcarnitine that were on average close to 1.94 μM lower than their male counterparts. Infants receiving formula alone or TPN / other undisclosed forms of nutrition had generally lower levels of total acylcarnitine than those reported to be exclusively breastfed or receiving a combination of breast milk and formula (coefficients -1.11 and -3.08, respectively). Infants fed on a combination of breast milk and formula had the highest average levels of total acylcarnitine (coefficient 0.21). Total acylcarnitine level increased with age at collection. Samples collected from newborns of ages 36-47 and 48-71 hours had generally higher levels of total acylcarnitine than those collected from 24-35 hours of age (coefficients 1.09 and 1.75, respectively). Total acylcarnitine level decreased with increasing socioeconomic quintile. Compared with newborns in the highest income quintile, those in lower quintiles had higher levels of total acylcarnitine (for lower to higher quintiles, relative to the highest income quintile, coefficients 1.39, 0.82, 0.50, and 0.23, respectively). Total acylcarnitine level decreased by approximately 0.32 μM for every unit increase in transit time (days).

In the bivariate analysis, in comparison with term infants all categories of premature neonates had lower levels of total acylcarnitine (coefficients -0.57, -0.24, -0.26, and -0.40, respectively). In comparison with infants in the highest birth weight quintile, those in quintiles 1, 2, 3, and 4 had on average lower levels of total acylcarnitine (coefficients -0.66, -1.46, -1.42, and -1.13, respectively), with the lowest average concentrations occurring in quintiles 2 and 3.

As with the other analytes reported, in the multivariate model the interaction between gestational age and birth weight was significantly associated ($P < 0.0001$) with the

concentration of total acylcarnitine in neonatal blood. Specifically, total acylcarnitine level varied considerably with birth weight quintile in extremely, very, and medium premature infants (Figure 8 & Appendix D). Again, this level of variation generally decreased with increasing gestational age. In term infants, the concentration of total acylcarnitine varied little across birth weight quintiles. Neonates in the lowest birth weight quintile demonstrated generally higher levels of total acylcarnitine than those in higher quintiles. In this group, total acylcarnitine level decreased with increased gestational age with the exception of the extremely premature category (where levels were similar to those in term neonates). In higher birth weight categories, levels of total acylcarnitines generally increased with gestational age (Figure 8), although results were quite variable.

4.2.1.5 Associations of Independent Variables with Short Chain Acylcarnitine

Multivariate R-Square: 0.026 ($P < 0.0001$)

Most important predictors: sex (0.8%) and age of collection (0.6%)

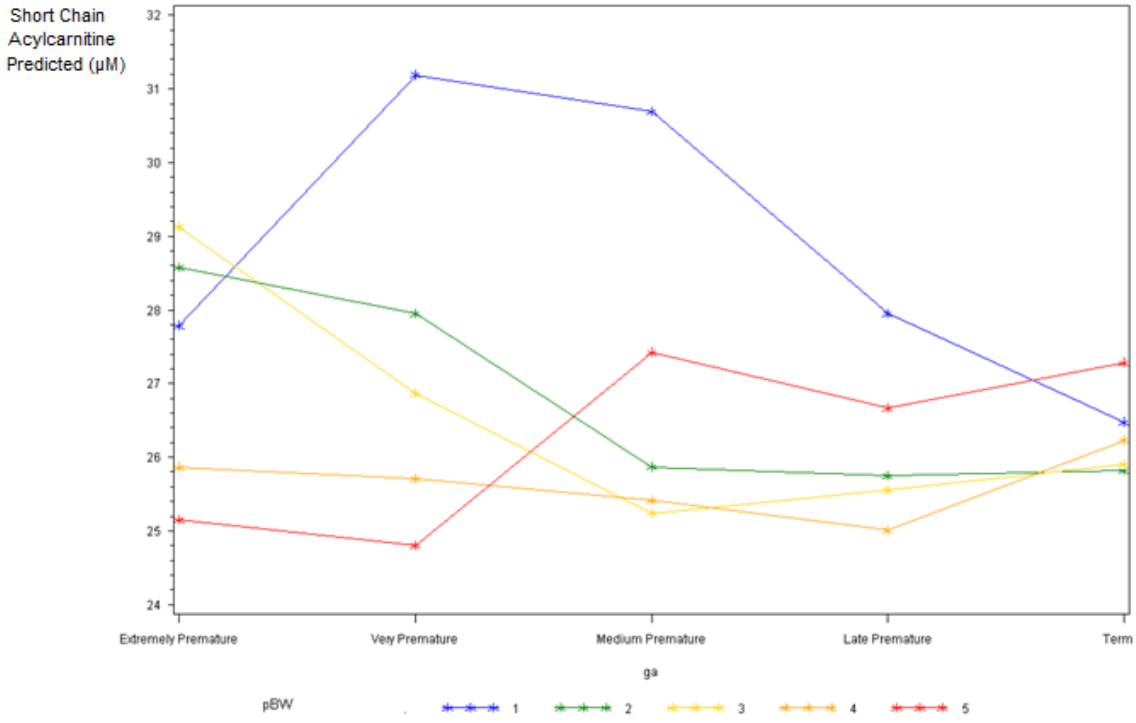
Interaction between birth weight & gestational age (trends):

- Short chain acylcarnitine level varied with birth weight quintile (level of variation decreased with increasing gestational age)
- Inverse association between short chain acylcarnitine level and birth weight quintile in extremely, very premature; no consistent association in later gestational age categories
- Short chain acylcarnitine level steady across gestational age

Table 22 – Factors Associated with Short Chain Acylcarnitine in Newborn Infants in Ontario (2007-2009)

Short Chain Acylcarnitine	R-Square	Intercept (µM)	Regression Coefficient (µM)		
Multivariate Model	0.026 (p < 0.0001)	28.235	**Categories**	**Bivariate Model**	**Multivariate Model**
Infant Feeding	0.001 (p < 0.0001)	26.317	Breast Milk & Formula	0.579	0.419
			Breast Milk Only	0.000	0.000
			Formula Only	-0.322	-0.461
			Other	-0.976	-2.228
Sex	0.008 (p < 0.0001)	27.105	Female	-1.546	-1.522
			Male	0.000	0.000
Gestational Age	0.000 (p < 0.0001)	26.378	Extremely Premature	1.017	-----
			Very Premature	0.908	-----
			Medium Premature	0.594	-----
			Late Premature	-0.181	-----
			Term	0.000	-----
Age of Collection	0.006 (p < 0.0001)	25.869	24-35 Hours	0.000	0.000
			36-47 Hours	1.030	0.998
			48-71 Hours	1.577	1.505
Birth Weight Quintile	0.004 (p < 0.0001)	27.256	Q1	-0.700	-----
			Q2	-1.434	-----
			Q3	-1.370	-----
			Q4	-1.077	-----
			Q5	0.000	-----
Interaction Term (BW*GA)	0.005 (p < 0.0001)	-----	(See Appendix D for conditional means)	-----	-----
Socio Economic Status Quintile	0.003 (p < 0.0001)	25.816	Q1	1.213	1.278
			Q2	0.692	0.773
			Q3	0.367	0.463
			Q4	0.158	0.218
			Q5	0.000	0.000
Transit Time	0.004 (p < 0.0001)	27.674	Transit Time	-0.304	-0.307

Figure 9 – Short Chain Acylcarnitine Level Predicted by Gestational Age & Birth Weight Quintile



There was very little difference in the bivariate and multivariate results (Table 22). Bivariate analyses of the association between short chain acylcarnitine level and each independent variable were statistically significant at $P < 0.0001$. These associations were individually able to account for between 0.0% - 0.8% of variation in short chain acylcarnitine. In the bivariate analysis, short chain acylcarnitine level was explained most strongly by sex (0.8%) and age of collection (0.6%). The multivariate analysis involving all independent variables (including the interaction term between gestational age and birth weight) was statistically significant at $P < 0.0001$ and accounted for 2.6% of variation in short chain acylcarnitine.

In the multivariable analysis, female infants had on average levels of short chain acylcarnitines that were 1.52 μM lower than their male counterparts. Infants receiving formula alone or TPN / other undisclosed forms of nutrition had generally lower levels of short chain acylcarnitine than those reported to be exclusively breastfed (coefficients -0.46 and -2.23, respectively). Infants fed on a combination of breast milk and formula had the highest levels of short chain acylcarnitine (coefficient 0.42). Short chain acylcarnitine level increased with age of collection. Samples collected from newborns of ages 36-47 and 48-71 hours had generally higher levels of short chain acylcarnitine than those collected from 24-35 hours of age (coefficients 1.00 and 1.51, respectively). Short chain acylcarnitine level decreased with increasing socioeconomic quintile. Compared with newborns in the highest income quintile, those in lower quintiles had higher levels of short chain acylcarnitine (coefficients for the lowest to second highest quintiles, 1.28, 0.77, 0.46, and 0.22, respectively). Short chain acylcarnitine level also decreased by approximately 0.31 μM for every unit increase in transit time (days).

In the bivariate analysis, in comparison with term infants, extremely, very, and medium premature neonates had generally higher levels of short chain acylcarnitine (coefficients 1.02, 0.91, and 0.59, respectively), while late premature infants had the lowest levels (coefficient -0.18). Infants in birth weight quintiles 1, 2, 3, and 4 had on average lower levels of short chain acylcarnitines than those in birth weight quintile 5 (coefficients -0.70, -1.43, -1.37, and -1.08, respectively). Neonates in birth weight quintiles 2 and 3 had the lowest levels of short chain acylcarnitines.

In the multivariable model, the interaction between gestational age and birth weight was a significant predictor of the analyte concentration ($P < 0.0001$). Short chain acylcarnitine concentrations varied considerably with birth weight quintile in extremely, very, and medium premature infants (Figure 9 & Appendix D). Again, this level of variation generally decreased with increasing gestational age and was not apparent in term infants. Infants in lower birth weight categories had generally elevated levels of short chain acylcarnitine as compared to those in higher birth weight quintiles, with the largest differences seen in quintile 1 versus the higher birth weight categories. Neonates of medium premature or higher gestational age in birth weight quintile 5 were the exception to this trend. This group had generally higher levels of short chain acylcarnitines than those of comparable gestational age in birth weight quintiles 2, 3, and 4. Short chain acylcarnitine rates remained fairly consistent across gestational age (Figure 9).

4.2.1.6 Associations of Independent Variables with Medium Chain Acylcarnitine

Multivariate R-Square: 0.060 ($P < 0.0001$)

Most important predictors: infant feeding status (4.9%) and sex (0.6%)

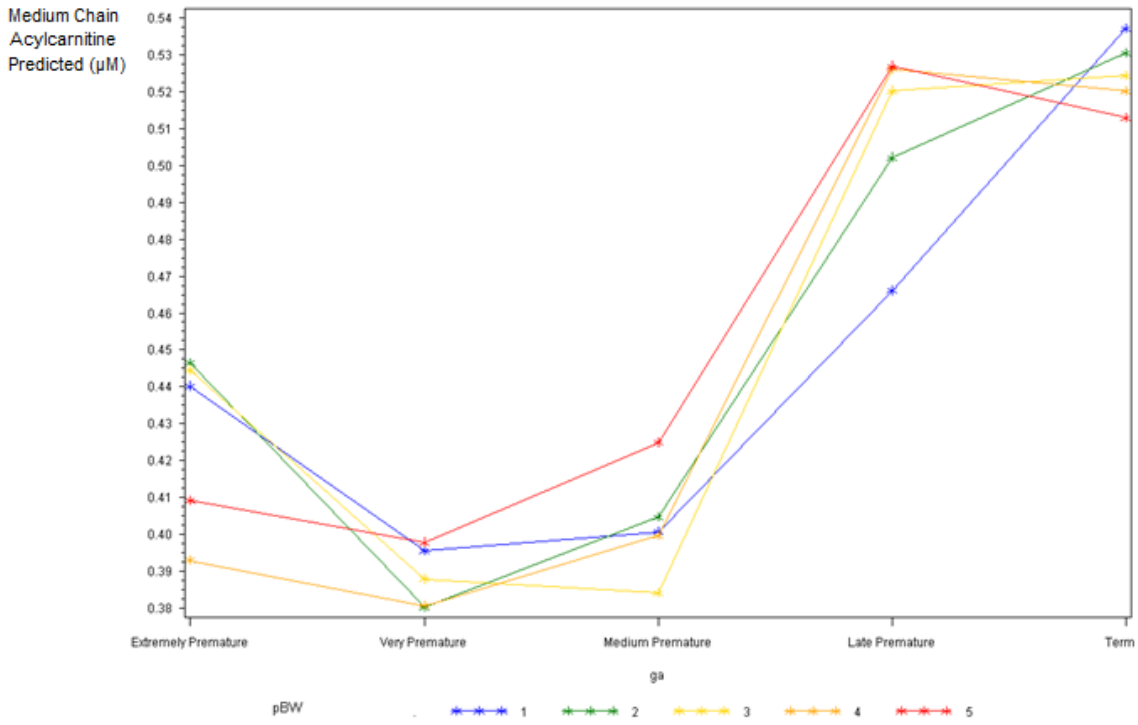
Interaction between birth weight & gestational age (trends):

- Medium chain acylcarnitine level increased with gestational age (with the exception of the extremely to very premature categories)
- Medium chain acylcarnitine steady across birth weight quintiles (except in late premature neonates where it increased with birth weight, and term neonates where an inverse association was present)

Table 23 – Factors Associated with Medium Chain Acylcarnitine in Newborn Infants in Ontario (2007-2009)

Medium Chain Acylcarnitine	R-Square	Intercept (µM)	Regression Coefficient (µM)		
Multivariate Model	0.060 (p < 0.0001)	0.544	**Categories**	**Bivariate Model**	**Multivariate Model**
Infant Feeding	**0.049 (p < 0.0001)**	**0.544**	Breast Milk & Formula	-0.052	-0.054
			Breast Milk Only	0.000	0.000
			Formula Only	-0.100	-0.100
			Other	-0.128	-0.115
Sex	**0.006 (p < 0.0001)**	**0.536**	Female	-0.025	-0.025
			Male	0.000	0.000
Gestational Age	0.004 (p < 0.0001)	0.525	Extremely Premature	-0.097	-----
			Very Premature	-0.137	-----
			Medium Premature	-0.121	-----
			Late Premature	-0.017	-----
			Term	0.000	-----
Age of Collection	0.000 (p = 0.3513)	0.524	24-35 Hours	0.000	0.000
			36-47 Hours	-0.001	0.002
			48-71 Hours	<0.001	0.007
Birth Weight Quintile	0.002 (p < 0.0001)	0.513	Q1	0.021	-----
			Q2	0.016	-----
			Q3	0.011	-----
			Q4	0.007	-----
			Q5	0.000	-----
Interaction Term (BW*GA)	0.007 (p < 0.0001)	-----	(See Appendix D for conditional means)	-----	-----
Socio Economic Status Quintile	0.001 (p < 0.0001)	0.532	Q1	-0.013	-0.003
			Q2	-0.012	-0.004
			Q3	-0.009	-0.003
			Q4	-0.005	-0.003
			Q5	0.000	0.000
Transit Time	0.000 (p = 0.4215)	0.524	Transit Time	<0.001	<0.001

Figure 10 – Medium Chain Acylcarnitine Level Predicted by Gestational Age & Birth Weight Quintile



There was very little difference in the bivariate and multivariate results (Table 23). Bivariate analyses of the association between medium chain acylcarnitine level and each independent variable were statistically significant at $P < 0.0001$ with the exception of the relationship between medium chain acylcarnitine level and age of collection ($P = 0.3513$), and medium chain acylcarnitine level and transit time ($P = 0.4215$). These associations were individually able to account for between 0.0% - 4.9% of variation in medium chain acylcarnitine. Medium chain acylcarnitine level was explained most strongly by infant feeding status (4.9%) and sex (0.6%) in the bivariate analyses. The multivariate analysis involving all independent variables (including the interaction term between gestational age

and birth weight) was statistically significant at $P < 0.0001$ and accounted for 6.0% of variation in medium chain acylcarnitine.

In the multivariable model, female infants had on average lower levels of medium chain acylcarnitines than their male counterparts (coefficient -0.025) but the effect size was small. Infants receiving formula alone, a combination of breast milk and formula, or TPN / other undisclosed forms of nutrition all had generally lower levels of medium chain acylcarnitine than those reported to be exclusively breastfed (coefficients -0.100, -0.054, and -0.115, respectively). Newborns receiving a combination of formula and breast milk had the lowest concentrations of medium chain acylcarnitines after adjusting for confounding factors in the multivariable model. In contrast to the bivariate results (where the association was not statistically significant), in the multivariable model, medium chain acylcarnitine level increased with age at time of collection, with samples taken from newborns of ages 36-47 and 48-71 hours having generally higher levels of medium chain acylcarnitine than those collected from 24-35 hours of age (coefficients 0.002 and 0.007, respectively), though the effect sizes were small. As in the bivariate analysis, income quintiles 1, 2, 3, and 4 each had lower levels of medium chain acylcarnitine than quintile 5 (coefficients -0.003, -0.004, -0.003, and -0.003, respectively). In the multivariate model, the lowest levels presented in neonates of socioeconomic quintile 2, whereas in the bivariate model the lowest concentrations were seen in quintile 1. Medium chain acylcarnitine level also increased with transit time but the amount increase for every unit increase in transit time (days) was less than 0.001 μM.

In the bivariate model, in comparison with term infants all categories of premature neonates had lower levels of medium chain acylcarnitine (coefficients -0.097, -0.137, -0.121, and -0.017, respectively). Infants in the lower categories of gestational age had generally lower levels of medium chain acylcarnitines. This level decreased as birth weight quintile increased, but effect sizes were small compared with the association with gestational age. Infants in birth weight quintiles 1, 2, 3, and 4 had on average higher levels of medium chain acylcarnitines than those in birth weight quintile 5 (coefficients 0.021, 0.016, 0.011, and 0.007, respectively).

In the multivariable results, the interaction between gestational age and birth weight was significantly associated with the concentration of medium chain acylcarnitines in the multivariable model. The mean concentration, adjusted for all of the other predictors in the analysis, generally increased with gestational age, with the exception of a small decline between extremely and very premature individuals (Figure 10 & Appendix D). In late premature neonates, medium chain acylcarnitine levels increased with birth weight, while in term neonates the opposite correlation was observed, although in both cases the relationship with birth weight appeared smaller relative to the differences across categories of gestational age. No relationship with birth weight was apparent in extremely, very, and medium premature neonates (Figure 10).

4.2.1.7 Associations of Independent Variables with Long Chain Acylcarnitine

Multivariate R-Square: 0.031 ($P < 0.0001$)

Most important predictors: sex (1.4%) and infant feeding status (1.2%)

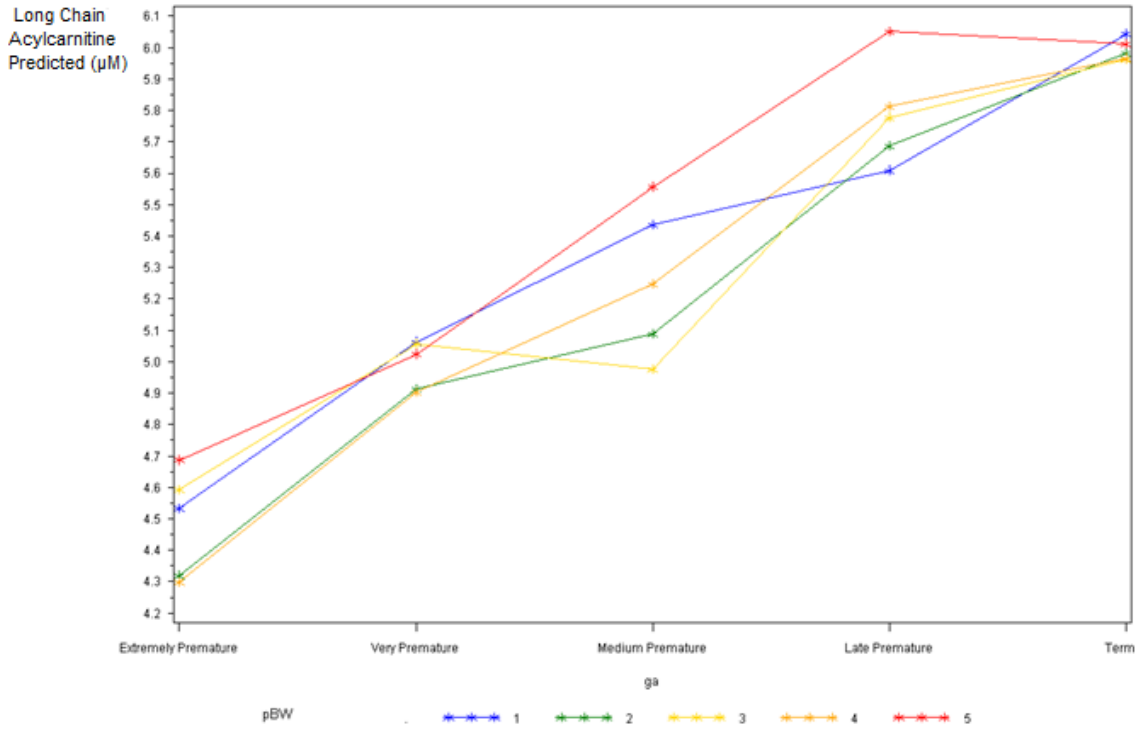
Interaction between birth weight & gestational age (trends):

- Long chain acylcarnitine level increased with gestational age
- Long chain acylcarnitine level stable across birth weight

Table 24 – Factors Associated with Long Chain Acylcarnitine in Newborn Infants in Ontario (2007-2009)

Long Chain Acylcarnitine	R-Square	Intercept (µM)	Regression Coefficient (µM)		
Multivariate Model	0.031 (p < 0.0001)	6.257	**Categories**	**Bivariate Model**	**Multivariate Model**
Infant Feeding	0.012 (p < 0.0001)	6.067	Breast Milk & Formula	-0.137	-0.154
			Breast Milk Only	0.000	0.000
			Formula Only	-0.548	-0.546
			Other	-0.910	-0.724
Sex	0.014 (p < 0.0001)	6.174	Female	-0.394	-0.396
			Male	0.000	0.000
Gestational Age	0.002 (p < 0.0001)	5.995	Extremely Premature	-1.506	-----
			Very Premature	-1.009	-----
			Medium Premature	-0.731	-----
			Late Premature	-0.206	-----
			Term	0.000	-----
Age of Collection	0.002 (p < 0.0001)	5.934	24-35 Hours	0.000	0.000
			36-47 Hours	0.066	0.091
			48-71 Hours	0.186	0.235
Birth Weight Quintile	0.000 (p < 0.0001)	6.009	Q1	0.013	-----
			Q2	-0.045	-----
			Q3	-0.061	-----
			Q4	-0.054	-----
			Q5	0.000	-----
Interaction Term (BW*GA)	0.003 (p < 0.0001)	-----	(See Appendix D for conditional means)	-----	-----
Socio Economic Status Quintile	0.000 (p < 0.0001)	5.961	Q1	0.062	0.111
			Q2	0.017	0.057
			Q3	0.010	0.042
			Q4	0.002	0.015
			Q5	0.000	0.000
Transit Time	0.000 (p < 0.0001)	6.067	Transit Time	-0.020	-0.020

Figure 11 – Long Chain Acylcarnitine Level Predicted by Gestational Age & Birth Weight Quintile



There was very little difference in the bivariate and multivariate results (Table 24). Bivariate analyses of the association between long chain acylcarnitine level and each independent variable were statistically significant at $P < 0.0001$. These associations were individually able to account for between 0.0% - 1.4% of variation in long chain acylcarnitine concentrations. Long chain acylcarnitine level was explained most strongly by sex (1.4%) and infant feeding status (1.2%) in the bivariate analyses. The multivariate analysis involving all independent variables (including the interaction term between gestational age and birth weight) was statistically significant at $P < 0.0001$ and accounted for 3.1% of variation in long chain acylcarnitine.

In the multivariable analysis, female infants had levels of long chain acylcarnitine that were approximately 0.40 μM lower on average than those found in their male counterparts. Infants receiving formula alone, a combination of breast milk and formula, or TPN / other undisclosed forms of nutrition all had generally lower levels of long chain acylcarnitine than those reported to be exclusively breastfed (coefficients -0.55, -0.15, and -0.72, respectively), with newborns receiving TPN / alternative forms of nutrition having the lowest concentrations of long chain acylcarnitine. Long chain acylcarnitine level increased with age at which the blood sample was collected. Samples taken from newborns of ages 36-47 and 48-71 hours had generally higher levels of long chain acylcarnitine than those collected from 24-35 hours of age (coefficients 0.09, and 0.24, respectively). Long chain acylcarnitine level decreased with increasing socioeconomic quintile. Compared with newborns in the highest income quintile, those in lower quintiles had higher levels of long chain acylcarnitine (coefficients for quintiles 1,2,3, and 4 relative to quintile 5: 0.11, 0.06, 0.04, and 0.02, respectively). Long chain acylcarnitine level also decreased by approximately 0.02 μM for every unit increase in transit time (days).

In the bivariate model, in comparison with term infants all categories of premature neonates had progressively (with increased prematurity) lower levels of long chain acylcarnitines (from extremely premature through to late premature infants, relative to term infants, coefficients -1.51, -1.01, -0.73, and -0.21, respectively). Infants in birth weight quintiles 2, 3, and 4 had on average lower levels of long chain acylcarnitine than those in birth weight quintile 5 (coefficients -0.05, -0.06, and -0.05, respectively), while infants in birth weight quintile 1 had the highest levels (coefficient 0.01), though the effect sizes were small.

In the multivariable model, the interaction of gestational age and birth weight was a significant predictor of the concentration of long chain acylcarnitines, but gestational age appeared to be a much stronger predictor than birth weight when comparing the conditional means (Figure 11 & Appendix D). The degree of variation in long chain acylcarnitines across birthweight quintiles remained relatively steady and small within each category of premature gestational age, with virtually no differences in concentration across birth weight quintiles among term infants. Long chain acylcarnitine levels increased with gestational age (Figure 11).

4.2.1.8 Associations of Independent Variables with the Ratio of Acylcarnitine to Free Carnitine

Multivariate R-Square: 0.110 ($P < 0.0001$)

Most important predictors: transit time (2.0%) and sex (1.4%)

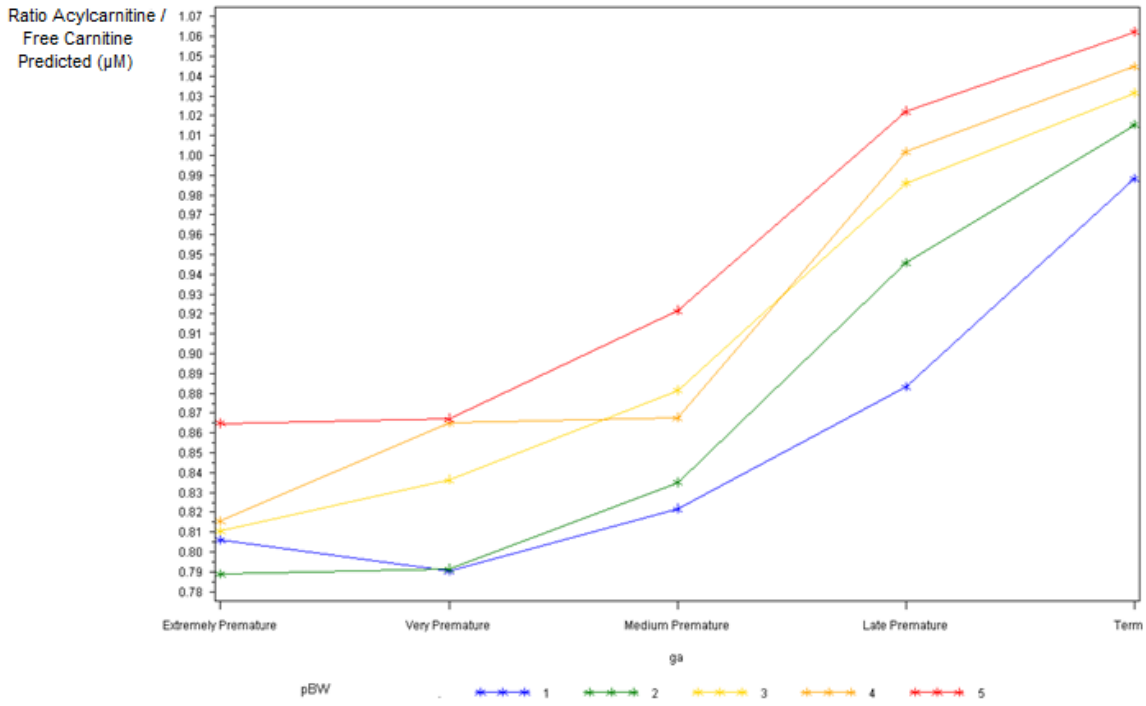
Interaction between birth weight & gestational age (trends):

- The ratio of total acylcarnitine to free carnitine increased with birth weight
- The ratio of total acylcarnitine to free carnitine increased with gestational age

Table 25 – Factors Associated with the Ratio of Acylcarnitine to Free Carnitine in Newborn Infants in Ontario (2007-2009)

Ratio Total Acylcarnitine / Free Carnitine	R-Square	Intercept	Regression Coefficient		
Multivariate Model	0.110 (p < 0.0001)	1.120	**Categories**	**Bivariate Model**	**Multivariate Model**
Infant Feeding	0.004 (p < 0.0001)	1.054	Breast Milk & Formula	-0.087	-0.080
			Breast Milk Only	0.000	0.000
			Formula Only	-0.114	-0.105
			Other	-0.173	-0.140
Sex	0.014 (p < 0.0001)	1.013	Female	0.025	0.026
			Male	0.000	0.000
Gestational Age	0.001 (p < 0.0001)	1.029	Extremely Premature	-0.212	-----
			Very Premature	-0.199	-----
			Medium Premature	-0.164	-----
			Late Premature	-0.061	-----
			Term	0.000	-----
Age of Collection	0.000 (p < 0.0001)	0.993	24-35 Hours	0.000	0.000
			36-47 Hours	0.079	0.079
			48-71 Hours	0.086	0.097
Birth Weight Quintile	0.004 (p < 0.0001)	1.060	Q1	-0.077	-----
			Q2	-0.048	-----
			Q3	-0.031	-----
			Q4	-0.018	-----
			Q5	0.000	-----
Interaction Term (BW*GA)	0.020 (p < 0.0001)	-----	(See Appendix D for conditional means)	-----	-----
Socio Economic Status Quintile	0.005 (p < 0.0001)	1.054	Q1	-0.048	-0.026
			Q2	-0.041	-0.021
			Q3	-0.030	-0.015
			Q4	-0.018	-0.010
			Q5	0.000	0.000
Transit Time	0.020 (p < 0.0001)	1.100	Transit Time	-0.017	-0.016

Figure 12 – Ratio Total Acylcarnitine to Free Carnitine Level Predicted by Gestational Age & Birth Weight Quintile



There was very little difference in the bivariate and multivariate results (Table 25). Bivariate analyses of the association between the ratio of acylcarnitine to free carnitine and each independent variable were statistically significant at $P < 0.0001$. These associations were individually able to account for up to 2.0% of variation in this ratio. The ratio of total acylcarnitine to free carnitine was explained to the greatest degree by transit time (2.0%) and sex (1.4%) in the bivariate analyses. The multivariate analysis involving all independent variables (including the interaction term between gestational age and birth weight) was statistically significant at $P < 0.0001$ and accounted for 11.0% of variation in the ratio of acylcarnitine to free carnitine.

In the multivariable analysis, female infants had on average a higher ratio of total acylcarnitine to free carnitine than their male counterparts (coefficient 0.03). Infants

receiving TPN / other undisclosed forms of nutrition, formula alone, or a combination of breast milk and formula all had generally lower ratios than those reported to be exclusively breastfed (coefficients -0.14, -0.11, and -0.08, respectively), with newborns receiving TPN or other forms of nutrition having the lowest ratios of acylcarnitine to free carnitine. This ratio increased with age at time of sample collection. Samples taken from newborns of ages 36-47 and 48-71 hours had generally higher ratios than those collected from 24-35 hours of age (coefficients 0.08, and 0.10, respectively). The ratio of acylcarnitine to free carnitine increased with socioeconomic quintile. Income quintiles 1, 2, 3, and 4 all had lower ratios than quintile 5 (coefficients -0.03, -0.02, -0.02, and -0.01, respectively). This ratio decreased by approximately 0.02 for every unit increase in transit time (days).

In the bivariate analysis, in comparison with term infants all categories of premature neonates had progressively (with increased prematurity) lower ratios of acylcarnitine to free carnitine (coefficients from extremely premature to late premature, relative to term, of -0.21, -0.20, -0.16, and -0.06, respectively). The ratio increased with birth weight quintile. Infants in quintiles 1, 2, 3, and 4 had on average lower ratios of acylcarnitine to free carnitine than those in birth weight quintile 5 (coefficients -0.08, -0.05, -0.03, and -0.02, respectively).

In the multivariable model, the interaction between gestational age and birth was a significant predictor of the ratio between total acylcarnitine level and free carnitine level ($P < 0.0001$). The conditional mean ratio varied with birth weight in all categories of gestational age (Figure 12 & Appendix D). Neonates in lower birth weight categories had generally lower ratios of total acylcarnitine to free carnitine than those in higher quintiles. On average,

this ratio increased with gestational age, although there was little difference in the mean ratio between extremely premature and very premature infants (Figure 12).

4.2.2 False Positives

With 1941 samples, false positives comprised approximately 0.5% of the main dataset. The false positive rate decreased with increased gestational age. In extremely premature individuals, 6.1% of samples were false positives. Overall, false positives comprised 8.2% of all very premature samples, 6.2% of medium premature, 2.4% of late premature, and 0.4% of term samples. The false positive rate was marginally increased in neonates in the lowest birth weight quintile (0.6%), but rates remained constant across the other four categories (0.5%). Infants receiving nutrition from sources other than breast milk and formula had a higher proportion of false positives at 4.5% versus 0.7% with formula alone, 0.6% with breast milk and formula, and 0.4% when fed exclusively on breast milk. False positive rates remained relatively steady across categories of sex, age at time of blood collection, socioeconomic quintile, and transit time. Infants who had received a transfusion had a higher proportion of false positives (2.5%) than those who had not (0.5%) (Table 26).

4.2.3 Sensitivity Analyses

4.2.3.1 Restricted by Feeding Status (No TPN / Other)

A sensitivity analysis was conducted in which all samples with a feeding status of TPN / other was removed. After this reduction, 387,671 samples remained. In this case the multivariate analysis explained 4.1% of variation in free carnitine and 1.7% of variation in C8 concentration (Table 27). The interaction between birth weight and gestational age was significant ($P < 0.0001$). The patterns of their combined associations with free carnitine were the same as in the main model. The interaction between birth weight and gestational age was also statistically significant ($P < 0.0001$) in relation to concentrations of C8. As in the main model, the mean C8 level conditional on all other independent variables in the analysis appeared to be strongly associated with birth weight quintile in extremely premature infants (Figure 14, Appendix E). In the main model, neonates in the highest quintile of birth weight had levels of C8 that were 20% - 25% lower than those in the other four categories. In this sensitivity analysis, C8 levels decreased steadily, with infants in the highest quintile of birth weight recording levels over 40% lower than those in the single lowest birth weight quintile.

4.2.3.2 Restricted by Transfusion Status (No Transfusions)

A sensitivity analysis was conducted in which all samples in which the neonate had received a blood transfusion were removed. After this reduction, 389,472 samples remained. In this case, the multivariate analysis explained 4.2% of variation in free carnitine and 1.7% of variation in C8 concentration (Table 27). The interaction between birth weight and gestational age was significant ($P < 0.0001$) in relation to concentrations of both free carnitine and C8. The patterns of their combined association with free carnitine and C8 were also the same as in the main model.

Table 26 – False Positive Rates

Variable	Categories	# Samples	# False Positives	% False Positives
Age of Collection	24-35 hours	235501	1040	0.4
	36-47 hours	83057	440	0.5
	48-71 hours	71156	461	0.6
Birth Weight Quintile	1	75508	433	0.6
	2	75017	342	0.5
	3	75561	380	0.5
	4	75323	379	0.5
	5	75362	358	0.5
Gestational Age	Extremely Premature	345	21	6.1
	Very Premature	601	49	8.2
	Medium Premature	1478	92	6.2
	Late Premature	14204	341	2.4
	Term	373086	1438	0.4
Infant Feeding	Breast milk only	264964	1054	0.4
	Formula only	41871	286	0.7
	Breast milk & formula	50389	312	0.6
	Other	2043	92	4.5
Infant Sex	Female	188893	819	0.4
	Male	196920	1109	0.6
Socioeconomic Quintile	1	83407	442	0.5
	2	74235	396	0.5
	3	75628	375	0.5
	4	75818	370	0.5
	5	59307	287	0.5
Transit Time (days)	0 - 4	228907	1146	0.5
	5 - 9	156009	772	0.5
	10 - 14	4797	23	0.5
Transfusion Status	No	389472	1935	0.5
	Yes	242	6	2.5

Table 27 – Interaction Term (BW & GA) Bivariate & Multivariate R-square Value Results

Sample Population	R-Square	
	C0	C8
Main Dataset (unrestricted)	0.042 ($p < 0.0001$)	0.017 ($p < 0.0001$)
Feeding Status Restricted (No TPN / Other)	0.041 ($p < 0.0001$)	0.017 ($p < 0.0001$)
Transfusion Status Restricted (No Transfused)	0.042 ($p < 0.0001$)	0.017 ($p < 0.0001$)

Figure 13 – Free Carnitine Level Predicted by Gestational Age & Birth Weight (Sensitivity Analysis: Feeding Status)

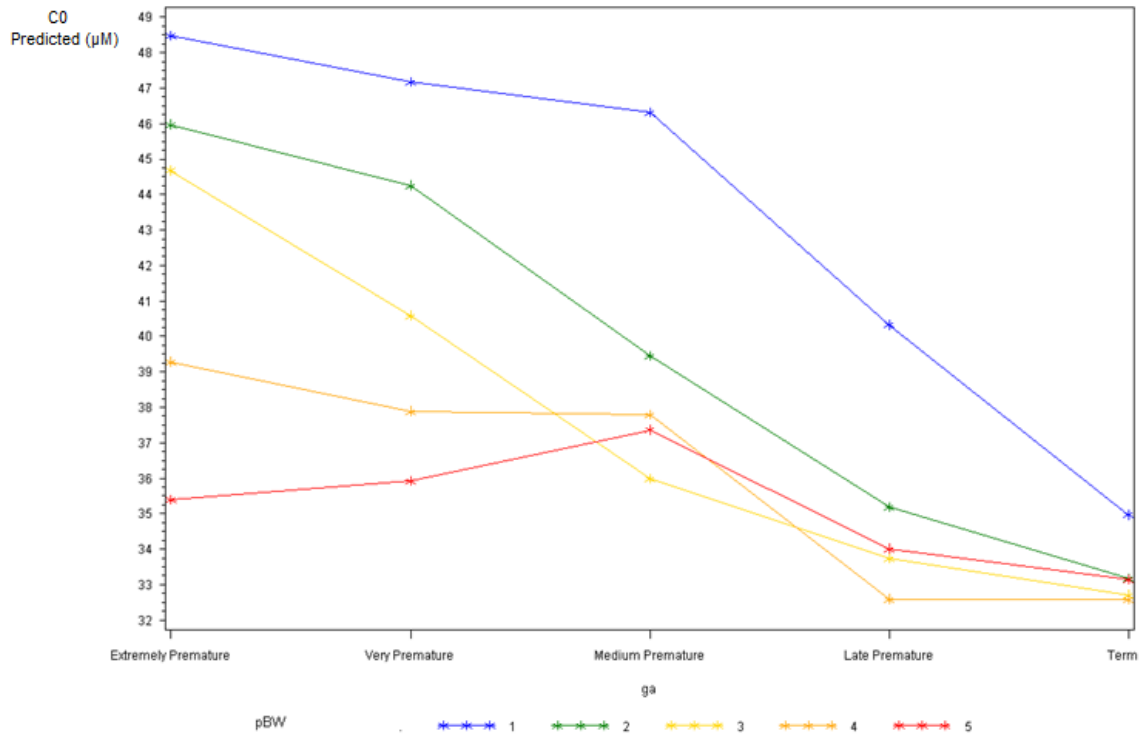


Figure 14 – C8 Level Predicted by Gestational Age & Birth Weight (Sensitivity Analysis: Feeding Status)

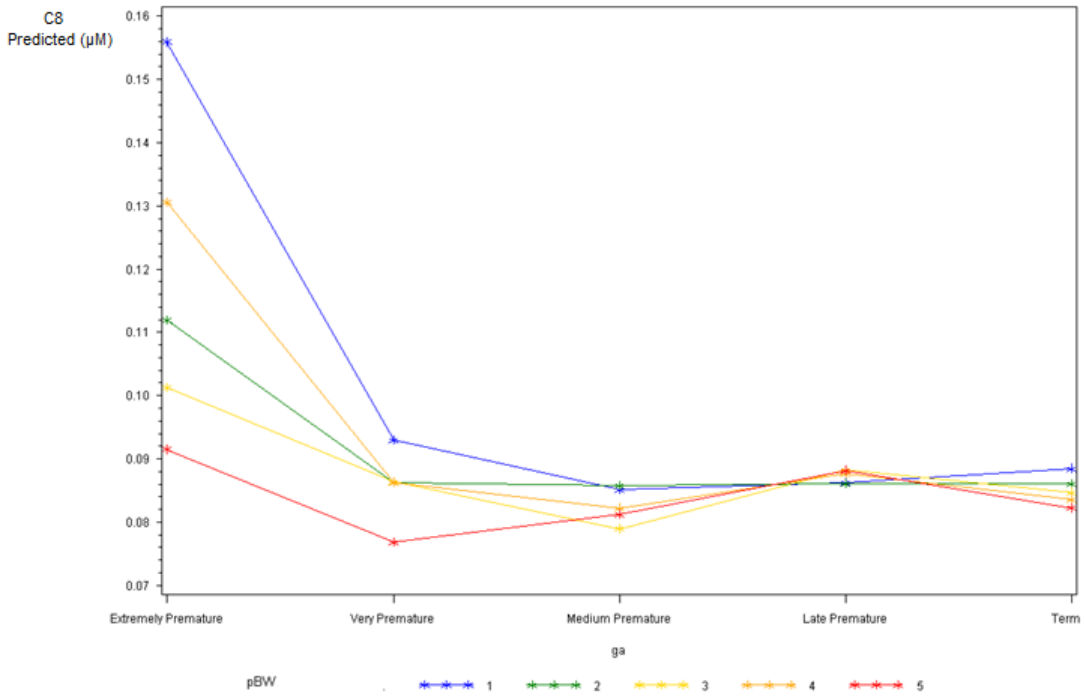


Figure 15 – Free Carnitine Level Predicted by Gestational Age & Birth Weight (Sensitivity Analysis: Transfusion Status)

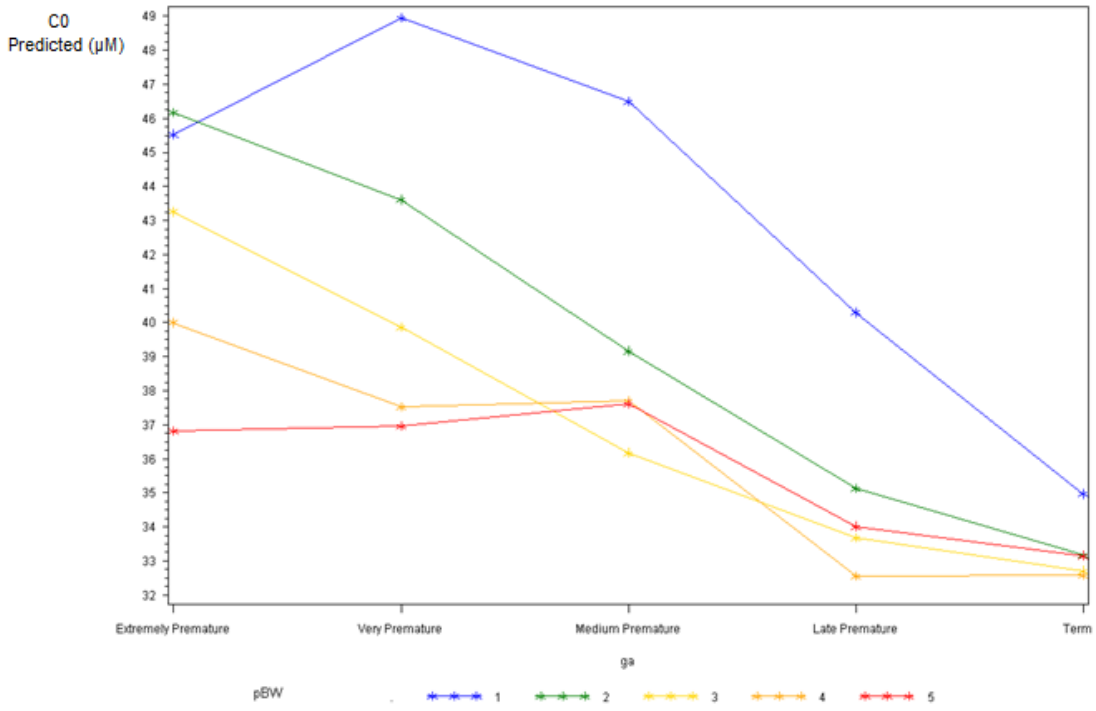
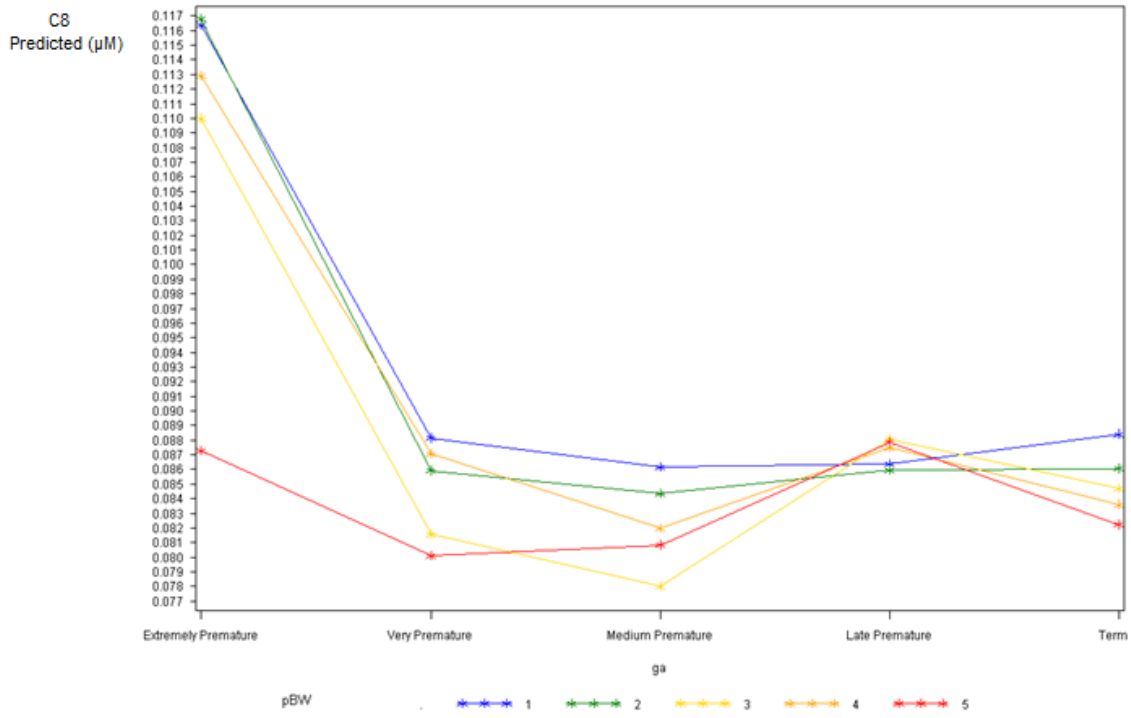


Figure 16 – C8 Level Predicted by Gestational Age & Birth Weight (Sensitivity Analysis: Transfusion Status)



4.3 Discussion of Clinical Database Analysis Results and Integration with Systematic Review Findings

4.3.1 Age of Collection & Gestational Age

During preliminary analysis, it became clear that the minority of samples obtained at an age of collection greater than 72 hours exhibited a considerably different metabolic profile than the majority, collected between 24 and 71 hours of age, with respect to the association between gestational age and analyte levels (Figures 3 & 4). As this discrepancy was present in nearly every carnitine or acylcarnitine level or ratio examined, and could potentially impact the associations between analyte levels and other independent variables (notably gestational age and the interaction between gestational age and birth weight), a post hoc

decision was made to eliminate samples collected at an age of 72 hours or higher from the main dataset.

4.3.2 Statistical & Clinical Significance

These analyses benefitted from a large sample size. As sample size increases, effect size can be measured with a greater degree of confidence. However, with the increase in statistical power, even minor differences may be found significant. In this study, nearly every association investigated was significant at a level of P = 0.05. Therefore, we relied not only on statistical significance, but also on describing the effect size and on the proportion of the variation in the outcome variable explained by a given predictor. In this study, the multivariate models (encompassing every independent variable including the gestational age & birth weight interaction term) explained from 1.7% to a maximum of 11% of all variability in the carnitine or acylcarnitine level being measured. This means that a minimum of approximately 90% of variability remained unexplained after accounting for all of the independent variables in our study.

4.3.3 Associations between Independent Variables and Analyte Levels

Both multivariate and bivariate models were best able to explain variation in the ratio of total acylcarnitines to free carnitine (multivariate R-squared = 0.110), followed by levels of medium chain acylcarnitines (multivariate R-squared = 0.060), and free carnitine (multivariate R-squared = 0.042). Only 1.7% of the variation in concentrations of C8 was explained by the independent variables in our analysis.

The low proportion of variation in analyte concentrations explained by the variables included in this study has implications for newborn screening. These factors were chosen as they are clinically considered important. The persistence of variation in analyte levels when these variables are accounted for may implicate unmeasured factors such as birth stress or may reflect fundamental differences within children. These results support the use of universal algorithms instead of ones tailored to the specific perinatal and newborn screening related characteristics we considered in order to determine cut-offs for carnitine and acylcarnitine concentrations and ratios.

This is particularly true of C8, the major screening marker for MCADD, which is the most common FAOD included in newborn screening. Variation in C8 was not well explained by either the bivariate or multivariate analyses. This was surprising, as C8 is a medium chain acylcarnitine central to fatty acid oxidation. The modest association of C8 with perinatal and infant characteristics or associated newborn screening related factors may be beneficial to newborn screening programs, suggesting that algorithms involving C8 are robust to differences across these characteristics. However, our findings also suggest that gestational age, specific forms of nutrition, and transfusion status are highly related to false positive status (Table 26) and may warrant consideration in interpretation of results by newborn screening programs.

4.3.4 False Positives

False positive rates (for all disorders included in neonatal screening in Ontario) were highest in infants of high prematurity and those having received a blood transfusion or nutrition from sources other than breast milk or formula. Proportionally, infants of a

gestational age of ≤ 33 weeks had the highest rates of false positives with 12.2 to 16.4 times the rate seen in the general population. False positive rates were generally stable across categories of age at time of blood spot collection, sex, socioeconomic status, and transit time.

False positive rates decreased with increasing gestational age. Rates of false positives were highest in extremely premature individuals, followed by very, medium, and late premature, and lowest in samples taken from term neonates. This may be due to the generally higher C0 and C8 levels (two major screening markers) in premature neonates as compared to term individuals (Tables 18 - 19).

Infants in the lowest birth weight quintile had slightly higher rates of false positive screening results as compared to those in the higher four categories of birth weight, but the differences were minor. In previous studies, very low birth weight individuals were found to account for significantly higher percentages of false positives [14]. Differences in the impact of low birth weight on false positive rates may be the result of variation in definitions of low birth weight between studies. Previous studies where significant differences were found have defined the lowest birth weight category with an objective cut off weight. In our study, birth weight categories were defined by quintiles within categories of sex and gestational age. Due to the low number of objectively defined extremely low birth weight individuals in the newborn screening population, the lowest birth weight quintile category included a range of birth weights with a mean that was likely higher than that of the objectively defined categories. In addition, objectively defined low birth weight categories used in other studies may not have taken gestational age into account, in which case the significant increase in false positives for low birth weight infants in those studies may have been influenced by the

overlap between extremely low birth weight and extremely premature individuals. Our study found little impact of birth weight on false positive results, after accounting for confounding by gestational age.

4.3.5 Correlations

Correlations among analyte levels were statistically significant with $p < 0.0001$. Significant correlations between analyte levels were to be expected, as long chain acylcarnitines are broken down into medium chain acylcarnitines, which are further metabolized into short chain acylcarnitines. Total acylcarnitine level is the combined sum of all short, medium, and long chain acylcarnitines, while free carnitine is used in the metabolism of fatty acids and the creation of acylcarnitines. The highest correlations were detected between total acylcarnitine and short chain acylcarnitine; and total carnitine with free carnitine, total acylcarnitine, and short chain acylcarnitine. Short chain acylcarnitines comprise approximately 80.2% of all acylcarnitines, while total carnitine is comprised of approximately 50.4% free carnitine and 49.6% total acylcarnitine. A surprisingly small correlation was found between total acylcarnitine and the ratio of total acylcarnitine to free carnitine (0.091). In the clinical database analysis, the strength and direction of associations with independent variables were generally similar for analytes that were more highly correlated. This was especially true of total acylcarnitine and short chain acylcarnitine (Figures 8 & 9, Tables 21 & 22).

4.3.5.1 Infant Feeding

Infant feeding status was not examined in the systematic review. In the clinical database analysis, infants receiving breast milk had on average the lowest levels of free

carnitine and C8, and relatively high levels of total acylcarnitine, short, medium, and long chain acylcarnitine, and the ratio of acylcarnitine to free carnitine. Infants receiving both breast milk and formula had high levels of all analytes including the ratio of acylcarnitine to free carnitine. Infants receiving formula exclusively had moderate levels of all analytes except for C8, where they had the highest levels. This may be due in part to the inclusion of C8 supplements in modern neonatal formula. Neonates receiving TPN / alternative forms of nutrition generally had the highest levels of free carnitine and relatively high levels of total carnitine, but the lowest levels of total acylcarnitine, short, medium, and long chain acylcarnitine, and the ratio of total acylcarnitine to free carnitine. In general, the effect of receiving TPN / alternative forms of nutrition was more important in the bivariate analyses than in the multivariate analyses. These effects were especially pronounced in free carnitine, C8, and total carnitine levels. The difference in this association may be the result of the multivariate analysis adjusting for potential confounding factors such as prematurity which are associated with the likelihood of a neonate receiving TPN / alternative forms of nutrition.

4.3.5.2 Sex

Infant sex consistently explained a relatively high degree of variation in analyte levels compared with other independent variables. This was surprising as sex was not found to be an important predictor of analyte concentration in studies included in the systematic review. This may be a result of generally low sample sizes in the systematic review, and small effect sizes in the clinical database analysis. With exception of the study by Khalid et al., no studies examining the association of sex and carnitine or acylcarnitine levels had a sample size exceeding 200 participants. With a sample population of 389,714, our clinical database analysis would likely have had better sensitivity to detect interactions. With small

effect sizes, sex did not necessarily explain a large degree of variation in any particular analyte concentration, but it was more important than some of the other infant and newborn screening related factors we examined. Female infants had on average lower levels of C0, C8, total carnitine, total acylcarnitine, and short, medium, and long chain acylcarnitines than their male counterparts. Females had on average higher ratios of total acylcarnitine to free carnitine.

4.3.5.3 Age of Collection

Age of the newborn at the time of blood sample collection was found to be significantly associated with analyte levels in both bivariate and multivariate analyses, though effect sizes tended to be smaller than for most other independent variables. In the systematic review, the presence and directionality of a possible association between age of collection and analyte levels varied between and within studies. The effect of age of collection was difficult to compare between studies in the systematic review, and between the systematic review and our clinical database analysis, because the range of age of collection is considerably different depending upon the study. In the database analysis, age of collection ranged from 24 to 71 hours of age. In the systematic review, age of collection ranged from birth to as late as 28 postnatal days.

The variable representing the age of the newborn at the time of blood sample collection consistently yielded relatively low r-square values in comparison with the other independent variables in predicting analyte levels. This is likely in part a result of the removal of all samples taken at or after 72 hours of age, thereby limiting the range of ages within this variable. Total acylcarnitine, short chain acylcarnitine, long chain acylcarnitine,

and the ratio of acylcarnitine to free carnitine increased with age at time of collection. Free carnitine and C8 levels were highest in samples collected between 24 and 35 hours of age and lowest in those collected between 36 and 47 hours, while total carnitine and medium chain acylcarnitine levels were generally highest in samples collected from 48 -71 hours of age and lowest in those collected from 36 – 47 hours.

4.3.5.4 Socioeconomic Status Quintile

No studies on socioeconomic status were identified in the systematic review. In the clinical database analysis, postal code was linked to 2006 census data and used as an area based measure of socioeconomic status. Despite relatively small effect sizes, most analytes demonstrated a relatively consistent trend with concentrations either increasing or decreasing steadily with each increase in income quintile. These trends remained generally unchanged between the bivariate and multivariate models. It is possible that this may be related to residual confounding whereby income quintile is related to maternal nutritional status, access to healthcare during pregnancy, ethnicity, or other unaccounted for variables.

In the clinical database analysis, free carnitine, total carnitine, total acylcarnitine, short chain acylcarnitine, and long chain acylcarnitine decreased progressively as socioeconomic quintile increased, with the highest levels occurring in quintile 1 and the lowest in quintile 5. Medium chain acylcarnitine levels and the ratio of acylcarnitine to free carnitine increased with income quintile, though effect sizes were small. C8 concentrations also increased with income quintile in the bivariate analysis but decreased in the multivariate analysis. The C8 effect sizes were also small.

4.3.5.5 Transit Time

No studies on transit time were identified in the systematic review. In the clinical database analysis, transit time was not generally a strong predictor of variation in analyte levels. Free carnitine and C8 levels increased on average with transit time. Total carnitine, total acylcarnitine, short chain acylcarnitine, long chain acylcarnitine, and the ratio of total acylcarnitine to free carnitine all decreased on average as transit time increased. Medium chain acylcarnitine decreased with increasing transit time in the bivariate model, though the effect size was small, and increased with transit time in the multivariate model. It is uncertain why this shift occurred though it may simply be a chance finding.

4.3.5.6 Gestational Age & Birth Weight

Gestational age was found to be significantly associated with analyte levels in both our clinical database analysis and in the majority of studies we reviewed, though in the systematic review the nature and presence of these associations varied between studies and little information was available on the association between gestational age and total short, medium, and long chain. In the clinical database analysis, the effect of gestational age on analyte levels was also variable. Total carnitine and total acylcarnitine concentrations were higher in every category of premature neonates than in term infants. Likewise, free carnitine decreased as gestational age increased, with the lowest levels occurring in the term category. Short chain acylcarnitine levels were also generally higher in preterm categories than in term, with the exception of late premature infants, who had the lowest levels of short chain acylcarnitine. C8 levels were generally higher in premature neonates than in term children, with the exception of those in the medium premature category, who had on average the lowest levels. The opposite was true of long chain acylcarnitines and the ratio of total

acylcarnitine to free carnitine, both of which increased as gestational age increased. For medium chain acylcarnitines, every preterm category had generally lower concentrations than term infants.

Birth weight was found to be significantly associated with analyte levels in our clinical database analysis as well as the majority of studies included in the systematic review. The directionality of the associations varied, likely as a result of variation in the additional characteristics of the sample populations among the different studies. In the clinical database analysis, C8 and medium chain acylcarnitine levels decreased with increasing birth weight. Infants in lower birth quintiles had on average lower levels of total acylcarnitine, short chain acylcarnitine, and the ratio of acylcarnitine to free carnitine than those in birth weight quintile 5. Likewise, levels of long chain acylcarnitine were lower in most low categories of birth weight than in quintile 5, with the exception of those in quintile 1 who had the highest. Free carnitine levels were highest in birth weight quintile 1 and lowest in quintiles 2, 3, and 4, while total carnitine levels were highest in quintiles 1 and 2, and lowest in quintiles 3 and 4.

The relationships between variables of birth weight and gestational age, and their association with carnitine and acylcarnitine levels and ratios are complex and confounded. Graphs provided a way to clarify these relationships by visually interpreting trends across both gestational age and birth weight. The interaction term between gestational age and birth weight was generally a comparably strong predictor of analyte levels. In the systematic review, Battistella et al. found that free carnitine level varied with birth weight in premature neonates, but that birth weight was not associated with significant variance in infants of term

gestational age or those in the highest category of birth weight regardless of gestational age. This was consistent with our clinical database analysis of the interaction between birth weight and gestational age: in general, the amount of variability in analyte levels across birth weight categories was considerably larger in extremely, very, and medium premature neonates and decreased as gestational age increased. This variability across birth weight quintiles was generally lowest in term neonates. Free carnitine and total carnitine levels were highest on average in earlier premature neonates and those of a low birth weight quintile. Total acylcarnitine and short chain acylcarnitine levels were fairly steady across gestational age but tended to be higher in low birth weight quintiles. C8 level was highest in extremely premature neonates and those in the lower quintiles of birth weight, but stabilized in the remaining four categories of birth weight. Medium chain acylcarnitine levels were lower in early premature categories of birth weight but increased rapidly between the medium and late premature groups, levelling out between late premature and term neonates. Infants in the higher categories of birth weight tended to have higher levels of medium chain acylcarnitine. Long chain acylcarnitine levels and the ratio of acylcarnitine to free carnitine both increased with gestational age. Birth weight had no clear impact on the ratio of acylcarnitine to free carnitine, but long chain acylcarnitine levels increased with birth weight quintile.

The fact that in extremely premature infants, analyte levels varied considerably with birth weight may reflect birth weight providing a proxy marker for metabolic developmental status. Extremely premature neonates of low birth weight are likely underdeveloped or ill in comparison to those of a higher birth weight quintile, thus levels of metabolic markers vary considerably with birth weight quintile. Also, premature infants with a higher birth weight may be metabolically closer to term neonates than those of lower birth weight. In neonates of

higher gestational age, we hypothesize that the lesser variation in analyte level associated with birth weight may be because the metabolic pathways in a higher percentage of term or late premature neonates have developed to the point that infant size and birth weight are no longer as important. This is consistent with the results of the bivariate and multivariate analyses, where the highest differences from median analyte levels were seen in extremely premature neonates with low birth weight, and late premature or term infants in the lowest birth weight quintile.

Term neonates comprised 96% of the sample population. This posed a challenge for creating predictive models, as only 4% of the population was spread over 4 of the 5 categories of gestational age. This may have been a factor in the small effect sizes found for gestational age in association with some analyte levels, such as C8, although the sample size for our study was large enough that the estimates were likely to be fairly robust. In addition, it is in the extremely premature or extremely low birth weight groups where we might expect a higher degree of variability, and this was supported by the graphs showing mean analyte concentrations adjusted for potential confounders.

Through Figure 6 it is clear that a large interaction was present between birth weight and gestational age in association with C8. In this case, the impact of gestational age (especially the extremely premature category) was much smaller in individuals of higher birth weight. This may be because individuals in higher birth weight categories were not being supplemented. This would be especially important in children of extreme prematurity, where rates of supplementation are highest, which is consistent with our findings. Another theory is that extremely premature neonates with low birth weight may have underdeveloped

metabolic pathways that are less capable of effectively metabolizing C8 to C6, therefore C8 levels remain high. A third theory involves extremely premature neonates and lower birth weights infants being more likely to experience a catabolic state. If this is the case, levels of C8 may be higher without additional supplementation and would reflect breakdown of body stores of fatty acids.

4.3.6 Sensitivity Analyses

Sensitivity analyses were conducted to assess the impact of feeding and transfusion status on analyte concentrations; specifically the effect of receiving TPN or other undisclosed nutrition or having received a blood transfusion. These interventions were examined due to their increased likelihood of being used in ill infants and the possibility that they may have a direct effect on analyte levels. In the restricted datasets, the multivariate analyses explained 4.1% (feeding status) and 4.2% (transfusion status) of variation in free carnitine concentration. In the unrestricted dataset, 4.2% of variation was explained. This indicates that the removal of these potentially ill neonates did not significantly impact the overall strength of the model for free carnitine. Multivariate analyses of each restricted dataset explained 1.7% of variation in concentrations of C8. The unrestricted dataset also explained 1.7% of variation. This indicates that the removal of these infants did not significantly impact the overall strength of the model for C8 either. The associations of the independent variables sex, age of collection, socioeconomic status, and transit time with C0 and C8 remained largely unchanged between the main dataset and the sensitivity analyses.

The interaction between birth weight and gestational age was also examined in each of the restricted datasets. When infants receiving TPN / other forms of nutrition besides

formula and breast milk were removed from the sample, little change was seen in this interaction. The primary difference occurred in neonates of extreme prematurity who were in birth weight quintile 1. In the main dataset, these infants had lower levels of free carnitine than those of very or medium prematurity in the same birth weight quintile. In the dataset restricted on feeding status, these neonates had the highest levels of free carnitine of any combination of birth weight and gestational age. These higher levels may be a result of the elimination of sick individuals in the lowest category of birth weight and gestational age. The individuals remaining in this category would then be those strong enough to receive traditional forms of nutrition (breast milk and formula). That the only obvious change occurred in this specific group of small, premature neonates highlights the fact that it is these individuals that are most likely to be ill and require TPN or other forms of alternative nutrition in the first place. When the sample was restricted on transfusion status, no appreciable changes (from the main unrestricted dataset) occurred in the interaction between gestational age and birth weight.

When infants receiving TPN / other forms of nutrition besides formula and breast milk were removed from the sample, a small degree of change was seen in the interaction between gestational age and birth weight in relation to concentrations of C8. Two main differences were observed in this dataset, both of which concern extremely premature neonates. In the unrestricted dataset, the range of average C8 concentrations in extremely premature neonates based upon birth weight quintile is between 0.091 - 0.156 μM (Figure 14). In the restricted dataset, the upper limit in extremely premature neonates is over 30% higher than the upper limit in the unrestricted dataset. This may be a consequence of eliminating samples in which sick infants were not receiving the same level of dietary C8 as

their breast or formula fed peers. By removing these potentially sick individuals, the neonates remaining in the extremely premature categories may have been both healthier and receiving higher amounts of exogenous C8 or C8 precursors, resulting in generally higher levels of C8 throughout the extremely premature categories. Infants in birth weight quintile 5 did not experience a strong increase in C8 level, possibly because larger infants may be naturally healthier and a smaller proportion of these neonates would have been receiving TPN or alternative forms of nutrition, leaving this section of the population less changed than those in lower birth weight quintiles of the same gestational age.

In addition to the generally higher concentrations of C8, in the feeding restricted dataset the difference between C8 levels in birth weight quintiles were much more evenly distributed than in the main dataset. In the unrestricted model, there was a large discrepancy between extremely premature neonates in the highest birth weight quintile and those in the lower 4. In the restricted model, mean analyte concentrations vary, but no one specific quintile is separated to a large degree from the rest. When the sample was restricted on transfusion status, no appreciable changes (from the main unrestricted dataset) occurred in the interaction between gestational age and birth weight.

4.3.7 Potential relationships to Metabolic Pathways and implications for combining Analytes

Short chain acylcarnitines (chain lengths C2-C5) are not exclusively the end products of fatty acid oxidation. They may also result from other metabolic pathways including branched chain amino acid catabolism [47]. As a result, medium and long chain acylcarnitines be more consistently representative of changes in the fatty acid oxidation pathways that were the main focus of our interest in this work. The short, medium, and long chain acylcarnitine

variables in this study were each created from the sum of all acylcarnitines within the specified range. One potential issue with this method is that within a category, certain specific acylcarnitines were present in much higher levels than others. In these cases, the higher individual acylcarnitine levels may overpower and mask differences in those with naturally lower levels.

The independent variables we studied were collectively better able to account for variation in the ratio of total acylcarnitine to free carnitine than the variation in any single analyte. This indicates that the relationship between analytes may be more important than individual analyte levels. Fatty acid metabolism is a dynamic process, and acylcarnitine levels are not independent of each other. Ratios are important because individual analyte marker levels may be affected by many different variables. For example, a high C8 level could be indicative of a catabolic state, lack of adequate nutrition, or interruption in the metabolic pathway between C8 and C6. If C8 is the only analyte level available, it is impossible to know what is causing this variation. Ratios are a comparison of analyte levels present at different stages of the metabolic process, thus providing a more complete picture of the metabolic state of the neonate.

4.3.8 Newborn Screening Thresholds

Newborn screening programs use algorithms and set cut-off points to detect FAODs in the neonatal population but do not generally account for differences in natural metabolic profile as a result of factors such as sex, gestational age, or birth weight. While this works well for term infants of average or high birth weight, high false positive rates among preterm individuals (and to a lesser extent those in the lowest birth weight quintile) indicate that a

tailored screening profile for these higher risk individuals may be beneficial. In particular, extremely premature neonates and low birth weight infants of gestational age ≥34 weeks (both of which indicate a higher likelihood of underdeveloped metabolic pathways or illness) may benefit from customized screening thresholds or special consideration in the interpretation of screening results. It is important to note, in this study false positive status was general and not specific to metabolic disorders. As a result, the associations between false positive status and perinatal and infant characteristics and newborn screening related factors are also not specific to FAODs and may be related to positive screens for other disorders. Even so, the high false positive rate associated with extreme prematurity or low birth weight indicates that specialized screening or interpretation may be beneficial in these individuals.

4.4 Conclusion

Our clinical database analyses indicate a statistically significant relationship between carnitine and acylcarnitine concentrations and all perinatal / infant characteristics and newborn screening related factors of interest. In the bivariate analyses, though strength and directionality of associations varied between analytes, the individual factors that demonstrated a consistently comparatively high impact on analyte levels were sex and infant feeding status, while age of collection, transit time, and gestational age were commonly among the least individually influential factors. We also identified clinically meaningful associations by investigating false positive rates in relation to independent variables; the results highlighted the potential importance of gestational age, transfusion status, and a nutritional source other than breast milk or formula.

The interaction between gestational age and birth weight had an important impact on carnitine and acylcarnitine levels, especially among extremely premature individuals. The effect of birth weight on these analytes was most pronounced in neonates of < 28 weeks gestational age, and decreased as gestational age increased. In neonates of high gestational age, birth weight was generally not a strong predictor of analyte concentrations. Free carnitine levels generally decreased with gestational age while the ratio of total acylcarnitine to free carnitine increased. Acylcarnitine concentrations tended to increase or remain steady as gestational age increased, with the exception of medium chain acylcarnitines in neonates of extremely premature gestational age.

Both bivariate and multivariate regression models were best able to account for variation in the ratio of total acylcarnitine to free carnitine (11.0%) and least able to account for variation in C8 concentrations (1.7%). The analyte that represents most closely the dynamic process of fatty acid metabolism was better explained than any individual carnitine or acylcarnitine concentration in this study. This supports the idea that proportions of carnitine and acylcarnitines may be more important in understanding an individual's metabolic functioning than individual analyte levels.

The low proportion of variation explained by all multivariate models does not support the use of algorithms in newborn screening tailored to specific perinatal and newborn screening related factors, as major diagnostic markers should ideally not be influenced to a large extent by external factors. However, high false positive rates associated with characteristics of premature or ill neonates (low gestational age, a positive transfusion status,

or TPN or alternative sources of nutrition) indicate that these cases may warrant additional consideration in the interpretation of results by newborn screening.

Understanding the ways in which perinatal and newborn screening related characteristics relate to metabolite levels has potentially important implications for the interpretation of newborn screening results and the results of associated diagnostic investigations [16]. This may be especially important in promoting understanding of results in cases where a child's carnitine or acylcarnitine levels are borderline according to current threshold values in disease screening, or where characteristics commonly associated with high false positive rates are present. The low proportion of variation explained by these results also suggest the need for further large scale empirical research targeted at previously unaccounted for perinatal factors such as birth stress and their association with carnitine and acylcarnitine levels in neonatal blood.

Appendix A

Ovid Medline Search Strategy

October 1st, 2009
Ovid MEDLINE(R) In-Process & Other Non-Indexed Citations and Ovid MEDLINE(R) 1950 to Present

#	Searches	Results
1	carnitine/ or acetylcarnitine/	6629
2	carnitine.mp.	10479
3	acylcarnitine.mp.	1083
4	acylcarnitine.nm.	306
5	carnitine.nm.	7854
6	or/1-5	10724
7	mass screening/ or neonatal screening/	70167
8	metabolism, inborn errors/ or exp lipid metabolism, inborn errors/	33613
9	newborn screening.mp.	1975
10	metabolism inborn error$.mp. [mp=title, original title, abstract, name of substance word, subject heading word]	18075
11	fatty acid oxidation.mp.	4304
12	or/7-11	114532
13	6 and 12	1944
14	limit 13 to "all infant (birth to 23 months)"	476

Search strategies in additional databases were adapted from the Ovid Medline strategy.

Appendix B

Data Abstraction Forms

Main Attributes of Paper						
Study ID#						
Data Extracted By						
Date Extracted						
Main Authors (up to 3)						
Journal Name/Reference Source						
Date Published						
Title						
Type of Publication	Full Paper	Short report	Abstract	Letter	Unpublished Report	Other
Funding Sources						

Main Attributes of Study			
Year study conducted	NR		If reported:
In which country/province/state was the study conducted?	NR		If reported:
Description of study design			
Study sample size			
Study Duration			
Single or multi-centre study	NR		If reported:
Age distribution of patients	NR		If reported:
	Mean:	SD:	Median:
Sex distribution of patients	NR		If reported:
	# Male (%):	# Female (%):	# Intersex (%):
Is there a comparison/control group			
Other factors of note (ex: multiple births, grouped ages)			

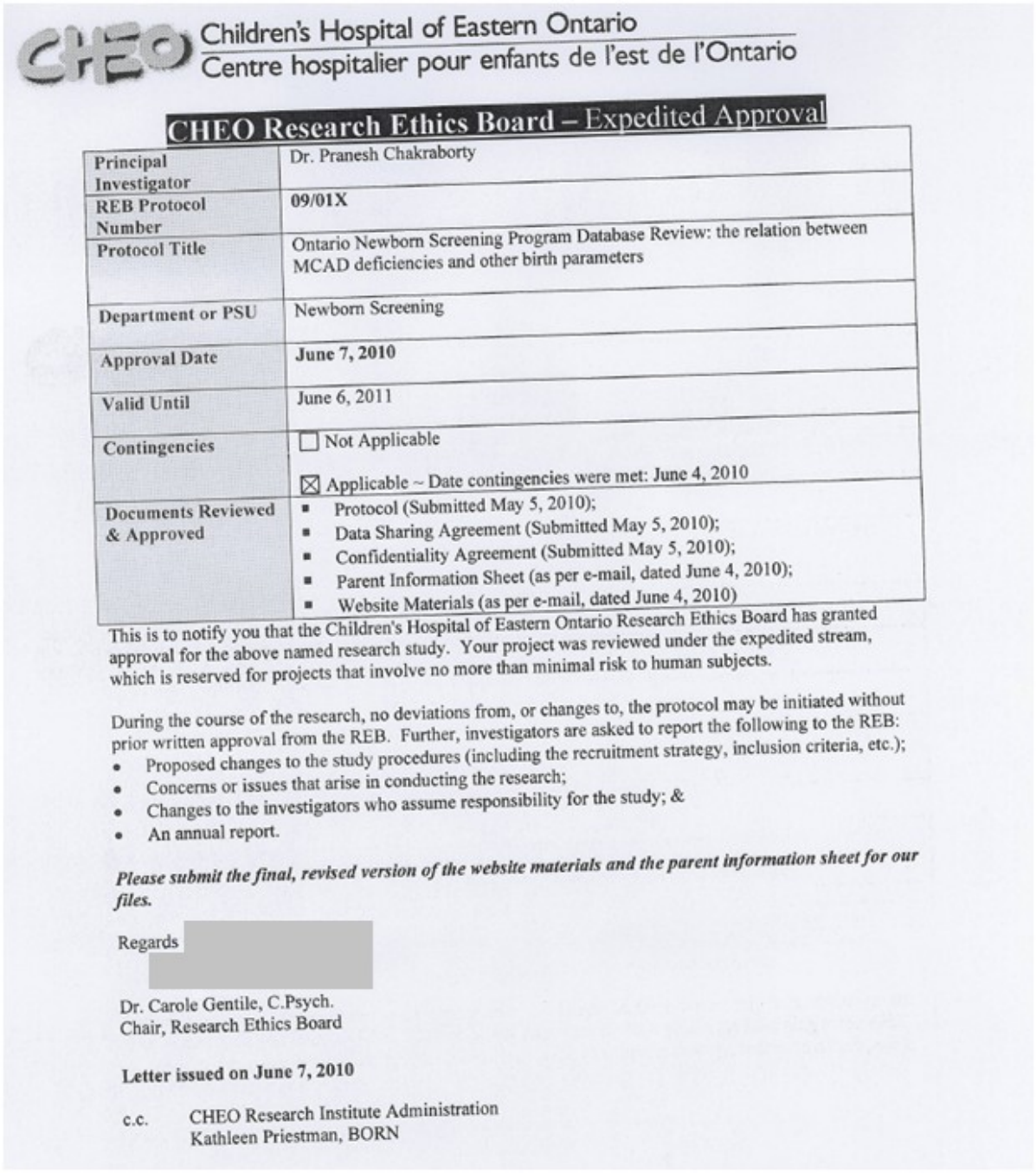
Carnitines, acylcarnitines and pre/perinatal factors assessed in the study	
Carnitines & Acylcarnitines	*Pre/Perinatal Factors*
C6	Socio-economic status
C8	Birth weight
C10	Gestational age
C8/C10	Feeding status
C14	Sex

Free carnitine	Complications during pregnancy	
Total carnitine	Complications during birth	
	Neonatal infection status	
	Ethnicity	
	Age at blood collection	
	Time between collection and analysis	
	Nature of sample	
	Analysis technique	
Describe how outcomes are assessed (follow-up period/s, number of assessments, units, how measured, etc.), distinguishing between multiple outcomes where appropriate		
Reporting quality for outcomes generally	Acceptable	Poor
Describe statistical analysis for examining relationship between factors and outcome/s (or indicate if none), distinguishing between multiple outcomes where appropriate		
Describe findings, distinguishing between multiple outcomes where appropriate		

Summary Comments	
Additional comments about study	
Key conclusions by authors (if reported)	

Appendix C

CHEO Ethics Approval



CHEO Children's Hospital of Eastern Ontario
Centre hospitalier pour enfants de l'est de l'Ontario

CHEO Research Ethics Board – Expedited Approval

Principal Investigator	Dr. Pranesh Chakraborty
REB Protocol Number	**09/01X**
Protocol Title	Ontario Newborn Screening Program Database Review: the relation between MCAD deficiencies and other birth parameters
Department or PSU	Newborn Screening
Approval Date	**June 7, 2010**
Valid Until	June 6, 2011
Contingencies	☐ Not Applicable ☒ Applicable ~ Date contingencies were met: June 4, 2010
Documents Reviewed & Approved	▪ Protocol (Submitted May 5, 2010); ▪ Data Sharing Agreement (Submitted May 5, 2010); ▪ Confidentiality Agreement (Submitted May 5, 2010); ▪ Parent Information Sheet (as per e-mail, dated June 4, 2010); ▪ Website Materials (as per e-mail, dated June 4, 2010)

This is to notify you that the Children's Hospital of Eastern Ontario Research Ethics Board has granted approval for the above named research study. Your project was reviewed under the expedited stream, which is reserved for projects that involve no more than minimal risk to human subjects.

During the course of the research, no deviations from, or changes to, the protocol may be initiated without prior written approval from the REB. Further, investigators are asked to report the following to the REB:

- Proposed changes to the study procedures (including the recruitment strategy, inclusion criteria, etc.);
- Concerns or issues that arise in conducting the research;
- Changes to the investigators who assume responsibility for the study; &
- An annual report.

Please submit the final, revised version of the website materials and the parent information sheet for our files.

Regards

Dr. Carole Gentile, C.Psych.
Chair, Research Ethics Board

Letter issued on June 7, 2010

c.c. CHEO Research Institute Administration
Kathleen Priestman, BORN

Appendix D

Analyte Level Conditional Means Dependent upon the Interaction Term (Birth Weight & Gestational Age)

Categories	Conditional Means							
Interaction Term (BW*GA)	C0 (µM)		C8 (µM)		Total Carnitine (µM)		Total Acylcarnitine (µM)	
	Bivariate	Multivariate	Bivariate	Multivariate	Bivariate	Multivariate	Bivariate	Multivariate
Extremely Premature BW Q1	41.912	41.356	0.117	0.112	74.921	75.139	32.706	33.496
Extremely Premature BW Q2	44.254	44.146	0.119	0.119	77.368	77.793	33.446	33.927
Extremely Premature BW Q3	43.594	43.279	0.114	0.121	77.516	78.253	34.203	35.293
Extremely Premature BW Q4	38.483	38.993	0.112	0.113	69.183	70.661	30.576	31.456
Extremely Premature BW Q5	36.704	36.874	0.089	0.087	66.895	66.839	30.211	29.983
Very Premature BW Q1	48.164	48.030	0.089	0.091	85.032	85.176	36.691	37.050
Very Premature BW Q2	43.503	42.043	0.086	0.087	76.562	74.979	33.309	33.164
Very Premature BW Q3	39.627	38.562	0.082	0.082	71.873	69.976	32.198	31.369
Very Premature BW Q4	37.370	37.650	0.087	0.085	68.491	68.922	31.033	31.150
Very Premature BW Q5	36.842	37.344	0.080	0.081	67.545	67.941	30.192	30.049
Medium Premature BW Q1	46.440	46.098	0.086	0.088	83.260	82.712	36.567	36.397
Medium Premature BW Q2	39.094	38.556	0.084	0.085	70.463	69.516	31.388	30.928
Medium Premature BW Q3	36.000	35.224	0.077	0.079	66.603	65.258	30.590	29.997
Medium Premature BW Q4	37.660	37.454	0.082	0.081	68.885	68.060	31.063	30.409
Medium Premature BW Q5	37.526	37.327	0.081	0.081	70.940	69.939	33.357	32.542
Late Premature BW Q1	40.259	40.170	0.086	0.086	74.297	73.490	34.031	33.317
Late Premature BW Q2	35.112	34.809	0.086	0.084	67.084	65.748	31.954	30.925
Late Premature BW Q3	33.675	33.837	0.088	0.085	65.533	64.875	31.849	31.047
Late Premature BW Q4	32.535	32.740	0.088	0.085	63.900	63.351	31.354	30.614
Late Premature BW Q5	33.998	34.448	0.088	0.085	67.251	67.053	33.248	32.617
Term BW Q1	34.962	35.233	0.088	0.084	68.026	67.713	33.061	32.493
Term BW Q2	33.171	33.665	0.086	0.081	65.506	65.470	32.334	31.816
Term BW Q3	32.703	33.325	0.085	0.080	65.085	65.195	32.385	31.883
Term BW Q4	32.582	33.254	0.084	0.079	65.300	65.436	32.715	32.191
Term BW Q5	33.148	33.803	0.082	0.077	66.965	67.013	33.810	33.213

Categories	Conditional Means							
Interaction Term (BW*GA)	Short Chain Acylcarnitine (µM)		Medium Chain Acylcarnitine (µM)		Long Chain Acylcarnitine (µM)		Ratio Total Acylcarnitine to Free Carnitine	
	Bivariate	Multivariate	Bivariate	Multivariate	Bivariate	Multivariate	Bivariate	Multivariate
Extremely Premature BW Q1	27.786	28.309	0.440	0.463	4.533	4.754	0.806	0.834
Extremely Premature BW Q2	28.577	28.805	0.446	0.471	4.318	4.512	0.789	0.804
Extremely Premature BW Q3	29.128	29.919	0.445	0.491	4.592	4.811	0.811	0.828
Extremely Premature BW Q4	25.865	26.563	0.393	0.424	4.298	4.439	0.815	0.819
Extremely Premature BW Q5	25.146	24.776	0.409	0.439	4.687	4.791	0.865	0.852
Very Premature BW Q1	31.187	31.444	0.395	0.424	5.062	5.140	0.790	0.803
Very Premature BW Q2	27.951	27.701	0.380	0.403	4.913	4.993	0.792	0.811
Very Premature BW Q3	26.866	25.968	0.388	0.399	5.057	5.008	0.836	0.833
Very Premature BW Q4	25.705	25.724	0.381	0.402	4.903	4.976	0.865	0.854
Very Premature BW Q5	24.799	24.674	0.398	0.402	5.024	5.003	0.867	0.850
Medium Premature BW Q1	30.689	30.503	0.401	0.416	5.436	5.433	0.822	0.827
Medium Premature BW Q2	25.866	25.446	0.405	0.413	5.088	5.014	0.835	0.833
Medium Premature BW Q3	25.240	24.755	0.384	0.389	4.975	4.859	0.881	0.881
Medium Premature BW Q4	25.414	24.905	0.400	0.399	5.247	5.107	0.868	0.851
Medium Premature BW Q5	27.423	26.722	0.425	0.417	5.556	5.462	0.922	0.901
Late Premature BW Q1	27.948	27.350	0.466	0.458	5.608	5.500	0.883	0.864
Late Premature BW Q2	25.750	24.947	0.502	0.480	5.687	5.487	0.946	0.922
Late Premature BW Q3	25.550	24.991	0.520	0.492	5.777	5.565	0.986	0.958
Late Premature BW Q4	25.011	24.514	0.526	0.494	5.812	5.606	1.002	0.975
Late Premature BW Q5	26.666	26.248	0.527	0.494	6.051	5.868	1.022	0.991
Term BW Q1	26.478	26.171	0.537	0.495	6.043	5.827	0.988	0.965
Term BW Q2	25.819	25.582	0.531	0.484	5.982	5.750	1.015	0.987
Term BW Q3	25.897	25.678	0.524	0.477	5.961	5.727	1.031	1.000
Term BW Q4	26.229	25.995	0.520	0.471	5.966	5.726	1.045	1.011
Term BW Q5	27.284	26.982	0.513	0.465	6.012	5.770	1.062	1.026

Appendix E

Analyte Level Conditional Means Dependent upon the Interaction Term (Birth Weight & Gestational Age) (Sensitivity Analysis)

Categories	Conditional Means							
	C0				C8			
Interaction Term (BW*GA)	Restricted on Feeding Status (No TPN / Other) (µM)		Restricted on Transfusion Status (No Transfused) (µM)		Restricted on Feeding Status (No TPN / Other) (µM)		Restricted on Transfusion Status (No Transfused) (µM)	
	Bivariate	Multivariate	Bivariate	Multivariate	Bivariate	Multivariate	Bivariate	Multivariate
Extremely Premature BW Q1	48.471	47.694	45.523	45.120	0.156	0.146	0.116	0.114
Extremely Premature BW Q2	45.969	44.205	46.168	46.143	0.112	0.097	0.117	0.117
Extremely Premature BW Q3	44.669	46.522	43.247	42.396	0.101	0.102	0.110	0.116
Extremely Premature BW Q4	39.274	40.799	39.992	40.424	0.131	0.137	0.113	0.114
Extremely Premature BW Q5	35.391	34.415	36.806	36.926	0.091	0.090	0.087	0.085
Very Premature BW Q1	47.170	45.718	48.938	48.682	0.093	0.095	0.088	0.090
Very Premature BW Q2	44.250	40.580	43.603	42.066	0.086	0.083	0.086	0.086
Very Premature BW Q3	40.582	39.081	39.852	38.679	0.086	0.082	0.082	0.081
Very Premature BW Q4	37.882	38.045	37.520	37.801	0.086	0.083	0.087	0.085
Very Premature BW Q5	35.922	36.401	36.961	37.498	0.077	0.078	0.080	0.081
Medium Premature BW Q1	46.308	46.050	46.484	46.222	0.085	0.087	0.086	0.088
Medium Premature BW Q2	39.450	39.048	39.157	38.592	0.086	0.088	0.084	0.085
Medium Premature BW Q3	35.976	35.027	36.166	35.457	0.079	0.081	0.078	0.079
Medium Premature BW Q4	37.783	37.504	37.690	37.510	0.082	0.081	0.082	0.081
Medium Premature BW Q5	37.358	37.043	37.603	37.416	0.081	0.082	0.081	0.081
Late Premature BW Q1	40.322	40.278	40.289	40.265	0.086	0.086	0.086	0.085
Late Premature BW Q2	35.187	34.931	35.117	34.885	0.086	0.084	0.086	0.083
Late Premature BW Q3	33.726	33.939	33.674	33.914	0.088	0.086	0.088	0.085
Late Premature BW Q4	32.587	32.865	32.538	32.824	0.088	0.086	0.087	0.084
Late Premature BW Q5	34.008	34.527	34.009	34.537	0.088	0.086	0.088	0.085
Term BW Q1	34.957	35.286	34.960	35.314	0.088	0.085	0.088	0.084
Term BW Q2	33.170	33.720	33.169	33.745	0.086	0.082	0.086	0.081
Term BW Q3	32.702	33.383	32.703	33.407	0.085	0.081	0.085	0.080
Term BW Q4	32.580	33.312	32.582	33.337	0.084	0.080	0.084	0.078
Term BW Q5	33.139	33.850	33.148	33.885	0.082	0.078	0.082	0.077

References

1. Voet D, Voet J. Lipid metabolism. In: *Biochemistry.* 3rd ed. New York: John Wiley & Sons; 2005:914-28.

2. Schuler AM, Wood PA, Schuler AM, Wood PA. Mouse models for disorders of mitochondrial fatty acid beta-oxidation. [Review] [49 refs]. *ILAR J*. 2002;43(2):57-65.

3. Meyburg J, Schulze A, Kohlmueller D, Linderkamp O, Mayatepek E. Postnatal changes in neonatal acylcarnitine profile. *Pediatr Res*. 2001;49(1):125-129.

4. Flanagan JL, Simmons PA, Vehige J, Willcox MD, Garrett Q. Role of carnitine in disease. *Nutr Metab*. 2010;7.

5. Chace DH, Hillman SL, Van Hove JLK, Naylor EW. Rapid diagnosis of MCAD deficiency: Quantitative analysis of octanoylcarnitine and other acylcarnitines in newborn blood spots by tandem mass spectrometry. *Clin Chem*. 1997;43(11):2106-2113.

6. Chace DH, Pons R, Chiriboga CA, et al. Neonatal Blood Carnitine Concentrations: Normative Data by Electrospray Tandem Mass Spectometry. *Pediatr Res*. 2003;53(5):823-829.

7. Angelini C, Federico A, Reichmann H, et al. Chapter 37: Fatty Acid Mitochondrial Disorders. In: Gilhus NE, Barnes MP, Brainin M, eds. *European Handbook of Neurological Management, Second Edition, Volume 1, Second Edition.* Oxford, UK: Wiley-Blackwell; 2011:501-511. 10.1002/9781444328394.ch37.

8. Newborn Screening Ontario. Newborn Screening Ontario: Information For The Public. . Updated 2012. Accessed May/11, 2012.

9. McHugh DM, Cameron CA, Abdenur JE, et al. Clinical validation of cutoff target ranges in newborn screening of metabolic disorders by tandem mass spectrometry: A worldwide collaborative project. *Genet in Med*. 2011;13(3):230-254.

10. Roe C, Ding J. Mitochondrial fatty acid oxidation disorders. In: Scriver C, Beaudet A, Sly W, Valle D, eds. *The Metabolic and Molecular Bases of Inherited Disease.* 8th ed. New York: McGraw-Hill; 2001:2297-326.

11. Millington DS, Roe CR. Medium-chain acyl-CoA dehydrogenase deficiency. *N Engl J Med*. 1989;320(18):1219.

12. Diaz R. Screening: Sensitivity versus specificity: Neonatal screening for congenital hypothyroidism. *Nat Rev Endocrionol*. 2010;6(10):534.

13. Kwon C, Farrell PM. The magnitude and challenge of false-positive newborn screening test results. *Arch Pediatr Adolesc Med*. 2000;154(7):714-718.

14. Slaughter JL, Meinzen-Derr J, Rose SR, et al. The effects of gestational age and birth weight on false-positive newborn-screening rates. *Pediatrics*. 2010;126(5):910-916.

15. Miller J. *Newborn Screening for Preterm, Low Birth Weight, and Sick Newborns: Approved Guideline.* Wayne, PA: Clinical and Laboratory Standards Institute; 2009:69.

16. Millington D. Tandem mass spectrometry in clinical diagnosis. In: Blau N, Duran D, Blaskovics M, Gibson K, eds. *Physician's guide to the laboratory diagnosis of metabolic diseases.* Berlin: Springer; 2003:57-75.

17. Shenai JP, Borum PR, Mohan P, Donlevy SC. Carnitine Status at Birth of Newborn Infants of Varying Gestation. *Pediatr Res*. 1983;17(7):579-582.

18. Schoderbeck M, Auer B, Legenstein E, et al. Pregnancy-related changes of carnitine and acylcarnitine concentrations of plasma and erythrocytes. *J Perinat Med*. 1995;23(6):477-485.

19. Novak M, Wieser PB, Buch M, Hahn P. Acetylcarnitine and Free Carnitine in Body Fluids before and after Birth. *Pediatr Res*. 1979;13(1):10-15.

20. Takiyama N, Matsumoto K. Age-and sex-related differences of serum carnitine in a Japanese population. *J Am Coll Nutr*. 1998;17(1):71-74.

21. Dalziel K, Round A, Stein K, Garside R, Castelnuovo E, Payne L. Do the findings of case series studies vary significantly according to methodological characteristics? *Health Technol Assess*. 2005;9(2):iii-iv, 1-146.

22. Katrak P, Bialocerkowski AE, Massy-Westropp N, Kumar S, Grimmer KA. A systematic review of the content of critical appraisal tools. *BMC Med Res Methodol*. 2004;4:22.

23. Sanderson S, Tatt ID, Higgins JP. Tools for assessing quality and susceptibility to bias in observational studies in epidemiology: a systematic review and annotated bibliography. *Int J Epidemiol*. 2007;36(3):666-676.

24. Von Elm E, Altman DG, Egger M, et al. The strengthening the reporting of observational studies in epidemiology (STROBE) statement: guidelines for reporting observational studies. *PLoS Med*. 2007(4):1623-7.

25. Meyburg J, Schulze A, Kohlmueller D, et al. Acylcarnitine profiles of preterm infants over the first four weeks of life. *Pediatr Res*. 2002;52(5):720-723.

26. Moher D, Liberati A, Tetzlaff J, Altman D, The PRISMA Group. Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. *PloS Med*. 2009;6(6):e1000097.

27. Battistella PA, Vergani L, Donzelli F. Plasma and urine carnitine levels during development. *Pediatr Res*. 1980;14(12):1379-1381.

28. Cavedon CT, Bourdoux P, Mertens K, et al. Age-related variations in acylcarnitine and free carnitine concentrations measured by tandem mass spectrometry. *Clin Chem*. 2005;51(4):745-752.

29. Khalid JM, Oerton J, Besley G, et al. Relationship of octanoylcarnitine concentrations to age at sampling in unaffected newborns screened for medium-chain acyl-CoA dehydrogenase deficiency. . Updated 2010. Accessed 6, 56.

30. Wilcken B, Wiley V, Sim KG, Carpenter K. Carnitine transporter defect diagnosed by newborn screening with electrospray tandem mass spectrometry. *J Pediatr*. 2001;138(4):581-584.

31. Christensen ML, Helms RA, Mauer EC, Storm MC. Plasma carnitine concentration and lipid metabolism in infants receiving parenteral nutrition. *J Pediatr*. 1989;115(5 Pt 1):794-798.

32. Schmidt-Sommerfeld E, Penn D, Wolf H. Carnitine deficiency in premature infants receiving total parenteral nutrition: effect of L-carnitine supplementation. *J Pediatr*. 1983;102(6):931-935.

33. Takahashi M, Sawaguchi S. Lipid metabolism in parenterally alimented neonates: carnitine blood concentrations and fat utilization. *Indian J Pediatr*. 1983;50(403):161-168. http://search.ebscohost.com/login.aspx?direct=true&db=lhh&AN=19841453272&site=ehost-live.

34. Yeh YY, Cooke RJ, Zee P. Impairment of lipid emulsion metabolism associated with carnitine insufficiency in premature infants. *J Pediatr Gastroenterol Nutr*. 1985;4(5):795-798.

http://search.ebscohost.com/login.aspx?direct=true&db=lhh&AN=19861480407&site=ehost-live.

35. Maier EM, Pongratz J, Muntau AC, et al. Validation of MCADD newborn screening. *Clin Genet*. 2009;76(2):179-187.

36. Strnadova KA, Holub M, Muhl A, et al. Long-term stability of amino acids and acylcarnitines in dried blood spots. *Clin Chem*. 2007;53(4):717-722.

37. Barns RJ, Bowling FG, Brown G, Clague AE, Thompson A. Carnitine in dried blood spots: a method suitable for neonatal screening. *Clin Chim Acta*. 1991;197(1):27-33.

38. Schmidt-Sommerfeld E, Werner D, Penn D. Carnitine plasma concentrations in 353 metabolically healthy children. *Eur J Pediatr*. 1988;147(4):356-360.

39. Martin J, Kung H, Mathews T, et al. Annual Summary of Vital Statistics: 2006. *Pediatrics*. 2008;121(4):788.

40. Berkowitz GS, Papiernik E. Epidemiology of Preterm Birth. *Epidemiol Rev*. 1993;15(2):414-443.

41. Newborn Screening Ontario. Newborn Screening Ontario: Information For Health Care Providers. . Updated 2012. Accessed May/17, 2012.

42. Ministry of Health and Long-Term Care. The Ontario Newborn Screening Program. Newborn Screening: A healthy start leads to a healthier life. . Updated 2012. Accessed June/03, 2012.

43. Morken N-, Källen K, Jacobsson B. Outcomes of preterm children according to type of delivery onset: A nationwide population-based study. *Paediatr Perinat Epidemiol*. 2007;21(5):458-464.

44. Amin SB, Harte T, Scholer L, Wang H. Intravenous lipid and bilirubin-albumin binding variables in premature infants. *Pediatrics*. 2009;124(1):211-217.

45. Lindsay RS, Dabelea D, Roumain J, Hanson RL, Bennett PH, Knowler WC. Type 2 and low birth weight: The role of paternal inheritance in the association of low birth weight and diabetes. *Diabetes*. 2000;49(3):445-449.

46. Statistics Canada. Geography Division. Postal Code Conversion File Plus (PCCF+)
. . 2010;5G.

47. Millington DS, Stevens RD. Acylcarnitines: analysis in plasma and whole blood using tandem mass spectrometry. *Methods Mol Biol*. 2011;708:55-72.