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RESEARCH REPORT

The Risk of Fatty Acid Oxidation Disorders and Organic Acidemias in Children with Normal Newborn Screening

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Abstract New Zealand has undertaken expanded newborn screening since 2006. During that period there have been no reported cases of fatty acid oxidation disorders or organic acidemias that have been diagnosed clinically that the screening programme missed. However there may have been patients that presented clinically that were not diagnosed correctly or notified.

In order to investigate the false-negative screening rate a case-control study was undertaken whereby the clinical coding data and relevant medical records were reviewed for 150 controls and 525 cases. The cases had normal newborn screening but with key analytes and/or ratios just below the notification level for individual disorders and thus in theory were most at risk of having metabolic disease.

Two cases had medical histories suggestive of metabolic disease and thus could represent a false-negative screen. One of these had marginally elevated octanoyl carnitine levels and thus possible medium-chain acyl Co-A dehydrogenase deficiency (MCADD) while the other had elevated isovaleryl carnitine and thus may have been a case of isovaleric acidemia (IVA). However, subsequent molecular analysis revealed that the diagnosis of MCADD and IVA was unlikely.

Despite relatively high cut-offs the New Zealand Newborn Metabolic Screening Programme does not appear to have missed any confirmed cases of fatty acid oxidation disorders and organic acidemias in its first 8 years of expanded newborn screening. This would suggest a similar low false-negative screening rate in centres with comparable screening protocols and would indicate that the risk of fatty acid oxidation disorders and classical organic acidemias in children who had normal newborn screening is low.

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Introduction

Expanded newborn screening (ENBS) refers to the early detection of disorders of intermediary metabolism by the measurement of acylcarnitines and amino acids in dried blood spots. By focusing on key analytes and ratios, 20 plus potentially clinically devastating diseases can be diagnosed and treated (Chace et al. 2003; Wilcken et al. 2009). A highly specific test is required in order to minimise the laboratory and clinical cost and workload, and more importantly family stress, involved in the follow-up of false positives (Schmidt et al. 2012). Screening laboratories have thus adopted particular key analytes and ratios that are generally accepted to offer the highest screening sensitivity, specificity and positive predictive value. The analyte cut-offs are similar between programmes albeit with some local variation in screening laboratories internationally (McHugh et al. 2011).

For each analyte there are children who at the time of screening had levels just below the cut-off and thus were never seen or at least never offered a secondary test and

others who were just above the cut-off and were thus notified to the local clinical metabolic service for follow-up, investigation and treatment. Children in the former group should be those that are most at risk of having false screen-negative metabolic disease.

The New Zealand newborn metabolic screening programme (NMSP) is responsible for the newborn screening of all children born in New Zealand. Testing occurs on days 2–3 and on average it takes 4–5 days for the samples to be transported and analysed in the screening laboratory. The NMSP has close to 100% coverage. ENBS was established in 2006 and over 20 metabolic diseases are screened for.

The NMSP has always adopted relatively high analyte cut-offs (https://www.clir-r4s.org/PartTools/tarCutComp.aspx?guiPartID=188). However apart from one child with asymptomatic carnitine uptake disorder who was diagnosed after a younger sibling was diagnosed, and one child with tyrosinaemia type 1, whose tyrosine was below the cut-off, the NMSP and the New Zealand National Metabolic Service are not aware of any known false screen-negative metabolic disease cases. Likewise, the two New Zealand biochemical genetics laboratories, both of whom have weekly teleconference meetings with the NMSP, have not diagnosed any new cases of fatty acid oxidation disorders (FAODs) or organic acidemia (OA) that were missed by ENBS. Despite this there may still be patients who have presented to hospital with signs and symptoms caused by underlying metabolic disease who remain undiagnosed.

For many years infants born in New Zealand have been assigned a unique health number at birth. This number is used on the newborn screening sample and for all subsequent healthcare episodes, hence it is relatively straightforward to correlate screening results with medical records.

This possibility, along with the ongoing process of reviewing the laboratory screening protocols for each condition, underlined the uncertainty regarding the risk of metabolic disease in those patients who had levels of screening analytes just below the cut-off and whom could be most at risk of a false-negative screen. The purpose of this study was to investigate the outcome in regard to metabolic disease signs and symptoms of children in that cohort and thus estimate the incidence of FAODs and OAs in those children with normal ENBS.

Study Methodology

A retrospective, case-control, observational analysis was performed with investigators blinded to case or control group through the random allocation of study numbers. The primary outcome measure was the prevalence of signs and/or symptoms suggestive of metabolic disease.

Clinical coding data for all hospital attendances or deaths were obtained from the New Zealand Ministry of Health for both cases and controls (as coded by the International Statistical Classification of Diseases and Related Health Problems, Tenth Revision, Australian Modification, ICD-10-AM). Coding criteria considered consistent with FAODs or OA included hypoglycaemia, lethargy, vomiting, seizures, coma, metabolic acidosis, hepatomegaly, increased transaminases, increased CK, muscle weakness, jaundice, cardiac arrhythmias, cardiomyopathy and unexplained death. Study numbers were noted and the hospital medical notes were reviewed for these admissions, allowing classification into either likely metabolic disease or no evidence of metabolic disease. Groups were then unblinded for analysis.

Newborn screening dried blood spot acylcarnitine profiles, the majority collected on days 2 and 3 of life, for both cases and control groups were measured using flow injection electrospray-tandem mass spectrometry.

Cases and controls were chosen from approximately 460,000 children born between December 2006 and December 2014 who weighed between 2.5 and 4.5 kg, were not premature (more than 37-week gestation) and whose initial screening samples had not come from a neonatal intensive care unit. Approximately 100 cases were chosen from each of six potential disease groups: children with possible (a) methylmalonic/propionic acidemia (MMA/PA), (b) isovaleric acidemia (IVA), (c) medium-chain acyl Co-A dehydrogenase deficiency (MCADD), (d) carnitine uptake deficiency (CUD), (e) carnitine-acylcarnitine transporter deficiency (CACT), carnitine palmitoyl transferase deficiency type II (CPTII), and (f) carnitine palmitoyl transferase deficiency type I (CPTI). The cases were identified by having both the ENBS key analyte and related analyte ratio/s just below (or just above in the case of CPT1) and closest to the cut-off for being classified as a positive screen at the time of screening. This case cohort was chosen as they were screen negative and yet were the most likely biochemically to have metabolic disease.

The analytes were, respectively, in group (a) C3: 7.5–15.5 μmol/L with the C3/C2 ratio 0.25–0.46; (b) C5: 0.7–1.5 μmol/L with the C5/C0 ratio 0.01–0.1; (c) C8: 0.60–0.93 μmol/L with the C8/C2 $\geq$0.02; (d) C0: 2–5 μmol/L; (e) C16: 11–14.75 μmol/L with the (C16 + C18.1)/C2 ratio 0.2–0.5; and (f) C0: 50–160 μmol/L with the C0/(C16 + C18) ratio 31–77 (Table 1).

The numbers in each group did not equal 100 but were as close to this as possible while also including all the patients that met the above criteria. Often there was a group of cases with exactly the same ENBS biochemistry for the particular analyte and/or ratio and one had to either include or exclude them all in order to get the number in the group closest to 100. In group g only 56 patients were included as expanding the criteria recruited very large numbers of patients. There were

Table 1 Key analyte level and ratio for cases

Disease	Analyte (μmol/L)	Ratio	Current NZ cut-off[a]	International 50%ile cut-off[b]	Number
MMA, PA	C3: 7.5–15.5	C3/C2: 0.25–0.46	C3 ≥5 and C3/C2 ≥0.25	C3 ≥5.35, C3/C2 ≥0.22	108
IVA	C5: 0.7–1.5	C5/C0 0.01–0.1	C5 ≥1.0	C5 ≥1.2	90
MCAD	C8: 0.60–0.93	C8/C2: ≥0.02	C8 ≥0.8 or C8 ≥0.6 and C8/C2 ≥0.04	C8 ≥0.32, C8/C2 ≥0.04	94
CUD	C0: 2–5.1		C0 ≤5	C0 ≤8	94
CPT II, CACT	C16: 11–14.75	(C16 + C18.1)/C2: 0.2–0.5	C16 ≥12.4	C16 ≥7	83
CPT I	C0: 50–160	C0/(C16 + C18): 31–77	C0 ≥122, and C0 (C16 + C18) ≥70	C0 ≥124	56

[a] These cut-offs have been slightly modified over the duration of screening and thus a small number of patients who may not have been screen positive at the time of screening would be screen positive now
[b] https://www.clir-r4s.org/PartTools/tarCutComp.aspx?guiPartID=188

in total 525 cases. Over the duration of the screening programme the criteria for a positive screen has evolved and thus a very small number of cases that at the time were screen negative would be considered screen positive now.

None of the cases or families had been seen by either the national metabolic or the local paediatric clinical services in regard to their newborn screening results. In some cases, especially with C3 and low CO the infant's initial screen had indicated, as per the NMSP protocol, a need for a second follow-up dried blood spot, and this had been performed. The resultant level was not within the notification range.

The control group were a selection of 150 randomly chosen patients with normal newborn screening who had the analytes between the 25th and 75th centiles.

C14.1, corresponding to the disease very-long-chain acyl Co-A dehydrogenase deficiency (VLCADD), was not included in this study as it was the subject of an extensive previous study (Ryder et al. 2016). C5DC, the key analyte for glutaric acidemia type 1 (GA1), was not included as there was a clear bimodal distribution with a very small group of GA1 patients who had very high levels of glutaryl carnitine (≥0.7 μmol/L) and the rest having normal levels (≤0.45 μmol/L) with only five patients having levels of 0.45–0.7 μmol/L. C5OH, the analyte for 3-methylcrotonyl carboxylase deficiency and related disorders, was not included as the NMSP no longer screens for these conditions due to the clinical uncertainty regarding a positive screening result.

The intracellular cobalamin defects were not specifically investigated in this study as the NMSP is currently assessing the use of homocysteine rather than methionine, as well as C3, for the screening of these conditions.

Results

Of the 525 cases, two had medical records that revealed admissions to hospital with events that were suggestive of an underlying metabolic disease.

Patient A had newborn screening C8 of 0.74 μmol/L and a C8/C2 ratio of 0.03 at day 2 screening (cut-off for positive screen C8 ≥0.8 μmol/L or C8 ≥0.6 μmol/L and C8/C2 ≥0.04). The patient presented aged 17 months after 2 days of upper respiratory tract viral symptoms and poor feeding. They had fed poorly the previous evening and were found the following morning lethargic and hypotonic. The admission blood sugar was 1.7 mmol/L (normal 3.5–5.4 mmol/L) with an appropriately elevated beta-hydroxybutyrate of 3.7 mmol/L. They were commenced on IV 10% dextrose and discharged the following day after a normal meal. An acylcarnitine profile revealed a slightly elevated C8 of 0.3 μmol/L (normal 0.1–0.2 μmol/L) with the laboratory commenting that it was not typical of MCADD but may reflect an MCADD carrier. No follow-up of this was made. The child had two subsequent hospital visits with viral vomiting illnesses and poor oral intake. During these events the child had been given carbohydrate drinks by the parents and was not hypoglycaemic. Sequencing of exons 1–12 of the ACADM gene as well as the intronic areas immediately bordering the exons along with deletion/duplication analysis from DNA obtained from the original dried blood spot revealed one copy of the common c.985A≥G (p.Lys329Glu) mutation with no other abnormality identified.

Patient B had a screening C5 of 0.9 μmol/L and a C5/C0 of 0.01 on day 3 of life (positive screen C5 ≥1.0 μmol/L). There were no other acylcarnitine abnormalities. At 4 months of age the child was found unresponsive in bed and required cardiopulmonary resuscitation. The previous day they had a vomiting illness with poor oral intake. Upon arrival to hospital they were hypotensive and unresponsive with a marked metabolic acidosis and moderately raised liver function tests. An MRI brain showed widespread severe ischemic changes. The child was initially ventilated but due to signs of brain death this was discontinued and the child died that evening. A postmortem and metabolic investigations were not performed. Sequencing of all 12

exons and flanking intronic regions of the isovaleryl Co-A dehydrogenase (IVD) gene from DNA obtained from the original dried blood spot revealed no mutations.

One of the control patients had a medical history suggestive of a possible FAOD. The baby presented in the early neonatal period with hypoglycaemia and required IV 10% dextrose. The baby had mild left ventricular hypertrophy. Both the hypoglycaemia and the hypertrophy resolved over the next few days and it was felt that they had transient hyperinsulinism. There was no maternal diabetes.

Discussion

Expanded newborn screening has been undertaken internationally for nearly 20 years and there are numerous reports from screening centres of their diagnostic results. Few screening services have specifically reported their experience with missed cases (Estrella et al. 2014; Wilcken 2013).

Since commencing ENBS in late 2006, the NMSP has diagnosed 67 cases of FAODs or OAs (Table 2). In addition, there have been five cases of long-chain fat disorders (three CACT deficiency, two VLCADD) and four cases of holocarboxylase synthase deficiency that presented clinically on day 1 of life, prior to newborn screening. One of the CACT cases and both the VLCADD cases died in the first few days of life. The service also had one case of tyrosinaemia type I who presented with severe liver disease at a 3 months of age and had a normal tyrosine level at the time of screening and was thus missed by the ENBS. There has been one missed asymptomatic case of CUD. Other than these, there have been no known missed cases in New Zealand.

The potential high local incidence of false-positive cases in diseases such as of VLCADD, CPT-1, 3-MCC, IVA, citrulinaemia and maternal CUD (Ensenauer et al. 2004; Vijay et al. 2006; Glamuzina et al. 2011; Ryder et al. 2016; Rips et al. 2016) and the local workforce capacity particularly in the first few years of that contributed to the NMSP adopting relatively high cut-off levels. This results in less work for the screening laboratory, fewer second tests and less false-positive cases, thus limiting unnecessary stressful notifications to families. However higher cut-offs bring the concern that clinically significant cases will be missed.

Table 2 Cases of FAOD and OAs diagnosed by New Zealand newborn screening (number screened approximately 460,000)

	Condition	Case count
Fatty acid oxidation	MCAD	28
	VLCAD	11
	CUD (maternal)	5
	CPT-I	3
	MADD	2
	CUD	1
Organic acidemia	IVA	5
	3-MCC	4
	MMA	3
	GA-I	3
	PA	2

Screening for the aminoacidopathies maple syrup urine disease, homocystinuria and tyrosinaemia using leucine/isoleucine, methionine and tyrosine, respectively, is known to be problematic with high rates of false-positive screening and missed cases being reported (Estrella et al. 2014; Naughten et al. 1998). In fact many patients with these conditions can have normal metabolites at the time of newborn screening (https://www.clir-r4s.org/ProjTools/DiseaseRangeComp.aspx) and thus these conditions were not part of this study. The NMSP plans to improve its screening for homocystinuria and tyrosinaemia type I by using total homocysteine and succinylacetone as key analytes, respectively.

This study investigates whether there have been any false-negative screening cases presenting with FAODs and OAs since New Zealand commenced ENBS in 2006. There were only two cases who presented with possible metabolic disease. Case A was a possible MCADD presentation. The baby/child had a typical clinical MCADD event but with significant ketosis. The latter is unusual but not unknown in MCADD (Christodoulou et al. 1995). They did have an acylcarnitine profile at the time suggestive of a possible 'MCADD carrier state'. They are now being treated with an emergency plan. Molecular analysis revealed only a carrier state and thus they are not likely to represent true MCADD. Case B had borderline ENBS levels of C5, the marker for IVA, and died after a short illness at 4 months. This was the only case in the cohort that died or suffered serious morbidity. While the clinical details were suggestive of IVA, the lack of specific metabolic investigations at the time of death and the absence of any disease-causing mutations in the *IVD* gene made the diagnosis of IVA unlikely. IVA is a difficult disease for screening laboratories as there are a number of relatively common variants with elevated screening levels who are at very low or no risk of clinical disease (Ensenauer et al. 2004). C5 is also elevated in the very rare disease 2-methylbutyryl-CoA dehydrogenase deficiency but this does not present with a severe hepatic metabolic decompensation as seen in Case B and thus seems unlikely.

A limitation of this study is that there may be cases who had metabolic disease signs and symptoms who were not captured in the analysis as they presented to their general

practitioners (family doctors) and/or private paediatrician and not the public hospital. However in New Zealand, any children with significant disease would be seen at a public hospital and have ICD-10-AM coding data sent to the Ministry of Health. Likewise any patient who had metabolic disease diagnosed would be known to the metabolic clinical service and/or the two biochemical genetics laboratories and thus be known to the investigators of this study. A further limitation is that very unusual and/or chronic presentations of these conditions may not have been captured by this study. It is also possible that milder forms of these conditions may present in later childhood or adulthood and thus would not yet be identified by this study. However, newborn screening in New Zealand aims to only diagnose patients whereby treatment makes a difference to outcome in early life. There is a small chance that patients may have emigrated from New Zealand and presented clinically with metabolic disease although it would seem likely that the NMSP would have been notified.

This study did not include premature, NICU and/or low-birth -weight babies as these children frequently have ongoing health issues and it was felt that including them would make the analysis of this study difficult. There is thus the possibility that they could have had metabolic disease missed by screening although neither the metabolic laboratory nor the clinical metabolic service is aware of such cases.

The only missed case of FAOD by the NMSP was a child who at day 2 ENBS had a C0 of 8.3 μmol/L, above our cut-off of 5 μmol/L. She was however subsequently diagnosed with CUD, after the family had moved to Australia and the screening service there diagnosed, by ENBS, the disorder in a younger sibling who had a newborn screening C0 of 3 μmol/L. The older child was asymptomatic and interestingly a review of her initial screening parameters showed that even using the Region 4 post-analytical tool (https://www.clir-r4s.org/PostAlyt-Tools/CondScoreSingle.aspx?pcID=1783) for CUD she would have been missed with a profile of 7, a score not informative for CUD. CUD is a difficult disease for a screening service as affected babies may have relatively high metabolites and thus be missed, there are a significant number of maternal cases diagnosed and the condition itself is likely to be benign in the majority of patients (Rasmussen et al. 2014). This study reflects the historic practice of the NMSP using a low free carnitine (C0) alone to screen for CUD. This is now known to be a less-than-optimal analyte by itself and the local experience of the NMSP is that a very low CO is more likely to reflect maternal CUD. The service has now adopted what is hoped to be a more sensitive and specific set of analytes using a combination of low C0, C3 and C16. Thus the results from this study regarding C0 and CUD are less reliable than for the other FAODs.

While not a focus of this study the number of false-positive cases diagnosed by the NZNMS is difficult to estimate as it depends on what one defines as a case. The relatively high cut-offs have resulted in the National Metabolic Service seeing very few screen-positive patients who based on follow-up biochemistry, enzymology and molecular have been subsequently shown to be a false positive. However the majority of the 32 patients with confirmed VLCADs, CUDs, CPT Is, IVAs and 3-MCCs have remained asymptomatic and it is possible, even likely, that many will remain so. This could be due to the treatment although apart from three patients with symptomatic VLCAD they receive a normal diet and only have an 'emergency plan' for times of illness.

It is essential that ENBS laboratories regularly audit their results, in particular the possibility of false-negative cases, and adjust their screening cut-off criteria accordingly. What is more debatable is whether the advantages of far fewer false-positive cases outweigh the harm of missing a small number of cases. This study along with the experience of the clinical NZNMS suggests that ENBS for clinically significant disorders of fatty acid and organic acid metabolism in New Zealand is highly sensitive as there are few missed cases. This is also likely to be the case in screening centres internationally where cut-offs similar to or more sensitive than those in New Zealand are used. ENBS however doesn't detect all metabolic disorders and there are some FAODs and OA that are not screened for. Accordingly, if clinically indicated, the appropriate diagnostic metabolic investigations and treatment should be undertaken even if the newborn screening was normal.

Synopsis

An audit of the medical records of the most at risk of those who were newborn screen negative suggests that New Zealand Newborn Metabolic Screening service has not missed any cases of clinically significant metabolic disease.

Compliance with Ethics Guidelines

Dr. Callum Wilson, Mr. Detlef Knoll, Mr. Mark de Hora, Dr. Campbell Kyle, Dr. Emma Glamuzina, and Dr. Dianne Webster declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000.

The study was anonymous and informed consent was not required. No identifying information about patients is included in this chapter. The study received approval from

the NZ Ministry of Health National Screening Unit (NSU), the National Health and Disability Ethics Committee and the Auckland District Health Board Research Committee.

Dr. Callum Wilson designed the study, obtained the ethical approval, helped with the initial collection and analysis of medical data and wrote the initial manuscript; Mr. Detlef Knoll, Mr. Mark de Hora and Dr. Dianne Webster collected and analysed the newborn screening data. Dr. Campbell Kyle and Dr. Emma Glamuzina helped with collection and analysis of the medical data. All the authors contributed to the final manuscript.

Dr. Callum Wilson serves as guarantor for this chapter and accepts full responsibility for the work.

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