Newborn screening for primary carnitine deficiency

Weighing benefits against harms

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Neonatale bloedspotscreening naar primaire carnitine deficiëntie
(met een samenvatting in het Nederlands)

Proefschrift

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geboren op 10 januari 1993
te 's Gravenhage

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Dr. G. Visser

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CHAPTER

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GENERAL INTRODUCTION

Carnitine and primary carnitine deficiency

Carnitine (3-hydroxy-4-N-trimethylaminobutyrate) is a quaternary amine involved in many biological processes.[1] A major function of carnitine is to shuttle long-chain fatty acids from the cytosol into the mitochondrial matrix where they are degraded via β-oxidation.[2] Carnitine enters the cell through the Organic Cation Transport Novel 2 (OCTN2) protein. Mediated by carnitine palmitoyltransferase 1 (CPT1), in the cell, carnitine binds to a long-chain acyl-CoA, forming an acylcarnitine. These acylcarnitines can then enter the mitochondrial matrix in exchange for free carnitine via carnitine-acylcarnitine translocase (CACT). There, the acylcarnitine is reconverted by carnitine palmitoyltransferase 2 (CPT2) into an acyl-CoA that enters β-oxidation and free carnitine that is ready to shuttle another fatty acid.[3] An overview of this pathway is presented in figure 1. Apart from the important role in mitochondrial β-oxidation, carnitine also plays a major role in peroxisomal β-oxidation, coenzyme A homeostasis and reducing toxic effects of residual acyl groups.[4,5]

The sources of carnitine in humans are dietary intake (roughly 75% with an ordinary omnivorous diet) and de novo biosynthesis (25%).[1] Foods high on carnitine include (red) meat (32-130 mg per 100 g), milk (3 mg per 100 ml) and fish (6 mg per 100 g).[6] Strict vegetarians and vegans obtain very little carnitine from dietary sources, and therefore mainly rely on biosynthesis as the source of carnitine (more than 90%).[6] Carnitine is synthesized from the amino acids lysine and methionine, which form the first metabolite in the carnitine-biosynthesis pathway: N^6-trimethyllysine.[5,7] Most tissues are able to convert N^6-trimethyllysine into the carnitine precursor, γ-butyrebetaine.[5] Carnitine synthesis can only be completed (conversion from γ-butyrebetaine to L-carnitine) in the kidney, brain and liver. In pregnant women, the placenta is, to a lesser extent, also able to fully synthesize carnitine, supporting foetal carnitine homeostasis.[8] The most notable contribution to the total human carnitine pool is the efficient renal reabsorption of carnitine. In the brush border membrane of the renal proximal convoluted tubule, 90-99% of filtered carnitine is reabsorbed by OCTN2.[9] The reabsorption rate decreases rapidly when plasma free carnitine values reach 40-60 µmol/L.[10] An overview of the sources of carnitine and their contribution to the total human carnitine pool is provided in figure 1.

Primary carnitine deficiency (PCD) (OMIM entry #212140; IEM0627; ICIMD 4.1.01.01) is an inborn error of metabolism (IEM) that is caused by pathogenic variations in the SLC22A5 gene, which encodes the OCTN2 protein.[11-13] Impaired function of OCTN2 leads to a severe decrease of intracellular carnitine concentrations, potentially impairing fatty acid oxidation.[14-16] PCD diagnosis is confirmed by identification of bi-allelic variants in the *SLC22A5* gene and/or by measuring a decreased residual carnitine transport (OCTN2) activity in patient fibroblasts.[12,14,17] Classically, patients are described to suffer from hepatic encephalopathy, cardiomyopathy and myopathy in childhood, and arrhythmia or sudden death in adulthood.[14] Treatment is simple, relatively cheap and consists of lifelong oral supplementation of carnitine (approx.

100-150 mg/kg daily for children and approx. 3-8 g daily for adults).[14,17] Sometimes, patients report a fishy odour as a side-effect of carnitine supplementation, which is caused by production of trimethylamine.[17] This can be alleviated by lowering the carnitine dose and/or prescribing riboflavin.[18,19] Other side-effects are rare and include increased gastrointestinal motility, diarrhoea and intestinal discomfort.[17]

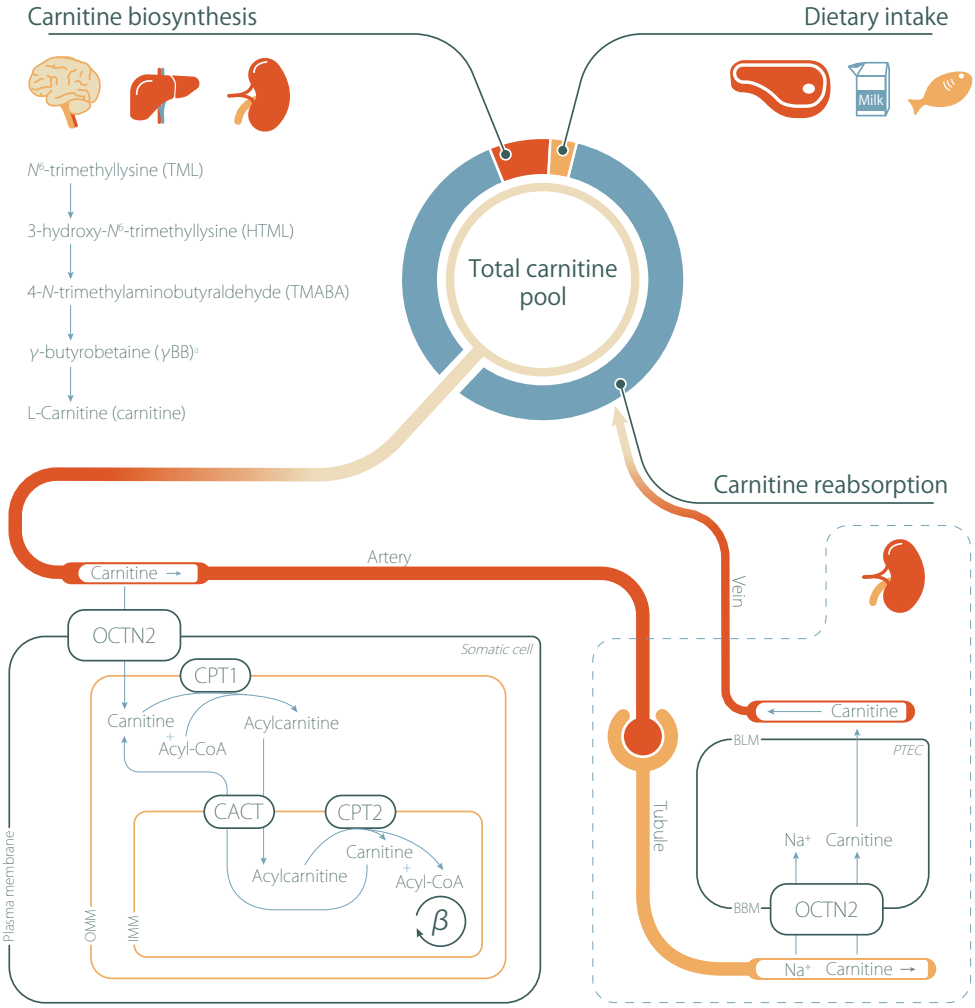

Figure 1 | Overview of different sources of carnitine, their contribution to the total carnitine pool in the human body and a schematic diagram of carnitine transport into the cell and its role in the mitochondrial carnitine-acylcarnitine shuttle.

Abbreviations: Acyl-CoA - Long-chain acyl-CoA (medium- and short-chain acyl-CoA can diffuse freely into the mitochondrial matrix); OMM - Outer mitochondrial membrane; IMM - Inner mitochondrial membrane; β - β-oxidation; PTEC - Proximal tubule endothelial cell; BLM - Basolateral membrane; BBM - Brush border membrane

[a] The final step of carnitine biosynthesis is only done in brain, liver and kidney.

Newborn screening

The goal of newborn screening (NBS) is to detect conditions that can cause irreversible damage at an early age. NBS enables an accelerated diagnosis, which results in early treatment initiation, thus preventing patients from developing serious symptoms. NBS is generally carried out by analyzing markers in a few drops of blood from the newborn preserved on a filter paper (dried blood spot (DBS)). Since the development of tandem mass spectrometry (MS/MS) in 1990, screening for multiple metabolites in DBS became efficient and affordable, which led to expansion of NBS programs in the years to follow. However, with the expansion of NBS, (ethical) issues of screening emerged.[23,24]

More patients are detected by NBS than detected clinically.[25] This might be due to detection of milder, possibly even benign phenotypes.[26,27] As prior to implementation of NBS these cases did not exist, scientific evidence on the health risks for these individuals is scarce, and thus, pragmatically, treatment is often initiated in all identified individuals with genetic variants. This raises ethical concerns, since identification might prevent serious signs and symptoms, but it simultaneously burdens asymptomatic individuals with the stigma of a diagnosis, the lifelong financial costs of treatment and follow-up and anxiety regarding their own health. Another concern of NBS is the possibility of unintended findings; i.e. identification of disorders not targeted by NBS (e.g. novel disorders or untreatable disorders), as well as identification of disorders in patients other than the newborn (e.g. maternal disorder, identified through NBS of their child).

To guide decision-making on adding disorders to screening programs, Wilson and Jungner proposed a set of 10 principles in 1968, now known as the Wilson and Jungner classic screening criteria.[28] In 2008 the World Health Organisation (WHO) adopted a revisited version of these criteria, and to this day, these adapted criteria are used as basis for policy-making for screening programs (Table 1).[29]

Primary carnitine deficiency and Dutch newborn screening

In 2007, several fatty acid oxidation disorders and certain organic acidemias have been added to the Dutch NBS panel. The identification of these disorders in the DBS is based on specific changes in the acylcarnitine profile. However, with insufficient available free carnitine, these changes might not appear, which can result in missed diagnoses.[30,31] Therefore, in the Netherlands, free carnitine in NBS dried blood spots should at least be 5 µmol/L for reliable interpretation of all acylcarnitine profile based screening results. Newborns with a value below this threshold, undergo a secondary heel prick and, if the low free carnitine levels persist, they are referred to a metabolic centre for evaluation.[32] Over the years, it became apparent that maternal carnitine deficiency (whether primary or secondary) could lead to decreased carnitine levels in NBS DBS.[33-35] Thus, mothers would regularly be evaluated as well. As a consequence, since expansion of the NBS program in 2007, PCD has been diagnosed as an unintended finding in newborns, as well as mothers from referred newborns, in the Netherlands.

In the current Dutch NBS program, PCD is considered an unintended finding. Since PCD is a potentially severe, easily treatable condition, it seems an obvious candidate for official implementation in the NBS

program. However, the outcome of NBS for PCD is controversial. In several countries with NBS for PCD, patients who do not develop any clinical symptoms at all are identified.[35,36] In contrast, severe events have been described in previously asymptomatic individuals.[37,38] Furthermore, NBS for PCD is known to have a fairly poor positive predictive value (PPV), ranging from 1.6 – 4.7%.).[39-41] Due to the high false positive rate, the limited knowledge on the natural history of PCD in an NBS-detected population and the high probability of overdiagnosis, the benefits of NBS for PCD are debatable.[40,42] These issues eventually led to discontinuation of screening for this disorder in New Zealand.[42]

Table 1 | Overview of the Wilson and Jungner classic screening criteria and the additional criteria as proposed by Andermann

Wilson and Jungner classic screening criteria[28]	
1.	The condition sought should be an important health problem.
2.	There should be an accepted treatment for patients with recognized disease.
3.	Facilities for diagnosis and treatment should be available.
4.	There should be a recognizable latent or early symptomatic stage.
5.	There should be a suitable test or examination.
6.	The test should be acceptable to the population.
7.	The natural history of the condition, including development from latent to declared disease, should be adequately understood.
8.	There should be an agreed policy on whom to treat as patients.
9.	The cost of case finding (including diagnosis) should be economically balanced in relation to possible expenditure on medical care as a whole.
10.	Case finding should be a continuing process and not a "once and for all" project.
Additional screening criteria as proposed by Andermann[29]	
1	The screening programme should respond to a recognized need.
2.	The objectives of screening should be defined at the outset.
3.	There should be a defined target population.
4.	There should be scientific evidence of screening programme effectiveness.
5.	The programme svhould integrate education, testing, clinical services and programme management.
6.	There should be quality assurance, with mechanisms to minimize potential risks of screening.
7.	The programme should ensure informed choice, confidentiality and respect for autonomy.
8.	The programme should promote equity and access to screening for the entire target population.
9.	Programme evaluation should be planned from the outset.
10.	The overall benefits of screening should outweigh the harm.

OUTLINE OF THE THESIS

This thesis aims to clarify multiple aspects of PCD, by evaluating if the screening criteria are met for its implementation in the Dutch NBS program. **Chapter 2** provides an overview of the clinical characteristics of PCD by reviewing literature on PCD cases. In order to correlate signs and symptoms with demographic, genetic and biochemical parameters, cases are assessed individually. In **chapter 3** we evaluate the postnatal course of free carnitine, using the free carnitine levels obtained by the first decade of newborn screening. We investigate the relation between free carnitine concentrations and gestational age and birth weight. In **chapter 4** we report on a severely affected patient with PCD that had recurrent cardiomyopathy after decades of treatment non-adherence. **Chapter 5** consists of a retrospective analysis of the clinical characteristics of PCD patients in the Netherlands. We survey all individuals referred for low plasma free carnitine in NBS (newborns and mothers). This survey provides information on the burden of disease in identified patients, as well as the accuracy of the NBS program as it was implemented up until now. In **chapter 6** we investigate the ratio of urine free carnitine over plasma free carnitine as an early test in the metabolic specialist centre for excluding PCD in referred newborns. In **chapter 7**, we discuss the results of all studies and reflect on the process of implementing a new disease in the newborn screening program. Methods for improving screening for PCD in the future are explored.

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CHAPTER

Clinical characteristics of primary carnitine deficiency – a structured review using a case by case approach

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ABSTRACT

A broad spectrum of signs and symptoms has been attributed to primary carnitine deficiency (PCD) since its first description in 1973. Advances in diagnostic procedures have improved diagnostic accuracy and the introduction of PCD in newborn screening programs (NBS) has led to the identification of an increasing number of PCD patients, including mothers of screened newborns, who may show a different phenotype compared to clinically diagnosed patients.

To elucidate the spectrum of signs and symptoms in PCD patients, we performed a structured literature review. Using a case by case approach, clinical characteristics, diagnostic data and mode of patient identification were recorded. Signs and symptoms were categorized by organ involvement. In total, 166 articles were included, reporting data on 757 individual patients. In almost 20% (N=136) of the cases, the diagnosis was based solely on low carnitine concentration which we considered an uncertain diagnosis of PCD. The remaining 621 cases had a diagnosis based on genetic and/or functional (i.e. carnitine transporter activity) test results. In these 621 cases, cardiac symptoms (predominantly cardiomyopathy) were the most prevalent (23.8%). Neurological (7.1%), hepatic (8.4%) and metabolic (9.2%) symptoms occurred mainly in early childhood. Adult onset of symptoms occurred in 16/194 adult patients, of whom 6 (3.1%) patients suffered a severe event without any preceding symptom (5 cardiac events, 1 coma).

In conclusion, symptoms in PCD predominantly develop in early childhood. Most newborns and mothers of newborns detected through NBS remain asymptomatic. However, though rarely, severe complications do occur in both groups.

INTRODUCTION

Primary carnitine deficiency (PCD) (OMIM #212140) is an inborn error of metabolism caused by pathogenic variants in the SLC22A5 gene, which encodes the Organic Cation Transporter Novel 2 (OCTN2) protein.[1] This protein is responsible for the transport of carnitine across the plasma membrane into cells, as well as the reabsorption in renal tubuli.[2] Impaired function of OCTN2 leads to a significant decrease of intracellular carnitine concentrations, potentially hampering mitochondrial fatty acid oxidation. In older publications, patients have been reported to suffer from hepatic encephalopathy, cardiomyopathy, myopathy and arrhythmia and/or sudden death.[3,4] However, with the introduction of PCD in newborn screening (NBS) programs, increasing numbers of individuals with biallelic SLC22A5 variants are identified, most of whom are asymptomatic.[5-7]

The diagnostic criteria for PCD have changed over the past decades due to advances in diagnostic techniques (see figure 1). Initially, patients were diagnosed solely based on low carnitine concentrations, either in muscle (termed "muscle carnitine deficiency") or blood/serum (termed "systemic carnitine deficiency"), distinguishing two forms of the disorder.[8,9] Currently, isolated muscle carnitine deficiency is no longer considered a form of PCD. In 1981, Gazzola et al. described a method to measure carnitine transporter activity in cultured fibroblasts, proving that impaired transport of carnitine across the plasma membrane is the cause of PCD.[10-12] In 1998, Tamai et al. successfully identified the transporter responsible for the transport of carnitine, the Organic Cation Transporter Novel 2 (OCTN2).[2] One year later, Nezu et al. found the encoding gene (SLC22A5) and showed that variants in this gene lead to impaired carnitine transport across the plasma membrane.[1] Subsequently, it has become common practice to diagnose PCD patients based on biallelic pathogenic variants in SLC22A5, and/or reduced carnitine transporter activity measured in fibroblasts.[3]

The clinical characterization of rare disorders relies on the combined data of reported cases. However, with advances in the diagnostic approach, revision of historical cases might uncover alternative underlying causes for the patients symptoms. A number of patients who were formerly diagnosed with PCD based on low serum carnitine levels, turned out to have secondary carnitine deficiency due to medium-chain acyl-CoA dehydrogenase (MCAD) deficiency.[13-17] Moreover, the first reported PCD patient widely cited as a benchmark case, showed elevated levels of C6, C7 and C8 dicarboxylic acids whilst on carnitine supplementation, which leads to a suspicion of MCAD deficiency.[9,18-20] These examples illustrate that historical case reports of a specific inborn error of metabolism need to be reappraised when determining the disorders clinical spectrum.

Introduction of PCD in NBS programs has enabled detection of newborns prior to the onset of symptoms.[21] However, NBS also detects many asymptomatic maternal cases, that might otherwise have remained unidentified.[22] It is uncertain what the health consequences for these individuals are, making counselling on the need of follow-up and treatment particularly difficult.

We aim to update the clinical characterization of PCD, based on current diagnostic standards, through a structured review of previous case reports. The phenotypic spectrum of PCD is described, including separate analyses of patients diagnosed clinically and through screening. This overview aids counselling and treatment decisions in PCD and can facilitate the discussion on the benefit of NBS for this disorder.

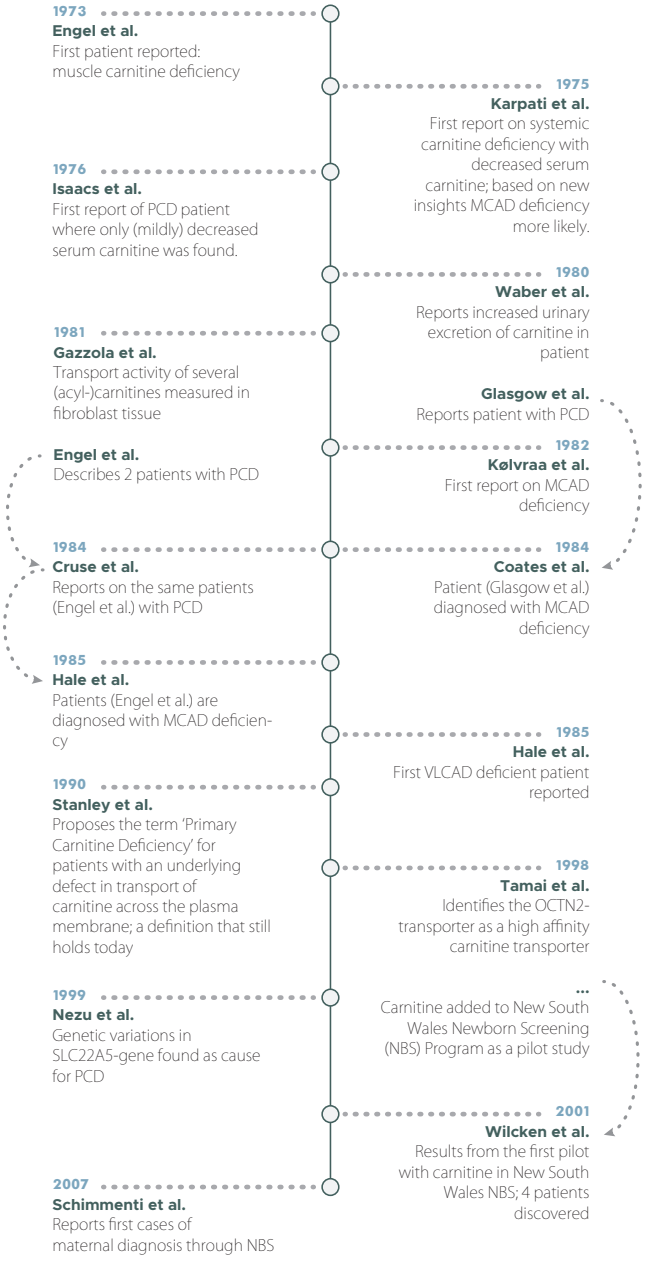

Figure 1 | Timeline of key moments that impacted the (differential) diagnosis of primary carnitine deficiency

METHODS

Data sources and search

PubMed and Embase were searched from inception to 31-12-2020. No restrictions were applied on publication date or article type. The search was constructed using terms and synonyms for carnitine deficiency or OCTN2 deficiency (see supplementary table 1). Duplicates were manually removed.

Study selection

Study selection was performed according to the PRISMA guidelines.[23] Title and abstracts were screened independently by LC and BS with use of Covidence (Covidence systematic review software, Veritas Health Innovation, Melbourne, Australia. available at http://www.covidence.org). The articles that passed for full-text assessments were judged for in- and exclusion by predefined criteria as listed in supplementary table 2. Articles were eligible for inclusion if a case of primary carnitine deficiency was reported and original data of the clinical characteristics were available in the article. A case of PCD was defined as a serum, plasma or blood carnitine level below the lower limit of the reference value at the study site reported in the article and/or confirmed low carnitine transporter activity in fibroblasts and/or the presence of biallelic variants in the SLC22A5 gene. Articles were excluded if the following criteria applied: diagnosis of muscle carnitine deficiency, diagnosis solely based on low post-mortem serum carnitine levels, incomplete diagnostics, low plasma carnitine levels more likely caused by a disorder other than PCD, the article did not report data of (an) individual case(s), the article did not concern PCD and finally, the article was written in a language other than English, Dutch, German, French or Danish. If full text was unavailable and the abstract contained a case description, the abstract was included in the study. If the abstract did not contain enough data, we requested the full text from corresponding authors for articles published from 1990 onwards, since from then, the diagnostic definition of PCD is in line with the currently used definition (see figure 1). If there was disagreement on whether or not to include an article, consensus was reached through discussion between LC and BS. A full list of included articles is available in supplementary data 1.

Data Extraction

The following data were extracted for individual cases from the included articles: age at diagnosis, diagnostic test results (plasma carnitine concentration, carnitine transport activity in fibroblasts, SLC22A5 variants), clinical symptoms, age at start of symptoms, additional tests performed for symptoms (e.g. imaging, laboratory tests), carnitine supplementation dose, effect of carnitine supplementation on symptoms. If the patient had died, all information on the cause of death and the results of post-mortem tests were extracted. If multiple articles described the same case (i.e. when referenced in the article or when multiple identifying data points and (part of) the research group overlapped), information from all articles was used but the case was included only once. In case of missing data, this was recorded.

General analysis of clinical characteristics

The entire study population was divided into several subgroups for analysis. Firstly, patients were grouped based on the reliability of the diagnosis of PCD, separating patients that were diagnosed solely based on low plasma/serum/blood carnitine concentration (unconfirmed PCD) from patients whose diagnosis was confirmed by either decreased OCTN2 transporter activity and/or biallelic variants in the SLC22A5 gene (confirmed PCD). Secondly, patients were grouped based on mode of detection of the disorder: clinical presentation, screening of high risk individuals (family screening or a nationwide adult screening campaign in the Faroe islands[24]), NBS or maternal diagnosis after NBS of the child. Patient characteristics and symptom prevalence were compared between these groups.

Categorization of signs and symptoms

To systematically categorize reported signs and symptoms, two groups were defined: (1) Sign/symptom reported by authors (description of a specific sign or symptom only without information on specific test results e.g. laboratory tests, cardiac ultrasound, etc). (2) Sign/symptom confirmed (data on additional test results on which the description of the sign or symptom was based were available, see supplementary table 3). If confirmatory tests and the reported sign/symptom contradicted each other, the conclusion of additional testing was used, overruling the sign/symptom reported by the authors. For instance: if authors reported dilated cardiomyopathy but the cardiac ultrasound showed normal ejection fractions, this was classified as cardiac involvement NOS, since ultrasound contradicts the presence of dilated cardiomyopathy.

All symptoms were registered verbatim in the database and translated to standardized terms using the human phenotype ontology (HPO version 1.7.0 http://www.human-phenotype-ontology.org). These standardized terms were consequently clustered based on organ involvement, resulting in the following groups: (1) cardiac function disorder (cardiomyopathy, cardiac involvement not otherwise specified (NOS)), (2) electrophysiological abnormalities (sustained ventricular tachycardia, ventricular fibrillation, cardiac arrest, sudden cardiac death, conduction disorder NOS, arrhythmia NOS). For cardiac events (ventricular fibrillation (VF), sustained ventricular tachycardia (sustained VT), cardiac arrest, sudden cardiac death), if multiple cardiac events occurred in one patient, only one was used for categorization, to prevent double entries in the dataset. For this, the most specifically documented cardiac event was used. For instance: a patient reported with cardiac arrest and VF, would be categorized as VF. (3) neurological symptoms (coma, encephalopathy, seizure, neurological involvement NOS), (4) hepatic symptoms (steatosis, hepatic failure, impaired liver function, elevated liver enzymes, hepatomegaly), (5) muscle symptoms (myopathy, rhabdomyolysis, atrophy, myalgia, weakness, muscle involvement NOS), (6) metabolic symptoms (hypoglycemia, hyperammonemia, metabolic acidosis), (7) other symptoms (anemia, motor delay, general developmental delay, dysmorphia, other/non-specific) and (8) death. For the full list of standardized terms that were applied and a summary of how these were designated in characteristic-groups, see supplementary table 4.

Genetic data

Extracted variants were annotated using the comprehensive overview of molecular changes in SLC22A5 by Frigeni et al. 2017, the OCTN2 database provided by ARUP Scientific Resource for Research and Education, and Alamut Visual V2.11 (Interactive Biosoftware).[25,26] For analysis of genotype-phenotype associations, variants were grouped into (1) missense variants and in-frame deletions/insertions, (2) nonsense, frameshift and splice site variants.

Statistics

Statistical analysis was performed using R (version 3.6.2, R Core Team, Vienna, Austria). For comparison between categorical data, the chi-square test was used. Mann-Whitney U tests were used for comparison between two groups with continuous data. Bonferroni correction was applied to correct for multiple testing. Significance was assumed for a p-value < 0.05.

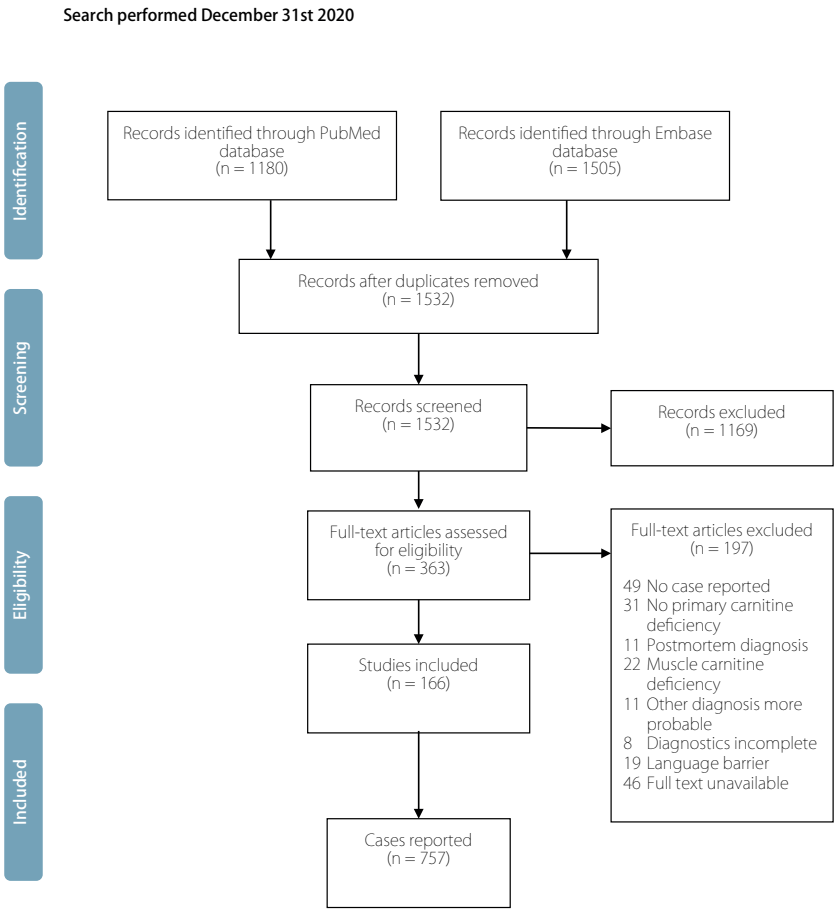

Figure 2 | PRISMA flow diagram of literature selection and inclusion

RESULTS

With the search, 1532 articles were identified for title and abstract screening of which 166 met the inclusion criteria for this review, yielding 757 unique cases. Of these, 53 cases were described in two or more articles. The results of data selection are presented in a PRISMA flow diagram (figure 2). 136 patients had unconfirmed PCD (diagnosis based on serum, plasma or blood carnitine concentration only), whilst the remaining 621 patients had confirmed PCD. Of those, 26 were diagnosed by decreased carnitine

Table 1 | Patient characteristics of patients with a possible and a certain diagnosis of PCD

		Possible diagnosis of PCD (diagnosis based on low plasma/serum/blood carnitine concentration only) N=136		**Certain diagnosis of PCD (diagnosis based on decreased activity and/or bialleic variants in the SLC22A5 gene)** N=621		*p*-value
		Median/N	**[Min - Max]/(%)**	**Median/N**	**[Min - Max]/(%)**	
Year published		2010	[1976 - 2020]	2014	[1979 - 2021]	**<0.001**
Sex (male)		45	(34.4%)	234	(42.4%)	
Age at diagnosis (years)		3.75	[0.0 - 45.0]	1.00	[0.0 - 79.0]	
Infancy		48	(35.6%)	286	(46.7%)	
Childhood		42	(31.1%)	133	(21.7%)	**0.025**
Adulthood		45	(33.3%)	194	(31.6%)	
Age at first symptom (years)		1.00	[0.0 - 40.0]	1.00	[0.00 - 39.0]	
Infancy		29	(42.6%)	63	(33.0%)	
Childhood		27	(39.7%)	112	(58.6%)	**0.003**
Adulthood		12	(17.6%)	16	(8.4%)	
Carnitine [a]						
Free Carnitine	(N samples)	82		470		
	(Ratio)	0.31	[0.01 - 095]	0.34	[0.00 – 1.00]	
Total Carnitine	(N samples)	50		68		
	(Ratio)	0.49	[0.00 - 1.37]	0.17	[0.00 - 3.58]	
Unspecified Carnitine	(N samples)	9		4		
	(Ratio)	0.59	[0.25 - 0.89]	0.13	[0.00 - 0.72]	
Transporter activity	(% of controls) [b]	-		5.0	[0.0 - 22.0]	
Cardiac symptoms		34	(25.0%)	148	(23.8%)	1.000
Neurological symptoms		21	(15.4%)	44	(7.1%)	**0.023**
Muscle symptoms		46	(33.8%)	64	(10.3%)	**<0.001**
Hepatic symptoms		22	(16.2%)	52	(8.4%)	0.071
Metabolic symptoms		27	(19.9%)	57	(9.2%)	**0.005**
Only non-specific symptoms		3	(2.2%)	72	(11.6%)	**0.012**
Asymptomatic		58	(42.6%)	342	(55.1%)	0.090
Deceased		10	(7.4%)	25	(4.0%)	0.057

Data presented as: N (%) or Median [Min – Max]. Significant p-values are in bold. For sex, age at diagnosis, age period at diagnosis, age at first symptom and age period at first symptom, the number of missing data were, respectively: Possible diagnosis: 5, 12, 1, 20, 12; Certain diagnosis: 69, 54, 8, 139,88.

[a] If possible, ratios were calculated for serum/plasma/blood carnitine concentrations as follows: $\frac{Carnitine\ concentration}{Lower\ reference\ value\ at\ study\ site}$. Provided data are: N available samples, ratio [Min – Max]

[b] If possible, results were converted to % of controls as follows: $\frac{Carnitine\ transporter\ activity}{Mean\ activity\ of\ controls} \cdot 100\%$. This conversion was possible for 67/87 samples.

transporter activity, 534 by the detection of biallelic variants in the SLC22A5 gene and 61 by results from both tests. An overview of patient characteristics of unconfirmed and confirmed PCD patients is provided in table 1. The median year of publication differed significantly between unconfirmed and confirmed PCD

(2010 [IQR 1990-2020] and 2014 [IQR 2010-2020] respectively, p <0.001). Neurological, muscle, hepatic and metabolic symptoms were all reported more often in unconfirmed PCD (15.4, 33.8, 16.2, 19.9% respectively) compared to the confirmed population (7.1, 10.3, 8.4, 9.2% respectively; p-values: 0.023, <0.001, 0.071, 0.005, respectively; only for hepatic symptoms was no significant difference observed). In the unconfirmed PCD

group, 42.6% of individuals were asymptomatic, versus 55.1% in the confirmed PCD group (p=0.090). Finally, mortality was higher in the unconfirmed PCD group (7.4 vs 4.0%; p=0.057), though this observation was not significant.

Analyses in the following paragraphs were performed in the confirmed PCD group only.

Clinical characteristics based on mode of detection

Of the 621 patients, 161 were identified after presenting with symptoms (clinical diagnosis), 102 maternal cases were identified through abnormal results in the NBS of their child (maternal cases), 249 cases were identified through NBS and finally, 109 patients were detected through high risk screening.

Clinically diagnosed patients (N=161) were mostly diagnosed in infancy/childhood (N=145; 90.1%). Only 5/161 patients (3.1%) experienced their first symptom after childhood. The median age at first symptom was 1 year for the clinically diagnosed patients [IQR 0.5 – 5 years]. In contrast, none of the maternal cases had experienced symptoms in childhood. Of the symptomatic maternal cases (17/102; 16.7%), 9 experienced exclusively pregnancy-related/non-specific symptoms (see table 2) and 8 patients developed the following symptoms: VF and hypotonia (N=1; 1.0%), sustained VT (N=1; 1.0%), cardiomyopathy with cardiac dilation (N=1; 1.0%), hepatic steatosis with hypoglycemia (N=1; 1.0%), muscle weakness (N=3; 3.9%) and fasting intolerance (N=1; 1.0%). The signs and symptoms in NBS patients (25/249; 10.0%) were respectively: non-specific (N=11), cardiac involvement (N=4; atrial/ventricular septal defect and/or mitral valve insufficiency), metabolic signs (N=1; metabolic decompensation), metabolic signs and neurological symptoms (N=3; hypoglycemia and coma/convulsions), hepatic symptoms (N=1; elevated ALAT), muscle symptoms (N=2; mild muscle weakness, elevated creatine kinase) and death (N=3).[6,27-32] In patients identified through high risk screening, the majority of individuals did not experience any (PCD-related) symptoms (30.3% asymptomatic and 44.0% only non-specific symptoms). But the prevalence of severe symptoms (cardiomyopathy, cardiac events and sudden death) was higher in this group than in patients identified through newborn screening (NBS and maternal cases). Genetic analysis of the SLC22A5 gene revealed more biallelic nonsense, frameshift or splice-site variants in patients that were diagnosed after symptomatic presentation, than in patients detected through high risk screening, maternal and NBS cases (38.5% vs 5.0, 2.1 and 6.4% respectively, p-value <0.001). Symptoms across all symptom groups were more prevalent in those that carry biallelic

Table 2 | Clinical characteristics of PCD patients based on mode of detection

		Symptomatic presentation		Risk screening		Maternal NBS		NBS	
		N=161		N=109		N=102		N=249	
Age at diagnosis (years)		3.0	[0.0 - 57.0]	29.0	[0.0 - 79.0]	29.0	[20.0 - 43.0]	0.0	[0.0 - 0.7]
Infancy		32	(19.9%)	5	(4.6%)	0	(0.0%)	249	(100%)
Childhood		113	(70.2%)	20	(18.3%)	0	(0.0%)	0	(0.0%)
Adulthood		13	(8.1%)	79	(72.5%)	102	(100%)	0	(0.0%)
Age at first symptom (years)		1.0	[0.0 - 39.0]	3.0	[0.5 - 34.0]	22.0	[20.0 - 34.0]	0.1	[0.0 - 4.0]
Infancy		51	(31.7%)	3	(3.9%)	0	(0.0%)	9	(3.7%)
Childhood		99	(61.4%)	10	(13.1%)	0	(0.0%)	3	(1.2%)
Adulthood		5	(3.1%)	4	(5.2%)	7	(7.4%)	0	(0%)
Free carnitine ratio	(N samples)	87		86		84		213	
	(Ratio)	0.12	[0.00 - 1.00]	0.44	[0.00 - 0.83]	0.20	[0.00 - 1.00]	0.41	[0.00 - 0.92]
Transporter activity	(N samples)	50		3		11		4	
	(% of control)	5.0	[0.0 - 22.0]	5.0	[5.0 - 6.0]	3.7	[0.1 - 9.0]	5.0	[5.0 - 16.0]
Genetic analysis performed		143		100		97		249	
MS.IF/MS.IF		52	(36.4%)	37	(37.0%)	65	(67.0%)	121	(48.6%)
MS.IF/NS.FS.SS		24	(16.8%)	11	(11.0%)	25	(25.8%)	90	(36.1%)
NS.FS.SS/NS.FS.SS		55	(38.5%)	5	(5.0%)	2	(2.1%)	16	(6.4%)
MS.IF/Missing		3	(2.1%)	0	(0.0%)	1	(1.0%)	7	(2.8%)
NS.FS.SS/Missing		3	(2.1%)	0	(0.0%)	0	(0.0%)	3	(1.2%)
Missing/Missing		6	(4.2%)	47	(47.0%)	4	(4.1%)	13	(5.2%)
Cardiac symptoms		118 [a]	(72.7%)	22	(20.2%)	3	(2.9%)	6 [b]	(2.4%)
Neurological symptoms		38	(23.6%)	3	(2.8%)	0	(0.0%)	3	(1.2%)
Muscle symptoms		55	(34.2%)	3	(2.8%)	4	(3.9%)	2	(0.8%)
Hepatic symptoms		49	(30.4%)	1	(0.9%)	1	(1.0%)	1	(0.4%)
Metabolic symptoms		49 [a]	(30.4%)	1	(0.9%)	2	(2.0%)	5	(2.0%)
Only unspecific symptoms		4 [c]	(2.5%)	48 [d]	(44.0%)	9 [e]	(8.8%)	11 [f]	(4.4%)
Asymptomatic		0	(0.0%)	33	(30.3%)	85	(83.3%)	224	(90.0%)
Deceased		21	(13.1%)	1	(0.9%)	0	(0.0%)	3	(1.2%)

Note: Data presented as: N (%) or median [Min–Max]. For age at diagnosis, age period at diagnosis, age at first symptom and age period at first symptom, the number of missing data were, respectively: symptomatic presentation: 29, 3, 45, 6; risk screening: 11, 5, 65, 59; maternal NBS: 9, 0, 14,10; NBS: 5, 0, 15, 13. Abbreviations: Maternal NBS - mothers identified through NBS of their child; MS.IF - missense/in-frame variant; NBS - newborn screening; NS.FS.SS - nonsense/frameshift/splice-site; PCD - primary carnitine deficiency ; Missing (under genetic analysis) – variant identified, but type was not reported.

[a] Includes one patient that was also diagnosed with NAGS deficiency.

[b] Includes one patient that was also diagnosed with NICCD.

[c] Failure to thrive without cardiac symptoms (N=2) | fatigue (N=1) | diabetes (N=1).

[d] Fatigue (N=17) | abdominal symptoms (N=10) | respiratory infection (N=4) | palpitations (N=3) | fever (N=2) | chest pain (N=2) | slow weight gain (N=1) | diabetes (N=1) | carotid dissection (N=1) | dizzynes (N=1) | oligomenorrhea (N=1) | pneumothorax (N=1) | psoriasis (N=1) | hypertension (N=1) | sinusitis (N=1) | testicular cancer (N=1).

[e] Decreased stamina during pregnancy, spontaneous miscarriages (N=1) | sinus tachycardia, palpitations, and preeclampsia (N=1) | hyperemesis gravidarum (N=1) | fatigue (N=6).

[f] Growth retardation (N=6) | cleft palate (N=2) | abdominal pain (N=1) | congenital double thumb (N=1) | fatigue (N=1).

nonsense, frame-shift and/or splice-site variants compared to those that carry missense or in-frame variants (supplementary table 5).

Treatment was initiated upon diagnosis in 133/161 (82.6%) of the clinically diagnosed patients (no treatment N=14, missing N=14), in 93/109 (85.3%) of the high risk screening patients (no treatment N=1, missing N=15), in 69/102 (67.6%) of the maternal cases (no treatment N=11, missing N=22) and in 200/249 (80.3%) of the newborns (no treatment N=7, missing N=42). The clinically detected patients and high risk screening patients that did not receive treatment died prior to diagnosis.

Cardiac symptoms

Cardiomyopathy was the most prevalent symptom, reported in 112 of 621 cases (18.0%), see table 3. In 59 patients (9.5%) cardiac dilation was described, with results of cardiac imaging available for 53 patients. Data on left ventricular ejection fraction (LVEF) were available for 23 patients, with a median LVEF of 34% (IQR: 25 – 39.5) without treatment. Fifteen of these received carnitine treatment with improvement of ejection fraction (median 66%; IQR: 61.5 – 73). Cardiomyopathy with hypertrophy was reported in 31 patients (5.0%) (imaging results available for 23 patients). For the remaining 22 (3.5%) patients with cardiomyopathy, imaging results were available for 6 patients, but no information of cardiac morphology was provided. Most patients that developed cardiomyopathy presented with symptoms in childhood (N=102; 95.3%; median age 1 year). Two patients (1.9%) developed cardiomyopathy in adulthood without any prior symptoms; 1 mother that was diagnosed through NBS of her child and 1 patient was diagnosed through family screening in childhood. The latter remained asymptomatic whilst on carnitine supplementation and developed cardiomyopathy after discontinuation in adulthood.[33,34] None of the PCD patients detected through NBS developed cardiomyopathy. In fifteen (2.4%) patients other cardiac signs or symptoms were described, namely: cardiomegaly (N=3), cardiac dilation with normal ejection fraction (N=3), mitral/aortic valve insufficiency (N=2), myocardial infarction (N=2), atrial septal defect (N=2), ventricular septal defect (N=1), cardiac fibroelastosis (N=1) and intramyocardial lipid deposition (N=1).

Twenty-three patients (3.7%) experienced cardiac events (table 4), namely: VF (N=7; 1.2%), sustained VT (N=2; 0.3%), cardiac arrest (N=6; 1.0%) and sudden cardiac death (N=8; 1.3%). In 14/23 patients with a cardiac event (60.8%), this event was the presenting symptom. In 8/23 patients (34.8%), the first cardiac event occurred in adulthood. Five of these adults (CE-1, CE-2, CE-4, CE-18 and CE-23) were completely asymptomatic before the cardiac event occurred. They had not received carnitine supplementation prior to the event. The 3 remaining patients had the following medical history prior to the cardiac event: coma 2 months before event (CE-14), laparotomy 4 days prior to event (CE-15) and NAGS-deficiency diagnosed at 5 years of age (CE-9) (dubious diagnosis, more details on case description in "deceased patients").[35] One patient (CE-6) experienced a cardiac event whilst on carnitine treatment; he died at the age of 26 years due to asystole after a history of multiple cardiac arrests in childhood, hypoglycemic coma with seizures, hyperammonemia, hepatomegaly, weakness and developmental delay.

Table 3 | Overview of cardiac symptoms

		Cardiac Function				Cardiac events							
		Cardiomyopathy N=112 (82)		Cardiac involvement NOS N=15 (11) [a, b]		Sus-VT N=2		VF N=7		Cardiac arrest N=6		Sudden death N=8	
Arrhythmia NOS [c]		6	(1 tachy, 4 brady, 1 ns-VT)	0		0		2	(1 brady, 1 ns-VT)	0		0	
Conduction disorder NOS [d]		4	(1 HB, 3 short QT)	2	(2 short QT)	1	(long QT)	1	(short QT)	0		0	
Deceased		6		3		1		2		4		8	
Age at first symptom (years)		1.0	[0.0-39.0]	1.0	[0.0 - 22.0]	23.0	[20.0 - 26.0]	28.0	[15.0 - 39.0]	0.5	[0.3 - 1.0]	5.0	[0.3 - 6.0]
Infancy		35		3		0		0		4		1	
Childhood		72		6		0		1		2		6	
Adulthood		3		3		2		6		0		0	
Detected through													
Symptoms		103		7		1		3		5		7	
Family screening		8		3		0		2		1		0	
Maternal NBS		1		1		1		2		0		0	
NBS		0		4		0		0		0		1	
Free carnitine ratio	(N samples)	66		9		1		4		4		3	
	(Ratio)	0.10	[0.00-1.00]	0.40	[0.11 - 0.76]	0.08	[-]	0.11	[0.04 - 0.20]	0.25	[0.09 - 0.41]	0.16	[0.14 - 0.16]
Transporter activity	(N samples)	35		2		1		1		4		1	
	(% of control)	5.00	[0.00-20.80]	3.47	[2.48 - 5.00]	4.8	[-]	9.6	[-]	5.0	[2.0 - 10.0]	5.00	[-]
Genetic analysis performed		99		13		2		6		5		7	
MS.IF/MS.IF		27	(27.3%)	6	(40.0%)	2	(100%)	4	(66.7%)	2	(40.0%)	2	(28.6%)
MS.IF/NS.FS.SS		17	(17.2%)	5	(33.3%)	0	(0.0%)	2	(33.3%)	1	(20.0%)	1	(14.3%)
NS.FS.SS/NS.FS.SS		44	(44.4%)	1	(6.7%)	0	(0.0%)	0	(0.0%)	2	(40.0%)	4	(57.1%)
MS.IF/Missing		2	(2.0%)	0	(0%)	0	(0.0%)	0	(0.0%)	0	(0.0%)	0	(0.0%)
NS.FS.SS/Missing		3	(3.0%)	0	(0%)	0	(0.0%)	0	(0.0%)	0	(0.0%)	0	(0.0%)
Missing/Missing		6	(6.0%)	1	(6.7%)	0	(0.0%)	0	(0.0%)	0	(0.0%)	0	(0.0%)
Carnitine treatment started		91		12		1		4		2		1	
Clinical improvement		86		7		1		3		2		0	

Note: Data presented as: N-total (confirmed) / N(%) or Median [Min – Max]. For age at first symptom and age period at first symptom, the number of missing data were, respectively: Cardiomyopathy: 30, 2; Cardiac involvement NOS: 8, 18; Sus-VT: 0, 0; VF: 1, 0; Cardiac arrest: 1, 0; Sudden death: 3, 1. Abbreviations: NBS – newborn screening; maternal NBS – Mothers identified through NBS of their child; MS.IF – missense/in-frame variant; NS.FS.SS – nonsense/frameshift/splice-site; Missing (under genetic analysis) – variant identified, but type was not reported; NOS – not otherwise specified; sus-VT – sustained ventricular tachycardia; VF – ventricular fibrillation; tachy – tachycardia; brady – bradycardia; ns-VT – non-sustained ventricular tachycardia; HB – first degree heart block

[a] Includes 1 patient that was also diagnosed with NAGS-deficiency.

[b] Includes 1 patient that was also diagnosis with NICCD.

[c] Total (N=11): brady (N=5) | atrial fibrillation (N=3) | ns-VT (N=1) | tachy (N=1) | arrhythmia, not otherwise specified (N=1)

[d] Total (N=13): short QT (N=7) | right bundle branch block (N=3) | left bundle branch block (N=1) | HB (N=1) | Long QT (N=1)

Table 4 | Patient characteristics of patients that suffered a cardiac event

Patient ID	Reference		Sex	Country	Detected through	Cardiac event	Age at first cardiac event	Age at Dx (years)	Cardiac event presenting symptom	Variant allele 1	Variant allele 2	Deceased
CE-1	Schimmenti[22]	2007	F	USA	Maternal NBS	susVT	20 years	25	Yes	c.95A>G	c.136C>G	
CE-2	Mazzini[53]	2011	F	USA	Maternal NBS	VF	22 years	22	Yes	c.424G>T/c.1463G>A	**c.1586+1G>T**	
CE-3	Rijlaarsdam[54]	2003	F	Iran	Symptomatic presentation	VF	15 years	15	Yes	NP. Transporter activity 9.6% of control		
CE-4	Rasmussen[55]	2014	F	Faroe	Symptomatic presentation	VF	39 years	39	Yes	c.95A>G	c.95A>G	
CE-5	Rasmussen[55]	2014	F	Faroe	Symptomatic presentation	Sudden cardiac death	NA	Postmortem	Yes	c.95A>G	c.95A>G	†
CE-6	Chapoy[56]	1980	M	Mexico	Symptomatic presentation	Cardiac arrest	3 months	3	Yes	**c.806delT**	**c.806delT**	†
CE-7	Tang[57]	1998	M	China	Symptomatic presentation	Cardiac arrest	6 months	Postmortem	Yes	**c.396G>A**	c.1433C>T	†
CE-8	Vaz[58]	1999	F	Cape Verde	Symptomatic presentation	Cardiac arrest	20 months	1	-	c.632A>G	c.632A>G	
CE-9	Hwu[35]	2007	M	Taiwan	Symptomatic presentation	Sudden cardiac death	20 years	Postmortem [a]	-	**c.760C>T**	**c.652+1G>A**	†
CE-10	Kishimoto[59]	2012	M	Japan	Symptomatic presentation	Sudden cardiac death	8 years	Postmortem	-	**c.-91_22del**	**c.-91_22del**	†
CE-11	Melegh[60]	2004	M	Hungary	Symptomatic presentation	Sudden cardiac death	6 years	Postmortem	Yes	**c.844delC**	**c.844delC**	†
CE-12	Han[61]	2014	NA	China	Symptomatic presentation	Sudden cardiac death	6 months	Childhood	-	**c.760C>T**	c.338G>A	†
CE-13	Grünert[62]	2020	M	Macedonia	Family screening	Cardiac arrest	4 years	7	-	c.1319C>T	c.1319C>T	
CE-14	Rasmussen[63]	2013	F	Faroe	Adult screening	VF	34 years	55	-	c.95A>G	c.95A>G	
CE-15	Rasmussen[63]	2013	F	Faroe	Symptomatic presentation	susVT	26 years	Postmortem	-	c.95A>G	c.95A>G	†
CE-16	Rasmussen[63]	2013	F	Faroe	Symptomatic presentation	VF	NA	Postmortem	Yes	c.95A>G	c.95A>G	†
CE-17	Dobrowolski[64]	2005	M	Australia	Symptomatic presentation	Sudden cardiac death	18 months	Postmortem	-	NP. Transporter activity < 5% of control		†
CE-18	Gélinas[65]	2019	F	China	Symptomatic presentation	Sudden cardiac death	26 years	Postmortem	Yes	c.51C>G	c.51C>G	†
CE-19	Rasmussen[63]	2013	F	Faroe	Symptomatic presentation	VF	NA	Postmortem	Yes	c.95A>G	c.95A>G	†
CE-20	Tang[57]	1998	M	China	Symptomatic presentation	Cardiac arrest	6 months	Postmortem	Yes	NP. Transporter activity 5% of control		†
CE-21	Lin[29]	2020	F	China	NBS	Sudden cardiac death	2 months	Infancy	Yes	**c.497+1G>T**	**c.517delC**	†
CE-22	Lin[29]	2020	F	China	NBS	Cardiac arrest	1 year	Infancy	NA	**c.760C>T**	**c.824+1G>A**	†
CE-23	Roussel[66]	2016	F	France	Family screening	VF	30 years	28	Yes	c.1411C>T	**exon 2 deletion**	

Note: Nonsense/frameshift/splice-site variants are in bold.

Abbreviations: Dx – diagnosis; M – male; F – female; NBS – newborn screening; maternal NBS – mothers identified through NBS of their child; NA – not available; NP – not performed; susVT – sustained ventricular tachycardia; VF – ventricular fibrillation; † – deceased; PCD – primary carnitine deficiency.

[a] diagnosed with NAGS-deficiency (9% residual activity) at age 5 years, PCD was confirmed postmortem, no variants in NAGS-gene could be identified

Neurologic, hepatic and metabolic signs and symptoms

Neurologic, hepatic and/or metabolic symptoms were reported in a total of 89 patients (14.3%), with more than one of these symptoms in 47 (52.8%) patients (table 5). Coma or encephalopathy occurred in 35 (5.6%) patients and was the presenting feature in 27 patients. In 14/35 patients (40%) an underlying cause was reported: hypoglycemia (N=8), hyperammonemia (N=1) or both (N=5). In 21/35 cases an underlying cause was not reported, but 13/21 did have a history with hypoglycemia and/or hyperammonemia. Hepatic symptoms (N=52; 8.4%) consisted of hepatomegaly (N=41; 6.6%) and hepatic steatosis (N=11; 1.8%), confirmed by liver biopsy in 8 patients. One girl presented in childhood with acute hepatitis, liver dysfunction and hypoketotic hypoglycemia.

At least 80/89 (89.9%) patients experienced their first symptom in childhood with a median age at onset of 1 year [IQR 0.4 – 2.8]. Adult presentation was reported in 6 patients of which 4 used pivalic acid, a compound known to decrease blood carnitine concentration[36,37], prior to development of symptoms (CE-14, CE-15, CE-16 and CE-19). Reported symptoms were hepatic steatosis (CE-15), coma (CE-14), hepatic steatosis and coma (CE-16), hepatomegaly (CE-19), metabolic acidosis and elevated liver enzymes (CE-4) and finally 1 patient with fasting intolerance.

Muscle symptoms

Muscle symptoms occurred in 64 patients (10.3%). The most frequently reported symptom was muscle weakness (N=46; 7.4%). In 10 patients (1.6%) rhabdomyolysis-like episodes were reported, with confirmatory laboratory results reported in only 4. Muscle biopsies were taken in 15 patients, which revealed lipid accumulation in 12 patients (1.9%). Motor developmental delay occurred in 6 patients (1.0%). Additional information is provided in supplementary table 6.

Deceased patients

An overview of characteristics of the 25 deceased patients (4.0%) is provided in table 6. In only 7 patients, the diagnosis of PCD was established prior to death. The age of death was <1 year in 5 patients, 1-8 years in 11 patients, >20 years in 6 patients and data on age of death were missing in 3 patients. In 7 patients, death was preceded by an episode of infection/vomiting. In 5 patients death ensued after treatment with pivampicillin. One third (N=8) of the deceased patients was asymptomatic until death. Three patients identified through NBS died (2 sudden death, 1 due to hypoglycemia) and were reported to have discontinued carnitine supplementation. One case was initially diagnosed with NAGS deficiency (9% residual activity, but no variations in the NAGS gene) after presenting with hypoglycemia, hyperammonemia, cardiomegaly and fatty liver at the age of 5 years, and he was known to have low serum carnitine levels at that time. Several weeks prior to his death, at 20 years of age, he discontinued his carnitine treatment. Postmortem genetic analysis revealed 2 null variants in the SLC22A5 gene, confirming PCD. The diagnosis of NAGS deficiency in this patient remains dubious. Finally, for one case no additional information on death was provided.

In 2 of the 25 deceased patients, no genetic analysis was performed, their diagnosis was based on decreased carnitine transporter activity. In 23 patients, 16 different genetic variants were identified, 11 of which were nonsense, frame-shift or splice-site variants (carried by 14 patients). Out of 9 patients that carried only missense variants, 7 were homozygous for the c.95A>G variant, a variant highly prevalent on the Faroe Islands[23], 1 patient was homozygous for c.51C>G, which is a frequently reported variant in Asia[29,30] and 1 patient was homozygous for c.849G>T, a variant only described in one symptomatic Japanese boy.[37] Frequencies of all variants per symptom group are provided in supplementary table 7.

Table 5 | Overview of neurologic, hepatic, and metabolic symptoms

	Only neurologic		Only hepatic		Only metabolic		Neurological and hepatic		Neurological and metabolic		Hepatic and metabolic		Neurological, hepatic and metabolic		Total	
	N=10		N=20		N=12		N=2		N=15		N=13		N=17		N=89	
Neurologic																
Coma [a]	4	(2 seizure)	-		-		1		5		-		11	(3 seizure)	21	(5 seizure)
Encephalopathy [b]	2		-		-		1		7	(1 seizure)	-		4		14	(1 seizure)
Neurological involvement NOS [c]	2		-		-		0		0		-		1		3	
Seizure [d]	2		-		-		0		3		-		1		6	
Coma/encephalopathy presenting feature (yes)	4		-		-		1		9		-		13		27	
Hepatic																
Elevated liver enzymes	-		4		-		0		-		5		9		18	
Impaired liver function	-		2		-		0		-		1		1		4	
Hepatic steatosis	-		1 (1)		-		2 (2)		-		2 (1)		6 (4)		11 (8)	
Hepatomegaly	-		16 (0)		-		1 (0)		-		10 (1)		14 (0)		41 (1)	
Hepatic failure	-		0 (0)		-		0 (0)		-		1 (0)		0 (0)		1 (0)	
Metabolic																
Hypoglycemia	-		-		8 (4) [e]		-		14 (2)		9 (0)		15 (4)		46 (10)	
Hyperammonemia	-		-		4 (3) [e]		-		6 (1)		5 (2)		10 (5)		25 (11)	
Acidosis	-				4 (1)		-		3 (0)		2 (2)		3 (1)		12 (4)	
Deceased	3		5		2		2		0		1		2		15	
Age at first symptom (years)	2.0	[0.0 - 34.0]	0.8	[0.3 - 26.0]	0.4	[0.0 - 5.0]	1.0	[1.0 - 1.0]	1.0	[0.1 - 12.0]	1.0	[0.3 - 39.0]	0.8	[0.3 - 5.0]	1.0	[0.0 - 39.0]
Infancy	1		10		6		0		5		4		11		37	
Childhood	8		6		5		1		10		7		6		43	
Adulthood	1		2		1		1		0		1		0		6	
Detected through																
Symptoms	8		18		9		2		11		12		17		77	
Risk screening	2		1		0		0		1		0		0		4	
Maternal NBS	0		0		1		0		0		1		0		2	
NBS	0		1		2		0		3		0		0		6	

Table 5 | (Continued)

	Only neurologic		Only hepatic		Only metabolic		Neurological and hepatic		Neurological and metabolic		Hepatic and metabolic		Neurological, hepatic and metabolic		Total	
	N=10		N=20		N=12		N=2		N=15		N=13		N=17		N=89	
Genetic analysis																
MS.IF/MS.IF	6	(60.0%)	7	(41.2%)	4	(33.3%)	2	(100%)	5	(33.3%)	2	(20.0%)	8	(53.3%)	35	(43.2%)
MS.IF/NS.FS.SS	0	(0.0%)	2	(11.8%)	2	(16.7%)	0	(0.0%)	3	(20.0%)	3	(30.0%)	1	(6.67%)	13	(16.0%)
NS.FS/NS.FS.SS	2	(20.0%)	5	(29.4%)	4	(33.3%)	0	(0.0%)	4	(26.7%)	4	(40.0%)	6	(40.0%)	22	(27.2%)
MS.IF/Missing	0	(0.0%)	2	(11.8%)	1	(8.33%)	0	(0.0%)	0	(0.0%)	0	(0.0%)	0	(0.0%)	3	(3.7%)
NS.FS.SS/Missing	1	(10.0%)	0	(0.0%)	0	(0.0%)	0	(0.0%)	0	(0.0%)	0	(0.0%)	0	(0.0%)	1	(1.2%)
Missing/Missing	1	(10.0%)	1	(5.9%)	1	(8.3%)	0	(0.0%)	3	(20.0%)	1	(10.0%)	0	(0.0%)	7	(8.6%)

Note: Data presented as: N-total (confirmed) / N (%) or Median [Min – Max]. For age at first symptom and age period at first symptom, the number of missing data were, respectively: Only neurologic: 2, 0; Only hepatic: 5, 2; Only metabolic: 2, 0; Neurological and hepatic: 1, 0; Neurological and metabolic: 4, 0; Hepatic and metabolic: 4, 1; Neurologic, hepatic and metabolic: 1, 0; Total: 19, 3.
Abbreviations: NOS – not otherwise specified; NBS – newborn screening; MS.IF – missense/in-frame variant; NS.FS.SS – nonsense/frameshift/splice-site; Missing (under genetic analysis) – variant identified, but type was not reported

[a] Hypoglycemic (N=5), hyperammonemic (N=1), hypoglycemic hyperammonemic (N=3), NOS (N=12)
[b] Hypoglycemic (N=3), hypoglycemic hyperammonemic (N=2), NOS (N=9)
[c] Encephalitis; hydrocephalus; patchlike abnormalities brain MRI
[d] Number of cases where seizure was the only reported neurological symptom
[e] Includes 1 patient that was also diagnosed with NAGS-deficiency.

Table 6 | Patient characteristics of deceased patients

Patient ID	Reference		Country	Sex	Consan-guinity	Family death	Age at Dx	Carnitine at diagnosis (ref)		Transporter activity
16 / CE-6	Chapoy[67]	1980	Mexico	M	No	1 brother	3.5 years	Unsp.	4.82 (28.11 - 44.57)	2% of control
52 / CE-9	Hwu[35]	2007	Taiwan	M	NA	NA	NA*[a]	Free	4 (29 - 45)	
086	Melegh[60]	2004	Hungary	M	No	2 cousins (180)	NA*	NP		
162_A / CE-10	Kishimoto[59]	2012	Japan	M	Yes	1 brother (163_A)	NA*	NP		
163_A	Kishimoto[59]	2012	Japan	M	Yes	1 brother (162_A)	NA*	NP		
164	Koizumi[68]	1999	Japan	M	No	0	NA*	NP		
168	Lund[69]	2007	Faroe	F	NA	0	NA*	Free	3.4 (median 27.8)	
169	Lund[69]	2007	Faroe	F	NA	0	NA*	Free	4.4 (median 27.8)	
182 / CE-11	Melegh[60]	2004	Hungary	M	No	1 sister	NA*	NP		
186 / CE-5	Rasmussen[55]	2014	Faroe	F	NA	0	NA*	NP		
293	Lee[33]	2010	Taiwan	F	NA	NA	1 year	NP		
90	Ohkuma[70]	2009	Japan	F	No	NA	Childhood	NP		
123 / CE-7	Tang[57]	1998	China	M	No	1 sister	NA*	Free	9 (22 - 50)	5% of control
180	Melegh[60]	2004	Hungary	M	No	2 cousins (86)	NA*	NP		
301 / CE-12	Han[61]	2014	China	NA	NA	1 sibling	Infancy	Free	1.62 (>10)	
98_B /CE-15	Rasmussen[63]	2013	Faroe	F	NA	1 sister (414_B)	NA*	NP		
414_B / CE-16	Rasmussen[63]	2013	Faroe	F	NA	1 sister (98_B)	NA*	NP		
408 / CE-17	Dobrowolski[64]	2005	Australia	M	NA	0	NA*	Free	3 (-)	5% of control
412 / CE-18	Gélinas[65]	2019	China	F	No	0	NA*	NP		
415	Rasmussen[63]	2013	Faroe	F	NA	NA	NA*	NP		
416 / CE-19	Rasmussen[63]	2013	Faroe	F	NA	NA	NA*	NP		
427 / CE-20	Tang[57]	1998	China	M	No	1 sister	NA*	Free	9 (22-50)	5% of control
507 / CE-21	Lin[29]	2020	China	F	NA	NA	1 month	Free	2.3 (10.3 - 54.2)	
572 / CE-22	Lin[29]	2020	China	F	NA	NA	1 month	Free	2.8 (10.3 - 54.2)	
724	Lin[27]	2021	China	NA	NA	NA	1 month	Free	2.8 (9-50)	

Note: Nonsense/frameshift/splice-site variants are in bold.

Abbreviations: Dx – Diagnosis; M – male; F – female; NA – not available; NP – not performed; Unsp – unspecified; CA – cardiac arrest; CMP – cardiomyopathy; CiNOS - cardiac involvement not otherwise specified; Seiz – seizure; Enc – encephalopathy; HG – hypoglycemia; HA – hyperammonemia; HepM – hepatomegaly; HepS – hepatic steatosis W – weakness; Dev – Developmental delay.

* Not applicable due to a postmortem diagnosis

[a] Diagnosed with NAGS-deficiency (9% residual activity) at age 5 years, PCD was confirmed postmortem, no variants in NAGS-gene could be identified

Table 6 | (continued)

Patient ID	Variant allele 1	Variant allele 2	Symptoms	Age at death	Comments on death
16 / CE-6	**c.806delT**	**c.806delT**	CA(childhood), CiNOS, HG, coma, Seiz, HA, HepM, W, Dev	26 years	Asystole.
52 / CE-9	**c.760C>T**	**c.652+1G>A**	CiNOS, HA, HG	20 years	Sudden death.
086	**c.844delC**	**c.844delC**	Asymptomatic	6 months	Admitted with mild respiratory tract infection, found dead one day after admission.
162_A / CE-10	**c.-91_22del**	**c.-91_22del**	CMP	8 years	Sudden death.
163_A	**c.-91_22del**	**c.-91_22del**	Asymptomatic	2 years	Died suddenly at 2 yrs of age after 1 day of diarrhea and vomiting.
164	c.849G>T	c.849G>T	Asymptomatic	18 months	Sudden death after 1 day episode of vomiting.
168	c.95A>G	c.95A>G		3 years	Encephalopathy with cerebral oedema and death.
169	c.95A>G	c.95A>G	Enc, Seiz, CMP	14 months	Febrile illness with encephalopathy, seizures and cardiac failure. Had been treated with pivampicillin.
182 / CE-11	**c.844delC**	**c.844delC**	Asymptomatic. CMP and HepS at autopsy	6 years	Died suddenly at age 6 years without any previous alarming sign.
186 / CE-5	c.95A>G	c.95A>G	Asymptomatic	NA	Died suddenly while sleeping.
293	**c.760C>T**	**c.760C>T**	Enc	NA	No additional info.
90	**c.396G>A**	**c.844C>T**	HepM, HA coma	NA	Died to heart failure after an infection.
123 / CE-7	**c.396G>A**	c.1433C>T	HepM	6 months	Cardiac arrest after 1 day of poor feeding due to URTI.
180	**c.844delC**	**c.844delC**	HepM, CiNOS	2 years	Respiratory tract infection followed by cardiac decompensation.
301 / CE-12	**c.760C>T**	c.338G>A	HG, HepM	6 months	Sudden death at 6 months of age.
98_B / CE-15	c.95A>G	c.95A>G	HepS	26 years	Underwent laparatomy and treatment with pivampicilin. 4 days later, developed ventricular tachycardia, then asystole and died.
414_B / CE-16	c.95A>G	c.95A>G	VF, coma, HepS	43 years	After treatment with pivalic acid: had two cardiac arrests, then myocardial infarction. Circulation worsened and she died.
408 / CE-17	NP	NP	Asthma, CMP	18 months	Died suddenly.
412 / CE-18	c.51C>G	c.51C>G	Asymptomatic	26 years	Died suddenly while sleeping.
415	c.95A>G	c.95A>G	Coma	3 years	Treated with pivampicilin, developed coma with seizures and died.
416 / CE-19	c.95A>G	c.95A>G	Bradycardia, CA, VF	30 years	Admitted after treatment with pivampicilin, developed ventricular fibrillation and then asystole. The patient died.
427 / CE-20	NP	NP	HepM	6 months	Admitted with respiratory tract infection, died due to cardiac arrest shortly after.
507 / CE-21	**c.497+1G>T**	**c.517delC**	Asymptomatic	2 months	Sudden death.
572 / CE-22	**c.760C>T**	**c.824+1G>A**	CA, HG	13 months	Sudden death.
724	**c.760C>T**	**c.1161T>G**	Asymptomatic	14 months	Death due to hypoglycemia.

DISCUSSION

We have studied the full spectrum of clinical characteristics, both in PCD patients with a diagnosis based only on low carnitine levels (unconfirmed diagnosis) as well as in PCD patients diagnosed by biallelic variants in the SLC22A5 gene and/or reduced carnitine transporter activity in fibroblasts (confirmed diagnosis). Overall symptoms were reported less frequently in the confirmed population compared to the unconfirmed population, although not statistically significant in all analyses. As unconfirmed cases were generally published earlier than confirmed cases (IQR 1990-2020 and 2010-2020, respectively), this might be partly explained by its introduction in NBS programs in several countries from 2001 onwards. In addition, the group with unconfirmed PCD likely comprises patients that do not meet the current diagnostic criteria for PCD and suffer from a different disorder; as illustrated by the fact that for some cases reported in literature, the diagnosis was reconsidered after re-evaluation.[13-17] In disorders that cause secondary carnitine deficiency (e.g. fatty acid oxidation disorders and organic acidemias) other organ systems may be more strongly affected than in PCD. This could have increased the prevalence of muscle and neurological symptoms in the unconfirmed population.[38,39] To ensure that the clinical spectrum of PCD cases as described in this study would not be affected by data of non-PCD patients, unconfirmed cases were not included in the further analyses. The most prevalent clinical characteristic in confirmed PCD was cardiomyopathy (18.0%), and this was the only reported clinical characteristic in 8.4% of patients. Neurologic, hepatic and/or metabolic signs and symptoms occurred in 14.3% and generally developed in early childhood. Four percent of patients died and one third of the deceased patients was completely asymptomatic up until death. Cardiac events in seemingly well patients were often preceded by provoking incidents such as a mild infection and/or initiation of pivalic acid containing antibiotics (13/33; 39.4%).

Symptoms were rarely (5.6%) observed in children identified by NBS. The most obvious explanation for this observation would be early initiation of treatment in NBS-identified patients, thus preventing symptoms. Another explanation could be detection of milder phenotypes in the patients identified by screening programs. The results of this study illustrate that the frequency of nonsense, frame-shift and splice-site variants was considerably lower in patients identified by screening than in patients identified after a symptomatic presentation. This suggests that the underlying variants may very well play a role in disease severity. In line with this hypothesis, Rose et al. previously reported 14 symptomatic and 14 asymptomatic women with PCD and showed that none of the asymptomatic women were homozygous or compound heterozygous for null-variants, whereas 9/14 of symptomatic women carried these variants.[40] Indeed, most maternal cases identified after NBS of their child, of whom the majority has remained asymptomatic throughout life, have missense variants on at least one allele. The identification of milder phenotypes by NBS for PCD is in line with phenotypes identified by screening programs for other diseases. [41-43]

The identification of milder or even asymptomatic maternal cases through NBS of their child raised a dilemma since implementation of PCD in NBS programs.[5,22,40] On the one hand, identification enables physicians to monitor and treat patients before they experience potentially lethal events. On the other

hand, there are ethical concerns of burdening asymptomatic mothers with a diagnosis of PCD, especially given the uncertainty regarding the health consequences and subsequent need for monitoring and treatment. To see if mothers identified through NBS of their child are at risk for acute events, we looked with particular interest at patients that were completely asymptomatic before suffering a (potentially) lethal event (i.e. cardiac event or coma) in adulthood. In our identified cohort, 3.1% (N=6) of adult patients (patients diagnosed in adulthood, N=194) experienced such an event (5 cardiac events, 1 coma) without any prior symptoms (of whom 2 were mothers identified through NBS of their child). This finding adds to the ethical dilemma of identifying asymptomatic mothers with PCD through NBS of their child, as it shows that the risk of a life threatening event is small, but not negligible. With this knowledge, most physicians will likely choose to continue follow-up and treatment in these mothers. However, the question remains whether the burden of lifelong follow-up and treatment and the knowledge of being at risk for a serious event is justified, considering the small risk of this event actually happening.

Previously, Rasmussen et al. published a retrospective analysis of sudden death in the Faroese population and uncovered 13 untreated PCD cases, all homozygous for the c.95A>G variant, suggesting a strong association between this variant and sudden death.[44] However, all these cases originate from a confined region with extremely high genetic homogeneity.[45] Therefore, other genetic traits predisposing for sudden cardiac death within this population cannot be ruled out. For PCD in general, it is difficult to establish a clear association between genotype and disease severity.[40,46-48] Typically, missense and in-frame deletion or insertion variants are less detrimental to protein integrity, theoretically resulting in a milder phenotype, as is also suggested by our data. However, in some cases, these types of variants can still substantially affect protein function (e.g. when the catalytic site is affected). Therefore, the practical variant categorization used in this review is not suited to truly assess functional consequence of variants, which requires in vitro studies such as gene expression studies. However, these approaches are only able to evaluate the effect of a single variant. Variants exist in numerous allelic combinations, each producing an OCTN2-protein with varying residual activity, making it impossible to predict disease outcome based on genotype alone. Unfortunately, at the time of writing, the other conventional diagnostic tool available – measurement of carnitine transporter activity in cultured fibroblasts – is not sensitive enough to reliably predict disease severity: our data showed a median residual transport activity of 5% of controls in asymptomatic (N=15; range 0.1 - 16) as well as symptomatic patients (N=52; range 0 - 22). Recently, a novel method for measuring carnitine transport activity, using D_3-labeled carnitine instead of radioactively labelled carnitine, was published by Ferdinandusse et al.[49] This novel assay is more sensitive and could reliably separate biallelic missense from biallelic nonsense variants (~26 vs ~2% of control, respectively). Further research is required to determine if this novel assay is able to distinguish those patients with symptoms from those individuals that remain asymptomatic.

This review is not without its limitations. Firstly, our approach involved retrieving data from previously reported cases, and is thus susceptible to publication bias. Secondly, some symptoms or signs may not have been reported in the case description (reporting bias). The latter might have resulted in an overestimation

of severe symptoms and/or an underestimation of milder clinical characteristics. However, the case by case approach in this study limited the risk of citation bias, and enabled us to approximate the prevalence of certain clinical traits within PCD patients as closely as possible, using only published data. Furthermore, this approach allowed us to focus specifically on severe outcomes and to identify clinical relevant differences between patients presenting with symptoms and asymptomatic individuals identified through screening.

The primary objective of our study was to identify the clinical spectrum of PCD. Although treatment data of PCD patients were collected, these data were often insufficient (e.g. no data on treatment duration, treatment dosage and/or treatment adequacy (i.e. plasma carnitine levels after treatment)) to draw reliable conclusions on the effect of carnitine supplementation on clinical symptoms. Furthermore, follow-up time could not be reliably collected for included patients. This is particularly important for evaluation of symptoms in the NBS group, as these newborns may have been too young to have developed symptoms when reported. However, the median age at first symptoms for the clinically identified population was 1 year [N=117; IQR 0.5 – 5 years] suggesting that, if left untreated, PCD-related symptoms generally develop throughout the first years of life. It might well be that part of the population of the NBS-group surpassed this age at the time of publication, but this could not be verified based on available data. Research in a controlled setting, preferably with a long follow-up time, is required for assessing symptoms incidence and treatment effect in future studies.

Inherent to the case by case approach, studies that only reported pooled data were excluded (4 articles; containing 57 cases).[40,44,50,51] Most of these cases had been described in other articles that were included in our study. This will therefore probably not have greatly influenced the outcome of this study. Vice versa, several large cohort studies did meet our inclusion criteria.[24, 29,52] As a consequence, there is an overrepresentation of specific geographical regions in our database. This creates a risk of bias in the symptoms reported in groups, based on background genetics, rather than mode of detection. For example 296 of included cases (48.5%) were Chinese. These cases contribute to 64.4% of the maternal cases identified by NBS of their child and to 79.4% of the NBS cases. Of the 14 symptomatic children identified through NBS, 13 were of Chinese heritage. Moreover, 97 cases (15.9%) are of Faroese heritage, representing 78.7% of the risk screening population.

In summary, this is the most extensive review on PCD to date. By reviewing individual clinical outcomes of patients diagnosed by current diagnostic standards, we provide an extensive overview of the clinical characteristics associated with PCD. Patients are predominantly at risk for developing cardiomyopathy. Neurological, metabolic, hepatic and muscle symptoms occurred in approximately 10% of patients. Patients identified through screening rarely developed symptoms, whether this is due to a treatment effect or the detection of milder phenotypes is not yet elucidated. Cardiac events are rare, but they have been reported as the first presenting symptom in a small number of PCD patients, including adults without a remarkable preceding medical history. Cohort studies in genetically diverse populations are needed to compare genetic traits and residual activity on the one hand, and disease outcome on the other to better

predict disease severity. This information is crucial to improve NBS programs, identifying those individuals with PCD requiring treatment and follow-up (actual disease), whilst reducing the number of individuals that have a genetic trait leading to reduced carnitine transporter activity, without clinical consequences (healthy individuals). Until more knowledge on the risk of developing serious events in asymptomatic individuals is gained, we recommend monitoring and treatment of all PCD cases identified.

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SUPPORTING INFORMATION

Primary data from this structured review are made publicly available through Figshare.
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Table S1 | Search terms

Pubmed	Embase
"Carnitine deficiency"[Title/Abstract]	'Carnitine deficiency':ab,ti
"Carnitine uptake defect"[Title/Abstract]	'Carnitine uptake defect':ab,ti
"OCTN2 deficiency"[Title/Abstract]	'OCTN2 deficiency':ab,ti
"OCTN2-deficiency"[Title/Abstract]	'OCTN2-deficiency':ab,ti
"Carnitine transporter deficiency"[Title/Abstract]	'Carnitine transporter deficiency':ab,ti
"Renal carnitine transport defect"[Title/Abstract]	'Renal carnitine transport defect':ab,ti
"Renal carnitine transporter defect"[Title/Abstract]	'Renal carnitine transporter defect':ab,ti
"carnitine uptake deficiency"[Title/Abstract]	'carnitine uptake deficiency':ab,ti
"carnitine membrane transporter deficiency"[Title/Abstract]	'carnitine membrane transporter deficiency':ab,ti
"carnitine uptake defect"[MeSH Terms]	'carnitine uptake defect'

Table S2 | Definition of in- and exclusion criteria

Inclusion	
Case of primary systemic carnitine deficiency	Serum, plasma or blood carnitine level below lower limit of reference value of study site, and/or
	Low carnitine transporter activity (reported as "low", or below lower reference range of study site), and/or
	Homozygous or compound heterozygous variants in SLC22A5 gene.
Original data available	Original data of clinical characteristics of described patient are reported.
Exclusion	
Muscle carnitine deficiency	Normal carnitine in serum but decreased carnitine in muscle tissue.
Post-mortem diagnosis	Primary carnitine deficiency is suggested as cause for death, however, this diagnosis is solely based on post-mortem low serum carnitine levels. If post-mortem diagnosis is confirmed with genetic analysis (bi-allelic SLC22A5 variants) or reduced transporter activity the case is included.
Other diagnosis more probable	Low serum carnitine, dubbed in article as primary carnitine deficiency, however based on new insights probably due to other defect (e.g. FAOD).
Diagnostics incomplete	Primary carnitine deficiency according to authors, however no information on diagnostics is reported in article (serum carnitine, genetic analysis, functional assay).
No case reported	No clinical data reported in the article.
No primary carnitine deficiency	Primary carnitine deficiency was not mentioned in article.

Table S3 | Criteria for confirming symptoms in patients where certain symptoms are reported

Symptom-group	Aditional testing Modality	Possible description in the articles
Cardiomyopathy with cardiac dilation	Ultrasound	LVEF < 50% or "reduced" and dilated cardiomyopathy in case description
		Dilated cardiomyopathy as conclusion of ultrasound
		Cardiac dilation and impaired myocardial function reported in case description
	Autopsy	Dilated cardiomyopathy as conclusion of autopsy
Cardiomyopathy with hypertrophy	Ultrasound	Cardiac hypertrophy as conclusion and impaired myocardial function reported in case description
		Increased LV wall thickness ≥15 mm
Cardiomyopathy	Ultrasound	LVEF ≤50% or "reduced" and no cardiac dilation reported in case description
		Cardiomyopathy as conclusion
		Left ventricular dysfunction
Cardiac involvement NOS	Ultrasound	Cardiac dilation with LVEF ≥ 50%
		Cardiac hypertrophy and no impaired myocardial function reported in case description
		Abnormalities in cardiac morphology without impaired myocardial function
Arrhythmia NOS	ECG	Arhhythmia witnessed on ECG[b]
Conduction disorder NOS	ECG	All forms/types of conduction disorders reported in case description[a]
Ventricular tachycardia	ECG	Ventricular tachycardia reported (without mention of duration <30sec)
Ventricular fibrilation	ECG	Ventricular fibrillation
Neurological involvement NOS	Brain MRI	Abnormalities reported [b]
	Brain CT-scan	Abnormalities reported [b]
Hepatomegaly	Ultrasound	Enlarged liver or hepatomegaly as conclusion of ultrasound
Hepatic fibrosis	Liver biopsy	Fibrosis reported as result of biopsy
Hepatic steatosis	Liver biopsy	Steatosis reported as result of biopsy
		Accumulation of lipids reported in biopsy
Elevated liver enzymes	Laboratory analysis	Mention of ALAT, ASAT, Alkaline phosphatase and/or Gamma-GT above reference range
		Elevated liver enzymes reported
Impaired liver function	Laboratory analysis	Serum albumin and/or prothrombine activity below reference value and or increased PT reported

Rhabdomyolysis	Blood CK concentration	Report of CK-value more than 5 times the upper reference value
Myopathy	Electromyography	Myopathic patterns described in EMG results
Muscle involvement NOS	Blood CK concentration	CK-concentration reported as elevated, but below 5 times the upper reference value
	Muscle biopsy	Reported as abnormal [c]
	Electromyography	Reported as abnormal [c]
Hypoglycemia	Serum glucose	Report of glucose concentration <2,5 mmol/L or <45 mg/dL
Hyperammonemia	Serum ammonia	Report of ammonia concentration above 80 umol/L

Criteria are guided by current practice guidelines, however, as often not all data required for complete diagnosis (according to guidelines) are provided, the criteria used for this review are more lenient.

[a] The conclusion of ECG is recorded in the case and discussed in results section (table 3)

[b] The conclusion of imaging is discussed in results section (table 5)

[c] Conclusions of muscle biopsy discussed in results section

Sources used in establishing these criteria:

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Newsome PN, Cramb R, Davison SM, Dillon JF, Foulerton M, Godfrey EM, Hall R, Harrower U, Hudson M, Langford A, Mackie A, Mitchell-Thain R, Sennett K, Sheron NC, Verne J, Walmsley M, Yeoman A. Guidelines on the management of abnormal liver blood tests. Gut. 2018 Jan;67(1):6-19. doi: 10.1136/gutjnl-2017-314924. Epub 2017 Nov 9. PMID: 29122851; PMCID: PMC5754852.

Kwo PY, Cohen SM, Lim JK. ACG Clinical Guideline: Evaluation of Abnormal Liver Chemistries. Am J Gastroenterol. 2017 Jan;112(1):18-35. doi: 10.1038/ajg.2016.517. Epub 2016 Dec 20. PMID: 27995906.

Table S4 | Overview of HPO-terms

HPO_code	HPO_name	Group
HP:0011675	Arrhythmia	Car_arrhythmia_NOS*
HP:0001662	Bradycardia	Car_arrhythmia_NOS*
HP:0001649	Tachycardia	Car_arrhythmia_NOS*
HP:0006543	Cardiorespiratory arrest	Car_cardiac_arrest
HP:0001671	Abnormal cardiac septum morphology	Car_cardiac_involvement_NOS*
HP:0001640	Cardiomegaly	Car_cardiac_involvement_NOS*
HP:0030148	Heart murmur	Car_cardiac_involvement_NOS*
HP:0001712	Left ventricular hypertrophy	Car_cardiac_involvement_NOS*
HP:0001653	Mitral regurgitation	Car_cardiac_involvement_NOS*
HP:0001642	Pulmonic stenosis	Car_cardiac_involvement_NOS*
HP:0001638	Cardiomyopathy	Car_CMP*
HP:0001635	Congestive heart failure	Car_CMP*
HP:0006670	Impaired myocardial contractility	Car_CMP*
HP:0012664	Reduced ejection fraction	Car_CMP*
HP:0012666	Severely reduced ejection fraction	Car_CMP*
HP:0001657	Prolonged QT interval	Car_conduction_disorder_NOS*
HP:0012232	Shortened QT interval	Car_conduction_disorder_NOS*
HP:0001644	Dilated cardiomyopathy	Car_DCM*
HP:0001639	Hypertrophic cardiomyopathy	Car_HCM*
HP:0004756	Ventricular tachycardia	Car_susVT*
HP:0001663	Ventricular fibrillation	Car_VF*
HP:0001699	Sudden death	Death_sudden_death
HP:0001903	Anemia	Gen_anemia
HP:0001891	Iron deficiency anemia	Gen_anemia
HP:0002312	Clumsiness	Gen_dev_delay
HP:0000750	Delayed speech and language development	Gen_dev_delay
HP:0001263	Global developmental delay	Gen_dev_delay
HP:0006889	Intellectual disability, borderline	Gen_dev_delay
HP:0001256	Intellectual disability, mild	Gen_dev_delay
HP:0025356	Psychomotor retardation	Gen_dev_delay
HP:0001999	Abnormal facial shape	Gen_dysmorphia
HP:0002648	Abnormality of calvarial morphology	Gen_dysmorphia
HP:0000175	Cleft palate	Gen_dysmorphia
HP:0000286	Epicanthus	Gen_dysmorphia
HP:0000316	Hypertelorism	Gen_dysmorphia
HP:0000298	Mask-like facies	Gen_dysmorphia
HP:0000252	Microcephaly	Gen_dysmorphia
HP:0000347	Micrognathia	Gen_dysmorphia
HP:0001177	Preaxial hand polydactyly	Gen_dysmorphia
HP:0000411	Protruding ear	Gen_dysmorphia
HP:0002000	Short columella	Gen_dysmorphia

HP:0031936	Delayed ability to walk	Gen_motor_delay
HP:0002194	Delayed gross motor development	Gen_motor_delay
HP:0001270	Motor delay	Gen_motor_delay
HP:0012758	Neurodevelopmental delay	Gen_motor_delay
HP:0002421	Poor head control	Gen_motor_delay
HP:0003535	3-Methylglutaconic aciduria	Gen_unspecific
HP:0012072	Aciduria	Gen_unspecific
HP:0002039	Anorexia	Gen_unspecific
HP:0007018	Attention deficit hyperactivity disorder	Gen_unspecific
HP:0000518	Cataract	Gen_unspecific
HP:0001289	Confusion	Gen_unspecific
HP:0004325	Decreased body weight	Gen_unspecific
HP:0001558	Decreased fetal movement	Gen_unspecific
HP:0000726	Dementia	Gen_unspecific
HP:0003215	Dicarboxylic aciduria	Gen_unspecific
HP:0031987	Diminished ability to concentrate	Gen_unspecific
HP:0002094	Dyspnea	Gen_unspecific
HP:0000969	Edema	Gen_unspecific
HP:0003546	Exercise intolerance	Gen_unspecific
HP:0002875	Exertional dyspnea	Gen_unspecific
HP:0001508	Failure to thrive	Gen_unspecific
HP:0012378	Fatigue	Gen_unspecific
HP:0002373	Febrile seizure (within the age range of 3 months to 6 years)	Gen_unspecific
HP:0011968	Feeding difficulties	Gen_unspecific
HP:0001945	Fever	Gen_unspecific
HP:0002020	Gastroesophageal reflux	Gen_unspecific
HP:0001290	Generalized hypotonia	Mus_weakness
HP:0012188	Hyperemesis gravidarum	Gen_unspecific
HP:0000737	Irritability	Gen_unspecific
HP:0000952	Jaundice	Gen_unspecific
HP:0001254	Lethargy	Gen_unspecific
HP:0003690	Limb muscle weakness	Mus_weakness
HP:0007185	Loss of consciousness	Gen_unspecific
HP:0002017	Nausea and vomiting	Gen_unspecific
HP:0001562	Oligohydramnios	Gen_unspecific
HP:0000980	Pallor	Gen_unspecific
HP:0011703	Sinus tachycardia	Gen_unspecific
HP:0001962	Palpitations	Gen_unspecific
HP:0002090	Pneumonia	Gen_unspecific
HP:0100602	Preeclampsia	Gen_unspecific
HP:0006699	Premature atrial contractions	Gen_unspecific
HP:0002719	Recurrent infections	Gen_unspecific
HP:0006532	Recurrent pneumonia	Gen_unspecific
HP:0002788	Recurrent upper respiratory tract infections	Gen_unspecific

HP:0004372	Reduced consciousness/confusion	Gen_unspecific
HP:0005972	Respiratory acidosis	Gen_unspecific
HP:0002098	Respiratory distress	Gen_unspecific
HP:0002878	Respiratory failure	Gen_unspecific
HP:0002093	Respiratory insufficiency	Gen_unspecific
HP:0100806	Sepsis	Gen_unspecific
HP:0004322	Short stature	Gen_unspecific
HP:0005268	Spontaneous abortion	Gen_unspecific
HP:0001873	Thrombocytopenia	Gen_unspecific
HP:0006682	Ventricular extrasystoles	Gen_unspecific
HP:0100502	Vitamin B12 deficiency	Gen_unspecific
HP:0002013	Vomiting	Gen_unspecific
HP:0001824	Weight loss	Gen_unspecific
HP:0030828	Wheezing	Gen_unspecific
HP:0001399	Hepatic failure	Hep_hepatic_failure
HP:0001397	Hepatic steatosis	Hep_hepatic_steatosis*
HP:0002240	Hepatomegaly	Hep_hepatomegaly*
HP:0001941	Acidosis	Met_acidosis
HP:0001942	Metabolic acidosis	Met_acidosis
HP:0001987	Hyperammonemia	Met_hyperammonemia*
HP:0003162	Fasting hypoglycemia	Met_hypoglycemia*
HP:0001943	Hypoglycemia	Met_hypoglycemia*
HP:0001985	Hypoketotic hypoglycemia	Met_hypoglycemia*
HP:0001958	Nonketotic hypoglycemia	Met_hypoglycemia*
-1	Metabolic decompensation	Met_acidosis
HP:0007210	Lower limb amyotrophy	Mus_atrophy
HP:0003560	Muscular dystrophy	Mus_atrophy
HP:0003202	Skeletal muscle atrophy	Mus_atrophy
HP:0003738	Exercise-induced myalgia	Mus_myalgia
HP:0003326	Myalgia	Mus_myalgia
HP:0003198	Myopathy	Mus_myopathy*
HP:0003236	Elevated serum creatine kinase	Mus_rhabdomyolysis*
HP:0003201	Rhabdomyolysis	Mus_rhabdomyolysis*
HP:0003327	Axial muscle weakness	Mus_weakness
HP:0001488	Bilateral ptosis	Mus_weakness
HP:0003551	Difficulty climbing stairs	Mus_weakness
HP:0009046	Difficulty running	Mus_weakness
HP:0003698	Difficulty standing	Mus_weakness
HP:0002355	Difficulty walking	Mus_weakness
HP:0003324	Generalized muscle weakness	Mus_weakness
HP:0009028	Generalized weakness of limb muscles	Mus_weakness
HP:0003391	Gowers sign	Mus_weakness
HP:0005216	Impaired mastication	Mus_weakness
HP:0007340	Lower limb muscle weakness	Mus_weakness

HP:0001324	Muscle weakness	Mus_weakness
HP:0001252	Muscular hypotonia	Mus_weakness
HP:0003323	Progressive muscle weakness	Mus_weakness
HP:0003701	Proximal muscle weakness	Mus_weakness
HP:0000508	Ptosis	Mus_weakness
HP:0003484	Upper limb muscle weakness	Mus_weakness
HP:0001259	Coma	Neuro_coma
HP:0001298	Encephalopathy	Neuro_encephalopathy
HP:0006929	Hypoglycemic encephalopathy	Neuro_encephalopathy
HP:0006582	Reye syndrome-like episodes	Neuro_encephalopathy
HP:0002637	Cerebral ischemia	Neuro_neurologic_involvement_NOS*
HP:0100021	Cerebral palsy	Neuro_neurologic_involvement_NOS*
HP:0002383	Encephalitis	Neuro_neurologic_involvement_NOS*
HP:0002301	Hemiplegia	Neuro_neurologic_involvement_NOS*
HP:0002445	Tetraplegia	Neuro_neurologic_involvement_NOS*
HP:0020221	Clonic seizure	Neuro_seizure
HP:0020219	Motor seizure	Neuro_seizure
HP:0001250	Seizure	Neuro_seizure
HP:0007359	Focal-onset seizure	Neuro_seizure

Table S5 | Overview of signs and symptoms in relation to genetic background

	MS.IF/MS.IF (N=275)		MS.IF/NS.FS.SS (N=150)		NS.FS.SS/NS.FS.SS (N=78)		MS.IF/Missing (N=10)		NS.FS.SS/Missing (N=6)		Missing/Missing (N=70)	
Transporter activity												
Number of samples (N)	21		14		15		NA		NA		1	
Median (% of control)	5.0	[0.0 - 20.8]	4.6	[2.0 - 16.0]	5.0	[0.0 - 10.7]					1.0	[-]
Cardiac symptoms	43	(15.6%)	25	(16.7%)	48	(61.5%)	2	(20.0%)	3	(50.0%)	10	(14.3%)
Neurologic symptoms	21	(7.6%)	4	(2.7%)	12	(15.4%)	0	(0.0%)	1	(16.7%)	4	(5.7%)
Muscle symptoms	20	(7.3%)	14	(9.3%)	13	(16.7%)	1	(10.0%)	1	(16.7%)	5	(7.1%)
Hepatic symptoms	19	(6.9%)	6	(4.0%)	15	(19.2%)	2	(20.0%)	0	(0.0%)	2	(2.9%)
Metabolic symptoms	19	(6.9%)	9	(6.0%)	18	(23.1%)	1	(10.0%)	0	(0.0%)	5	(7.1%)
Only nonspecific symptoms	27	(9.8%)	10	(6.7%)	3	(3.9%)	0	(0.0%)	0	(0.0%)	30	(42.9%)
Asymptomatic	179	(65.1%)	106	(70.7%)	13	(16.7%)	7	(70.0%)	3	(50.0%)	25	(35.7%)
Deceased	9	(3.3%)	2	(1.3%)	12	(15.4%)	0	(0.0%)	0	(0.0%)	0	(0.0%)

Data presented as: N (%) or Median [Min – Max].

Abbreviations: MS.IF – missense/in-frame; NS.FS.SS – nonsense/frameshift/splice-site; Missing (under genetic analysis) – Variant identified, but type was not reported

Table S6 | Overview of muscle symptoms

		Muscle weakness		Myalgia		Atrophy		Rhabdomyolysis		Myopathy		Muscle involvement NOS	
		N=42		N=1		N=2		N=11 (4)		N=7 (7)		N=14 (14)	
Motor developmental delay (N=7)		5		0		0		0		0		2	
Age at first symptom	(years)	1.25	[0.00 - 22.00]	NA	[-]	2.50	[2.00 - 3.00]	2.00	[0.08 - 30.00]	1.00	[0.25 - 3.00]	1.50	[0.08 - 30.00]
Infancy		15				0		2		3		4	
Childhood		27				2		10		4		11	
Adulthood		1				0		1		0		1	
Deceased		1		0		0		0		0		1	
Free carnitine ratio	(N)	34		1		1		11		2		13	
	(Ratio)	0.17	[0.00 - 0.65]	0.57	[0.57 - 0.57]	0.07	[-]	0.10	[0.00 - 0.80]	0.34	[0.24 - 0.44]	0.19	[0.00 - 0.80]
Transporter activity	(N)	14		0		1		4		4		6	
	(% of control)	7.50	[1.10 - 22.00]			4.00	[-]	4.05	[1.10 - 16.00]	5.00	[2.80 - 16.00]	2.24	[0.36 - 5.63]
Genetic analysis		39		1		2		13		5		12	
MS.IF/MS.IF		15	(38.5%)	0	(0.0%)	1	(50.0%)	2	(15.4%)	2	(40.0%)	4	(33.3%)
MS.IF/NS.FS.SS		8	(20.5%)	0	(0.0%)	1	(50.0%)	6	(46.2%)	2	(40.0%)	4	(33.3%)
NS.FS.SS/NS.FS.SS		12	(30.8%)	0	(0.0%)	0	(0.0%)	3	(23.1%)	0	(0.0%)	3	(25.0%)
MS.IF/Missing		1	(2.6%)	0	(0.0%)	0	(0.0%)	1	(7.7%)	0	(0.0%)	0	(0.0%)
NS.FS.SS/Missing		0	(0.0%)	0	(0.0%)	0	(0.0%)	1	(7.7%)	0	(0.0%)	1	(8.3%)
Missing/Missing		3	(7.7%)	1	(100%)	0	(0.0%)	0	(0.0%)	1	(20.0%)	0	(0.0%)
Carnitine treatment started		37		1		2		6		8		15	
Clinical improvement after treatment		28		1		2		6		7		14	

Data presented as: N-total (confirmed) / N(%) or Median [Min – Max].]. For age at first symptom and age period at first symptom, the number of missing data were, respectively: Muscle weakness: 14, 3; Myalgia: 1, 1; Atrophy: 0, 0; Rhabdomyolysis: 7, 0; Myopathy: 3, 1; Muscle involvement NOS: 1, 0.

Abbreviations: MS.IF – missense/in-frame; NS.FS.SS – nonsense/frameshift/splice-site; Missing (under genetic analysis) – Variant identified, but type was not reported

Table S7 | Allele frequencies of variants that were detected in deceased patients, in the entire study cohort

	c.-91_22del (N=8)		c.338G>A (N=24)		**c.396G>A** (N=8)		**c.497+1G>T** (N=9)		c.51C>G (N=101)		**c.760C>T** (N=156)	
Deceased	4	(50.0%)	1	(4.17%)	2	(25.0%)	1	(11.1%)	2	(1.98%)	6	(3.85%)
Cardiac	6	(75.0%)	8	(33.3%)	3	(37.5%)	2	(22.2%)	8	(7.92%)	46	(29.5%)
Neurologic	0		0		2	(25.0%)	0		1	(0.990%)	13	(8.33%)
Muscle	4	(50.0%)	1	(4.17%)	1	(12.5%)	0		6	(5.94%)	12	(7.69%)
Hepatic	0		1	(4.17%)	3	(37.5%)	1	(11.1%)	1	(0.990%)	5	(3.21%)
Metabolic	0		2	(8.33%)	2	(25.0%)	1	(11.1%)	1	(0.990%)	13	(8.33%)
Only unspecific	0		1	(4.17%)	0	0%	0		4	(3.96%)	11	(7.05%)
Asymptomatic	0		14	(58.3%)	4	(50.0%)	6	(66.7%)	84	(83.2%)	83	(53.2%)
	c.844C>T (N=19)		**c.844delC** (N=12)		c.849G>T (N=3)		c.95A>G (N=134)		**c.1161T>G** (N=2)		c.1433C>T (N=4)	
Deceased	1	(5.26%)	6	(50.0%)	2	(66.7%)	14	(10.4%)	1	(50.0%)	1	(25.0%)
Cardiac	8	(42.1%)	10	(83.3%)	0		30	(22.4%)	0		1	(25.0%)
Neurologic	1	(5.26%)	2	(16.7%)	1	(33.3%)	16	(11.9%)	0		0	
Muscle	3	(15.8%)	0		1	(33.3%)	9	(6.72%)	0		0	
Hepatic	1	(5.26%)	6	(50.0%)	1	(33.3%)	14	(10.4%)	1	(50.0%)	1	(25.0%)
Metabolic	2	(10.5%)	2	(16.7%)	1	(33.3%)	8	(5.97%)	1	(50.0%)	0	
Only unspecific	0		0		0		59	(44.0%)	0		1	(25.0%)
Asymptomatic	10	(52.6%)	0		0		31	(23.1%)	0		2	(50.0%)
	c.806delT (N=3)		**c.517delC** (N=3)		**c.652+1G>A** (N=5)		**c.824+1G>A** (N=3)				Total (N=494)	
Deceased	2	(66.7%)	1	(33.3%)	1	(20.0%)	1	(33.3%)			46	(9.31%)
Cardiac	3	(100%)	2	(66.7%)	2	(40.0%)	1	(33.3%)			130	(26.3%)
Neurologic	2	(66.7%)	0		0		0				38	(7.69%)
Muscle	2	(66.7%)	0		1	(20.0%)	0				40	(8.10%)
Hepatic	2	(66.7%)	1	(33.3%)	0		0				38	(7.69%)
Metabolic	2	(66.7%)	0		1	(20.0%)	1	(33.3%)			37	(7.49%)
Only unspecific	0		0		0		0				76	(15.4%)
Asymptomatic	0		1	(33.3%)	3	(60.0%)	2	(66.7%)			240	(48.6%)

Nonsense/frameshift/splice-site variants are in bold.

CHAPTER

Neonatal carnitine concentrations in relation to gestational age and weight

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ABSTRACT

Background: Free carnitine is measured in the Dutch newborn screening (NBS) program since 2007 with a referral threshold of ≤ 5 µmol/L, regardless of gestational age or birthweight. However, several studies suggest that carnitine concentrations may depend on gestational age and birthweight. We evaluated differences in postnatal day-to-day carnitine concentrations in newborns based on gestational age (GA) and/or weight for gestational age (WfGA).

Methods: A retrospective study was performed using data from the Dutch NBS. Dried blood spot (DBS) carnitine concentrations, collected between the 3rd and 10th day of life, of nearly 2 million newborns were included. Individuals were grouped based on GA and WfGA. Median carnitine concentrations were calculated for each group. Mann-Whitney U tests and chi-square tests were applied to test for significant differences between groups.

Results: Preterm, postterm and small for gestational age (SGA) newborns have higher carnitine concentrations at the 3rd day of life compared to term newborns. The median carnitine concentration of preterm newborns declines from day 3 onwards, and approximates that of term newborns at the 6th day of life, while median concentrations of postterm and SGA newborns remain elevated at least throughout the first 10 days of life. Carnitine concentrations ≤5 µmol/L, were found less frequently in SGA newborns and newborns born between 32 and 37 weeks of gestation, compared to term newborns.

Conclusion: Median carnitine concentrations in NBS DBS vary with day of sampling, gestational age and weight for gestational age. It is important to take these variables into account when interpreting NBS-results.

INTRODUCTION

Carnitine (3-hydroxy-4-N-trimethylaminobutyrate) is a quaternary amine that has an important role in energy metabolism. It facilitates the transport of activated long-chain fatty acids into the mitochondrion where they can be degraded via β-oxidation.[1] Long-chain fatty acids either derived from exogenous, dietary sources or generated endogenously are readily activated to their coenzyme A (CoA) esters which explains why the bulk of fatty acids is present in cells as acyl-CoAs. Importantly, acyl-CoAs are not able to enter the mitochondrion and instead need to be converted into the corresponding acylcarnitine as mediated by the enzyme carnitine palmitoyltransferase 1 (CPT1). Acylcarnitines can enter the mitochondrion in exchange for free carnitine via the mitochondrial carnitine acylcarnitine carrier (CACT) after which the acylcarnitine can be reconverted back into the original acyl-CoA and subsequently beta-oxidized. Apart from the important role of carnitine in mitochondrial beta-oxidation, carnitine also plays a major role in peroxisomal beta-oxidation and coenzyme A homeostasis.

Several inborn errors of metabolism result in abnormal plasma acylcarnitine profiles, which can be used as a sensitive diagnostic first-tier test.[1] In 2007, the Dutch newborn screening (NBS) program was expanded with several of these inborn errors of metabolism. In order to perform a reliable screen, the free carnitine (i.e. non-acylated carnitine) concentration should be sufficiently high, as low carnitine concentrations hinder acylcarnitine formation, and screening profiles thus cannot be reliably interpreted. For this reason, free carnitine is also measured and evaluated. A free carnitine concentration > 5 µmol/L in the dried blood spot (DBS) is used as cut-off for reliable interpretation of the acylcarnitine profile. In newborns with a lower value, the measurement is repeated in a second DBS and if low carnitine levels persist they are referred to a paediatric metabolic specialist. The referral threshold of ≤ 5 µmol/L is used for all newborns, regardless of gestational age, birthweight or day of sampling.

Several studies, however, have shown that normal free carnitine concentrations depend on gestational age (GA), weight for gestational age (WfGA) and day of sampling. In 1980, higher free carnitine concentrations at birth were reported in preterm infants of 30-36 weeks of gestation.[2] This finding was reproduced in several other studies.[3-7] Higher free carnitine concentrations in infants that are small for gestational age (SGA) have also been reported.[7,8] Only one study assessed the course of the carnitine concentration during the first weeks of life in preterm infants, comparing newborns with a GA of 22-27 weeks and 28-31 weeks.[9] In both groups a rapid decrease in carnitine concentration within the first 7 days of life was observed. A detailed overview of the data from previous studies can be found in the supplementary materials (Supplementary Table 1). However, data on the day-to-day free carnitine concentrations in preterm and/or SGA infants compared to term infants during the first week of life are still lacking.

The Dutch screening protocol allows for a rather wide sampling window, namely 72-168 hours after birth. Between 2007 and 2017 96,9% of NBS DBS samples were collected within that window. Thus, the time- and GA-dependency of free carnitine levels is relevant for correct interpretation of screening results. We therefore examined the day-to-day changes in carnitine concentrations in NBS bloodspots of infants of various gestational ages and birthweights during the 3rd up to and including 10th day of life.

METHODS

Study population

Data were extracted retrospectively from the registry used by the Dutch National Institute for Public Health and the Environment, Department for Vaccine Supply and Prevention Programmes (RIVM-DVP). The following data were collected anonymously for each carnitine measurement that was performed between January 2007 and December 2017: gender, GA at birth, birth weight, carnitine concentration, age when DBS sample was taken (sample day) and registered remarks concerning reliability of the DBS (e.g. insufficient filling of blood spot). Data retrieval was approved for by the Commission Data applications Praeventis of the RIVM-DVP.

In- and exclusion of samples

Samples obtained between 72 and 264 hours after birth were included, in order to represent the most common period for NBS, as during 2007-2017 98,2% percent of newborns were screened within this window (96,9% within 72-168 hours after birth). One percent of the newborns was screened < 72 hours. However, the main reason to perform a screening this early is severe illness, resulting in unrepresentative results. Therefore, these samples were not included in the study. Exclusion criteria were: carnitine > 100 µmol/L, unreliable quality of DBS based on registered remarks on the DBS card and GA > 308 days. Missing data were handled by listwise deletion, as the data were missing at random (Supplementary Figure 1) and consisted of < 0,07% of cases. Finally, records of all repeat samples from individual neonates were excluded from further analysis to avoid selection bias towards low carnitine concentrations, as repeat samples are often taken when the carnitine concentration in first sample is ≤ 5 µmol/L.

Newborns were divided into groups based on their gestational age at birth: preterm: ≤ 27^{+6} weeks ($GA_{<28}$), 28 ≤ 29^{+6} weeks ($GA_{28\text{-}30}$), 30 ≤ 31^{+6} weeks ($GA_{30\text{-}32}$), 32 ≤ 36^{+6} weeks ($GA_{32\text{-}37}$), term: 37 ≤ 41^{+6} weeks ($GA_{37\text{-}42}$) and finally postterm: 42 ≤ 44 weeks ($GA_{42\text{-}44}$).

Using the standardised Dutch birthweight charts provided by Perined (Dutch perinatal audit and perinatal registration)[10], included children were categorized according to WfGA into the following categories; small for GA (SGA; weight ≤10 percentile for GA), appropriate for GA (AGA; weight 10<90 percentile for GA) or large for GA (LGA; weight ≥90 percentile for GA). Children with a GA exceeding 294 days (42 weeks), were categorized using the standardised birthweights of newborns with a GA of 294 days.

Carnitine analysis in DBS

Carnitine concentrations were determined using 3,2 mm punches from NBS DBS cards with quantitative ESI-MS/MS-methods: from 01-01-2007 until 01-10-2008 the Neogram MSMS kit and from 01-10-2008 until 31-12-2017, the NeoBase™ Non-derivatized MSMS kit (both kits Perkin Elmer, Turku, Finland - extraction and sample preparation performed according to kit-insert) in combination with a Waters Micro tandem MS instrument (Waters, Milford, MA, USA).

Statistics

Statistical analysis was performed using SPSS (version 25.0.0.2, SPSS IBM, New York, NY) and GraphPad Prism (version 8.00, GraphPad Software, La Jolla California USA). Mann-Whitney U tests were used to compare the difference in carnitine concentrations between two groups. The chi-square test was used for comparison of the amount of children that had a carnitine concentration below 5 µmol/L between groups. Multiple comparisons were adjusted for using the Bonferroni correction. Significance was assumed for $p<0.05$.

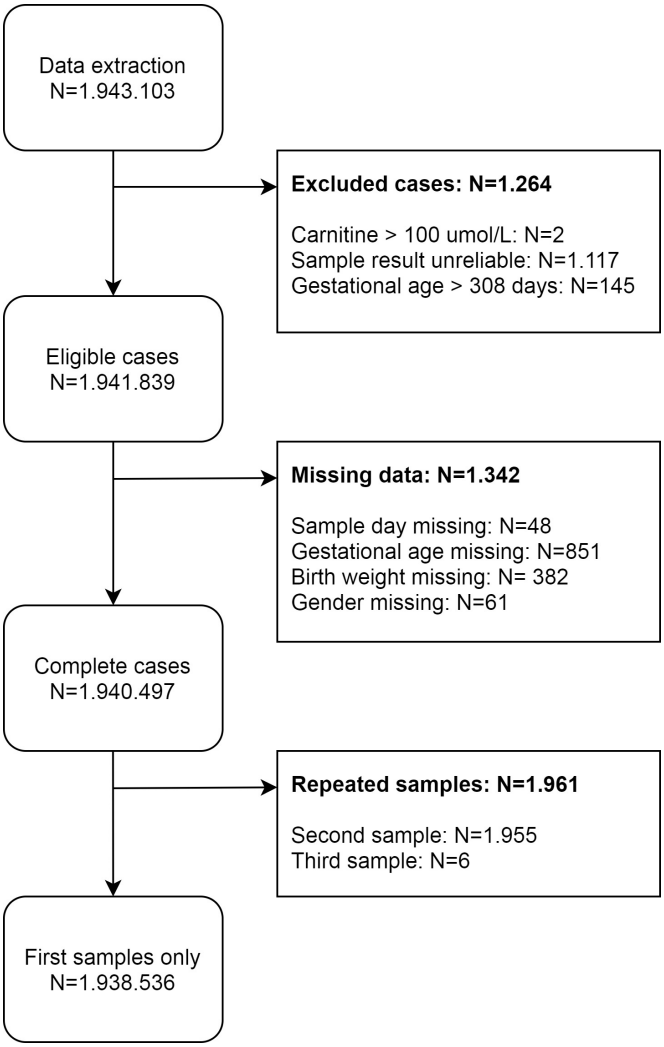

Figure 1 | Flowchart of data inclusion

RESULTS

In total, 1.943.103 DBS were available for this study, of which 1.938.536 unique cases (99.8%) were included (Figure 1). Characteristics of the included population are summarised in table 1. Median age at sampling was 4 days (interquartile range (IQR) 4-5). The median carnitine concentration of the complete cohort was 18,4 µmol/L (IQR 14,1-24,0).

Table 1 | Baseline characteristics of newborns included

		Median	***(IQR)***	**Range**
Gestational age (days)		279	*(271-285)*	140 – 308
Birthweight (grams)		3450	*(3100-3790)*	390 – 8000
Sample day (days)		4	*(4-5)*	3 – 10
Carnitine concentration (µmol/L)		18,4	*(14,1-24,0)*	0,0 – 99,9
		N	***(%)***	
Total		1.938.536	*(100)*	
Gender	Male	992.770	*(51,2)*	
	Female	945.766	*(48,8)*	
Gestational age category	≤ 27^{+6} weeks	4.482	*(0,2)*	
	28 ≤ 29^{+6} weeks	4.849	*(0,3)*	
	30 ≤ 31^{+6} weeks	8.513	*(0,4)*	
	32 ≤ 36^{+6} weeks	113.723	*(5,9)*	
	37 ≤ 41^{+6} weeks	1.766.215	*(91,1)*	
	42 ≤ 44 weeks	40.754	*(2,1)*	
Weight for gestational age	SGA	226.167	*(11,7)*	
	AGA	1.508.118	*(77,8)*	
	LGA	204.251	*(10,5)*	

Abbreviations: SGA - Small for gestational age; AGA –Appropriate for gestational age; LGA – Large for gestational age; IQR – Interquartile range.

Carnitine concentration per group

The median carnitine concentration was significantly higher in all preterm groups, as well as in postterm newborns when compared to term newborns. Median free carnitine concentrations for all groups are presented in Table 2. Median concentrations were significantly higher in the $GA_{<28}$, GA_{28-30} and GA_{30-32} groups compared to term newborns (21,5 (21,1-22,0); 22,9 (22,6-23,3); 24,0 (23,8-24,3) vs 18,2 (18,4-18,5) respectively (µmol/L, 95% CI in parenthesis)). Median free carnitine concentration of all preterm neonates combined was 20,6 (20,6-20,7). Median carnitine concentration in SGA newborns was significantly higher

Table 2 | Overall median carnitine concentrations

		Carnitine concentration (µmol/L)			Percentile	
		Median	95% CI	*p*-value[a]	1st	99th
Total		18,4	18,4-18,5	-	7,3	48,60
Gender	Male	19,0	19,0-19,1	<0.001	7,10	45,60
	Female	17,8	17,8-17,9		7,60	51,00
Gestational age category	$GA_{<28}$	21,5	21,1-22,0	<0.001	6,50	64,00
	GA_{28-30}	22,9	22,6-23,3	<0.001	7,70	65,50
	GA_{30-32}	24,0	23,8-24,3	<0.001	8,50	66,50
	GA_{32-37}	20,2	20,2-20,3	<0.001	8,00	58,00
	GA_{37-42}	18,2	18,2-18,3	-	7,30	47,30
	GA_{42-44}	19,8	19,7-20,0	<0.001	7,50	51,10
Weight for gestational age	SGA	21,4	21,4-21,5	<0.001	8,40	59,40
	AGA	18,1	18,1-18,2	-	7,30	46,30
	LGA	17,3	17,3-17,4	<0.001	7,00	45,80
Gestational age and SGA compiled	Term, non-SGA	18,0	18,0-18,1	-	7,20	45,60
	Preterm, non-SGA	19,5	19,5-19,6	<0.001	7,70	52,90
	Postterm, non-SGA	19,6	19,6-19,8	<0.001	7,40	50,00
	Term and SGA	20,9	20,9-21,0	<0.001	8,30	56,10
	Preterm and SGA	25,3	25,2-25,5	<0.001	9,20	69,80
	Postterm and SGA	22,2	21,9-22,6	<0.001	8,40	58,20

Abbreviations: CI – Confidence interval; SGA – Small for gestational age; AGA – Appropriate for gestational age; LGA – Large for gestational age.

[a]Mann-Whitney U test after Bonferroni correction. Gestational age categories compared against term category. SGA and LGA compared against AGA. Compiled subgroups were compared to term, non-SGA newborns.

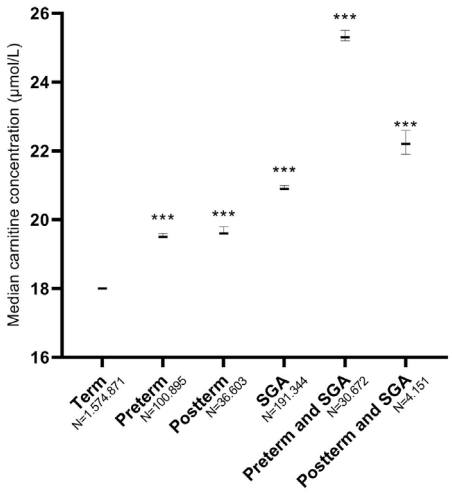

Figure 2 | Median carnitine concentrations of preterm, postterm, and/or SGA newborns. All groups were separately compared to term newborns. P value was calculated using the Mann-Whitney U test, after Bonferroni correction. abbreviations: SGA - Small for gestational age.

Asterisks indicate a *p*-value <.001

than that in AGA newborns (21,4 (21,4-21,5) vs 18,1 (18,1-18,2)), whereas the median carnitine concentration was significantly lower in LGA newborns (17,3 (17,3-17,4)). To analyse if there was an effect of birthweight on top of the effect of gestational age, the preterm and postterm groups were separated into a group that was only preterm/postterm and a group that was both preterm/postterm and SGA (Figure 2). Carnitine concentrations were 25,3 (25,2-25,5) in newborns that were both preterm and SGA versus 19,5 (19,5-19,6) in normal weight preterm babies and 22,2 (21,9-22,6) in newborns that were both postterm and SGA versus 19,6 (19,6-19,8) in normal weight post term babies, indicating an additive effect of birthweight and gestational age. The 1st and 99th percentile were calculated for all groups and are described in table 2.

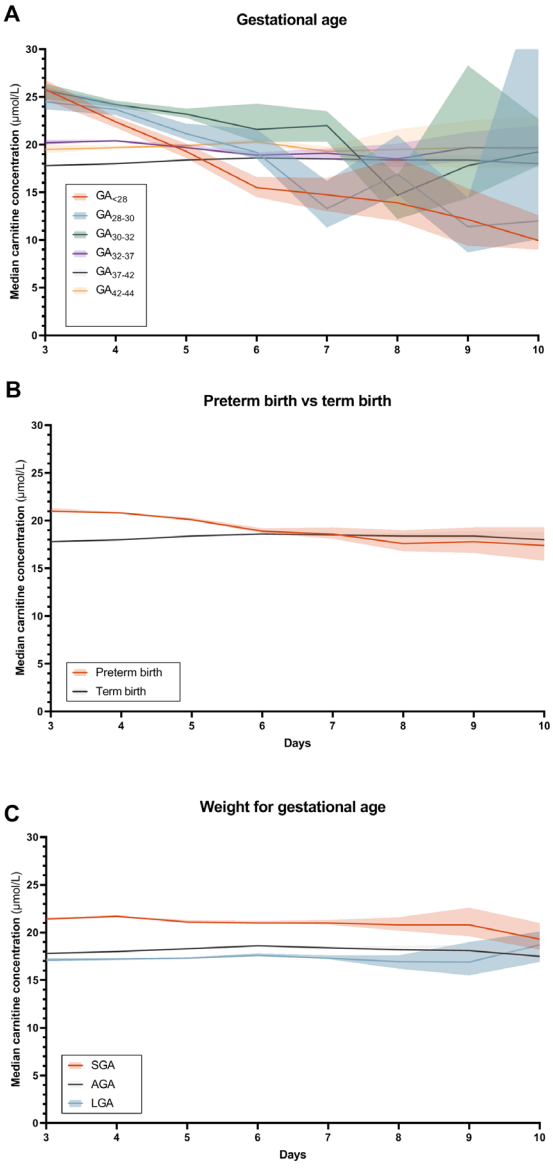

Figure 3 | Median carnitine concentration course. Median carnitine concentration courses from days 3 to 10 in groups of various, (A) gestational age, (B) gestational age with preterm births compiled, compared to term births and, (C) weight for gestational age. Transparent areas represent 95% confidence intervals. Abbreviations: AGA - appropriate for gestational age; LGA - large for gestational age; SGA - small for gestational age.

Day-to-day carnitine concentrations per group

Carnitine concentrations in the first 10 days of life in all groups are presented in figure 3. On the 3rd day of life, $GA_{<28}$, GA_{28-30} and GA_{30-32} showed the highest median carnitine concentrations (25,8 µmol/L, 24,5 µmol/L and 25,7 µmol/L respectively) versus 17,8 µmol/L in term infants. In the preterm children median carnitine concentration in group $GA_{<28}$ and GA_{28-30}, intersect median carnitine of term newborns at day 5 and 6 respectively, ultimately reaching a median carnitine concentration of 9,9 µmol/L and 12,0 µmol/L at day 10, respectively. Medians of GA_{32-37} and GA_{42-44} were mildly elevated compared to term newborns throughout the entire period. To assess the effect of prematurity in a broader sense, all preterm groups were compiled (GA ≤36^{+6} weeks) in figure 3B, revealing an initially increased median carnitine concentration (21,0 µmol/L), approximating the median of term newborns at day 6. These changes in median carnitine concentrations during day 3 to 10 were not observed within groups of differing WfGA (Figure 3C), where carnitine concentration remained more stable over time. Median carnitine in SGA newborns was roughly 2,8 µmol/L higher than in AGA newborns, whereas the median in LGA newborns was approximately 0,7 µmol/L lower than in AGA newborns. The median carnitine course of the combined groups is presented in figure 4. It shows, again, a cumulative effect when both attributes are present.

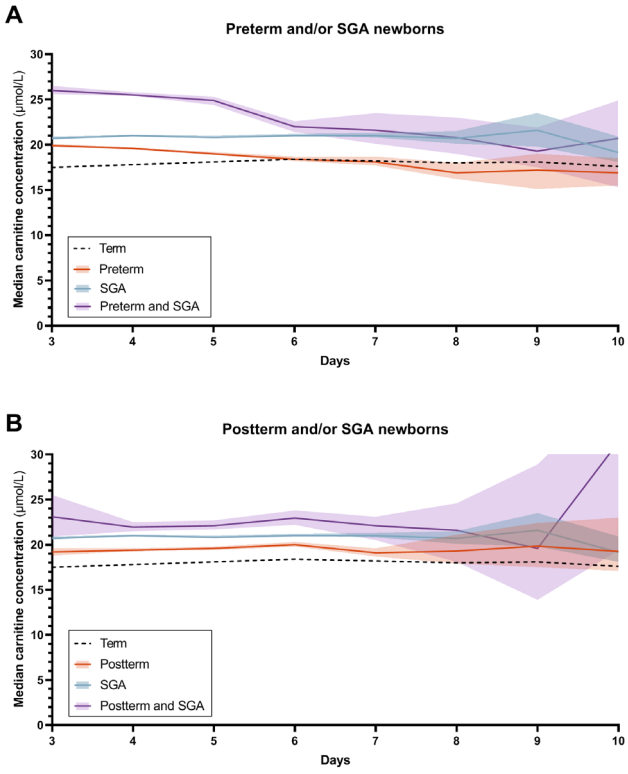

Figure 4 | Median carnitine concentration course. Median carnitine concentration courses from days 3 to 10 in (A) preterm and/or SGA newborns and (B) postterm and/or SGA newborns. Transparent areas represent 95% confidence intervals. Abbreviations: SGA - small for gestational age.

Analysis of carnitine concentration threshold

To analyse whether the relatively high carnitine concentrations in newborns with differing GA or WfGA influenced the detection rate of carnitine deficiency, we determined the number of children that had a NBS carnitine level below the screening cut off value of 5 µmol/L (table 3). The percentage of newborns with a carnitine ≤ 5 µmol/L was 0,062% for the entire cohort. Newborns with such a low carnitine level were significantly underrepresented in the group with a GA between 32 and 37 weeks as well as even more pronounced in the group of newborns that were SGA. Interestingly, extremely preterm infants were significantly overrepresented (0,201%). However, for the newborns in this group that had carnitine concentration below the threshold, the median sample day was day 7 (IQR 6-9) (data not shown), when carnitine concentrations have already decreased (figure 3A). In postterm children and children that are LGA, carnitine concentrations below the threshold were found more often than in the entire population (0,083% and 0,096% respectively).

Table 3 | Number of newborns with carnitine concentration below cut-off value

	Count (N)				Sample day
	Carnitine ≤ 5 µmol/L	**Carnitine > 5 µmol/L**	**% ≤ 5 µmol/L**	***p*-value**[a]	**Median *(IQR)***
Total	1.179	1.937.357	0,061%	-	4 *(4-5)*
$GA_{<28}$	9	4.473	0,201%	**.0010**	4 *(4-5)*
GA_{28-30}	4	4.845	0,083%	2.811	4 *(4-5)*
GA_{30-32}	3	8.510	0,035%	1.626	4 *(4-4)*
GA_{32-37}	38	113.685	0,033%	**.0008**	4 *(4-4)*
GA_{37-42}	1.091	1.765.124	0,062%	-	4 *(4-5)*
GA_{42-44}	34	40.720	0,083%	.4156	4 *(4-5)*
SGA	56	226.111	0,025%	**<.0001**	4 *(4-5)*
AGA	927	1.507.191	0,062%	-	4 *(4-5)*
LGA	196	204.055	0,096%	**<.0001**	4 *(4-5)*
Term, non-SGA	1.038	1.573.833	0,066%	-	4 *(4-5)*
Preterm, non-SGA	51	100.844	0,051%	.3173	4 *(4-4)*
Postterm, non-SGA	34	36.569	0,093%	.2391	4 *(4-5)*
Term and SGA	53	191.291	0,028%	**<.0001**	4 *(4-5)*
Preterm and SGA	3	30.669	0,010%	**.0007**	4 *(4-4)*
Postterm and SGA	0	4.151	0,000%	.4900	4 *(4-5)*

The amount and corresponding percentage of children that had a carnitine ≤ 5 µmol/L in primary blood sample in groups based on gestational age and/or weight for gestational age.

Abbreviations: carnitine – Carnitine concentration; IQR – Interquartile range; SGA – Small for gestational age; AGA – Appropriate for gestational age; LGA – Large for gestational age.

[a] Chi-square test after Bonferroni correction. Gestational age categories compared against term category. SGA and LGA compared against AGA. Compiled subgroups were compared to term, non-SGA newborns.

DISCUSSION

To our knowledge, this is the first study to evaluate the pattern in carnitine concentrations during the first 10 days of life in relation to GA and WfGA. We demonstrate that carnitine concentration in preterm, postterm and SGA newborns is higher compared to term newborns on the third day of life. In addition, our data show that on the sixth day of life, median carnitine concentrations of premature infants approximate those of term infants, whereas the concentrations of postterm and SGA infants remain elevated compared to AGA term infants. This corresponds with previously reported data.[2-9]

We evaluated whether the relatively increased median carnitine concentrations in certain groups lead to a lower number of referrals for a free carnitine ≤ 5 µmol/L. Indeed we found that relatively fewer SGA infants and children born after 32-37 weeks of gestation were referred.

The reason for the initially relatively higher concentrations of carnitine in these groups is not fully understood. It is plausible that placental transport of carnitine is facilitated in a phase where foetuses are not yet capable of supporting their own carnitine demand, resulting in relatively high carnitine concentrations in preterm infants that are born during this phase.[11] Previous research demonstrated a crucial role for fatty acid oxidation in foetal development and in late pregnancy, highlighting the need for carnitine in these stages.[12-16] In addition, renal development is incomplete in extremely preterm infants, resulting in an increased postpartum renal wasting of carnitine. [17,18] This is reflected by the finding of a relatively rapid decrease of median carnitine concentrations in extremely premature newborns compared to late preterm newborns (figure 3A). Theoretically, the elevated carnitine levels in preterms might also be caused by overestimation of the measured carnitine level in DBS. It is known that a higher haematocrit can increase certain metabolite concentrations when measured in DBS, thus giving rise to higher carnitine levels in infants that are prone to higher haematocrit levels, such as SGA and post-term infants. [19-22] However, preterm children are known to have lower haematocrit at birth; the relatively higher carnitine concentration in this group, make this a less likely explanation.[21,22]

It is possible to automatically adjust for covariates with the use of Collaborative Laboratory Integrated Reports (CLIR). CLIR is a multivariate pattern recognition software and interactive web tool, that uses a database containing 60 different NBS conditions, 7,8 million reference profiles and 16.363 true and false positive cases collected from 239 NBS programs in 70 countries (as of January 3rd 2020). With these data, CLIR is able to accurately create moving percentiles, adjusted for the variables gestational age, birth weight, sample day and gender. CLIR eliminates the need for multiple variable-dependent reference ranges and replaces such ranges with a single adjusted score, reflecting the likelihood of a given disease being present. However, for programs currently unable to use CLIR in the routine interpretation of NBS results, the findings of this study indicate that variable-dependent reference ranges are desirable in order to accurately evaluate carnitine concentrations in NBS.

By using data from the Dutch NBS program, large sample sizes could be reached for all groups, making a robust analysis of the effects of all attributes on the median carnitine concentration feasible. However, this study has some limitations. Since data were collected anonymously, it was not possible to confidently determine if all cases were healthy individuals. As illness is known to lower carnitine concentrations, we attempted to minimize the impact of this confounder by excluding samples that probably originated from ill individuals.[23] Still, it is probable that at least part of our data concerns ill newborns, with a majority likely in the preterm group. We believe this does not alter our conclusion, since, despite the presence of this potential confounder, we still found higher carnitine concentrations in preterm groups.

In conclusion, median carnitine concentrations in DBS of newborns vary with day of sampling, gestational age and weight for gestational age. It is important to take these covariates into account when interpreting NBS-results.

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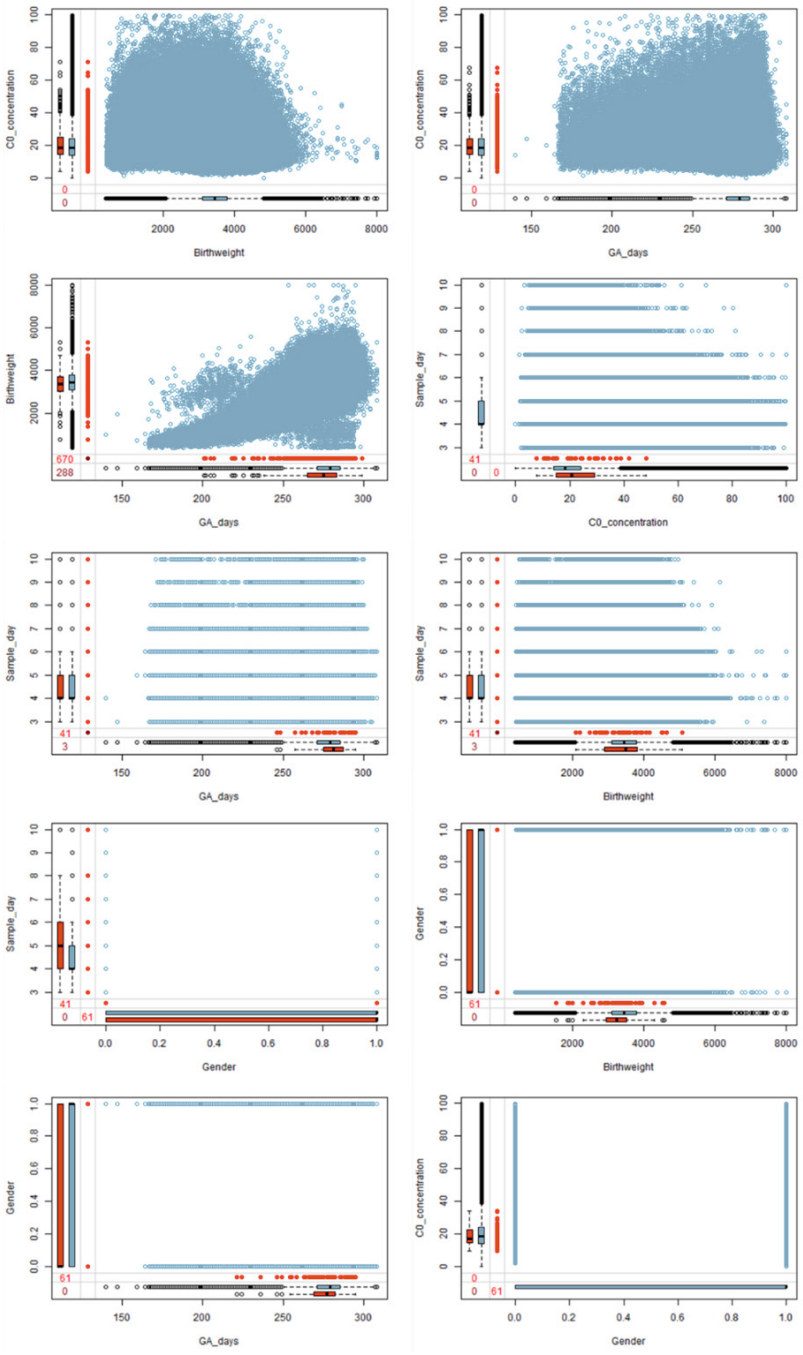

Figure S1 | Marginplots of missing data. Marginplots comparing variables from missing data (red) to those of non-missing data (blue).

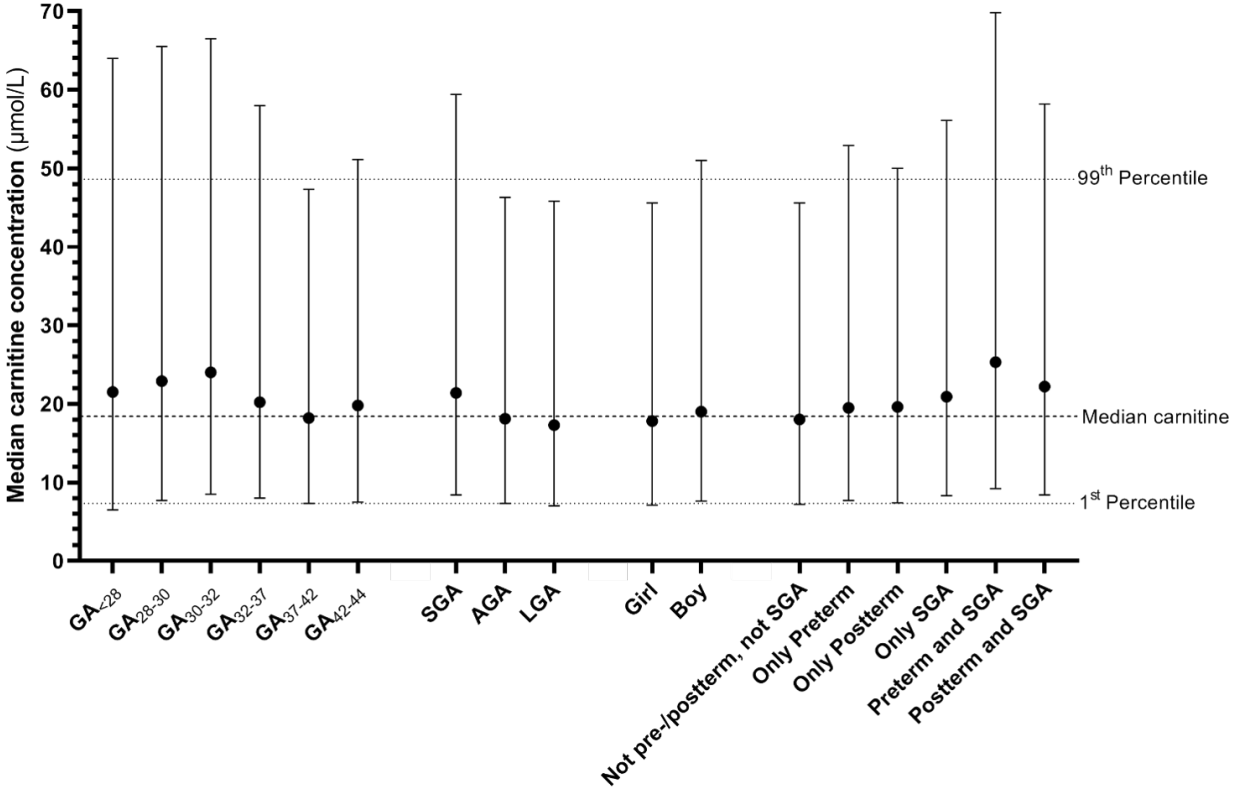

Figure S2 | Median carnitine concentrations. Graph of median carnitine concentrations and 1st and 99th percentiles. Abbreviations: SGA – Small for gestational age; AGA – Appropriate for gestational age; LGA – Large for gestational age.

Table S1 | Overview of previous research on postnatal carnitine concentrations in (A) SGA vs AGA newborns (B) preterm vs term newborns

A

	AGA		SGA		
Author	N	C0 Concentration (µmol/L)*	N	C0 concentration (µmol/L)*	Sample day (days)
Sánchez-Pintoz et al. 2016	73**	25.7 ± 16.0	71**	32.2 ± 14.4	3-5
		20.8 ± 11.2		23.5 ± 12.2	15
		24.9 ± 16.2		25.0 ± 13.6	30
		36.1 ± 19.5		30.4 ± 9.5	40
Liu et al. 2017	8264	15.4 (p5-95: 9.2-26.2)	715	16.4 (p5-95: 9.7-28.5)	3
* Mean ± SD or Median (p5-p95) ** not all sample days included the complete cohort. Total cohort size: day 3-5, N=108; day 15, N=81; day 30, N=57; day 40, N=17.					

B

	Term		Preterm				
Author	N	C0 Concentration (µmol/L)*	Preterm definition (Weeks of Gestation)	N	C0 concentration (µmol/L)*	Sample day (days)	Matrix
Battistella et al. 1980	15	31.2 ± 2.5 (SE)	30-32	29	43.0 ± 5.6 (SE)	0	Cord blood
			33-36		37.5 ± 3.1 (SE)		
Shenai et al. 1983	72	22.4 ± 0.8 (SE)	≤36	53	29.0 ± 1.8 (SE)	0	Cord blood
Watkins et al. 2019	150	19.9 (IQR 6.3-29.5)	23-36	150	21.7 (IQR 12.3-32.1)	0	Cord blood
Novak et al. 1981	7	~5.0 ± 2.5 (SE)	30-37	10	~16.0 ± 4.0 (SE)	0	Cord blood
Mandour et al 2013	143	26.3 (IQR 18.9-33.9)	27-36	131	30.0 (IQR 22.4-42.9)	5	DBS
		-			21.5 (IQR 16.1-30.2)	14	DBS
Liu et al. 2017	8914	15.5 (p5-95: 9.2-26.4)	<37	65	18.2 (p5-p95: 10.7-32.4)	3	DBS
Meyburg et al. 2002	30	28.0 ± 10.0 (SD)	22-27	30	41.0 ± 17.0 (SD)	5	DBS
					~35.0 ± 8.0 (SE)	7	
					~20.0 ± 4.0 (SE)	14	
			28-31	30	34.0 ± 12.0 (SD)	5	DBS
					~27.0 ± 2.0 (SE)	7	
					~37.0 ± 3.0 (SE)	14	
			32-36	30	37.0 ± 15.0 (SD)	5	DBS
* Mean ± SE/SD or Median (IQR) or Median (p5-p95) ~ exact data not provided, data estimated from published graphs.							

CHAPTER

Primary carnitine deficiency is a life-long disease

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ABSTRACT

Background: Primary carnitine deficiency is a rare autosomal recessive disease associated with acute hypoketotic hypoglycemia, cardiomyopathy and sudden cardiac death. Effective treatment with carnitine supplementation is available.

Case summary: An 18 months old boy, who presented with cardiomyopathy was diagnosed with primary carnitine deficiency, and carnitine supplementation resulted in a full recovery. At age 13 years, he discontinued his medication and at 20 years, he discontinued clinical monitoring. Nine years later, age 29, he presented with heart failure and atrial fibrillation and was admitted to an intensive care unit, where he was treated with furosemide, enoximone and intravenous carnitine supplementation, this lead to improved cardiac function within 2 weeks, and with continued oral carnitine supplements, his left ventricular ejection fraction normalised. The last 8 years were uneventful and he continued to attend his regular follow-up visits at a specialised metabolic outpatient clinic.

Discussion: We report recurrent reversible severe heart failure in a patient with primary carnitine deficiency, it was directly related to non-compliance to carnitine supplementation (and monitoring). This case report emphasizes first, the importance of continued monitoring of metabolic disease patients, second, the potential reversibility of cardiomyopathy in an adult patient, and third, the potential risks in the period of transition from the paediatric to adult care. This is an age where young adults desire to be healthy and ignore the need for ongoing medical treatment, even as simple as oral suppletion. Before they reach this age, adequate disease insight and self-management of the disease should be promoted.

INTRODUCTION

Primary carnitine deficiency (OMIM entry #212140) is a rare autosomal recessive metabolic disease caused by pathogenic variations in the *SLC22A5* gene, encoding the Organic Cation Transporter Novel 2 (OCTN2) protein.[1] This transporter protein maintains intracellular carnitine concentrations by transporting carnitine across the plasma membrane into cells, as well as reabsorbing carnitine in the proximal tubule of the kidney.[2 3] Defects in OCTN2 lead to significant decreases in intracellular carnitine availability which may impair mitochondrial fatty acid oxidation.[4-6] The disease can present early in life with acute hypoketotic hypoglycemia, cardiomyopathy and sudden cardiac death.[7] The condition is treatable with lifelong carnitine supplementation.

CASE REPORT

This report provides an update on a patient with primary carnitine deficiency that was reported earlier in 2 published case reports, 20 and 30 years ago.[8 9] A previously healthy 18 month old boy was admitted with lower respiratory tract infection (likely viral) followed by congestive heart failure. Cardiac ultrasound revealed a dilated left ventricle and poor contractility. Upon further investigation, serum free carnitine was extremely low 1.4 µmol/L (reference range 19-59 µmol/L) and urinary carnitine concentration was increased, which was indicative of primary carnitine deficiency. Due to advances in diagnostic procedures, the diagnosis primary carnitine deficiency was later genetically and functionally confirmed, identifying homozygous pathogenic variations in the *SLC22A5*-gene (c.632A>G) and decreased carnitine transporter activity in cultured fibroblasts (activity level under 10% of mean control values).[9] Therapy with diuretics, antibiotics and L-carnitine (3TD 500 mg daily) resulted in normalized cardiac functions within 3 months after which only carnitine supplementation was continued. The following years were uneventful, with no change in his normal left ventricular ejection fraction (LVEF). From the age of 13 onwards, the patient became non-compliant with his medication. Considering the patient decided to stop medication on a regular basis, his primary caretaker and parents repeatedly urged him to at least take carnitine supplements during illness. Despite not being on carnitine supplements, he participated in Ramadan for years without symptoms. Ramadan means fasting and abstinence from all food or drink, including water, from dawn to sunset (10-12 hours per day), every year, during a period of 29-30 days. At the age of 20 years, he stopped visiting his metabolic specialist and only sporadically attended his cardiologist. At the age of 27, he withdrew himself from medical care completely. His last measured LVEF was 50% (normal is ≥ 50%), and serum free carnitine level was 4.0 µmol/L (reference range 22.3-54.8). A timeline of functional cardiac parameters and treatment data is presented in table 1.

At the age of 29 years the patient presented a second time with heart failure and atrial fibrillation, this was likely provoked by a viral respiratory tract infection. He was admitted to the intensive care unit. Ultrasound imaging revealed a dilated heart with severely decreased cardiac function and severe mitral valve

insufficiency, LVEF could not be determined due to poor image quality. His free carnitine concentration was not measured upon admission. Treatment consisted of intravenous diuretics (furosemide), inotropes (enoximone) and L-carnitine (6 grams bolus and then 6 grams daily continuously). After two weeks, he was discharged with a moderately improved LVEF of 30% (table 1) and enrolled in a cardiac rehabilitation programme. In the following years he was followed up by his cardiologists and, with continued carnitine supplementation (varying from 4TD 1,5 - 3g daily), his LVEF improved to 57% with only moderate mitral valve insufficiency five years later.

At the age of 38 years, he was approached for participation in scientific research on primary carnitine deficiency, this resulted in a new appointment with an internist- metabolic consultant. His carnitine levels were again decreased: free carnitine 14 µmol/L, with carnitine supplementation of 4TD 3g. His most recent cardiac evaluation showed a slightly dilated left ventricle, LVEF of 55%, and no arrhythmias. He was still considered unfit for work.

Table 1 | Overview of cardiac parameters and carnitine status over time in the presented patient with primary carnitine deficiency

Age	Events	Prescribed carnitine suppletion dosage	Plasma free carnitine concentration (µmol/L)	LVEF (%)	Shortening fraction (%)
			Normal value 22.3 - 54.8	*Normal value ≥ 50%*	*Normal value ≥ 25%*
18 months	Presented with severe cardiac failure	-	1		16-18
21 months	PCD diagnosed	3TD 0.5g	NA		30
12 years		4TD 3.3g	NA		31
13 years	Non-compliance for medication	4TD 3.3g	NA		
18 years		4TD 3.3g	1	50	
19 years	Withdrew from metabolic specialist care	-	2		
27 years	Withdrew from all care	-	4	50	26
29 years	Admission ICU with severe cardiac failure	-	NA	Low[a]	
week 1		Intravenous: 6g/24 h	35	30	
week 2		Intravenous: 6g/24 h	49		
week 2	Discharge from ICU	4TD 1.5g	NA		
31 years		4TD 2g	30	42	21
33 years		4TD 3g	26	54	
35 years		4TD 3g	NA		33
37 years		4TD 3g	14	55	

Abbreviations: LVEF - left ventricular ejection fraction, PCD - primary carnitine deficiency, NA - not available, ICU - intensive care unit, TD – times daily.

[a] Very low, the ejection fraction was not quantified due to the poor image quality during severe cardiac failure, it will have been substantially lower than 30 % since the next measurement with an LVEF of 30% was reported as a marked improvement.

DISCUSSION

This case demonstrates that in diseases such as primary carnitine deficiency, where treatment is geared towards prevention, treatment adherence is a considerable challenge for both the patient and the caregiver, as the benefits from treatment are not immediately apparent.[10-16] The consequence of discontinued carnitine supplementation was life threatening but only after years of discontinuation. Especially young adults are at risk of withdrawing from follow-up, as they generally do not identify themselves as 'sick', having only a recollection of a symptom free life with good health. In these cases, treatment adherence may be improved with patient education aimed at improving disease insight.[11 17] We like to suggest to approach these patients as healthy and that our role as caregiver is to keep them healthy. Furthermore, patient engagement with the healthcare system may be improved with written information about the disease for other health care providers (*e.g.* the general practitioner), so they, too, can intervene and continue to urge restarting medication. In most expert centres there are special programs for safe and continued transition. Our patient indicated he discontinued his treatment before adolescence, as he believed there was no benefit from taking his medication and felt it was inconvenient. It is likely that, with continued treatment and follow-up, the second cardiac decompensation could have been prevented.

This patients history illustrates the severe end of the phenotypic variation of primary carnitine deficiency. In an effort to prevent complications like these, primary carnitine deficiency was implemented in several newborn screening programs. After its introduction, asymptomatic individuals with primary carnitine deficiency are diagnosed in increasing numbers.[18-20] It remains unclear if carnitine supplementation is beneficial for all individuals. Specifically in those that have had an uneventful childhood, being diagnosed by family counselling or as incidental finding following their child's newborn screening, the risk for severe disease outcome is unknown.[21 22] There have been reports of sudden cardiac death in seemingly asymptomatic adult PCD patients in the Faroese population, this will urge us to treat all diagnosed individuals.[23 24] The presented case, however, illustrates that continued carnitine supplementation seems essential for patients with initial symptomatic presentation, like heart failure. Even 'healthy' patients, without symptoms for decades while off treatment, can again develop severe complications.

Interestingly, at least for this patient, Ramadan related fasting did not provoke symptoms possibly related to PCD, despite very low free carnitine levels. This is surprising, as one would expect hypoketotic hypoglycemia to occur during fasting. Fasting is an important provoking factor for disorders of mitochondrial long-chain fatty acid oxidation and the carnitine shuttle.[4 25] In the presented case, both cardiac events were provoked by a viral upper respiratory tract infection. This is in line with other previously reported primary carnitine deficiency cases with severe cardiac decompensation, that were also preceded by (mild) upper respiratory tract infection.[26-29] Infections can be considered important provoking factors, and patients with low compliance should be reminded to take medication every day, but especially during (mild) infection.

In conclusion, patients with IEM that feel "healthy" following successful treatment during childhood years, may be prone to disregard their disease and important medication to maintain their health. Complications may occur after decades of non-compliance. The role of caregivers to counsel young adults and to prevent non-compliance, is of the utmost importance. Treatment cessation in primary carnitine deficiency can lead to severe heart failure, even decades later, and is unlike typical heart failure, completely reversible. In the event of cardiac decompensation all efforts should be made to bridge the gap to cardiac recovery.

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CHAPTER

Newborn screening for primary carnitine deficiency: who will benefit from treatment?

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ABSTRACT

Importance: Newborn screening (NBS) programs frequently identify newborns with diverse disease phenotypes, which raises the question whether early identification and subsequent treatment is beneficial for all. As treatment is generally immediately initiated in all diagnosed newborns, this question is difficult to address. For primary carnitine deficiency (PCD), a rare disorder caused by deficient activity of the carnitine transporter (OCTN2, coded by the *SLC22A5* gene) this situation differs. NBS for PCD not only identifies newborns with PCD, but also previously undiagnosed mothers. This provides a unique opportunity to study the long-term clinical course of untreated individuals that were diagnosed through NBS, enabling evaluation of the benefits of NBS for this condition.

Objective: To compare the clinical, genetic and functional (carnitine transport activity) characteristics of PCD patients diagnosed by NBS with those of clinically diagnosed patients.

Design: Retrospective cohort study.

Setting: Multicenter study in the Netherlands.

Participants: All individuals referred with low free carnitine by NBS (newborns and mothers) and clinically diagnosed PCD patients (before NBS) until end of 2020.

Exposures: NBS for PCD.

Main Outcomes and Measures: Clinical, genetic and functional data.

Results: PCD was confirmed in 19 out of 131 (19/131) referred newborns, 37/82 referred mothers and 5 clinically diagnosed patients. Severe clinical symptoms were found in all clinically diagnosed patients, 1 newborn and none of the mothers. Disease severity, based on clinical observations and published data on *SLC22A5* variants was classified as severe for 3/19 newborns (16%), 1/37 mothers (3%) and all clinically diagnosed patients (5/5, 100%); as likely benign in 8 newborns (42%), 36 mothers (97%); and unknown in 8 newborns (42%). Carnitine transport activity in patients with a severe phenotype was significantly lower than in those with a benign phenotype (median [range]: 4.3% [4.0 - 5.0] versus 26% [9.5 – 42.5]).

Conclusions and Relevance: The majority of individuals with low carnitine transport activity identified through NBS are likely to remain asymptomatic throughout life without treatment. Conversely, a small proportion with severe PCD is likely to greatly benefit from early treatment. The *SLC22A5* gene variants and carnitine transport activity can be used to distinguish these groups.

Trial Registration: Netherlands Trial Register, Identifier: NL7905

INTRODUCTION

Newborn screening (NBS) programs enable an accelerated diagnosis of severe conditions, which allows early treatment initiation and prevention of potentially irreversible signs and symptoms. Expansion of NBS programs often results in the detection of newborns with previously unreported genotypes, not found in clinically diagnosed patients before inclusion of the disease in NBS panels.[1-3] As a result, there are little or no data allowing prediction of the disease course in individuals with clinically unreported genotypes.[4,5] Nevertheless treatment is generally initiated in all diagnosed newborns, which complicates evaluation of the natural, untreated, disease course in this group.

Primary carnitine deficiency (PCD) (OMIM #212140) is an autosomal recessive disorder caused by variants in the *SLC22A5* gene, which encodes the Organic Cation Transporter Novel 2 (OCTN2) protein.[6] OCTN2 maintains the intracellular carnitine concentrations through transport of carnitine into cells, including reabsorption of carnitine in the renal tubuli.[7,8] Impaired function of OCTN2 leads to decreased intracellular free carnitine concentrations. In case of carnitine deficiency, activated long-chain fatty acids are not efficiently transported into the mitochondrial matrix for oxidation and ketone body production. PCD diagnosed based on symptoms (clinically identified) is a potentially fatal condition that can present with myopathy, (hepatic) encephalopathy, cardiomyopathy and/or arrhythmia, which develops during childhood in 90% of patients.[9] Sudden cardiac death has been observed in adulthood.[9] Treatment is simple and consists of lifelong oral supplementation of carnitine.

Because of the severity of the symptoms and the availability of an effective treatment, PCD has been included in NBS panels in a number of countries.[10,11] Screening is conducted by the measurement of the free carnitine concentrations in dried blood spots (DBS). Unexpectedly, inclusion of PCD in NBS panels led to the detection of mothers with PCD, diagnosed when their child (unaffected by PCD) was referred because of low free carnitine concentration in the NBS DBS, caused by a low free carnitine concentration in the mother.[10,12,13] Most of these mothers were asymptomatic at the time of diagnosis and without previous symptoms that might be related to PCD.[9,14] Nevertheless, since cardiac events have been reported in a small number of previously asymptomatic adult PCD-patients, these newly diagnosed mothers were treated and followed-up in regular care.[9,15,16] It may well be that a large proportion of these mothers with low carnitine transporter activity will never become symptomatic without treatment. The high number of patients identified by NBS with previously unreported genotypes hampers prognostication. In addition, the detection of mothers with mild disease or no disease at all raises doubts regarding the benefit of including PCD in NBS programs. The incomplete understanding of benefits of screening, weighed against potential harm caused by identification and treatment of asymptomatic individuals, led to discontinuation of NBS for PCD in New Zealand.[17]

In the Dutch NBS program, free carnitine levels are monitored in all DBS as a control for the quality of the acylcarnitine measurements: low carnitine in DBS may cause unreliable screening results for a number

of disorders relying on the acylcarnitine profile, including long- and medium-chain fatty acid oxidation disorders. Individuals with low carnitine levels are therefore referred even though PCD is not officially included in the Dutch NBS panel. Now, after more than a decade of detecting PCD as an incidental finding of NBS in the Netherlands, we can evaluate its results. The detection of maternal cases provides a unique opportunity to assess the natural course of PCD detected by screening. The presented study aims to evaluate patient characteristics that can be used to identify those patients that benefit from follow-up and treatment and those that do not require medical care and can be regarded as healthy individuals with a benign metabolic trait.

METHODS

Study design

The study protocol for this retrospective cohort study was approved by the Medical Research Ethics Committee Utrecht (METC protocol number 19-234/M). Written informed consent was obtained from all participants and/or their caregivers prior to enrolment. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Participants and data collection

The first group eligible for inclusion were all patients in the Netherlands diagnosed with PCD (further referred to as clinically diagnosed patients). PCD diagnosis was based on a genetic confirmation of bi-allelic variants in *SLC22A5*, and/or a functional confirmation with a reduced residual carnitine transport activity defined as < 50% transport activity of controls in cultured skin fibroblasts. The second eligible group consisted of all newborns referred because of low carnitine concentration in NBS (further referred to as newborns). The third group were all mothers referred because of unexplained low carnitine in the DBS of their child (further referred to as mothers). Inclusion ended on December 31st 2020. The following data were collected retrospectively: medical history, perinatal data, family history, physical examinations, education and occupation, diet, blood/serum/urine free carnitine concentrations, DNA diagnostics and additional test results performed in the context of evaluation of PCD (e.g. electrocardiogram or cardiac ultrasound). In addition, those diagnosed with PCD were either seen or contacted by phone by the researcher for an additional questionnaire on their current health status.

Newborn screening in the Netherlands

During the inclusion period (2007-2020) 2,464,710 children were screened (99.4% of total births) and 98.5% of NBS-samples were obtained within 72 to 168 hours after birth (the target range for NBS-sampling in the Netherlands). The acylcarnitine profile was determined in DBS. In case of a low free carnitine concentration (≤ 4 µmol/L in the period of January 2007 until June 2009 and ≤ 5 µmol/L from July 2009 onwards), a repeat sample was taken within 10 days. When low free carnitine concentration was also present in the second sample (applying the same cut-off value), the newborn was referred to a regional metabolic centre for follow-up, preferably within 24 hours. As a result of the evaluation at the metabolic centre, the mother of the newborn could be secondarily referred to an adult metabolic specialist. The referral rate of mothers increased over time as more reports of maternal PCD cases identified through NBS were published.[10,13,14]

Carnitine transport assay

Residual carnitine transport activity was analysed in cultured skin fibroblasts according to the method described by Ferdinandusse et al. (novel assay).[18] All cell lines were analysed in two independent experiments and the measurements within the experiments were performed in duplicate. Reported values are the mean of all measurements and are expressed as percentage of the mean activity in two or three control cell lines analysed in parallel.

Genetic data

Analysis of the *SLC22A5* gene was performed by sequence analysis of all exons and flanking intronic sequences amplified by polymerase chain reaction from genomic DNA isolated from either fibroblasts or blood from patients. Variants were annotated using Alamut Visual V2.11 (Interactive Biosoftware). All variants with classification 3 or higher were recorded. These variants were subsequently grouped into (1) missense variants and (2) nonsense, frameshift and splice-site variants.

We annotated the identified *SLC22A5* variants as either *classic variants*; variants previously identified in patients diagnosed following clinical symptoms, or as *screening variants*; variants identified in patients following NBS screening diagnosis only. (Supplementary table 1)

Disease severity classification

Based on the genotype, reported observations in literature and observed disease course into adulthood within our study population, three PCD disease severity categories were defined: (1) *Severe*: patients with severe disease are those who suffered severe symptoms (this cohort) and patients from this study cohort with genotypes previously reported in patients that suffered symptoms (2) *Likely benign*: individuals with a likely benign metabolic trait are adult patients in this study cohort who did not suffer any events or severe disease symptoms and NBS children in this cohort with genotypes reported in literature only in patients with an uneventful childhood (untreated) (3) *Unknown*: Individuals for whom the significance of the metabolic trait is unknown are treated NBS children (this cohort) with genotypes never reported in untreated patients in literature.

For this classification, severe symptoms were considered those that may cause irreversible damage: sudden death, cardiac arrest, ventricular fibrillation, sustained ventricular tachycardia (ventricular rhythm faster than 100 bpm, with a QRS-complex > 120 ms, lasting at least 30 seconds), cardiomyopathy with cardiac failure (left ventricular ejection fraction < 50%), encephalopathy, coma (without a discernible external cause), hypoglycaemia (blood glucose < 2.5 mmol/L), sepsis-like presentation and severe rhabdomyolysis (plasma Creatine Kinase (CK) > 5 times the upper reference range). A history of fatigue and myalgia (without elevated plasma CK) was annotated as mild symptoms. Because of the high risk of observational selection bias, new complaints of fatigue and/or myalgia that were mentioned only after specific enquiry by the physician/researcher were recorded, but not considered a symptomatic involvement in the context of this study.

Statistics

Statistical analyses were performed using R (version 3.6.2, R Core Team, Vienna, Austria). Continuous data was tested for normality with Shapiro-Wilk's method. The two-sided T-test was used to compare normally distributed continuous data between two groups and the Mann-Whitney U test was used to compare non-normally distributed continuous data between two groups. The chi-square test was used for comparison of categorical data. Multiple comparisons were adjusted with the Bonferroni correction. Significance was assumed for $p < 0.05$.

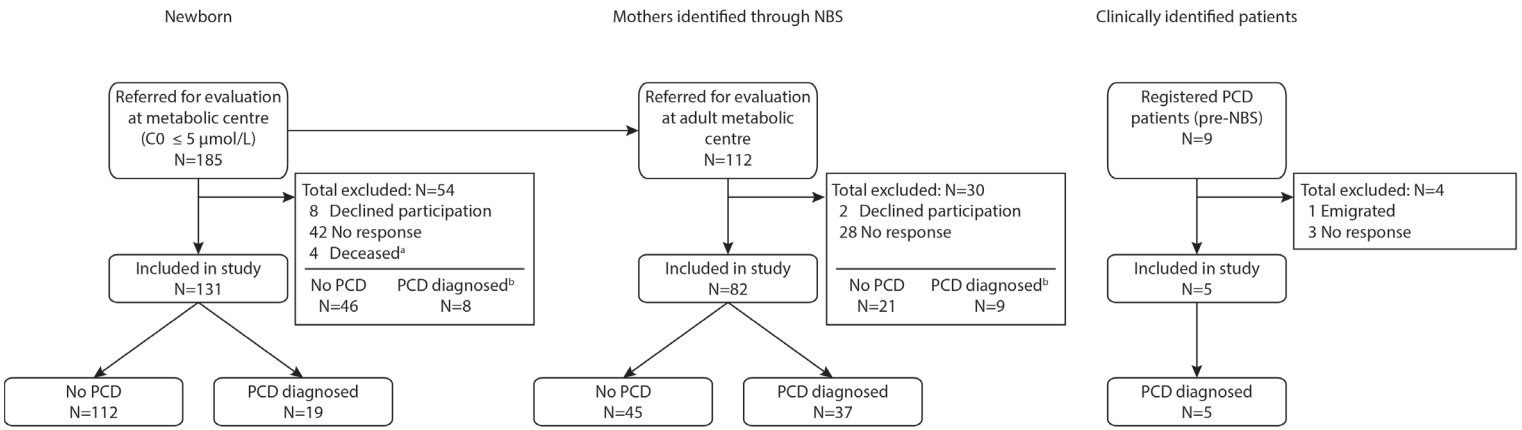

Figure 1 | Flowchart of patient inclusion

[a] All 4 infants died from complications explained by extremely preterm birth

[b] Based on national registries, validity of diagnosis could not be confirmed

RESULTS

From 2007 until 2020, 185 newborns were referred because of low free carnitine concentrations in NBS and 112 mothers were referred secondary to their newborn, see Figure 1. Of 131 newborns and 82 mothers consent for study participation was obtained. Five of in total nine known clinically diagnosed Dutch patients (all diagnosed before free carnitine was included in the Dutch NBS program) were included in the study.

Table 1 | Clinical, biochemical and functional characteristics of PCD patients in Dutch cohort

	Newborns (n=19)		Mothers (n=37)		Clinically diagnosed (n=5)	
Sex (male)	9 (47.4%)	{0}	0 (0%)	{0}	2 (40.0%)	{0}
Gestational age at birth (weeks)	40.1 [37.3, 41.7]	{1}	-		-	
Birth weight (grams)	3390 [2490, 4300]	{0}	-		-	
Sibling death	0 (0%)	{5}	1 (3.2%)	{6}	1 (20.0%)	{0}
Age at diagnosis (years)	0 [0, 0]	{0}	31.0 [21.0, 40.0]	{6}	1.0 [1.0, 15.0]	{0}
Age at last follow-up (years)	4.6 [1.0, 14.1]	{0}	34.6 [24.8, 48.6]	{0}	20.7 [19.6, 37.4]	{0}
Symptoms		{0}		{0}		{0}
Asymptomatic	15 (78.9%)		29 (78.4%)		0 (0%)	
Self-reported signs[a]	3 (15.8%)		8 (21.6%)		0 (0%)	
Severe symptoms[b]	1 (5.3%)		0 (0%)		5 (100%)	
SLC22A5 variants		{0}		{0}		{0}
Missense	13 (68.3%)		33 (89.2%)		3 (60.0%)	
Missense/Null	4 (21.1%)		2 (5.4%)		0 (0%)	
Null	2 (10.5%)		0 (0%)		2 (40.0%)	
Missense, single variant identified	0 (0%)		2 (5.4%) [d]		0 (0%)	
Variant reported		{0}		{0}		{0}
2 classic variants	5 (26.3%)		1 (2.7%)		5 (100%)	
2 screening variants	7 (36.8%)		31 (83.7%)		0 (0%)	
1 classic and 1 screening variant	7 (36.8%)		3 (8.1%)		0 (0%)	
Single variant identified (screening variant)	0 (0%)		2 (5.4%) [d]		0 (0%)	
Transport activity in fibroblasts (% of controls)	12.5 [8.50, 27.0]	{14}	26.0 [4.0, 42.5]	{17}	4.25 [4.00, 5.00]	{1}
Free carnitine concentration (blood; μmol/L)						
First NBS DBS Sample[c]	3.50 [1.80, 4.80]	{0}	3.70 [1.90, 5.00]	{2}		
Second NBS DBS Sample[c]	3.05 [1.30, 4.70]	{1}	3.80 [2.00, 5.00]	{2}		
First sample at metabolic centre (plasma)	6.87 [1.97, 12.0]	{0}	6.80 [2.09, 18.8]	{3}	3.00 [1.00, 4.40]	{0}

Data presented as n (%) or median [Min-Max]. Missing data points are presented in grey, within braces. Abbreviations: PCD - Primary carnitine deficiency; NBS - Newborn screening; DBS - Dried blood spot; IEM - Inborn error of metabolism.

[a] Fatigue, myalgia or corrected motor developmental delay.

[b] Sepsislike-presentation, cardiomyopathy, arrhythmia and/or coma.

[c] For mothers: concentration in the NBS sample of their child.

[d] Diagnosis based on reduced transport activity (30.5% and 40.5% respectively).

Table 2 | Overview of patients with severe genotypes

Patient ID	Identified	Sex	Age initial symptoms	Age at last follow-up	Initial symptoms	Clinical course at follow-up	Treatment effect	Variant allele 1	Variant allele 2	Transport activity
8	Clinically	M	11 months	19 years	Sepsis-like presentation, Coma, Cardiomyopathy	Difficulty feeding	Complete resolution.	**c.396G>A**	**c.396G>A**	NA
10	Clinically	M	6 months	37 years	Cardiomyopathy	Cardiomyopathy at 30Y	Complete resolution within 1Y. After suspending treatment for 12Y cardiomyopathy recurred with severe cardiac failure.	c.632A>G	c.632A>G	4%
11	Clinically	F	15 years	19 years	Ventricular fibrillation	NA	Defib. Implanted, normal ECG after 1Y.	c.1232G>T	c.1232G>T	5%
12	Clinically	F	3 years	22 years	Cardiomyopathy	Fatigue	Complete resolution.	**c.844C>T**	**c.844C>T**	4%
193	Clinically	F	9 months	20 years	Cardiomyopathy, CMV infection, Failure to thrive	Hypoglycaemic coma with VF which lead to diagnosis at 21M. Learning disability. Sudden death at 20Y	With conventional treatment (digoxine, diuretics) no improvement. Upon carnitine supplementation complete resolution.	c.632A>G	c.632A>G	4.5%
502	NBS	F	2 weeks	12 years	Sepsis-like presentation	Learning disability	Fast recovery after initiating carnitine supplementation.	**c.597delG**	**c.597delG**	NA
131	Mother	F	38 years	38 years	None	Asymptomatic	None	c.248G>T[a]	c.248G>T[a]	4%
597[b]	NBS	F	NA	7 years	-	Asymptomatic	-	c.248G>T[a]	c.248G>T[a]	NA
628[b]	NBS	M	9 years	13 years	None	Myalgia in legs with normal serum free carnitine	None	c.248G>T[a]	c.248G>T[a]	NA

Null variations are in bold. Abbreviations: NBS – Newborn screening; M – Male; F – Female; NA – Not available.

[a] Makhseed 2004[22]: c.248G>T homozygous patient, presented with axonal neuropathy at 16 months, at 3Y unresponsive to stimuli with hypoketotic hypoglycemia. Sibling 11Y (also homozygous), no symptoms.

[b] Siblings

Newborn screening results

An overview of clinical, biochemical and genetic characteristics of all confirmed PCD patients is provided in Table 1. Diagnosis of PCD was confirmed in 19/131 (15%) of the referred newborns and 37/82 (45%) of the referred mothers. No inherited metabolic disorders other than PCD were identified in newborns. Five mothers were diagnosed with another inherited metabolic disorder, causing the low carnitine concentration in the DBS of their child (medium-chain acyl-CoA dehydrogenase deficiency, n=2; glutaric aciduria type 1, n=2; 3-methylcrotonyl-CoA carboxylase deficiency, n=1). Data on referred individuals is provided in supplementary table 2.

Clinical characteristics of confirmed PCD patients

The median age at last follow-up of the 19 newborns was 4.6 years. Fifteen (79%) were asymptomatic, three (21%) had self-reported signs (fatigue n=2; myalgia n=1) and one patient presented with a sepsis-like episode with negative microbial cultures at two weeks of age. As she presented at the emergency room, her NBS results came in, after which she immediately received carnitine supplementation, and quickly recovered. Of the mothers (n=37), median age at diagnosis was 31 years, median age at last follow-up was 34.6 years, 29 (78%) were asymptomatic, eight (22%) had self-reported signs (fatigue n=6; myalgia n=1; non-specific pain in legs n=1), and none had experienced a severe event.

Clinically diagnosed patients (n=5) all presented in childhood (range 3 months to 15 years) with severe events (Table 2, patient 8, 10, 11, 12 and 193). The median age at diagnosis was 1 year and median age at last follow-up was 20.7 years. Four patients presented with cardiomyopathy that completely resolved with carnitine supplementation, of whom two were previously reported in literature (patient 10 and 193).[19,20] One patient presented with ventricular fibrillation at 15 years of age. After defibrillation, episodes of non-sustained ventricular tachycardia persisted, and a cardiac defibrillator was implanted. Carnitine supplementation resulted in complete normalization of cardiac parameters within one year.[21] She moved abroad and was lost to follow-up three years later.

All patients received treatment upon diagnosis. However, seven mothers were not immediately diagnosed at the time of referral because of relatively high plasma carnitine concentrations and the presence of only one variant in the *SLC22A5* gene. Genetic re-evaluation after several years revealed a second variant (c.-149A>G) in all cases. These cases were therefore not treated and follow-up data were not available (Table 3; cases 127, 136, 164, 166, 167, 191, 258).

Disease severity assessment based on genetic classification

Disease was classified as severe in nine cases (clinically identified n=5, newborn n=3, mothers n=1) (Table 2), likely benign in 44 cases (newborn n=8, mothers n=36) (Table 3) and as of unknown severity in 8 newborns (Supplementary table 3).

The allele frequencies of variants identified in the different disease severity groups were: *classic variants*:

severe n=18 (100%), likely benign n=5 (6%) and unknown n=9 (56%); *screening variants*: severe n=0, likely benign n=81 (92%) and unknown n=7 (44%) (*p*-value <0.001).

The carnitine transport activity in cultured fibroblasts (median (range)) in the different groups was as follows: severe (n=5) 4.0 % (4.0 - 5.0), likely benign (n=23) 26.9 % (9.5 – 42.5) and unknown (n=1: case 528) 8.5%, presented in figure 2. The carnitine transport activity differed significantly between the severe and likely benign groups (*p*-value <0.001).

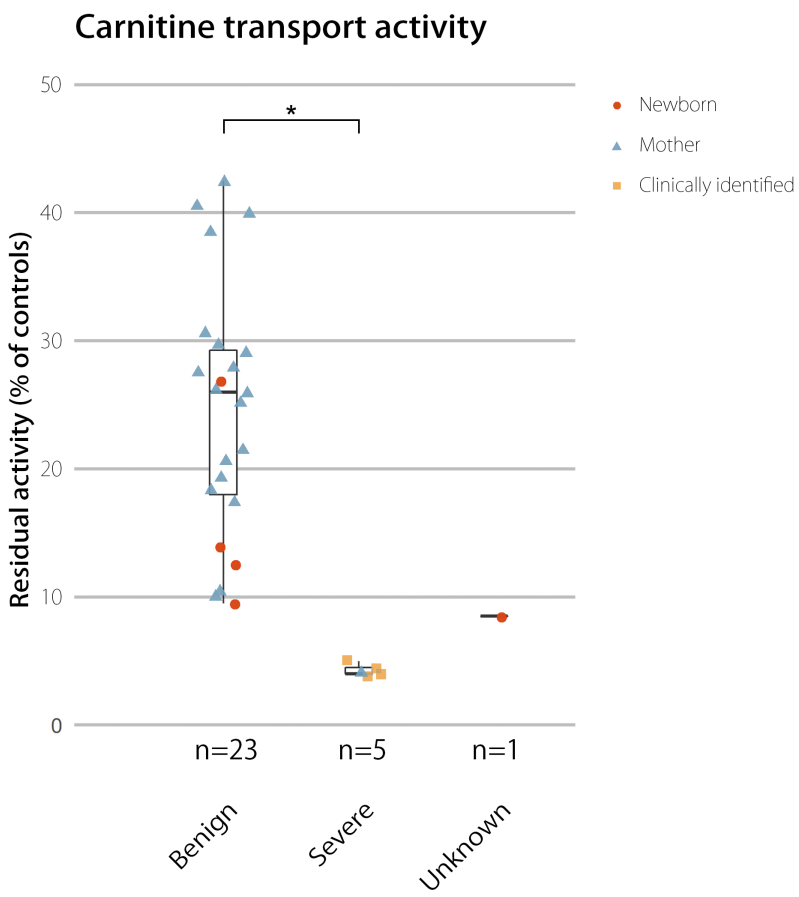

Figure 2 | Residual carnitine transport activity of Dutch PCD patients. The residual carnitine transport activities of PCD patients identified by Dutch newborn screening (newborn and mothers) or clinical presentation (clinically identified) are presented. The results are grouped by disease severity based on genotypes.

*p-value <0.001 (two-sided t-test)

Table 3 | Overview of patients with likely benign genotypes

Patient ID	Identified	Age at Dx	Age initial symptoms	Age at last follow-up	Symptoms	Treatment effect	Clinical course in literature	Variant allele 1	Variant allele 2	Transport activity
132	Mother	40Y	40Y	44Y	Asymptomatic[a]	None	Verbeeten[30] : family through NBS; 3 adults with fatigue in hindsight	c.-149G>A	c.-149G>A	20.5%
143	Mother	35Y	NA	37Y	Asymptomatic	-		c.-149G>A	c.-149G>A	27.5%
164	Mother	-	NA	30Y	Asymptomatic	-		c.-149G>A	c.-149G>A	40.0%
198	Mother	35Y	Puberty	39Y	Fatigue	None, Fishy odor		c.-149G>A	c.-149G>A	42.5%
136	Mother	-	NA	30Y	Asymptomatic	Not started		c.136C>T	c.-149G>A	NA
145	Mother	37Y	48Y	48Y	Asymptomatic[a]	Not started		c.136C>T	c.-149G>A	21.5%
156	Mother	32Y	37Y	38Y	Asymptomatic[a]	None		c.136C>T	c.-149G>A	NA
160	Mother	31Y	NA	31Y	Asymptomatic	Not started		c.136C>T	c.-149G>A	NA
166	Mother	-	36Y	41Y	Asymptomatic[a]	NA		c.136C>T	c.-149G>A	29.5%
174	Mother	34Y	NA	37Y	Asymptomatic	-		c.136C>T	c.-149G>A	NA
215	Mother	25Y	NA	27Y	Asymptomatic	Nausea		c.136C>T	c.-149G>A	NA
244	Mother	30Y	NA	35Y	Asymptomatic	-		c.136C>T	c.-149G>A	NA
266	Mother	31Y	Puberty	30Y	Myalgia	None, Fishy odor		c.136C>T	c.-149G>A	NA
551	Newborn	0M	6Y	6Y	Asymptomatic[a]	NA		c.136C>T	c.-149G>A	27.0%
101	Mother	25Y	28Y	30Y	Asymptomatic[b]	None	Li[31] : Mother through NBS, 26Y, asymptomatic. de Boer[32] :Patient 632	c.136C>T	c.136C>T	29.0%
150	Mother	25Y	NA	25Y	Asymptomatic	Not started		c.136C>T	c.136C>T	NA
247	Mother	26Y	Puberty	33Y	Fatigue	None, Fishy odor		c.136C>T	c.136C>T	NA
616	Mother	35Y	Childhood	38Y	Myalgia	None		c.136C>T	c.136C>T	NA
515	Newborn	0M	3Y	6Y	Asymptomatic[a]	Less fatigued		c.136C>T	c.136C>T	12.5%
529	Newborn	0M	NA	14Y	Asymptomatic	Fishy odor		c.136C>T	c.136C>T	14.0%
561	Newborn	0M	NA	2Y	Asymptomatic	-		c.136C>T	c.136C>T	NA
577	Newborn	0M	7M	1Y	Asymptomatic[c]	-		c.136C>T	c.136C>T	NA
632	Newborn	0M	1Y	13Y	Asymptomatic[c]	-		c.136C>T	c.136C>T	NA

Table 3 | (Continued)

Patient ID	Identified	Age at Dx	Age initial symptoms	Age at last follow-up	Symptoms	Treatment effect	Clinical course in literature	Variant allele 1	Variant allele 2	Transport activity
147	Mother	21Y	NA	31Y	Asymptomatic	-		c.640_641delinsTT	c.-149G>A	28.0%
191	Mother	-	NA	27Y	Asymptomatic	Not started		c.640_641delinsTT	c.-149G>A	NA
202	Mother	38Y	NA	44Y	Asymptomatic	None, Fishy odor		c.640_641delinsTT	c.-149G>A	25.0%
120	Mother	40Y	43	43Y	Asymptomatic[a]	None	Li[31] : 1 Mother through NBS, 37Y, asymptomatic but with three spontaneous miscarriages	c.136C>T	c.695C>T	10.0%
546	Newborn	0M	NA	3Y	Asymptomatic	-		c.136C>T	c.695C>T	NA
282	Mother	35Y	36Y	36Y	Asymptomatic[a]	None		c.-149G>A	c.707G>A	NA
167	Mother	-	34Y	35Y	Asymptomatic[a]	NA		c.1101C>G	c.-149G>A	38.5%
119	Mother	32Y	Childhood	36Y	Fatigue	None, Fishy odor		c.1354G>A	c.-149G>A	19.5%
242	Mother	27Y	34Y	34Y	Asymptomatic[a]	None		c.136C>T	c.457G>C	NA
235	Mother	33Y	NA	33Y	Asymptomatic	Not started		c.1421G>A	c.1421G>A	NA
144	Mother	32Y	16Y	36Y	Fatigue	None		c.34G>A	c.1340A>C	26.0%
127	Mother	-	NA	26Y	Asymptomatic	Not started		c.34G>A	c.-149G>A	NA
208	Mother	28Y	NA	32Y	Asymptomatic	-		c.797C>T	c.797C>T	18.5%
213	Mother	24Y	NA	28Y	Asymptomatic	-		c.680G>A	c.-149G>A	NA
205	Mother	29Y	NA	33Y	Asymptomatic	None, Fishy odor		c.718G>A	c.-149G>A	26.0%
258	Mother	-	NA	26Y	Asymptomatic	Not started		**c.825-1G>C**	c.-149G>A	NA
125	Mother	27Y	Adolescense	29Y	Fatigue	Less fatigued		**c.825-1G>C**	c.136C>T	10.5%
158	Mother	29Y	NA	30Y	Asymptomatic	Not started		c.95A>G	c.-149G>A	17.5%
504	Newborn	0M	NA	13Y	Asymptomatic	-	Sarafoglou[33] : Mother through NBS; 35Y, asymptomatic. Schimmenti[10] : Mother through NBS 28Y, fatigue.	c.136C>T	**c.844C>T**	9.5%
115	Mother	28Y	Adolescense	31Y	Fatigue	Less fatigued		c. 136C>T	Not identified	40.5%
196	Mother	29Y	29Y	35Y	Asymptomatic[b]	Reduced myalgia		c.640_641delinsTT	Not identified	30.5%

Null variations are in bold. Additional information on identified variants is provided in supplementary table 1. Abbreviations: NBS – Newborn screening; Mother – Mothers identified through screening of their child; Dx – Diagnosis; M – Months; Y – Years; NA – Not available.

[a] Only mentioned fatigue after specific inquiry by treating physician

[b] Only mentioned myalgia after specific inquiry by treating physician

[c] Initial slight motor developmental delay, corrected before 2Y of age

DISCUSSION

In this study we evaluated the results from 14 years of identifying PCD by the Dutch NBS program. We demonstrate that NBS for PCD is definitely beneficial to a small subset of identified newborns. In this study, at least 16% of the diagnosed newborns (n=3/19) were designated as patients with severe PCD. This group may be larger, as not all referred newborns were included in this study and 8 included newborns had an unknown disease severity. One newborn presented with severe symptoms, and recovered quickly after treatment, which was initiated promptly when results of the NBS came in. She continues to have a normal life. Furthermore, two NBS patients that received treatment since diagnosis (approx. at two weeks of age) have remained symptom free, whereas an untreated patient with a similar genotype developed axonal neuropathy at 16 months and hypoketotic hypoglycemia at 3 years (Table 2).[22]

The benefits of treatment and follow-up are not apparent for all NBS identified individuals. At least 78% of individuals diagnosed with PCD through NBS (newborns and mothers) are likely to remain free of severe disease symptoms during childhood, regardless of treatment. They are either asymptomatic or report complaints such as fatigue or myalgia, that often did not improve after treatment and may well be due to causes other than PCD. Our results show that these individuals differ significantly from patients with severe PCD in the type of *SLC22A5* variants they carry and their residual carnitine transport activity. Similar findings have been reported for other inborn errors of metabolism (*e.g.* for very long-chain acyl-CoA dehydrogenase deficiency, isovaleric acidemia and β-ketothiolase deficiency), where, after introduction to NBS programs, individuals with screening variants and biochemically milder defects of unknown significance are often identified.[1,2,23,24] The present study is the first to show that this group of individuals, at least for PCD, is unlikely to suffer disease symptoms early in life if left untreated. This prompts the discussion whether all individuals identified by NBS should be treated, or whether NBS should target only those in whom treatment improves quality of life early on. The first approach, treatment of all, ensures prevention of severe outcome, but comes at the cost of overmedicalisation. The second approach achieves effective treatment for those that may benefit, without burdening individuals with a benign metabolic trait. With the second approach, however, the potential of developing disease symptoms, possibly later in life, cannot be fully ruled out. How these advantages and disadvantages are weighed is influenced by ethical, cultural and economic viewpoints. Such discussions become even more relevant as technological developments (e.g. next generation sequencing) are likely to lead to further expansion of NBS programs.[25,26]

Previously, a small number of asymptomatic adult PCD patients were reported who presented with a sudden cardiac event.[10,27-29] Additionally, a retrospective analysis of sudden death cases in the Faroese population revealed 13 PCD patients, all homozygous for the c.95A>G variant. These reports in literature led to the follow-up and treatment of asymptomatic mothers detected through NBS of their child in the Netherlands and worldwide. In our study, after 14 years of follow up of individuals referred because of low carnitine in the NBS DBS, only one of the 37 identified mothers had potentially severe disease (based on genotype) and none had any sign of cardiac disease. Thus far, worldwide, two mothers identified through

NBS of their child have been reported with (a history of) cardiac events, namely ventricular fibrillation ([c.424G>T, c.1463G>A];[c.1586+1G>T])[28] and ventricular tachycardia ([c.95G>A;c.136C>G])[10] Both carried one classic variant (c.424G>T/c.1463G>A (identified on the same allele) and c.95A>G) and one screening variant (c.1586+1G>T and c.136G>C). It remains unclear if there was a secondary cause or risk factor for ventricular arrhythmias in these two mothers. Based on the limited number of observed cardiac events in our cohort and in literature in NBS identified patients, we believe this risk does not justify identification and subsequent lifelong follow-up and treatment of mothers diagnosed through NBS of their child.

In the presented cohort, disease severity remains unknown for eight newborns, since there is no natural history data available for their specific genotypes (Supplementary table 3). With the availability of a novel carnitine transport activity assay[18], that can differentiate between a severe deficiency associated with severe clinical symptoms and a mild deficiency associated with only mild or no clinical symptoms, the risk for newborns with unknown disease severity can be predicted. Since data on transport activity of only a limited number of patients is available at this moment, this does require further substantiation. We propose an independent international open registry for genetic, functional and phenotypic data of PCD patients of various genetic backgrounds, to assist phenotype prediction in newly identified patients.

In conclusion, the current NBS program in the Netherlands mainly identifies individuals with reduced carnitine transporter activity, both newborns and mothers, that are likely to remain asymptomatic without treatment throughout childhood and early adult life. However, a small number of newborns are likely to develop a severe outcome if left untreated and would therefore undeniably benefit from identification by NBS. The genotype as well as the residual carnitine transport activity in patients with a benign phenotype differ significantly from those that have a propensity to develop severe disease symptoms. Standardising the use of these parameters in the NBS follow-up protocol enables more specific identification of those patients that need early treatment and follow-up.

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Table S1 | *SLC22A5* variants encountered in Dutch patients

Mutation	Protein	Exon	Class.	Type	Allele freq.	First published	Other publications (homozygous)	Previously identified
c.125T>A	p.Leu42Gln	1	3	Missense	NA	Novel		Screening variant
c.136C>T	p.Pro46Ser	1	5	Missense	4.28e-4	Schimmenti (2007)	de Boer (2013)	Screening variant
c.248G>T	p.Arg83Leu	1	5	Missense	2.68e-4	Makhseed (2004)	el Hattab (2010); Li (2010); Lin (2020)	Clinically (Makhseed 2004)
c.34G>A	p.Gly12Ser	1	4	Missense	6.95e-4	Li (2010)	Jakoby 2021	Screening variant
c.95A>G	p.Asn32Ser	1	5	Missense	2.50e-5	Lamhonwah (2002)		Clinically (Lamhonwah 2002, Rasmussen)
c.396G>A	**p.Trp132Ter**	2	5	Nonsense		Tang (1999)		Clinically (Tang 1999)
c.448T>C	p.Phe150Leu	2	3	Missense	NA	Novel		Screening variant
c.457G>C	p.Val153Leu	2	3	Missense	NA	Novel		Screening variant
c.506G>C	p.Arg169Pro	3	5	Missense	NA	Frigeni 2017		Screening variant
c.597delG	**p.Phe200Leufs**	3	5	Frameshift	NA	Yilmaz 2015		Clinically (Yilmaz 2015)
c.610G>A	p.Gly204Ser	3	3	Missense	NA	Novel		Screening variant
c.632A>G	p.Tyr211Cys	3	5	Missense	7.95e-6	Vaz (1999)	Frigeni 2017	Clinically (Vaz 1999)
c.640_641delinsTT	p.Ala214Leu	3	3	Missense	NA	Novel		Screening variant
c.646G>C	p.Val216Leu	3	3	Missense	NA	Novel		Screening variant
c.680G>A	p.Arg227His	4	5	Missense	5.96e-5	Li (2010)	Yang 2013	Screening variant
c.695C>T	p.Thr232Met	4	5	Missense	9.90e-5	Dobrowolski (2005)	Li (2010); Lin (2021)	Clinically (Li et al. 2010)
c.707G>A	p.Cys236Tyr	4	3	Missense	NA	Novel		Screening variant
c.718G>A	p.Ala240Thr	4	5	Missense	NA	Li (2010)		Screening variant
c.760C>T	**p.Arg254Ter**	4	5	Nonsense	1.13e-4	Tang (2002)		Clinically (Tang 2002)
c.797C>T	p.Pro266Leu	4	4	Missense	4.38e-5	Han (2014)	Chen 2013; Lin 2020	Screening variant
c.825-1G>C		5	5	Splice	NA	Novel		Screening variant
c.844C>T	**p.Arg282Ter**	5	5	Nonsense	3.98e-5	Burwinkel 1999	Wang 1999	Clinically (Burwinkel 1999)
c.934A>G	p.Ile312Val	5	3	Missense	8.63e-4	Amat di San Filippo (2008)	Li (2010)	Clinically (Li et al. 2010)
c.1088T>C	p.Leu363Pro	7	5	Missense	3.98e-6	Akpinar (2010)		Screening variant
c.1101C>G	p.Asn367Lys	7	3	Missense	NA	Novel		Screening variant
c.1232G>T	p.Gly411Val	7	5	Missense	NA	Kilic (2011)		Clinically (Kilic 2011)
c.1340A>C	p.Tyr447Ser	8	3	Missense	NA	Novel		Screening variant
c.1354G>A	p.Glu452Lys	8	5	Missense	2.78e-5	Wang (2000)	Bijarnia-Mahay (2015); Deswal (2010)	Clinically (Wang 2000)
c.1421G>A	p.Ser474Asn	8	3	Missense	3.98e-6	Novel		Screening variant
c.424G>T & c.1463G>A	p.Ala142Ser & p.Arg488His	9	5	Missense	3.19e-3	Amat di San Filippo (2006)	Frigeni 2017	Clinically (Amat di san Filippo 2006)
c.-149G>A		5'UTR	4	5UTR	NA	Ferdinandusse (2019)		Screening variant

Null-variants are in bold. screening variants - variants identified in patients following screening diagnosis, and not in clinically diagnosed PCD. Clinically – variants identified in patients that have presented clinically (the reference of patients with this variant is provided). Allele freq – Allele frequencies, as provided by Gnomad (last consulted 30-5-2022).

Table S2 | Baseline data of all individuals referred because of low carnitine in two subsequent NBS DBS's

	NBS				Mothers referred after NBS			
	PCD		**Non-PCD**		**PCD**		**Non-PCD**	
	(n=19)		**(n=112)**		**(n=37)**		**(n=45)**	
Gender (male)	9 (47.4%)	{0}	46 (41.1%)	{0}	0 (0%)	{0}	0 (0%)	{0}
Gestational age (weeks)	40.1 [37.3, 41.7]	{1}	39.9 [24.4, 42.0]	{6}	-		-	
Birth weight (grams)	3390 [2490, 4300]	{0}	3480 [560, 4600]	{4}	-		-	
Sibling death	0 (0%)	{10}	1 (0.9%)	{50}	1 (3.2%)	{6}	0 (0%)	{24}
Other IEM after referral	0 (0%)	{0}	0 (0%)	{0}	0 (0%)	{0}	5 (8.2%)	{0}
Age at last follow-up (years)	4.56 [1.03, 14.1]	{0}	0.1 [0, 8.6]	{7}	34.6 [24.8, 48.6]	{0}	31.6 [20.4, 38.3]	{3}
Free carnitine concentration (blood)								
First NBS Sample[a]	3.50 [1.80, 4.80]	{0}	4.10 [1.00, 5.00]	{5}	3.70 [1.90, 5.00]	{2}	4.10 [1.00, 5.00]	{4}
Second NBS Sample[a]	3.05 [1.30, 4.70]	{1}	4.30 [1.60, 5.00]	{8}	3.80 [2.00, 5.00]	{2}	4.50 [1.30, 5.00]	{6}
First sample at metabolic centre	6.87 [1.97, 12.0]	{0}	11.0 [3.10, 79.7]	{9}	6.60 [2.09, 18.8]	{3}	16.0 [2.75, 35.0]	{4}

Data presented as N (%) or median [Min-Max]. Missing data points are presented in grey, within braces. Abbreviations: PCD - Primary carnitine deficiency; NBS - Newborn screening; IEM - Inborn error of metabolism.

[a]For mothers: concentration in the NBS sample of their child

Table S3 | Case overview of patients with genotypes of uncertain clinical significance

Patient ID	Identified	Age at Dx	Age initial symptoms	Age at last follow-up	Symptoms	Treatment effect	Variant allele 1	Variant allele 2	Transport activity
528	Newborn	0	NA	2Y	Asymptomatic	-	c.136C>T	c.248G>T	8.5%
554	Newborn	0	NA	2.5Y	Asymptomatic	-	c.506G>C	c.1088T>C	NA
603	Newborn	0	NA	5Y	Asymptomatic	-	c.95A>G	c.136C>T	NA
606	Newborn	0	NA	3Y	Asymptomatic	-	c.448T>C	**c.760C>T**	NA
609	Newborn	0	NA	4.5Y	Asymptomatic	-	**c.844C>T**	c.-149G>A	NA
627	Newborn	0	NA	1Y	Asymptomatic	-	**c.844C>T**	**c.825-1G>C**	NA
667	Newborn	0	NA	1Y	Asymptomatic	-	c.610G>A	c.-149G>A	NA
681	Newborn	0	7M	1Y	Asymptomatic[a]	-	**c.760C>T**	c.1354G>A	NA

Null variations are in bold. Additional information on identified variants is provided in supplementary table 1. Abbreviations: NBS – Newborn screening; Dx – Diagnosis; M – Months; Y – Years; NA – Not available.

[a]Febrile convulsions with normal serum carnitine

CHAPTER

Assessment of carnitine excretion and its ratio to plasma free carnitine as a biomarker for primary carnitine deficiency in newborns

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ABSTRACT

Background: In the Netherlands, newborns are referred by the Newborn Screening (NBS) Program when a low free carnitine (C0) concentration (< 5 µmol/L) is detected in their NBS dried blood spot. This leads to approximately 85% false positive referrals who all need an invasive, expensive and lengthy evaluation. We investigated whether a ratio of urine C0 / plasma C0 ($Ratio_{U:P}$) can improve the follow-up protocol for primary carnitine deficiency (PCD).

Methods: A retrospective study was performed in all Dutch metabolic centres, using samples from newborns and mothers referred by NBS due to low C0 concentration. Samples were included when C0 excretion and plasma C0 concentration were sampled on the same day. $Ratio_{U:P}$ was calculated as (urine C0 (µmol/mmol creatinine))/(plasma C0 (µmol/L)).

Results: Data were available for 59 patients with genetically confirmed PCD and 68 individuals without PCD. The $Ratio_{U:P}$ in PCD patients was significantly higher (p-value < 0.001) than in those without PCD, median [IQR], respectively: 3.4 [1.2 – 9.5], 0.4 [0.3 – 0.8], AUC 0.837. Classified for age (up to 1 month) and without carnitine suppletion (PCD; N= 12, Non-PCD; N=40), medians were 6.20 [4.4 – 8.8] and 0.37 [0.24 – 0.56], respectively. The AUC for $Ratio_{U:P}$ was 0.996 with a cut-off required for 100% sensitivity at 1.7 (yielding 1 false positive case).

Conclusion: $Ratio_{U:P}$ accurately discriminates between positive and false positive newborn referrals for PCD by NBS. $Ratio_{U:P}$ is less effective as a discriminative tool for PCD in adults and for individuals that receive carnitine suppletion.

INTRODUCTION

Acylcarnitines are valuable metabolites used in newborn screening (NBS), as abnormal plasma acylcarnitine profiles are indicative of several inborn errors of metabolism (IEM).[1,2] In case of carnitine depletion, theoretically, the measured concentration of other acylcarnitines may also be reduced, which in turn could give rise to false negative reporting.[3,4] C0 is therefore considered essential for reliable interpretation of the acylcarnitine profile. All newborns with a C0 concentration < 5 µmol/L are referred to a paediatric metabolic specialist for evaluation.[5]

In the past 15 years, in the Netherlands approximately 15% of newborns referred for follow up of low C0 were diagnosed with primary carnitine deficiency (PCD) (OMIM #212140). In the remaining 85% no underlying cause for the low C0 was identified and they were able to retain normal plasma C0 concentrations without carnitine supplementation. Furthermore, no other IEM was identified in any of these false positive referrals. Follow-up of these false-positive referrals is challenging, requiring a long period of confirmatory testing, which may include measurement of residual carnitine transporter activity in cultured fibroblasts and/or sequencing of the *SLC22A5* gene.[6] The prolonged uncertainty regarding the health of their newborn child during this extensive evaluation, can cause significant anxiety in the families concerned.[7] A faster exclusion of the diagnosis PCD may reduce harm by false-positive referral.

PCD is an autosomal recessive disorder caused by variations in the *SLC22A5* gene, encoding the Organic Cation Transporter Novel 2 (OCTN2) protein.[8,9] OCTN2 maintains intracellular C0 concentrations by transporting carnitine into cells, and reabsorbing C0 in the renal tubuli.[10-12] Defects in OCTN2 cause a decrease of plasma (and intracellular) C0 concentrations and an increased renal waste by inadequate tubular reabsorption.[10] This causes a shifted balance of plasma C0 versus C0 excretion in PCD patients, which can be determined by calculating the fractional carnitine excretion (FCE) as: ((urine C0 x plasma creatinine(Cr))/(plasma C0 x urine Cr)) x 100%. FCE has been shown to be increased in PCD patients, ranging from 3-190%, with proposed normal ranges of less than 4%. Whilst FCE may be a promising marker for early differentiation of healthy newborns and PCD affected newborns, information on FCE is limited, as most case descriptions at the time of writing are derived from conference abstracts, with restricted descriptions of control groups, timing of sampling, and circumstances at sampling (*e.g.* with or without carnitine supplementation).[13-17]

In the Netherlands, plasma Cr is not routinely measured in newborns referred for low C0. However, in most Dutch metabolic specialist centres, plasma C0, C0 excretion and urine Cr are measured upon referral. We investigated whether a ratio of C0 excretion to plasma C0 could be an effective diagnostic tool at the time of referral to improve and simplify the follow-up protocol for PCD.

MATERIALS AND METHODS

Study design

Urine and plasma C0 results were retrospectively collected. Inclusion criteria were: 1) C0 excretion and plasma C0 were sampled on the same day and 2) sampled individuals were referred after identification by NBS (newborns and mothers of newborns referred by NBS). The following data were collected: C0 and Cr excretion, plasma C0 concentration, age at sampling, sex, received carnitine suppletion at the time of sampling and PCD diagnosis (confirmed genetically, presented in supplementary table 1). Written informed consent was obtained from all participants and/or their caregivers (METC protocol number 19-234/M). The study was conducted in accordance with the principles of the Declaration of Helsinki.

Calculation of carnitine excretion in relation to plasma carnitine

The following equation was used to calculate the ratio of urine C0 to plasma C0 ($Ratio_{U:P}$):

$$Ratio_{U:P} = \frac{Urine\ C0\ (\mu mol/mmol\ Cr)}{Plasma\ C0\ (\mu mol/L)}$$

Diagnostic accuracy analysis

Receiver Operator Characteristic (ROC) curve analysis was performed for plasma C0, C0 excretion and $Ratio_{U:P}$, with PCD as the classifier. ROC curve analysis was then repeated in data classified for carnitine suppletion (with or without suppletion at time of sampling) and age (age at sampling up to 1 month and age at sampling above 1 month).

Statistics

All analyses were performed using R (version 4.1.3, R Core Team, Vienna, Austria). DeLong's test was used to compare two ROC curves. The Mann-Whitney U test was used to compare continuous data between PCD and non-PCD. The chi-squared test was used to compare two categorical data between two groups. Significance was assumed for $p < 0.05$

RESULTS

Baseline characteristics are provided in Table 1. In the non-PCD (n=68) versus the PCD (n=59) population, respectively, the median [IQR] plasma C0 concentration was 11.1 [8.6 – 17.7] and 9.0 [6.7 – 22.0] µmol/L, median C0 excretion was 5.0 [3.0 – 11.0] and 34.5 [9.6 – 127.6] µmol/mmol Cr and median $Ratio_{U:P}$ was 0.4 [0.3 – 0.8] and 3.4 [1.2 – 9.5]. The non-PCD population contained more samples from newborns (N=54 (79.4%) vs PCD: N=27 (45.8%)) and a lower age at sampling (13 days vs PCD: 9215 days (25 years)). In the non-PCD population, six (8.8%) received carnitine suppletion at the time of sampling, versus 22 (37.3%) in the PCD population.

Table 1 | Baseline characteristics

	No PCD N=68			PCD N=59			
	N/Median	*(%)/[IQR]*	*Range*	*N/Median*	*(%)/[IQR]*	*Range*	*p-value*
Gender (male, N)	19	(27.9)		12	(20.3)		0.431
Referral							**<0.001**
Newborn (N)	54	(79.4)		27	(45.8)		
Maternal (N)	14	(20.6)		32	(54.2)		
Age at sampling							
Age (median, days)	13	[10 – 92.3]	7 - 13400	9215	[53 - 12319]	5 - 14600	**<0.001**
Age < 1 month (N)	40	(58.8)		15	(25.4)		**<0.001**
Plasma C0 concentration (median, µmol/L)	11.1	[8.6 - 17.7]	3.2 - 79	9.0	[6.7 - 22.0]	3.6 - 73.0	0.087
C0 excretion (median, µmol/mmol Cr)	5.0	[3.0 - 11.0]	0 - 1460	34.5	[9.6 - 127.6]	0.7 - 1520	**<0.001**
$Ratio_{U:P}$ (median)	0.4	[0.3 - 0.8]	0 - 31.6	3.4	[1.2 – 9.5]	0.06 - 31.0	**<0.001**
On carnitine suppletion (yes, N)	6	(8.8)		22	(37.3)		**<0.001**

Abbreviations: PCD - primary carnitine deficiency; C0 - free carnitine; Cr – creatinine

Diagnostic accuracy of $Ratio_{U:P}$

Unclassified, AUC's [95%CI] for plasma C0, C0 excretion and $Ratio_{U:P}$ were: 0.588 [0.485 – 0.691], 0.783 [0.701 – 0.866] and 0.837 [0.763 – 0.912], respectively (Figure 1A). To further evaluate the accuracy of $Ratio_{U:P}$ in the target population (non-supplemented newborns), data were classified for carnitine suppletion (with or without suppletion; Figure 1B) as well as age (age ≤ 1 month and age >1 month; Figure 1C). AUC for $Ratio_{U:P}$ increased in an unsupplemented population (0.867 [0.790 – 0.943] vs 0.705 [0.437 – 0.973]) and in a population with an age up to 1 month (0.992 [0.977 – 1] vs 0.691 [0.464 – 0.917] for newborns sampled at age above 1 month and 0.868 [0.763 – 0.974] for maternal samples).

The final analysis, in an unsupplemented population with an age up to 1 month (median age at sampling: 11 days), demonstrated the following median [IQR] for non-PCD (n=40) and PCD (n=12), respectively: plasma C0 9.8 [7.8 – 12.1] and 8.0 [5.9 – 8.9] (p=0.005), C0 excretion 4.0 [2.8 – 5.4] and 43.0 [35.1 - 50.2]

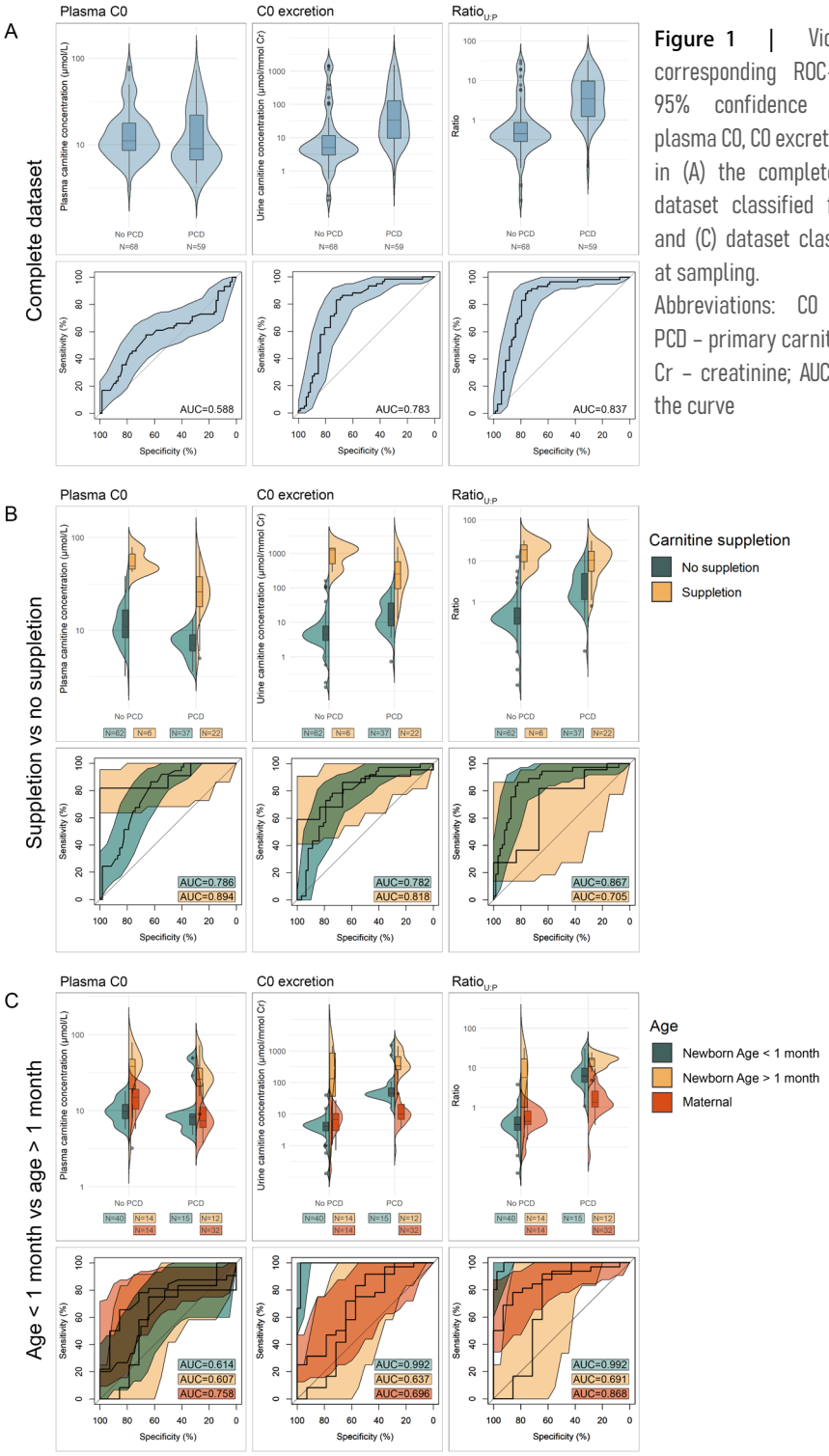

Figure 1 | Violin plots and corresponding ROC-curves (with 95% confidence interval) for plasma C0, C0 excretion and $Ratio_{U:P}$ in (A) the complete dataset, (B) dataset classified for suppletion and (C) dataset classified for age at sampling.

Abbreviations: C0 – carnitine; PCD – primary carnitine deficiency; Cr – creatinine; AUC - area under the curve

(p<0.001), $Ratio_{U:P}$ 0.37 [0.24 – 0.56] and 6.2 [4.4 – 8.8] (p<0.001). The AUC's [95% CI] for plasma C0, C0 excretion and $Ratio_{U:P}$ were respectively: 0.768 [0.634 - 0.901], 0.990 [0.968 – 1], 0.996 [0.986 – 1] (Figure 2). The corresponding thresholds for 100% sensitivity were 9.4 µmol/L, 15.2 µmol/mmol Cr, 1.7, respectively. The specificity [95% CI] at 100% sensitivity for C0 excretion and $Ratio_{U:P}$ both was 92% [92.5 – 100%] (yielding one false positive).

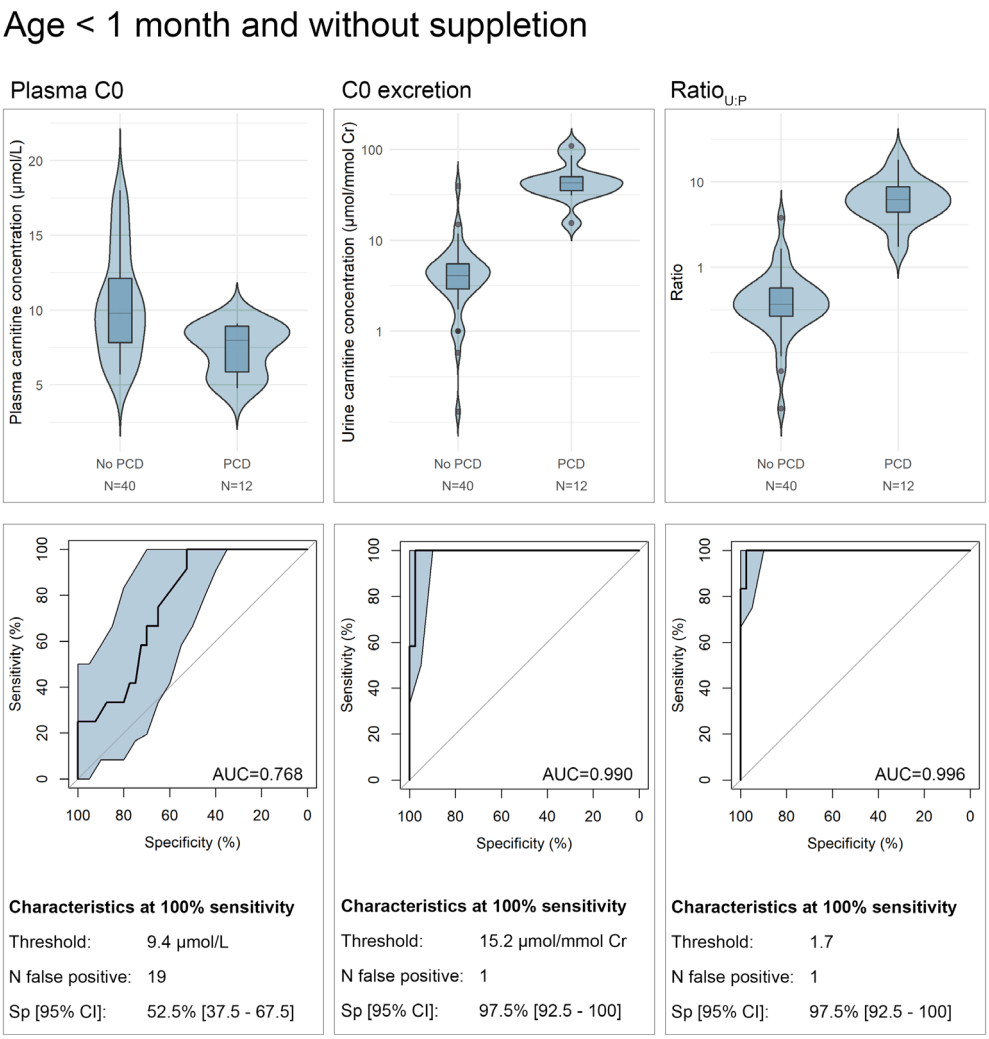

Figure 2 | Violin plots and corresponding ROC-curves (with 95% confidence interval) for plasma C0, C0 excretion and $ratio_{U:P}$ in newborns referred for low C0 in NBS, sampled before age 1 month and without carnitine suppletion at time of sampling. Provided with ROC analysis for each test are the diagnostic characteristics at the threshold for 100% sensitivity.

Abbreviations: C0 – carnitine; PCD – primary carnitine deficiency; Cr – creatinine; AUC - area under the curve; Sp – specificity; CI – confidence interval

DISCUSSION

The present study demonstrates that the ratio of C0 excretion to plasma C0 can be used as an early discriminative test for PCD in newborns referred by NBS. In our study population, applying a threshold of 1.7 the $Ratio_{U:P}$ yields 12 true positive cases, 1 false positive case and correctly identifies as negative 39 newborns that do not have PCD (median ratio in healthy newborns is 0.37). Using the $Ratio_{U:P}$ significantly decreases the duration of follow-up, cost and harm of false positive referrals by NBS.

PCD is implemented in several NBS programs across the world[1,18-20] and NBS for PCD is known to have a poor positive predictive value (PPV), ranging from 1.6 – 4.7%.[21,22] In part due to the high false positive rate, the benefit of screening for PCD remains in question.[22,23] The high false positive rate, in addition to the identification of many asymptomatic individuals, led to discontinuation of screening for PCD in New Zealand.[23] Detection of PCD following NBS in the Netherlands performs slightly better (PPV 14.6%), which might be due to a different NBS procedure. If C0 concentration is low in the DBS, a second NBS sample is taken and newborns are only referred when the low C0 concentration persists.[5] However, this algorithm comes at the cost of timeliness. In the Netherlands initial DBS's are obtained 72-168 hours after birth and in the event a second sample is requested, there is a further delay of 2-3 days before a newborn is referred.[1,18-20] $Ratio_{U:P}$ does not improve the PPV of NBS for PCD, but it can mitigate the harm of the high amount of false positive referrals by quickly ruling out PCD.

The difference in relative carnitine excretion is less pronounced in supplemented individuals (Figure 1B). This is expected, as the plasma concentration threshold for tubular carnitine reabsorption is 40-60 µmol/L.[24] Therefore, supplemented healthy individuals could quickly increase their relative carnitine excretion, approaching values of PCD patients (with or without suppletion). Indeed, the median plasma C0 concentration of healthy supplemented individuals was 49 µmol/L (range 42.6-79), with a $Ratio_{U:P}$ of 18.8 (range 5.9 – 31.6) (Supplementary table 2). Therefore, we recommend using the $Ratio_{U:P}$ as a test prior to initiating carnitine supplementation.

$Ratio_{U:P}$ is less effective as a discriminative test in a population above the age of 1 month (Figure 1C). It is important to note that many of the newborns in this category received carnitine suppletion when sampled (11/12 PCD and 6/14 non-PCD), and as discussed above, this may have confounded the results in this population. The other part of this population consists of mothers, sampled approximately 1-2 weeks after delivery (N=46 out of 72). During the first trimester, glomerular filtration rate increases, and slowly recovers 6-8 weeks after delivery.[25] This may cause an underestimation of $Ratio_{U:P}$ in PCD affected mothers. Furthermore, maternal PCD cases identified through NBS likely have milder phenotypes, as many are reported to be completely asymptomatic at the time of diagnosis.[26-29] A milder defect may result in less pronounced renal C0 wasting, leading to a lower $Ratio_{U:P}$ (median $Ratio_{U:P}$ maternal PCD: 1.35, newborn PCD: 9.83. Supplementary table 3). Lastly, in general, with increasing age, the population becomes less homogenous, with more diverse renal functions, body masses and diets that may all differently impact carnitine homeostasis.

Thus far, only FCE has been investigated as a diagnostic parameter expressing relative carnitine excretion.[14-17] Theoretically, FCE may be superior to $Ratio_{U:P}$, as it corrects for Cr excretion by taking plasma Cr levels into account. However, for the purpose of fast exclusion of PCD, this correction is only beneficial (compared to $Ratio_{U:P}$) when Cr-excretion is increased due to elevated plasma levels of Cr – in that case, C0 excretion and $Ratio_{U:P}$ would appear low, while FCE would still be elevated, due to the high plasma Cr in the numerator of the equation. Causes for primarily elevated plasma Cr include increased muscle degradation (*e.g.* due to a myopathy) and strenuous exercise, which are extremely rare in newborns and are therefore unlikely to cause false negatives when using $Ratio_{U:P}$.[30] Other causes for Cr disturbances are renal insufficiency, where plasma Cr rises, as urine Cr decreases[31], which would lead to a perceived increase of both FCE and $Ratio_{U:P}$ or glomerular hyperfiltration, where urine Cr is increased and plasma Cr decreases, which would lead to a perceived decrease of both FCE and $Ratio_{U:P}$.[25,32] Unfortunately, we could not compare FCE to $Ratio_{U:P}$ in our NBS population, as plasma Cr was often not available. Future research comparing FCE to $Ratio_{U:P}$ as a marker for PCD is required.

We acknowledge the limitations of using the $ratio_{U:P}$ as a discriminative tool for PCD. First, carnitine in urine is not routinely available for clinical decision makers and larger studies are necessary to validate the threshold. Finally, immaturity of renal absorption may result in decreased ability of kidneys to preserve carnitine. Interestingly, solely C0 excretion can accurately discriminate PCD from false-positives in an unsupplemented, newborn population (with an AUC of 0.990). The added benefit of C0 excretion in NBS has been reported previously in a conference abstract by Gallant et al, demonstrating the following C0 excretion (nmol/mg Cr) (median [range]): PCD 401 [45-1030], false positives 28 [7-516].[33] They report increased accuracy when combining newborn plasma and urine C0 concentrations. As C0 excretion and plasma C0 are generally sampled simultaneously we would recommend using the $ratio_{U:P}$ rather than urine excretion values.

In conclusion, the ratio of urinary free carnitine over plasma free carnitine can effectively discriminate between true and false positive referrals from NBS for PCD reducing time to diagnosis and mitigating the negative effects of a false positive referral. The ratio is most effective in neonates under one month of age and prior to carnitine supplementation

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SUPPLEMENTARY MATERIAL

Table S1 | Genetic variants of the PCD patients in the study cohort

Case number	Case type	Coding DNA change allele 1	Protein change allele 1	Coding DNA change allele 2	Protein change allele 2
1*	Maternal	c.136C>T	p.Pro46Ser	c.136C>T	p.Pro46Ser
2	Maternal	c.1354G>A	p.Glu452Lys	c.-149G>A	
3	Maternal	c.136C>T	p.Pro46Ser	c.695C>T	p.Thr232Met
4	Maternal	c.34G>A	p.Gly12Ser	c.-149G>A	
5	Maternal	c.-149G>A		c.-149G>A	
6*	Maternal	c.-149G>A		c.-149G>A	
7	Maternal	c.34G>A	p.Gly12Ser	c.1340A>C	p.Tyr447Ser
8	Maternal	c.95A>G	p.Asn32Ser	c.-149G>A	
9	Maternal	c.136C>T	p.Pro46Ser	c.-149G>A	
10	Maternal	c.136C>T	p.Pro46Ser	c.-149G>A	
11*	Maternal	c.-149G>A		c.-149G>A	
12*	Maternal	c.640_641delinsTT	p.Ala214Leu	c.-149G>A	
13	Maternal	c.718G>A	p.Ala240Thr	c.-149G>A	
14*	Maternal	c.797C>T	p.Pro266Leu	c.797C>T	p.Pro266Leu
15*	Maternal	c.680G>A	p.Arg227His	c.-149G>A	
16	Maternal	c.136C>T	p.Pro46Ser	c.-149G>A	
17*	Maternal	c.136C>T	p.Pro46Ser	c.-149G>A	
18*	Maternal	c.-149G>A		c.707G>A	p.Cys236Tyr
19	Maternal	c.136C>T	p.Pro46Ser	c.136C>T	p.Pro46Ser
20	Newborn	c.597delG	p.Phe200Leufs	c.597delG	p.Phe200Leufs
21*	Newborn	c.136C>T	p.Pro46Ser	c.136C>T	p.Pro46Ser
22*	Newborn	c.136C>T	p.Pro46Ser	c.248G>T	p.Arg83Leu
23*	Newborn	c.136C>T	p.Pro46Ser	c.136C>T	p.Pro46Ser
24*	Newborn	c.136C>T	p.Pro46Ser	c.695C>T	p.Thr232Met
25	Newborn	c.136C>T	p.Pro46Ser	c.-149G>A	
26	Newborn	c.506G>C	p.Arg169Pro	c.1088T>C	p.Leu363Pro
27	Newborn	c.136C>T	p.Pro46Ser	c.136C>T	p.Pro46Ser
28*	Newborn	c.136C>T	p.Pro46Ser	c.136C>T	p.Pro46Ser
29	Newborn	c.248G>T	p.Arg83Leu	c.248G>T	p.Arg83Leu
30*	Newborn	c.95A>G	p.Asn32Ser	c.136C>T	p.Pro46Ser
31	Newborn	c.448T>C	p.Phe150Leu	c.760C>T	p.Arg254Ter
32*	Newborn	c.844C>T	p.Arg282Ter	c.-149G>A	
33	Newborn	c.248G>T	p.Arg83Leu	c.248G>T	p.Arg83Leu
34*	Newborn	c.610G>A	p.Gly204Ser	c.-149G>A	
35	Newborn	c.760C>T	p.Arg254Ter	c.1354G>A	p.Glu452Lys

Variants were detected in the SLC22A5 gene using Sanger sequencing.

* From these patients multiple samples were used in the study; before and after suppletion.

Table S2 | Characteristics of individuals with and without suppletion

	No suppletion				Suppletion			
	No PCD (N=62)		PCD (N=37)		No PCD (N=6)		PCD (N=22)	
Sex (male, N)	45	(72.6)	30	(81.1)	4	(66.7)	17	(77.3)
Age at sampling								
Age (median, days)	12.0	[7.00 - 13400]	10300	[5.00 - 14600]	58.5	[36.0 - 3040]	1650	[18.0 - 13400]
Age < 1 month (N)	40	(64.5)	12	(32,4)	0	(0)	3	(13.6)
Plasma C0 concentration (median, µmol/L)	10.9	[3.20 - 38.4]	7.11	[3.58 - 13.0]	49.0	[42.6 - 79.0]	26.1	[5.00 - 73.0]
C0 excretion (median, µmol/mmol Cr)	4.50	[0 - 159]	12.6	[0.721 - 109]	1260	[285 - 1460]	253	[12.1 - 1520]
Ratio$_{U:P}$ (median)	0.4	[0 - 12.5]	1.74	[0.06 - 18.2]	18.8	[5.9 - 31.6]	10.4	[0.8 - 31.0]

Values presented as: median [range] or N (%). Abbreviations: PCD - primary carnitine deficiency; C0 - free carnitine; Cr - creatinine

Table S3 | Characteristics of individuals identified by newborn screening (Newborn) or by newborn screening of their child (Maternal)

	Newborn				Maternal			
	No PCD (N=54)		PCD (N=27)		No PCD (N=14)		PCD (N=32)	
Plasma C0 concentration (median, µmol/L)	10.9	[3.20 - 79.0]	15.6	[4.77 - 73.0]	15.2	[5.77 - 22.2]	7.39	[3.58 - 39.0]
C0 excretion (median, µmol/mmol Cr)	4.58	[0 - 1460]	109	[15.4 - 1520]	6.79	[0.176 - 27.6]	10.0	[0.721 - 237]
Ratio$_{U:P}$ (median)	0.430	[0 - 31.6]	9.83	[1.08 - 31.0]	0.445	[0.00921 - 1.37]	1.35	[0.0619 - 9.81]
Age at sampling								
Age (median, days)	12.0	[7.00 - 3040]	18.0	[5.00 - 3680]	12000	[9510 - 13400]	12100	[9060 - 14600]
Age < 1 month (N)	40	(74.1)	15	(55.6)	0	(0)	0	(0)
Age > 1 month (N)	14	(25.9)	12	(44.4)	14	(100)	32	(100)
On carnitine suppletion (yes, N)	6	(11.1)	14	(51.9)	0	(0)	8	(25.0)

Values presented as: median [range] or N (%). Abbreviations: PCD - primary carnitine deficiency; C0 - free carnitine; Cr - creatinine

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Table S4 | AUC's and 100% sensitivity cut-off values for all classified groups

Unclassified	PCD (N)	No PCD (N)	Newborn (N)	Maternal (N)		
	59	68	81	46		
	AUC	(95% CI)		Threshold 100% sensitivity	N false positive at 100% sensitivity	
Plasma C0	0,588	0,485	0,691	73,04	66	97%
C0 excretion	0,783	0,701	0,866	0,71	62	91%
$Ratio_{U:P}$	0,837	0,763	0,912	0,06	63	93%
No suppletion	**PCD (N)**	**No PCD (N)**	**Newborn (N)**	**Maternal (N)**		
	37	62	61	38		
	AUC	(95% CI)		Threshold 100% sensitivity	N false positive at 100% sensitivity	
Plasma C0	0,786	0,698	0,874	13,07	38	61%
C0 excretion	0,782	0,688	0,875	0,71	56	90%
$Ratio_{U:P}$	0,867	0,790	0,943	0,06	57	92%
Suppletion	**PCD (N)**	**No PCD (N)**	**Newborn (N)**	**Maternal (N)**		
	22	6	20	8		
	AUC	(95% CI)		Threshold 100% sensitivity	N false positive at 100% sensitivity	
Plasma C0	0,894	0,773	1,000	73,0	4	67%
C0 excretion	0,818	0,651	0,986	Inf	6	100%
$Ratio_{U:P}$	0,705	0,437	0,973	31,3	5	83%
Age < 1 month	**PCD (N)**	**No PCD (N)**	**Newborn (N)**	**Maternal (N)**		
	15	40	55	0		
	AUC	(95% CI)		Threshold 100% sensitivity	N false positive at 100% sensitivity	
Plasma C0	0,614	0,421	0,807	Inf	40	100%
C0 excretion	0,992	0,974	1,000	15,22	1	3%
$Ratio_{U:P}$	0,992	0,977	1,000	1,07	3	8%

Age > 1 month	PCD (N)	No PCD (N)	Newborn (N)	Maternal (N)		
	44	28	26	46		
	AUC	**(95% CI)**		**Threshold 100% sensitivity**	**N false positive at 100% sensitivity**	
Plasma C0	0,689	0,563	0,815	73,04	24	86%
C0 excretion	0,567	0,419	0,714	0,71	24	86%
Ratio$_{U:P}$	0,662	0,515	0,808	0,04	25	89%
Final	**PCD (N)**	**No PCD (N)**	**Newborn (N)**	**Maternal (N)**		
	12	40	52	0		
	AUC	**(95% CI)**		**Threshold 100% sensitivity**	**N false positive at 100% sensitivity**	
Plasma C0	0,768	0,634	0,901	9,37	19	48%
C0 excretion	0,990	0,968	1,000	15,22	1	3%
Ratio$_{U:P}$	0,996	0,986	1,000	1,70	1	3%

Abbreviations: C0 – free carnitine; Ratio$_{U:P}$ – Ratio urine plasma C0 (urine C0 (µmol/mmol creatinine)/ plasma C0 (µmol/L)); PCD – primary carnitine deficiency; AUC – area under the curve.

CHAPTER

General discussion and future perspectives

GENERAL DISCUSSION

Primary carnitine deficiency (PCD) is currently considered an unintended finding of the Dutch newborn screening (NBS) program. Nevertheless, since 2007 newborns as well as mothers, detected though the abnormal NBS result of their child, have been diagnosed with PCD. Now, after more than a decade, we need to decide whether PCD should officially be included in the Dutch NBS panel.[1] To reach a decision, NBS for PCD can be evaluated using Wilson and Jungner's classic screening criteria and Andermann's additional criteria (Table 1).[2,3] Introduction of PCD into NBS programs in other countries revealed that a critical criterion was not fully met: *"The natural history of the condition, including development from latent to declared disease, should be adequately understood"*.[4,5] NBS identifies a broader disease spectrum of PCD than previously documented (**chapter 2**), and for most individuals identified by NBS, it is unclear what their place in the disease spectrum is. This directly necessitates re-evaluation of the following criteria: *"There should be an agreed upon policy on whom to treat as patients"*, *"There should be a defined target population"*, *"There should be a suitable test or examination"* and finally, *"The overall benefits of screening should outweigh the harm"*. In the following section, we address these criteria and discuss how the results presented in this thesis may support decision-making regarding implementation of PCD in the Dutch NBS. Next, persisting gaps in knowledge concerning PCD are addressed and future opportunities to further improve NBS for PCD are explored. Finally, we discuss the lessons learned from this project, and outline how the results from this thesis may be used when considering expanding NBS for other disorders.

Changing perspectives on the natural history of PCD

The structured review performed in **chapter 2** demonstrates that our perception of a disease is highly dependent on the definitions we use for said disease. Historically, detection of a decreased plasma carnitine concentration was considered enough for the diagnosis PCD. Patients often suffered neurological and muscle involvement, as well as hypoglycemia and/or hyperammonemia. In retrospect, in these patients other inherited errors of metabolism might have caused the symptoms, confounding the perceived natural history of PCD. Since 1999, genetic (bi-allelic variants in the *SLC22A5* gene) and/or functional confirmation (reduced carnitine transport activity) of a PCD diagnosis is required. Patients with a confirmed diagnosis mostly suffer from cardiomyopathy, though they could also present with coma, encephalopathy, hepatomegaly, hypoglycemia and rarely, but most critically, severe cardiac events such as ventricular fibrillation (VF) or sudden cardiac death. At least 93% of these patients present in childhood, with a median age at first symptom of 1 year. In addition to the diagnostic criteria, the mode of detection plays an important role in the type of patient that is diagnosed. Ever since PCD was introduced in NBS, multiple adult women with genetically and/or functionally confirmed PCD were diagnosed. However, in contrast to the clinically identified PCD patients who predominantly present in childhood, 92% of these mothers remained completely asymptomatic throughout childhood and early adult life.[6-8]

Table 1 | Overview of the Wilson and Jungner classic screening criteria and the additional criteria as proposed by Andermann

Wilson and Jungner classic screening criteria[3]	
1.	The condition sought should be an important health problem.
2.	There should be an accepted treatment for patients with recognized disease.
3.	Facilities for diagnosis and treatment should be available.
4.	There should be a recognizable latent or early symptomatic stage.
5.	There should be a suitable test or examination.
6.	The test should be acceptable to the population.
7.	The natural history of the condition, including development from latent to declared disease, should be adequately understood.
8.	There should be an agreed policy on whom to treat as patients.
9.	The cost of case finding (including diagnosis) should be economically balanced in relation to possible expenditure on medical care as a whole.
10.	Case finding should be a continuing process and not a "once and for all" project.
Additional screening criteria as proposed by Andermann[2]	
1	The screening program should respond to a recognized need.
2.	The objectives of screening should be defined at the outset.
3.	There should be a defined target population.
4.	There should be scientific evidence of screening program effectiveness.
5.	The program should integrate education, testing, clinical services and program management.
6.	There should be quality assurance, with mechanisms to minimize potential risks of screening.
7.	The program should ensure informed choice, confidentiality and respect for autonomy.
8.	The program should promote equity and access to screening for the entire target population.
9.	Program evaluation should be planned from the outset.
10.	The overall benefits of screening should outweigh the harm.

In **chapter 5**, we demonstrate that nearly all maternal PCD cases identified by Dutch NBS were asymptomatic and have genotypes that were never reported in clinically identified PCD. Furthermore, the residual carnitine transport activity in these mothers was significantly higher compared to the patients identified clinically, suggesting that these genetic and functional features contribute to an attenuated, perhaps even benign form of PCD, unlikely to cause severe disease in childhood. This provides valuable insight in our understanding of the development of the disease for individuals with such features, especially given the goal of Dutch NBS, stated by the Health Council of the Netherlands as follows:

"The purpose of neonatal screening is to detect disorders for which interventions shortly after birth have obvious benefits over interventions that either cannot take place without screening or can only take place at a later stage."[9]

At least 8 newborns diagnosed with PCD through Dutch NBS had relatively high residual carnitine transport activity and/or genotypes identical to asymptomatic maternal cases and are therefore likely to remain healthy without intervention and do not fit within the goal of the Dutch NBS. However, NBS also identified at least 3 newborns with very low residual carnitine transport activity and genotypes identical to historically reported patients with severe outcome. These children most likely rightfully fall within the category "patient", and require life-long treatment. The importance of adhering to life-long treatment for patients with severely decreased residual carnitine transport activity is emphasized by the case reported in **chapter 4**. Finally, a subgroup of newborns diagnosed after NBS has a disease severity of yet unknown significance. For them, treatment benefit remains unclear and it is uncertain whether NBS should aim to identify them. Until more knowledge on their disease severity is gained, we err on the side of caution.

Recommendation: Concerning the criterion *"There should be an agreed policy on whom to treat as patients"* we recommend treatment in all patients with severe PCD and those with currently unknown disease severity.

Maternal PCD identified through NBS

Obviously, mothers are not the target population of NBS, but if health benefits can be gained, without interfering with the original goal of the program, it is worth considering whether investigation of mothers in the follow-up of an unexplained result in their child is desirable. Even though most mothers identified by NBS are asymptomatic, some may be at risk for severe cardiac events. **Chapter 2** showed that at least 8 PCD patients had their first cardiac event in adulthood, of whom 5 were completely asymptomatic prior to this event.[6,10-15] Importantly, all of them carried at least 1 *SLC22A5* variant that was either a null-mutation ([c.760C>T], [c.1586+1G>T] or exon 2 deletion) or a missense variant widely reported in severely affected patients ([c.95A>G] or [c.51C>G]).[8,16,17] Two maternal PCD cases with cardiac events have been reported in literature: 1 mother experienced sustained ventricular tachycardia at 20 years of age (variants: [c.95A>G];[c.136C>G]) (diagnosed with PCD 5 years later)[6] and 1 mother suffered from VF at the age of 22 years, 6 weeks postpartum (variants: [c.424G>T, c.1463G>A];[c.1586+1G>T]).[10] Besides these 2 patients, multiple other maternal cases have been reported who also carry at least 1 null-variant (compound heterozygous), though they have not suffered any cardiac events.[8,18-20] This raises the question whether they are indeed at risk for a cardiac event or whether there is a secondary cause in the 2 reported patients leading to the cardiac events? At the time of writing, this question cannot be fully answered. It is possible that PCD had some role in the development of cardiac events, as no further events occurred in both patients after the start of treatment with carnitine, but the evidence remains anecdotal. Currently, there seems little evidence that asymptomatic adults with PCD without null-mutations or those missense variants widely reported in severely affected patients, carry a significant risk for developing cardiac events.

If mothers can be diagnosed with PCD as a result of NBS of their child, this possibility (of a maternal diagnosis) should be included in the information provided prior to screening.[21,22] At the time of expansion of the Dutch NBS program in 2007, the fact that mothers could be diagnosed with inborn errors of metabolism through

NBS of their child was unknown and as a result this information was not included in the brochure that parents received prior to NBS. Understandably, these mothers were surprised, and sometimes displeased, that they were labeled "patient" as a result from NBS (information based on preliminary data from a national study on the psychologic burden of newborn screening (PANDA-study, ZonMw grant number 543002006, principal investigator L. Henneman)). However, it is essential to be aware that if parents are informed that a disorder can be detected in mothers as a result of NBS of their child, this might dissuade them from participating in NBS altogether.[21,22] Well-balanced information is therefore warranted.

Recommendation: Considering the limited benefit of identifying maternal cases through NBS of their child, juxtaposed to the harm it might cause (*i.e.* reduction of NBS participation and overmedicalization), diagnosing PCD in this population does not seem favorable. Hence, concerning the criterion *"There should be a defined target population"* in our opinion the Dutch NBS program should aim to identify newborns with severe PCD only, in line with its original goal.

Reviewing the NBS referral algorithm for PCD

Currently in Dutch NBS, newborns with low carnitine in DBS, are scheduled for a second DBS sampled within 10 days. When low carnitine levels persist, they are referred for evaluation at one of the metabolic centers. This referral algorithm leads to many false positive referrals reflected in the low positive predictive value (PPV), currently 14.6%. For other countries, where a second DBS sample is not used, the PPV is even lower (1,6 - 4,7%).[4,23] Although secondary sampling increases PPV for PCD, it also generates a risk of missing severe PCD due to referral delay, meaning severely affected patients may die between first and second sampling. Due to privacy concerns, this could not be investigated, but a pilot study is currently in preparation. Ideally, the screening test employed by NBS would only target patients with a severe form of PCD, ignoring individuals with a benign genetic trait. This may be possible in the future, as is discussed in the section future perspectives. Currently, the number of newborns with confirmed severe PCD identified by Dutch NBS is too low, therefore designing a strategy based on the available data is not yet possible.

Until it is possible to improve the referral algorithm we can strive to accelerate the diagnostic process for those that are referred. In **chapter 6**, we show that a ratio of the concentration of excreted free carnitine and plasma free carnitine ($Ratio_{U:P}$) can quickly distinguish newborns with PCD (both severe and benign) from newborns without PCD. In the study, 97% of false positive referrals could have been discharged immediately after the first evaluation at the metabolic centre, based on this $ratio_{U:P}$.

Recommendation: Currently, an NBS referral algorithm with improved PPV and without the need of a second DBS sample, is not yet feasible. Therefore, concerning the criterion *"There should be a suitable test or examination"* we recommend employing the same NBS referral algorithm as has been used in the past decade and continue research on improving the PPV. We also recommend to study the feasibility of the use of the $ratio_{U:P}$ as an early test in the follow-up protocol, to rapidly identify false positive PCD in referred newborns.

The overall benefits of screening should outweigh the harm

Here, we evaluate the benefits and harms of NBS for PCD, if implemented according to the recommendations above. This means (1) NBS would seek to identify newborns with PCD only, mothers will not be investigated (2) follow-up aims to diagnose and treat severe PCD, while accepting treatment of PCD of unknown severity and (3) NBS employs the same test as it did the past decade.

The benefit of NBS for PCD is the prevention of a severe, potentially life-threatening disease outcome (**chapter 2, 4 and 5**). Since only patients with the severe form of PCD should be targeted by NBS, treatment benefits are undisputed and patients will likely be more receptive to the idea that life-long treatment adherence is necessary (**chapter 4**).[24]

If implemented according to the recommendations above, some harm can still be done. Firstly, some infants with a disease severity that cannot be predicted will be diagnosed and subsequently treated. As a few of these individuals may actually have a benign genetic trait, they would be better off without going through life as a "patient". Furthermore, the PPV of NBS for PCD remains low, leading to high numbers of false positive referrals. Although this can be mitigated by accelerated diagnosis with an improved follow-up protocol, this still will cause stress and anxiety in the families involved.[25] Also, a second NBS DBS is still required, leading to delayed referral, where treatment initiation might be too late. Lastly, and partly caused by the low PPV, there is a high chance of recurrent false positive referral by NBS in case of multiple pregnancies, since mothers are no longer investigated for maternal (asymptomatic) PCD.

Recommendation: Due to the seriousness of symptoms of severe PCD and the efficacy, simplicity and low cost of treatment, we believe the overall benefits outweigh the harm of NBS for PCD if implemented as suggested above. However, ongoing research and follow-up is needed to improve NBS further, further tilting the scale towards the benefits of NBS for PCD.

FUTURE PERSPECTIVES

Improve understanding of natural history

It is still unclear which of the asymptomatic adults with PCD are at risk for cardiac events. To accurately assess the relative risk that PCD adds to developing severe cardiac events, research with extensive follow-up time is required in untreated individuals, since PCD is rare, and events can occur after a long period of time. Such expensive and labor-intensive prospective research is unlikely to be realized, and it could take decades before results are available. Alternatively, one could perform a retrospective cross-sectional cohort study in patients that suffered severe cardiac events, searching the *SLC22A5* gene for variants, comparing variant allele frequencies of the study cohort with that of the general population. However, this study setting is not suited for proving causality. In 2019, Rasmussen et al. performed such a study and evaluated 65 patients with unexplained death in the Faroese population, a genetically homogenous population from a confined region.[16,26] Thirteen of them turned out to be homozygous for the [c.95A>G] variant.[16] This shows a strong association between this variant and sudden death, however the presence of other (genetic) risk factors for sudden cardiac death were not reported and therefore cannot be ruled out. Similar studies in other regions may identify other variants that are associated with cardiac events, further improving our understanding of the natural history of PCD in different types of patients. Unpublished preliminary data from a Dutch cohort of 1286 patients who suffered either cardiomyopathy or sudden cardiac death, revealed that 16 patients carried 1 *SLC22A5* variant, and none had variants on both alleles. However, large deletions and intronic variants (such as the [c.-149G>A] variant) in the *SLC22A5* gene could not be detected, and can therefore not be ruled out.

Towards a dichotomous divide

Ideally, in the future, there would be a dichotomous divide of PCD: those with a predicted severe disease and those with a benign genetic trait. In the past however, for PCD, genotype and residual activity could not be correlated to phenotype.[27] This, in part, may be due to the assay that was used to measure the residual carnitine transport activity. The assay used in the past had low sensitivity, especially if residual carnitine transport is very low. For instance, previous research performed using that assay, showed that residual transport of [c.725G>T] expressed in Chinese Hamster Ovary (CHO) cells was 0%, but in fibroblasts from homozygous, symptomatic patients, it ranged from 2.8 – 20.8%.[28-30] Meanwhile, a similar range of activity, determined with that same assay, was reported in completely asymptomatic mothers.[6,27,31] Likewise, in the Dutch cohort this previously used assay showed no differences in carnitine transport activity in fibroblasts of severe PCD patients and individuals with a likely benign genetic trait. In contrast, the assay newly developed at our institute demonstrated a clear separation between carnitine transporter activity in severe PCD patient cells (4-5%) and those of individuals with a benign genetic traits (median 26%), making it a promising marker for disease severity. However, this requires further validation in international patient cohorts, as several genotypes that have been reported to cause symptomatic PCD are not present in the

Dutch population. These include, among others, homozygosity for [c.95A>G], [c.760C>T], [c.844delC], [c.-91_22del] and [c.806delT].[8,11,12,32-34] It would be interesting to investigate the residual transport activities with the novel assay in patients with these genotypes - are they consistent with the findings in Dutch patients with severe PCD and can we further confirm the transport activity range indicative of severe PCD? We propose the development of an international PCD database. This would expand upon the work previously performed by Frigeni *et al*, cataloguing different patient genotypes, their symptoms (with or without treatment) and their transport activity (novel assay).[28] The suggested database can be readily updated to add information on known variants and include data on novel variants. Such a database will provide a convenient and complete overview of observed clinical and functional consequences of PCD variants, allowing classification of disease severity based on genotype and residual carnitine transport activity for most individuals identified.

Increasing the positive predictive value of NBS for PCD

Once we can specifically identify those patients with severe PCD, the NBS referral algorithm can be improved to identify only those patients. This will drastically decrease the number of false positive referrals, further reducing the harm of NBS for PCD. Additionally, the requirement of a second NBS DBS sample might be eliminated. One could retrospectively evaluate DBS's from patients with severe PCD, then search their DBS's for significant markers (or even patterns of multiple markers) compared to the DBS's from healthy newborns (*e.g.* with multivariable regression analysis). This analysis could also include covariates as sex, gestational age at birth, birth weight and age at sampling, as they, too, influence the carnitine concentration measured in NBS DBS (**chapter 3**). Tools such as *Collaborative Laboratory Integrated Reports* (CLIR), can present NBS results as a single score, adjusted for all covariates, reflecting the likelihood of PCD being present, rather than using a complicated referral diagram containing different cut-off values for separate covariates (*i.e.* multiple age groups, birth weights etc.). Alternatively, in the future, alongside DBS, dried urine spots could be created to determine the free carnitine excretion.[35-37] Newborn DBS's are known to reflect maternal carnitine status[38,39], but newborn dried urine spots will invariably show the newborn's carnitine excretion. **Chapter 6** shows that carnitine excretion is significantly increased in newborns with PCD. However, reference values for this application are absent, and we cannot assume the results from the highly selected population in **chapter 6** apply to the general population with a younger age at sampling (median age 4 days vs 11 days) Also, this strategy would be expensive to implement, since procedures for dried urine sampling are not in place and it would require a complete overhaul of the current NBS protocol.

Lessons learned

The first signs of overdiagnosis of inborn errors of metabolism (IEM's) by NBS were reported in 2003.[40] Since then, the yield of multiple screening programs was evaluated, and many showed an increased incidence of an IEM after its introduction in NBS.[41-43] For some IEM's, certain genetic variants were solely identified by NBS and caused only mild defects (based on residual protein function), compared to patients diagnosed pre-NBS.[44-46] This suggests that NBS for some disorders may be too sensitive, and diagnoses individuals

with certain biochemical and genetic traits who should not be considered patients and do not require treatment at all.[43] To counteract overdiagnosis, one could define true disease within the target disease[47], or NBS for a given IEM could be discontinued altogether.[5] Still, such an undertaking is not without risk, as the health consequences for mildly affected patients remain unknown. The work in this thesis underscores that, at least for PCD, mild functional defects, and genetic variants only identified by NBS, are unlikely to cause significant health risks in untreated children. Therefore, we are able to identify true disease (severe PCD) within the target disease. It is probable that the same applies to NBS populations with other IEM's (*e.g.* isovaleric acidemia, beta-ketothiolase deficiency and very-long-chain acyl-CoA dehydrogenase deficiency).[41-43] This emboldens us to reconsider true disease within NBS populations in a broader sense.

We argue that genetic disorders in general contain a disease spectrum, and screening lays bare the part of the spectrum that was hidden within asymptomatic individuals. As screening becomes more accessible through modern modalities, such as next generation sequencing, more will walk the thin line between patient and individual with a benign genetic trait. It is important to recognize this issue prior to implementing disorders in NBS, and prospectively gather data on the characteristics of individuals diagnosed through NBS, to establish a feedback loop. This way, the benefits of NBS for public health are maintained, while potential harms can be signaled and counteracted.

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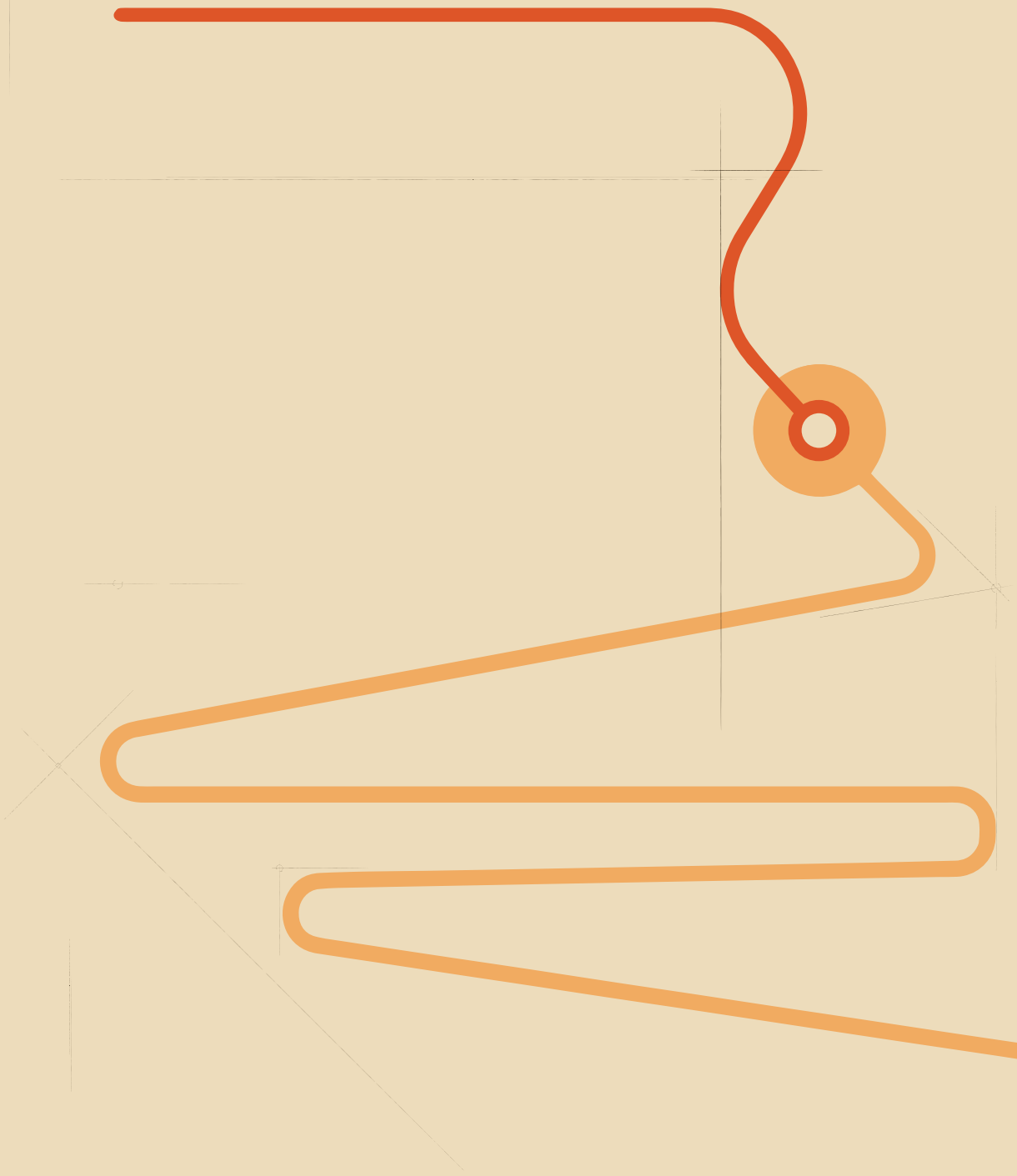
APPENDIX



SUMMARY

Primary carnitine deficiency (PCD) is a treatable inborn error of metabolism that has been associated with hepatic encephalopathy, cardiomyopathy, arrythmia and sudden death, and it is therefore included in newborn screening (NBS) programs. It not only identifies newborns but also an increasing number of, predominantly asymptomatic maternal cases with PCD. The high number of patients identified by NBS with previously unreported genotypes and the incomplete understanding of benefit of screening, weighed against potential harm caused by identification and treatment of asymptomatic individuals raises the question whether NBS for PCD is desirable. This thesis aims to clarify whether NBS for PCD, meets all the needed criteria for implementation in the Dutch NBS program.

In **chapter 2** we perform a structured review to evaluate the clinical characteristics associated with PCD. We compare the clinical spectrum based on certainty of diagnosis (unconfirmed: diagnosis solely based on low plasma carnitine vs confirmed: diagnosis confirmed genetically and/or functionally) and mode of detection (presented clinically vs NBS). Using a case by case approach we gather data on 136 cases with unconfirmed PCD and 621 cases with confirmed PCD. We show that the clinical spectrum of confirmed PCD is less diverse than that of unconfirmed (often historically reported) PCD patients. Symptoms are mainly reported in clinically diagnosed individuals; predominantly consisting of cardiac manifestations that may lead to sudden death. Other signs and symptoms include coma, encephalopathy, hepatomegaly, hypoglycaemia and myalgia. At least 93% of clinically diagnosed PCD patients present in childhood, with a median age of first symptoms at 1 year. Patients identified through NBS (whether they are newborns , or maternal cases identified through the screening of their child) generally are asymptomatic at the time of diagnosis. However rare, severe cardiac events have been described in previously asymptomatic individuals.

In **chapter 3** we provide the day-to-day courses of carnitine concentrations in newborns of various gestational ages and birth weights during the 3rd to 10th day of life. We analyse dried blood spot carnitine concentrations of nearly 2 million newborns, using Dutch NBS data from more than a decade of screening. With these data we demonstrate the day-to-day median carnitine concentrations for newborns in general, but also smaller sub-populations, grouped by gestational age and weight for gestational age. Our results show that carnitine concentrations depend on gestational age and weight for gestational age, and most particularly of age at sampling. These variables may be accounted for when interpreting carnitine concentrations from newborn screening results.

We report a case of PCD in **chapter 4.** The patient presented with severe cardiomyopathy in childhood, which, after decades of treatment non-adherence, recurred in adulthood. The case demonstrates the importance of continued monitoring of metabolic disease patients and emphasizes that the transition from the paediatric to adult care is a challenging period, both for patients and caregivers. It is likely that, with continued treatment, his second cardiac decompensation could have been prevented. Furthermore, this case demonstrates that treatment cessation in primary carnitine deficiency can lead to severe heart

failure. Unlike typical heart failure, cardiac decompensation caused by primary carnitine deficiency is completely reversible.

In **chapter 5**, we evaluate the clinical, genetic and functional characteristics of Dutch PCD patients identified by NBS and those identified by clinical presentation. Our study demonstrates a clear discrepancy between clinically identified patients and patients identified by NBS. Differences were observed in the clinical presentation, the genetic variants and the residual carnitine transport activity. The majority of patients identified by NBS (maternal cases and newborns) have genetic and functional features that likely constitute asymptomatic or very mild disease. However, a small part of newborns identified by NBS have features similar to PCD patients that presented clinically, and likely will benefit from early treatment. Our results encourage using a narrowed down definition of the patients with the target disease when implemented in screening programs, in order to combat overmedicalization caused by NBS.

NBS for PCD yields a high numbers of false positive referrals. It is a lengthy and complex process to exclude PCD as a diagnosis in these newborns, which can cause significant anxiety and stress in the families involved. In **chapter 6**, we evaluate the use of a ratio of urine free carnitine to plasma free carnitine ($Ratio_{U:P}$) as an early discriminative test in newborns referred by NBS for low free carnitine. $Ratio_{U:P}$ is significantly higher in newborns with PCD than in those without PCD. This finding is more pronounced in newborns that are sampled before 1 month of age and that are not on carnitine suppletion. $Ratio_{U:P}$ accurately discriminates between positive and false positive newborn referrals for PCD by NBS, without misidentifying true positive cases. This test can be applied as a first test in referred newborns and may allow early exclusion of PCD as a diagnosis, greatly reducing the harm and cost of follow-up diagnostics.

In **chapter 7**, we discuss how the results from this thesis may be used to advance the implementation of PCD in Dutch NBS, according to the screening criteria recommended by the world health organization. Our recommendations are: (1) NBS should seek to identify newborns with PCD only, mothers will not be investigated (2) follow-up aims to diagnose and treat severe PCD, while accepting treatment of PCD of unknown severity and (3) NBS employs the same test as it did the past decade. If implemented this way, we believe the overall benefits will outweigh the harm of NBS for PCD. We conclude with future perspectives on how to further improve the balance between the benefit and harm by NBS for PCD.

NEDERLANDSE SAMENVATTING

Primaire carnitine deficiëntie (PCD) is een goed behandelbare metabole ziekte die gepaard gaat met hepatische encefalopathie, cardiomyopathie, aritmie and plotse (hart)dood. De ziekte is in veel landen opgenomen in de neonatale bloedspot screening (NBS). Via deze screening worden niet alleen kinderen, maar ook in toenemende mate asymptomatische moeders met PCD ontdekt. Binnen het Nederlandse NBS programma is PCD een toevalsbevinding. Het is op dit moment nog niet duidelijk of NBS voor PCD in Nederland aanvaardbaar is. Dit komt door het hoge aantal patiënten dat wordt gevonden via NBS met nooit eerder beschreven genetische varianten, in combinatie met onduidelijkheid over de voordelen van deze screening, afgewogen tegen de mogelijke nadelen die volgen uit het vinden en behandelen van asymptomatische personen. Met dit proefschrift evalueren we of NBS voor PCD voldoet aan de criteria die nodig zijn om de ziekte officieel op te nemen in het Nederlandse NBS programma.

Hoofdstuk 2 bevat een gestructureerde literatuur review waarbij we de klinische eigenschappen van PCD uiteen zetten. We vergelijken het klinische spectrum van PCD (d.w.z. de klachten beschreven bij deze ziekte) op basis van de zekerheid van de diagnose (onbevestigde diagnose: enkel gebaseerd op een verlaagd plasma carnitine, of bevestigde diagnose: gebaseerd op genetische en/of functionele afwijkingen) maar ook op basis van de manier waarop de patiënt ontdekt is (klinische presentatie met symptomen, of via NBS). We verzamelen data van individuele PCD casus beschreven in literatuur, 136 patiënten hadden een onbevestigde diagnose en 621 een bevestigde diagnose. Het klinische spectrum van bevestigde PCD patiënten is minder divers dan dat van de (vaak historisch beschreven) onbevestigde patiënten. Symptomen worden vooral beschreven bij patiënten die zich klinisch presenteerden, en bestaan met name uit cardiale manifestaties die tot plotse dood kunnen leiden. Andere symptomen waren coma, encefalopathie, hepatomegalie, hypoglycemie en spierklachten. Ten minste 93% van deze patiënten ontwikkelde de eerste klachten in de kindertijd, waarbij de mediane leeftijd 1 jaar is. Patiënten die ontdekt zijn via NBS (kinderen dan wel de moeders van kinderen ontdekt via screening van hun kind) zijn meestal asymptomatisch ten tijde van de diagnose. Cardiale incidenten zijn, zij het zeer zelden, beschreven in asymptomatische volwassenen.

In hoofdstuk 3 tonen we het verloop van de carnitineconcentratie van zuigelingen tussen de 3e en 10e levensdag. Daarbij houden we rekening met de zwangerschapsduur en het geboortegewicht van de zuigelingen. We analyseren de bloedspot carnitine concentraties van bijna 2 miljoen zuigelingen die tussen 2007 en 2019 aan het NBS programma deel hebben genomen. Onze resultaten laten zien dat de carntineconcentratie afhangt van de zwangerschapsduur, het geboortegewicht, maar bovenal de leeftijd bij de bloedafname. Men kan rekening houden met deze variabelen bij de interpretatie van de NBS uitslagen.

In **hoofdstuk 4** wordt een PCD casus beschreven. De patiënt presenteerde zich als kind met een ernstige cardiomyopathie, welke na jarenlange therapie-ontrouw recidiveerde op volwassen leeftijd. Deze casus laat zien dat doorlopende monitoring bij patiënten met een metabole ziekte essentieel is en toont aan

dat de transitie van de kindergeneeskunde naar de volwassen zorg een uitdagende periode is, zowel voor de patiënt als de zorgverlener. Waarschijnlijk zou, met onafgebroken therapie, de tweede cardiale decompensatie voorkomen kunnen worden. In tegenstelling tot typische cardiale decompensatie, is een decompensatie veroorzaakt door PCD volledig reversibel.

In **hoofdstuk 5** analyseren we de klinische, genetische en functionele eigenschappen van Nederlandse PCD patiënten, enerzijds van degenen ontdekt via NBS en anderzijds van degenen die gediagnosticeerd zijn nadat zij zich klinisch (met symptomen) presenteerden. Ons onderzoek laat een duidelijke scheiding zien tussen klinisch geïdentificeerde patiënten en patiënten ontdekt na NBS. De verschillen zijn te zien in klinische presentatie, de genetische varianten die de patiënten bij zich dragen en de resterende functie van het aangedane eiwit in PCD (OCTN2). De meerderheid van patiënten ontdekt via NBS (zowel moeders als kinderen) hebben genetische en functionele eigenschappen die waarschijnlijk leiden tot een zeer milde vorm van de ziekte. Echter, een klein deel van de kinderen ontdekt door NBS lijkt qua eigenschappen sterk op de patiënten die gediagnosticeerd werden nadat zij zich meldden met (ernstige) klachten. Het is aannemelijk dat deze kinderen baat hebben bij het snel starten van medicatie. Onze resultaten sporen aan om een nauwere definitie van "patiënt" te hanteren als het aankomt op ziekten ontdekt via NBS. Op die manier kunnen we overmedicalisatie door NBS tegengaan.

NBS voor PCD gaat gepaard met een hoog aantal vals positieve verwijzingen. Het is een langdurig en lastig traject om de diagnose PCD vervolgens bij hen uit te sluiten. Tijdens deze periode waarin onzeker is of het pasgeboren kind aan de ziekte lijdt, kunnen families angst en stress ervaren. In **hoofdstuk 6** onderzoeken wij of de ratio tussen urine en plasma vrij carnitine ($Ratio_{U:P}$) kan dienen als een vroege test in kinderen die verwezen zijn vanwege een laag carnitine in NBS. Deze ratio is significant hoger bij de kinderen met PCD dan bij de kinderen zonder PCD. Wanneer de kinderen tijdens afname van het materiaal jonger zijn dan 1 maand en geen carnitinesuppletie ontvangen is het verschil het meest uitgesproken. Met $Ratio_{U:P}$ kan goed onderscheid worden gemaakt tussen kinderen met en kinderen zonder PCD. Daarbij wordt geen enkel kind onterecht beschouwd als "geen PCD". De $Ratio_{U:P}$ kan gebruikt worden als een eerste test in het ziekenhuis bij verwezen kinderen om snel de diagnose PCD uit te sluiten. Zo worden de schade en kosten van vervolgdiagnostiek beperkt.

In **hoofdstuk 7** bespreken we hoe de resultaten van dit proefschrift gebruikt zouden kunnen worden om de implementatie van PCD in het Nederlandse NBS programma vorm te geven. Onze aanbevelingen ten aanzien van NBS voor PCD zijn: (1) NBS voor PCD is enkel gericht op het identificeren van kinderen met de ziekte, moeders worden niet langer onderzocht (2) het vervolg na verwijzing is gericht op het diagnosticeren en behandelen van ernstige PCD, daarbij aanvaarden we dat kinderen met onbekende ernst ook behandeld worden en (3) de methode van testen die men gebruikt bij NBS blijft hetzelfde als dat hij was. Als de screening op deze manier wordt uitgevoerd, zijn wij ervan overtuigd dat de voordelen van NBS voor PCD opwegen tegen de nadelen. Tot slot bediscussiëren we enkele toekomstperspectieven die de balans de tussen voor- en nadelen van NBS voor PCD kunnen verbeteren.

ABBREVIATIONS

AGA	Appropriate for gestational age
AUC	Area under the curve
C0	Free carnitine
CACT	Carnitine-acylcarnitine translocase
CI	Confidence interval
CoA	Coenzyme A
CPT1	Carnitine palmitoyltransferase 1
CPT2	Carnitine palmitoyltransferase 2
Cr	Creatinine
DBS	Dried blood spot
DVP	Dienst Vaccinvoorziening en Preventieprogramma's
FCE	Fractional carnitine excretion
GA	Gestational age
HPO	Human phenotype ontology
ICU	Intensive care unit
IEM	Inborn error of metabolism
IQR	Interquartile range
LGA	Large for gestational age
LVEF	Left ventricular ejection fraction
MCAD	Medium-chain acyl-CoA dehydrogenase
METC	Medisch ethische toetsingscommissie
MS/MS	Tandem mass spectrometry
NA	Not available
NBS	Newborn screening
NOS	Not otherwise specified
OCTN2	Organic cation transport novel 2
PCD	Primary carnitine deficiency
PPV	Positive predictive value
$Ratio_{U:P}$	Ratio of urine free carnitine to plasma free carnitine
RIVM	Rijksinstituur voor volksgezondheid en milieu
ROC	Receiver Operator Characteristic
SGA	Small for gestational age
TD	Times daily
VF	Ventricular fibrillation
WfGA	Weight for gestational age
WHO	World health orginization

LIST OF PUBLICATIONS

Crefcoeur LL, de Sain-van der Velden MGM, Ferdinandusse S, Langeveld M, Maase R, Vaz FM, Visser G, Wanders RJA, Wijburg FA, Verschoof-Puite RK, Schielen PCJI. Neonatal carnitine concentrations in relation to gestational age and weight. JIMD Rep. 2020 Sep 8;56(1):95-104. doi: 10.1002/jmd2.12162. PMID: 33204600; PMCID: PMC7653253.

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Crefcoeur LL, Dekkers EHBM, Ferdinandusse S, Fuchs SA, Huidekoper HH, Janssen MCH, Langendonk JG, Maase RE, de Sain-van der Velden MGM, Rubio-Gozalbo ME, van Spronsen FJ, Vaz FM, Verschoof-Puite RK, de Vries MC, Wijburg FA, Visser G, Langeveld M. Newborn screening for primary carnitine deficiency: who will benefit from treatment?

CURRICULUM VITAE

Loek Crefcoeur was born on January 10th 1993 to Paul and Paula Crefcoeur as the youngest member of the family. He grew up alongside his four brothers and two sisters in the Hague. Loek graduated from Gymnasium Sorghvliet in 2011 and in the same year moved to Utrecht where he studied medicine at University Medical Centre Utrecht. During his internships, Loek developed a keen interest in the fields of pediatrics and internal medicine. After obtaining his medical doctor degree in 2017, Loek worked for 1 year at the department of cardiology as a medical education assistant, to improve his academic teaching skills. Here, he was responsible for the teaching of all cardiological topics mandated by the curriculum of Medicine (CRU and SUMMA) at University Medical Centre Utrecht. He then applied for a Ph.D project at the department of metabolic diseases in the Wilhelmina Children's Hospital, where pediatrics, internal medicine and cardiology were all represented. Under the supervision of Professor E.E.S. Nieuwenhuis, Professor F.A. Wijburg and co-promoteres Dr. G. Visser and Dr. M. Langeveld, he started his Ph.D. project focusing on the implementation of primary carnitine deficiency in the Dutch newborn screening programme.

Loek is currently working as an internal medicine, pulmonology, gastroenterology and cardiology resident at the Tergooi Hospital in Hilversum and Blaricum.

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