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Review Article

20 Years After Discovery of the Causative Gene of Primary Carnitine Deficiency, How Much More Have We Known About the Disease?

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Abstract We review the development of molecular diagnosis of primary carnitine deficiency 卡尼丁缺乏症, also known as carnitine uptake defect. Knowledge of mutations of the causative gene, *SLC22A5* (previously called *OCTN2*), greatly enhanced our understanding of the physiology, pathogenesis and therapy. DNA diagnosis and newborn screening are very useful in early diagnosis and early therapy which leads to favourable prognosis. With the latest incidence data, primary carnitine deficiency turns out as one of the most common inherited metabolic disease in Chinese (the incidence as high as 1 in 10,000) and up to a thousand patients have been diagnosed and treated in the Greater China region during the last 20 years.

Key words Carnitine; Carnitine uptake defect; Fatty acid oxidation defects; Primary carnitine deficiency; *SLC22A5*

Introduction

In 1996, a family with recurrent unexplained infant deaths were investigated with all available pathology and genetic methods possible at that time. After a thorough examination, the diagnosis of primary carnitine deficiency was made for the first time in this locality.[1] As it was the first case, there was little insight how prevalent this disease could be in Chinese. There was also no prior experience in greater China region. After 20 years, this disease now turns out to be one of the most prevalent inherited metabolic disease (IMD) in Chinese and up to 1,000 cases have been diagnosed and treated all over China. This article reviews and highlights special features about primary carnitine deficiency 卡尼丁缺乏症 which is also commonly known as carnitine uptake defect (CUD).

Early reports of "Primary carnitine deficiency" were in fact mis-diagnosed cases of medium-chain acyl-CoA dehydrogenase (MCAD) deficiency and the carnitine deficiency was secondary to blockage of fatty acid β-oxidation. It was not until the development of an in-vitro carnitine uptake assay on cultured fibroblast, a clear-cut definitive diagnosis of CUD could be made.[2] However, in-vitro functional assay has many limitations and the most obvious one is the unavailability of living fibroblast as fibroblast culture from skin biopsy is not a routine investigation that is readily available. Furthermore, failure to setup a culture sometimes happens which makes it less reliable as a diagnostic test. To overcome these troubles, we and two other teams identified the causative gene which was called *OCTN2* historically, now known as *SLC22A5,* around the same time in 1999.[3-5] The discovery allows making use of a simple DNA mutation test to make a definitive diagnosis. A battery of early mutations reported by the three papers were archived in the OMIM database (#MIM:603377, https://www.omim.org/entry/603377). As we were the only one of few laboratories performing

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mutation analysis for *SLC22A5* at that time, we have the privilege to work with colleagues from Asia regions and other part of the World to understand the mutation spectrum and also describe new features of this disease.

Physiological Role of the Plasma Membrane Carnitine Transporter

Intracellular carnitine concentration is much higher than extracellular concentration which is achieved by this plasma membrane sodium carnitine co-transporter with ATPase activity (Figure 1). This transporter belongs to a large family of active organic ion transporters which share similar protein structure and common molecular evolution. Other member transporters play important role in transport and absorption of drugs and other endogenous organic molecules.

Carnitine is an essential compound in fatty acid oxidation pathway. Fatty acid is a key energy source for cardiac muscle and skeletal muscle. Therefore, carnitine transporter is highly expressed in muscle tissues to maintain a high carnitine concentration in the cytoplasm. Kidney tubular epithelial cells also have a high expression of the carnitine transporter. As carnitine has a low molecular weight, it is passively filtered across glomeruli. Over 90% of filtered carnitine are reabsorbed presumably in the proximal tubule by this active transporter (Figure 1). If carnitine transporter is defective, extensive renal wastage of carnitine causes a very low plasma carnitine concentration and subsequently carnitine deficiency.

In addition to support fatty acid metabolism of muscular tissue itself, fatty acid oxidation in liver produces the emergency energy currency, ketone bodies. Central nervous system is dependent on ketone bodies as its primary energy after depletion of body carbohydrate stores and during hypoglycaemia. This phenomenon happens in patients with various fatty acid oxidation defects and manifests as hypoketotic hypoglycaemia, which is a common hallmark of this group of IMDs. As infants have small body carbohydrate stores, they are prone to hypoglycaemia when any coincidental illness results in poor feeding. The inability to generate sufficient ketone bodies from fatty acid leads to a state of acute energy deficiency and patients could become comatose as soon as 6-8 hours after stopped feeding.

On the other hand, cardiomyopathy may take time to develop. A form of dilated cardiomyopathy typically occurred among patients who survived acute metabolic attacks during infancy particularly among those patients who had not been treated with carnitine. This was also the case of the referral from Macau who was a 10-year-old proband with dilated cardiomyopathy.[6]

Regional Disease Prevalence and Geographic Distribution of Common Mmutations, R254X and S467C

Founder Mutation R254X

In 2002, we reported for the first time a common mutation, R254X which was found in referral cases from Macau, Taipei, and Kaohsiung.[6] R254X is a mutation changing the codon 254 from Arginine to a stop codon which causes a loss of function of the transporter. This mutation also had an unusually high carrier frequency of 1 in 125 in local Chinese. This carrier frequency predicts a disease incidence of 1 in 62,500 and suggested carnitine uptake defect (CUD) was a common IMD in Hong Kong.

We do not have good statistics of the disease incidence in Hong Kong as territory wide newborn screening NBS commenced only recently. A high disease incidence of CUD (~1 in 40,000) was confirmed in Taiwan where NBS for fatty acid oxidation defect (FAOD) had been started in 2002. In fact, CUD was the most prevalent FAOD in Taiwan. Similarly high incidence has been recently reported in Chinese provinces south of the Yangtze River (Figure 2), including Guangdong, Fujian, Hunan, Shanghai and Xuzhou in Zhejiang.[7-10] In fact, the highest incidence of CUD may be in Fujian at around 1 in 10,000 newborns.[9] On the other hand, incidence was lower in Northern China, like Shangdong.[11]

The high disease incidence in this locality is due to common mutations. R254X is common in Southern China. It is also found in Xuzhou and Shanghai but it is less frequent in Beijing and Vietnam.[7,8,12] Interestingly, the same nonsense mutation was found as far as in Western Asia countries including Lebanon, Turkey and Saudi Arabia (Figure 2). There are 2 alternate possibilities to account for the geographical distribution of R254X mutation. Firstly, these mutations might arise separately in history through independent mutation events happened to different ancestors. Alternatively, the mutation happened to a single ancestor and descended through generations and spread to new locations with people movement or migration. It is called a founder mutation. It is possible to differentiate the two scenarios by looking at the neighbour segments of the chromosome carrying the mutation. If the mutation arose

only once to a single common ancestor supporting the scenario of a founder mutation, the neighbour segments of the chromosome of nowadays patients carrying the R254X mutation should be descended from this common ancestor and thus identical among nowadays patients. This is called haplotype analysis. We showed that R254X among Southern Chinese indeed shared the same haplotype presumably that of the common ancestor and so this is a founder mutation.

Shortly after our publication, very much to our surprise, R254X was also found in 3 different countries as far as in Western Asia and Middle East.[13-16] The research team in Lebanon followed our protocol and showed that R254X in Lebanon patients had the same haplotype as ours. It suggests that R254X in Lebanese patients shared the same ancestor as Chinese patients. On the other hand, R254X in Saudi Arabia patients had a different haplotype (our unpublished data) and it arose independently (marked as triangle in Figure 2). Haplotype of R254X in Turkish patients was not examined so its origin remained as a question. At first sight, it was incomprehensible how Chinese and Lebanese could share a mutation descended from one common ancestor. Historically, both Lebanon and Turkey formed the western end of Silk Road through which heavy trade traffic happened up to the 14th Century. Macro Polo was the prototype historical figure who

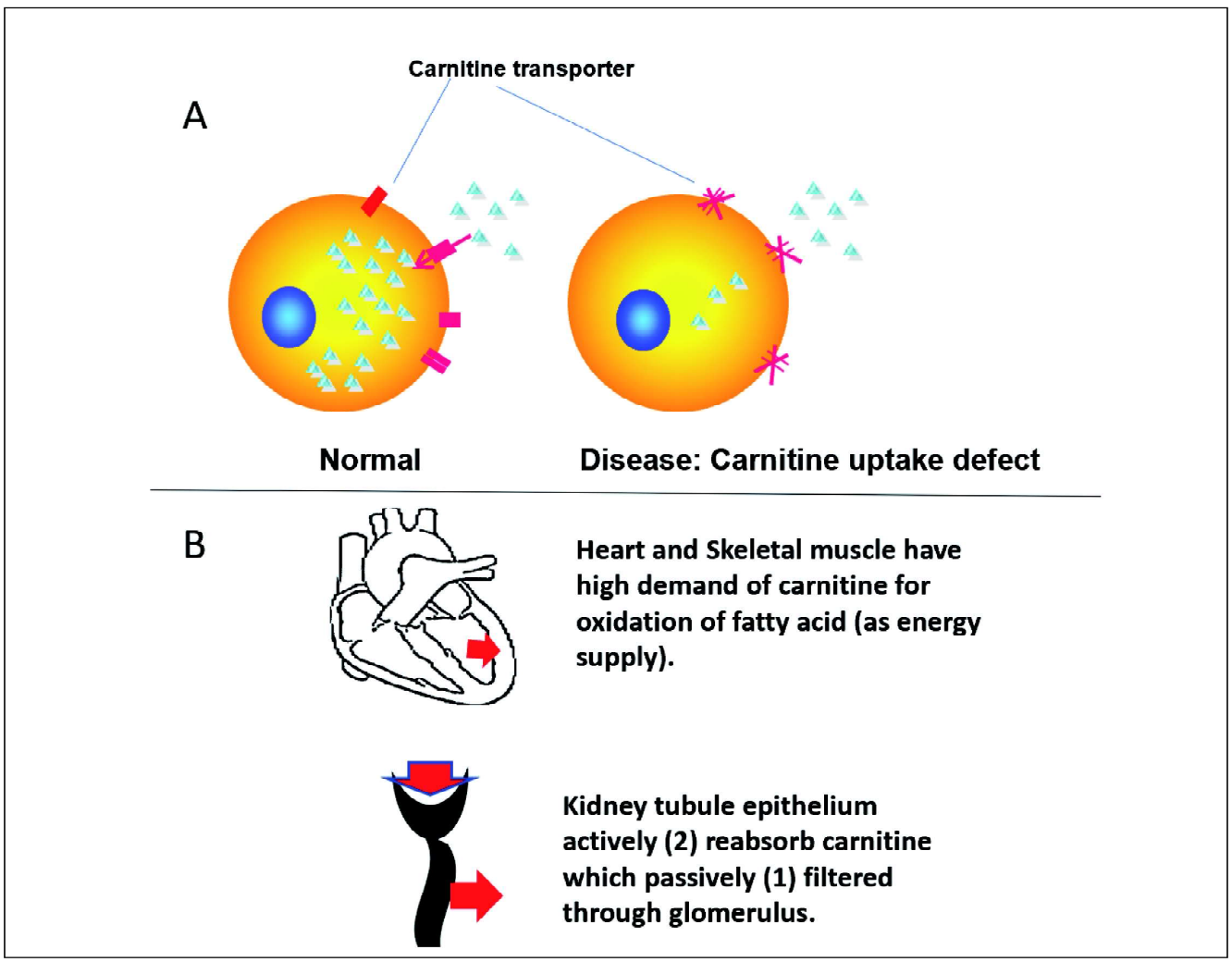

Figure 1 (A) The primary function of the plasma membrane carnitine transporter. It is an active sodium-carnitine co-transporter which pumps carnitine into cytoplasm against a concentration gradient. Mutations of its encoding gene *SLC22A5* (previously called *OCTN2*) result in impairment of carnitine uptake and significant reduction of intracellular carnitine level. (B) This plasma membrane carnitine transporter is crucial for uptake of carnitine into heart and skeletal muscle which are dependent on fatty acid oxidation for energy supply. It is also required in reabsorption of carnitine from tubular fluid back to the circulation in renal tubules.

travelled Silk Road between 1271-1295. After his departure from Venice, he landed at the city Arce which is next to the current border of Lebanon. On his return, he took a route through Turkey (Wikipedia).

By analysis of the length of shared segments of nowadays mutation chromosomes that were descended from the putative common ancestor, an estimation of age of the founder mutation can be performed. The principle is based on probability of recombination is related to (1) length on the chromosome and (2) number of generation (number of meiosis). The longer the shared segment of common haplotype the shorter the history (passed fewer generations) of the mutation. The shared haplotype segment of Chinese and Lebanese R254X extended over 6 million base pairs next to the *SLC22A5* gene on chromosome 5. Age calculation suggested that the mutation arose 620 years ago (confident interval: 375 to 1075 years ago).[17] This coincided with Macro Polo's historical period when the Silk Road traffic was also most active. Of course, this hypothesis is pending for further confirmation, until then it remains speculative.

Maternal Carnitine Uptake Defect (mCUD) and S467C

While we performed mutation analysis for NBS screen-positive CUD neonates referred from Taiwan screening centres, there were occasional newborns with whom only one mutation was found. As CUD is an autosomal recessive disease, affected patients are expected to have two mutations, one on each of two chromosomes. In a putative affected case, missing one mutation represent a major diagnostic dilemma. Steps are needed to differentiate among several scenarios, and importantly each of them leads to a different diagnosis and management. These scenarios may represent (a) false negative mutation analysis, (b) wrong diagnosis, or (c) mosaicism and other rare genetic aberrations.

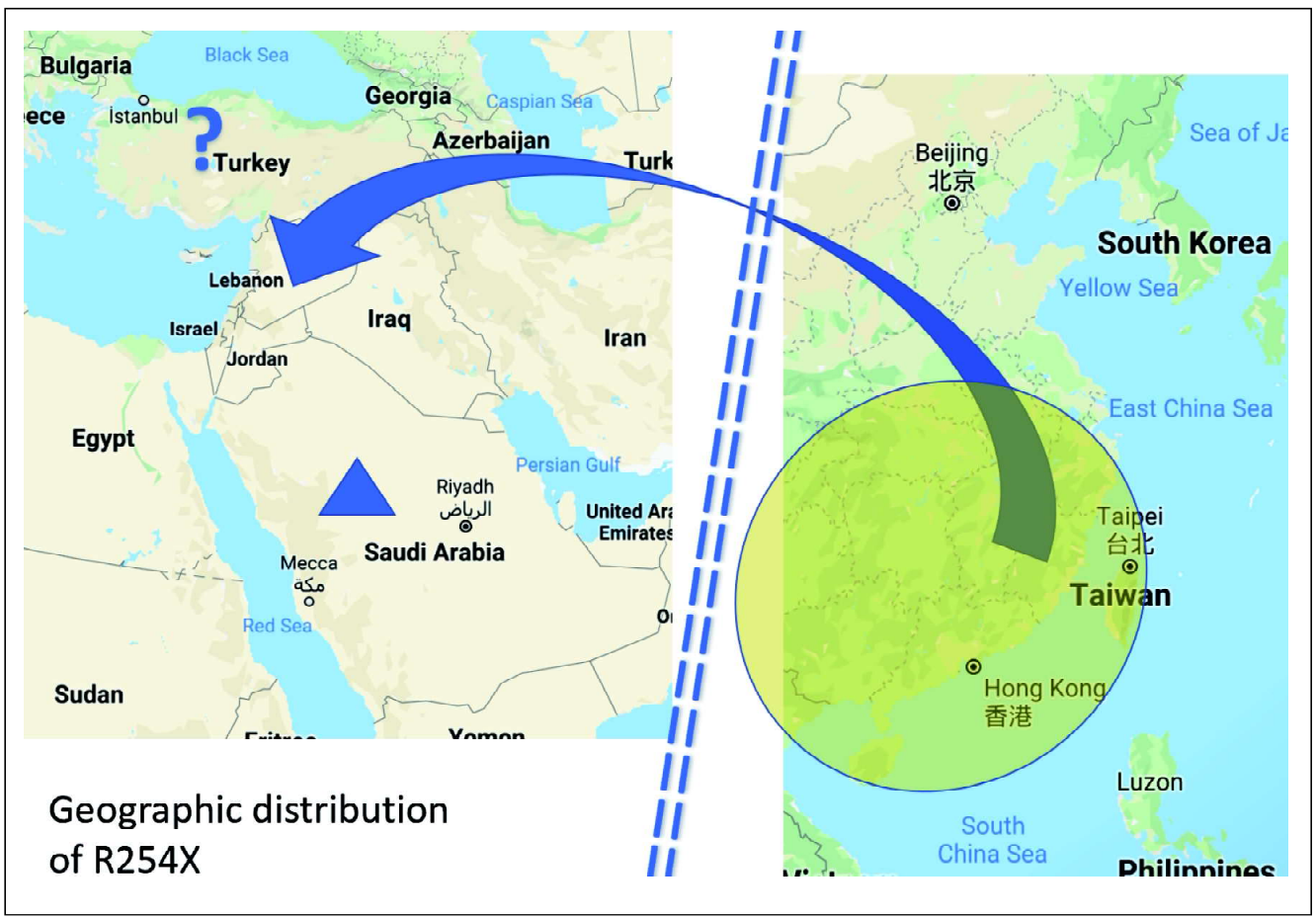

Figure 2 R254X mutation is particularly prevalent in the Southern China region. Molecular evolution analysis indicates that the mutation was originated from a single founder in the past. Interestingly this founder mutation was also found in Lebanese patients. It is uncertain R254X reported in Turkey is the same founder mutation while the mutation in Saudi Arabia arose as a separated origin (not from the same founder).

Colleagues from the referring centre found that several mothers of these cases had unusually low serum carnitine concentration which were comparable to that of CUD patients. Mutation analysis of maternal samples of these cases readily confirmed that the mothers had two mutations in *SLC22A5* gene. The results indicated that the real patients were in fact the mothers and those children picked up by NBS were just obligate carriers.

When we first looked at the list of mutations in these maternal CUD cases, it took only a quick glance to spot an eye-catching pattern. All except one case were carriers of the same mutation, S467C.[18] This is change of Serine to Cysteine at codon 467. In fact, S467C was among the first batch of mutations ever diagnosed in CUD.[19] Among the Japanese patients, this mutation was prevalent in Akita region where carrier frequency was as high as 1 in 100. As it is a common mutation in Japanese, functional study had been performed to evaluate the biochemical function of mutated carnitine transporter. In-vitro study confirmed that S467C had a substantial residual function.[20] Examples in other diseases indicate that mutants with residual function may lead to a milder phenotype. S467C may serve as an example to illustrate this point.

S467C was first reported as mutations found in Akita region of Japan where is well known for a high incidence of CUD. Asymptomatic adults were screened for serum carnitine levels and those with low carnitine were analysed for mutations and carriers of S467C were found. Although residual transporter function, almost up to 10% of normal update capacity, was demonstrated, its particular role in mCUD was not aware at that time.[19-20] Pregnancy leads to major changes in physiological function and adaptation, like increased cardiac output, renal filtration etc. Many of them demand for additional calories/energy supply, and thus represent major stress to cellular function and also fatty acid oxidation. It is unexpected to have a CUD patient (the mother in this case) to go through all the biochemical challenges of the 40-week pregnancy without any clinical presentation. This could be related to such residual function of S467C which allows the necessary fatty acid oxidation to proceed. As one mutant with residual function would be sufficient to support the cell function in recessive disease, it does not matter which mutant is found on the other chromosome. So the most common genotype of mCUD was (S467C; R254X). Another allele F17L was also more frequently found among mCUD than clinical presented cases, and it may represent another mild mutant contributing to mCUD.[9,18,21]

Excellent Treatment Response But Good Compliance Is the Key

High dose supplement therapy with carnitine is the key treatment for patients. Cederbaum et al reported favourable prognosis after carnitine supplement of the first three ever-diagnosed CUD patients.[22] As some of their patients diagnosed after symptomatic attacks or cardiomyopathy, such already established pathology were not reversible. Subsequent to the discovery of *OCTN2* gene (*SLC22A5*), molecular analysis has become a routine test to establish definitive diagnosis when patients present with symptoms (clinical symptomatic patients). Latest implementation of NBS allows the treatment to start even earlier. As early diagnosis and treatment prevent development of metabolic crisis and cardiomyopathy, most NBS-diagnosed patients are expected to live a normal life span free of symptoms.

However, good prognosis is dependent on good compliance to carnitine supplement, irrespective of age. CUD patients are at risk of sudden death if carnitine supplement is abruptly stopped. It might take as short as a few day after cessation of carnitine, cardiac arrhythmia and cardiac arrest might come suddenly leading to fatal outcome.[23,24] On the other hand, it is uncertain if asymptomatic carrier (with one mutation) should be treated with carnitine. Some reports suggested improve cardiac function indicator after carnitine supplement[25] but many clinicians are not convinced that they should be treated.

Newborns Are Now Screened for CUD But…

Many colleagues and us have been advocates for implementations of NBS in Hong Kong for many years. The Hong Kong government started NBS in 2015 for a battery of inherited metabolic diseases including CUD and other fatty acid oxidation defects. Given the population allelic frequencies of common mutations, the incidence of CUD in Hong Kong is estimated to be ~1 in 60,000.[26] This estimate matches with early results from our NBS data published in this issue and reported recently.[27,28]

However, NBS is not 100% sensitive. Early report indicated that sensitivity of NBS for CUD was not perfect.[29] So, there is a small proportion of clinical CUD cases that might be missed by NBS and will present at the time of metabolic derangement or cardiomyopathy. Paediatricians need to be alerted of such possibility and carry out appropriate investigations to make the diagnosis when face with such scenario. In fact, this is a very difficult situation. For example, doctors and parents would not readily challenge a negative NBS report. Therefore, clinical

vigilance and readily available investigation algorithms are helpful. A diagnostic algorithm for hypoglycaemia which is the most common presentation of CUD, is shown here as a quick reference (Figure 3).

Hypoglycaemia is a common presentation of many differential diagnoses. From the perspective of IMD, hypoglycaemia could be broadly categorised by presence of urine reducing substances and ketone bodies. These tests are very important bedside dipstick test with good utility in early triage of patients into various diagnostic groups which might need different management. Of course, bedside dipstick urine test results need to be confirmed by subsequent definitive assays in laboratory. It is also very important to collect pre-treatment specimen or any specimen as early as possible for laboratory investigation as treatment might alleviate or alter the biochemical derangement and thus the laboratory results. Hypoketotic hypoglycaemia is the typical feature for the group of IMDs belonging to fatty acid oxidation defect, and CUD is one of them. The diagnosis of CUD may preliminary be made with results of serum free carnitine, acylcarnitine profile, and urine metabolic screening while the definitive diagnosis is made after DNA mutation analysis. Carnitine treatment should be given if clinical suspicious is high or as soon as a preliminary diagnosis is available because it may be lifesaving and CUD patients typically have good response if treated early in the course of biochemical derangement.

Declaration of Interest

The authors declare that there is no conflict of interest.

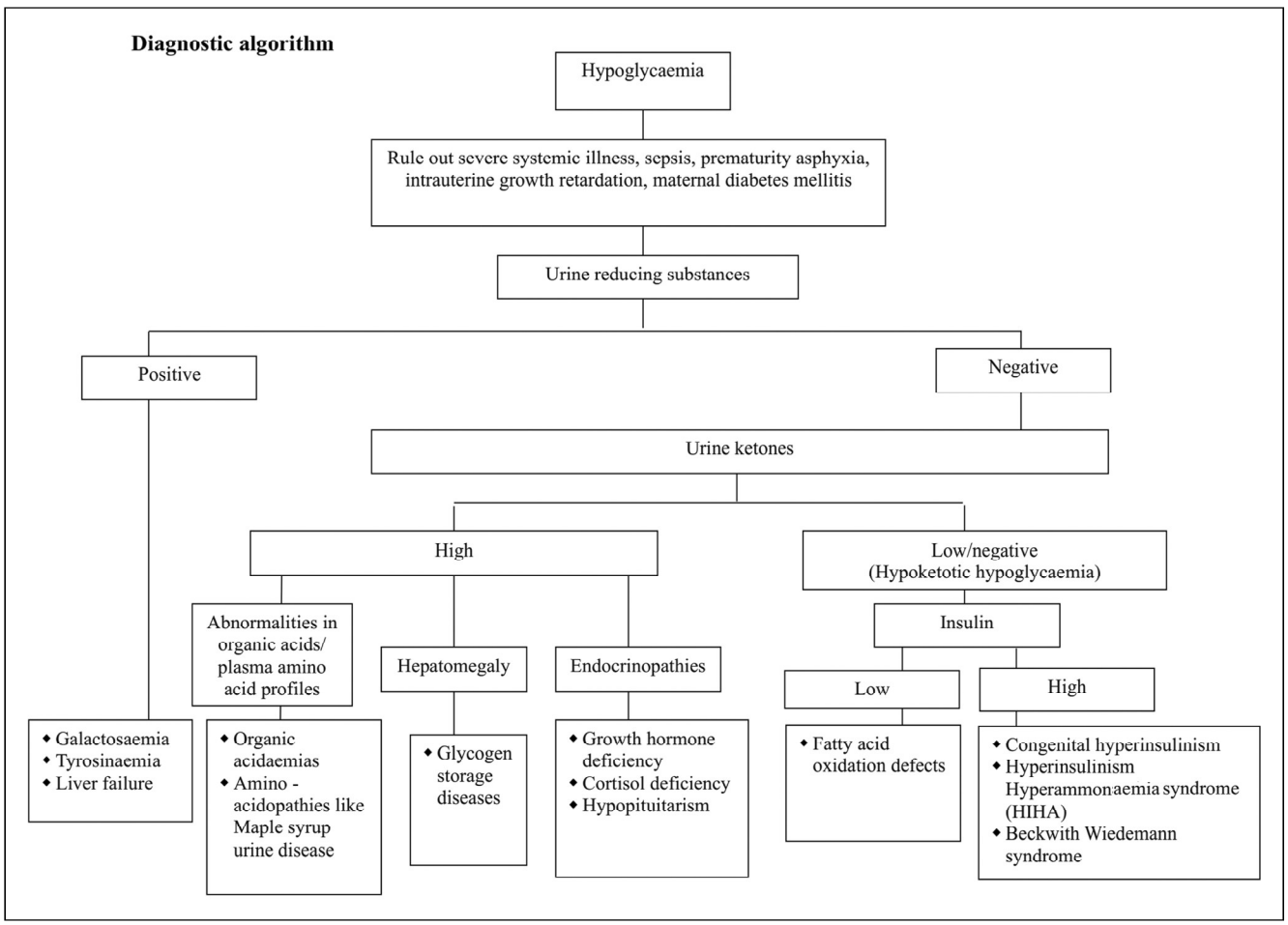

Figure 3 Diagnostic algorithm of hypoglycaemia. CUD and other fatty acid oxidation defects are characterised by hypoketotic hypoglycaemia.

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