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## **Estimation of the prevalence of Cholesteryl Ester Storage Disorder (CESD) in a cohort of patients with clinical features of Familial Hypercholesterolaemia.**

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Key words: familial hypercholesterolaemia, cholesteryl ester storage disease, lysosomal acid lipase deficiency

### **Highlights:**

**An alternative diagnosis for the FH phenotype was investigated.**

**663 no-mutation FH patients were examined.**

**The common *LIPA* splice site mutation (E8SJM) was genotyped.**

**3 carriers were identified for an allele frequency of 0.0023.**

**CESD is not common in a Welsh cohort of patients with an FH phenotype.**

### **Abstract**

**Background and aim.** Familial Hypercholesterolaemia (FH) is caused by variants in the LDL-C metabolic pathway involving *LDLR*, *APOB* and *PCSK9* genes. A national genetic testing service in Wales, UK has observed that no FH variant is found in almost 80% patients with the FH phenotype. It has recently been suggested that some adult patients with an FH phenotype may have Cholesteryl Ester Storage Disease (CESD) which can also present as a mixed hyperlipidaemia. The commonest genetic cause of CESD is an exon 8 splice junction variant in the *LIPA* gene (rs116928232, c.894G>A;E8SJM) previously found to have an allele frequency of 0.0011 (1 in 450 individuals) in a large European population. This study investigated the prevalence of the E8SJM in patients with an FH phenotype in Wales, United Kingdom.

**Method.** 1203 patients with a clinical suspicion of FH, but no FH variant were invited to participate. Of these, 668 patients provided informed, written consent. Stored DNA samples from 663 patients were genotyped for the E8SJM variant.

**Results.** Three heterozygotes were identified (allele frequency 0.0023). Whole gene sequencing of the *LIPA* gene was undertaken in these 3 individuals, but no other variants were found. Therefore there were no CESD patients (homozygote or compound heterozygote) identified in this cohort.

**Conclusion.** The allele frequency 0.0023 (1 in 221 individuals) for the E8SJM variant was more prevalent in this cohort than in a European population study, however, no CESD homozygotes were identified. We found no evidence to support routine testing for CESD in adult patients with an FH phenotype.

## Introduction

Familial Hypercholesterolemia (FH) is an inherited condition characterised by lifelong exposure to elevated Low Density Lipoprotein Cholesterol (LDL-C) and increased risk of premature coronary heart disease (CHD). Classical FH is caused by variants in the metabolic pathway for low density lipoprotein (LDL), most commonly the LDL receptor gene (*LDLR*). FH can also be caused by variants in other genes involved in the metabolism of cholesterol e.g. the *APOB* and *PCSK9* genes. FH caused by variants in *LDLR*, *APOB* and *PCSK9* behaves as an autosomal dominant monogenic disorder and is estimated to affect 1 in 250 of the general population in United Kingdom (approximately 262,000 people), though it is significantly underdiagnosed in clinical practice.<sup>1,2</sup> In the UK and elsewhere guidelines recommend genetic cascade testing from a genetically diagnosed proband to improve detection rates in younger patients so that they may be offered lipid lowering treatment.<sup>3</sup> A very rare autosomal recessive form of FH caused by variants in the *LDLRAP1* gene is also recognized.<sup>4</sup>

## The Wales FH Service

Wales has a well-established service for diagnosis and family testing of FH (since October 2010). This offers genetic testing to individuals with hypercholesterolaemia who present to lipid clinics across Wales on the basis of clinical signs, symptoms and family history<sup>5</sup>.

Patients with a clinical diagnosis of possible or definite FH are offered genetic testing for common FH mutations. If a variant is found in an index patient then family cascade testing is offered to relatives. Since October 2010, over 2000 index patients have undergone DNA testing for possible FH. A recognized pathogenic variant was found in approximately 21% of patients, and a further 7% of patients had genetic variants of uncertain significance (uncertain whether variant is pathogenic or non-pathogenic).<sup>6</sup> Seventy-two percent did not have variants in any of the genes being tested, which is a similar value to studies of lipid clinic patients reported in the United Kingdom where the majority of patients tested had possible FH as defined by the Simon Broome criteria<sup>7</sup>. Individuals who do not have a pathogenic variant are managed with lipid lowering treatment, but currently no further genetic testing is offered to their relatives. Their hyperlipidaemia is considered to be due to FH-causing variants in other genes not included in the test panel, for example in *ABCG5*<sup>8,9</sup> or

possibly in *APOE*<sup>10</sup> or in *STAP1*.<sup>11</sup> In the majority of cases it is believed that the cause is polygenic in nature.<sup>12</sup>

### **Cholesteryl Ester Storage disease (CESD)**

CESD is a lysosomal storage disease that is also known as Lysosomal Acid Lipase Deficiency (LAL-D). In CESD a deficiency of this enzyme leads to hyperlipidaemia and, in many patients, accumulation of lipids in the liver. There is a wide spectrum of severity, ranging from a severe early onset infantile form (Wolman Disease) through to a less severe, later onset adult form which is generally known as CESD. The majority of LAL-D patients present in childhood,<sup>13</sup> but there are some case reports of individuals presenting in adulthood with raised LDL-C similar to FH.<sup>14</sup>

In CESD the major problem is accumulation of cholesterol esters and triglycerides in lysosomes with multi-system effects including hepatic steatosis, hyperlipidaemia and premature atherosclerosis leading to early onset heart disease.<sup>15</sup> The frequency of shared symptoms with other conditions means that CESD is underdiagnosed. It has been reported that some patients with CESD may present with a lipid profile similar to that of FH.<sup>16,17</sup> Standard lipid lowering treatments have been shown to reduce the hyperlipidaemia in patients with CESD, but hepatic damage may still persist.<sup>18</sup> Therefore, it is important to accurately diagnose and treat CESD. Enzyme replacement therapy has been shown to effectively improve dyslipidaemia and liver abnormalities associated with LAL-D and has now been approved for use in both adults and children.<sup>19</sup>

CESD is an autosomal recessive condition resulting from variants in the *LIPA* gene. The most common variant associated with CESD is an exon 8 splice junction mutation (E8SJM; c.894G>A), which encodes mostly inactive LAL with approximately 3-8% of normal activity.<sup>20</sup> This mutation has been estimated to account for 51%-69% reported variants among multi-ethnic CESD patients.<sup>21</sup>

This variant was reported to have an allele frequency of 0.25% (1 in 200 persons) in a population cohort of 2023 German individuals enriched with patients attending Italian and German outpatient lipid clinics.<sup>22</sup> CESD heterozygotes in this study tended to have higher LDL cholesterol compared to controls and this reached statistical significance in men. A much larger study by Stitzel *et al* reported the allele frequency to be lower: 0.11% (1 in 450 persons out of 27,472 tested) in individuals of European ancestry.<sup>14</sup> In contrast to the findings of Muntoni *et al*, they found no significant difference in lipids (LDL-C, Triglycerides (Tg), HDL-C) between individuals heterozygous for E8SJM and control subjects.<sup>18</sup> A recent Portuguese study found four LAL-D homozygotes within a sample of patients with a clinical, but not a genetic diagnosis of FH. All four patients were found to have the common E8SJM variant.<sup>23</sup>

The current study aimed to determine whether the CESD (E8SJM variant) has been overlooked as a cause of hypercholesterolaemia in a Welsh cohort of adult patients with a clinical diagnosis of FH, but in whom the common FH genetic variants were absent.

### **Patients and methods**

Participants were adult probands ( $\geq 18$  years) with a clinical phenotype suggestive of familial hypercholesterolaemia who had undergone genetic testing for FH by the All Wales FH Testing Service and had a negative genetic test for FH. All participants provided written, informed consent for their stored DNA samples to be re-analysed for the current study. The study protocol conforms to the ethical guideline of the 1975 Declaration of Helsinki. A favourable ethical opinion was obtained from National Institute for Social Care and Health Research, Research Ethics Service: reference 14/WA/1008.

Stored samples were retrieved and aliquots sent to Cardiovascular Genetics, BHF Labs, Institute Cardiovascular Science, and University College London for analysis. DNA quantification was performed using the Nanodrop 800 (Thermo-Fisher Scientific, UK). The DNA was standardised in distilled water (Sigma-Aldrich (Gillingham, UK) to 15ng/ul then a further dilution down to 2.25ng/uL in Nunc polypropylene 96 well plates (Sigma-Aldrich). A positive DNA control heterozygote for rs116928232; c.894C>T was used. Genotyping was performed using TaqMan technology (Life Technology, Thermo-Fisher Scientific UK)). TaqMan primer and probes were provided by Synageva BioPharma Corp, Lexington, MA, USA.

#### **Further investigation of E8SJM heterozygotes.**

The coding region of the *LIPA* gene of identified c.984C>T heterozygotes was sequenced to check for the presence of another pathogenic variant to account for their phenotype. Primers were designed for sequencing the coding region and intron exon boundaries of the *LIPA* gene using Primer3 (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>). PCR was carried out using ACQUA PRC Mix (Applied Biosystems, Thermo-Fisher Scientific UK) in a 20  $\mu$ l final volume using a final concentration 0.4pmol/ul of each primer on a Bio Rad C1000 thermal cycler. PCR products were verified by electrophoresis on a 1.5% agarose gel and subsequently purified by GFX column (illustra GFX PCR DNA and Gel Band Purification Kit, (GE Healthcare, Cambridge, UK) before sequencing. Purified samples were sent to Source BioScience (Cambridge, UK) for sequencing. Subsequently, any amino acid changes observed were assessed in silico using Polyphen2, <http://genetics.bwh.harvard.edu/pph2/>, SIFT <http://sift.jcvi.org/> and Mutation Taster (<http://www.genomatix.de/matinspector.html>)

LAL enzyme activity in c.984C>T heterozygotes was also determined from dried blood spots by the Biochemistry Department, Yorkhill Hospital, Yorkhill, Glasgow, United Kingdom.<sup>24</sup>

#### **Results**

One thousand, two hundred and three FH variant negative index patients were invited to participate. Of these, 668 provided written consent and 663 had a sufficient stored sample for analysis.

Of the 663 genotyped only three were found to be c.984C>T heterozygotes.

Sequencing of the *LIPA* gene of these three participants showed that all three had one or more variants in Exon 2: rs1051338 and rs1051339. However, these two variants are frequent and in the signal peptide (amino acids 1-23) which is cleaved in the mature protein.

*In silico* assessment using Polyphen2, SIFT and Mutation Taster as shown (Table 1) indicates that these are not disease causing variants. Therefore, we conclude that none of the participants were found to have other variants in the *LIPA* gene that would give rise to compound heterozygosity.

Dried blood spot analysis showed that all three heterozygotes had reduced LAL activity, typical of heterozygote carriers, but not in the significantly deficient range. All three patients had normal liver function tests. Lipid profiles (pre and on treatment) are shown (Table 2). The allele frequency of the E8SJM c.984C>T in this cohort was 0.0023 or 1 in 221.

## Discussion

This study was undertaken to investigate the hypothesis that CESD may be overlooked as a possible cause of lipid alterations in a cohort of patients with clinically suspected FH, but where no FH variant has been identified. There are examples of other genes that have been identified as causing an FH like clinical presentation including *ABCG5*, *APOE*, and *STAP1* variants.<sup>8,10,11</sup> The range of symptoms associated with CESD is sufficiently diverse that an overlap with FH and potential masquerading as this condition is conceivable and was first suggested by a European study which included a general population sample and hyperlipidaemic patients.<sup>17</sup> Later that same year the suggestion of lipid alterations in E8SJM heterozygotes was challenged in a much larger European population cohort which reported no association of the E8SJM variant with either plasma lipid levels or MI/CAD risk and reported a lower carrier frequency of 0.16%.<sup>14</sup>

In the current study we have observed an allele frequency of 0.0023 or a carrier frequency of 1 in 221. This is similar to the observed allele frequency of 0.0025 in Muntoni's German, population based study.<sup>22</sup> Pullinger *et al*, 2015 used a similar methodology to the current study to determine the prevalence of the common E8SJM variant in 1357 patients with hyperlipidaemia in the USA.<sup>25</sup> These authors identified six CESD E8SJM heterozygotes (1 in 226), one of whom was found to be a compound heterozygote (frameshift variant involving an exon 4 deletion). A recent report from the Netherlands which included 213 adults and 63 children with a clinical, but no genetic diagnosis of FH, reported six heterozygous carriers of a potentially pathogenic variant in the *LIPA* gene.<sup>26</sup> Two of these patients were heterozygous carriers of c.894G>A variant. These authors reported finding no homozygous or compound heterozygous carriers of potentially pathogenic *LIPA* variants. A recent Norwegian study recently concluded that CESD was a very rare cause of hypercholesterolaemia when they observed only two patients with the E8SJM variant in a cohort of 3027 adult patients with an FH phenotype and none of the common variants in *LDLR*, *PCSK9* and *APOB*.<sup>27</sup>

In contrast to the studies discussed above, a recent Portugese study of patients with a clinical diagnosis of FH (n=492) and patients with dyslipidaemia and altered liver function (n=258) identified 26 different variants in the *LIPA* gene.<sup>23</sup> These included 10 common polymorphisms identified in several individuals, 16 rare variants, 15 not described before in LAL-D patients, and three homozygotes with the c.894 E8SJM. All three E8SJM homozygotes

were referred to the service as children and were in the FH variant-negative group. Following cascade screening, one child was found to have a homozygous sibling. No adults with the homozygous E8SJM were found. In all families with LALD patients, some, but not all carriers had altered lipid metabolism. This reflects the inconsistencies regarding the association between hyperlipidaemia and heterozygous E8SJM reported in previous studies. Moreover, this study illustrates the importance of establishing care pathways for unexplained hyperlipidaemia in children. In the absence of a genetic diagnosis of FH it may be pertinent to consider dried blood spot testing for LALD as a diagnostic test especially if there are disturbed liver function tests and raised triglyceride.

The strategy of screening for the most common CESD variant (E8SJM) and subsequent full sequencing in carriers has been shown to be an efficient means of detecting carriers and combined heterozygosity at the *LIPA* locus.<sup>25</sup> Therefore, our results suggest that the CESD E8SJM does not appear to be enriched in this Welsh cohort of hyperlipidaemic patients with a clinical, but no genetic diagnosis of FH. We conclude that testing for this variant in the *LIPA* gene is not clinically helpful in our cohort of adult FH patients and should not be added to our routine panel of genetic tests for hypercholesterolaemic patients with a phenotype of FH. It should be reserved for children with raised triglyceride and cholesterol with unexplained liver steatosis.

### **Conflict of interest statement**

Pauline Ashfield-Watt and Ian McDowell received funding from Synageva BioPharma in the form of a project grant as described below. Steve Humphries is the Medical Director and minority shareholder of a UCL spin-out company called StoreGene, which uses a 20 SNP genetic test, in combination with the classical risk factor profile, for estimating an individual's future risk of CVD, and which offers FH genetic testing via an accredited DNA diagnostic laboratory. No other authors have declared any conflict of interest.

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**Ethical approval:** A favourable ethical opinion was obtained from National Institute for Social Care and Health Research, Research Ethics Service, Wales, UK: reference 14/WA/1008.

**Guarantor:** IMcD

**Contributorship:** IMcD, PAW and KH researched the literature and developed the protocol. PAW and IMcD obtained ethical approval. PAW contacted the patients and co-ordinated sample retrieval and transport between labs. RE, DT and RG liaised with patients for additional study specific blood samples. SEH and KWL analysed the samples. PAW drafted the manuscript and all authors reviewed it and approved the final version.

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