

Whole exome sequence analysis reveals a homozygous mutation in *PNPLA2* as the cause of severe dilated cardiomyopathy secondary to neutral lipid storage disease

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Neutral lipid storage disease with myopathy (NLSD-M; *PNPLA2*) is a rare, mostly autosomal recessive disorder, caused by mutations in the gene encoding patatin-like phospholipase domain-containing protein 2 (*PNPLA2*), also known as adipose triglyceride lipase (ATGL). ATGL catalyses the breakdown of triacylglycerol (TAG) to diacylglycerol. Dysfunction of ATGL leads to accumulation of TAG-containing cytoplasmic vacuoles in most tissues [1]. Only about 40 patients have been described with NLSD-M worldwide. Clinical features include progressive myopathy, TAG-containing cytoplasmic vacuoles in leukocytes in peripheral blood smears (Jordan's anomaly) and raised CK levels. Cardiomyopathy is reported in 44% of patients [2]. Other clinical manifestations are hypertriglyceridemia, diabetes mellitus and pancreatitis.

A 22 year old computer engineer with consanguineous Pakistani parents presented with syncope and shortness of breath. Further questioning revealed that he had never been able to keep pace with his friends and experienced muscle aches after exercise. Several years previously the patient's alanine transaminase (ALT) and aspartate transaminase (AST) had been elevated at 360 IU/L (reference range 0 – 52) and 93 IU/L (0 – 40) respectively on routine blood tests. A raised creatinine kinase (CK) level had also been noted at 2341 U/L another time. On both occasions no cause was identified. The patient is married to his first cousin. There is a family history of sudden death at a young age in his wife's mother and elder sister, who also suffered from muscle weakness. Furthermore, the patient's aunt is known to have dilated cardiomyopathy (see Figure 1a).

Physical examination revealed a pan-systolic murmur loudest in the mitral area. There was winging of the scapulae, weakness of the small muscles of the hand, deltoid and spinati with relative sparing of biceps and triceps muscles. Lower limb examination revealed weakness of hip and knee flexion, with preservation of hip extension, abduction and adduction. He

had marked weakness of ankle dorsiflexion, eversion and inversion, and mild weakness of planter flexion (4/5). Blood results included a raised CK at 2221 U/L (30 – 210), troponin I at 0.48 µg/L (0 – 0.05), NT-PRO BNP at 2717 ng/L (2.5 – 110.0) and ALT at 89 U/L (0 – 52). A 12 lead Electrocardiogram (ECG) showed sinus rhythm at a rate of 61 bpm, left axis deviation, a QRS duration of 100 ms, poor R wave progression across the chest leads and T wave inversion in lead V6. A transthoracic echocardiogram showed a dilated (diastolic dimension 60 mm) and severely impaired left ventricle with an ejection fraction of 20% and restrictive filling pattern. There was moderate mitral regurgitation. The right ventricle was not dilated, but had severely impaired long-axis and radial function (see Figure 1b). Cardiac MRI scan confirmed a severe dilated biventricular cardiomyopathy with wall thinning and akinesia of the left inferolateral wall. There was a moderate, posteriorly directed jet of mitral regurgitation due to restriction of the posterior leaflet secondary to LV dilatation. Sub-endocardial Gadolinium enhancement was noted in the anterior, lateral and inferior left ventricular walls with circumferential extension of the enhancement in the apex (see Figure 1c). A 24 hour holter-monitor recorded one run of non-sustained ventricular tachycardia. Electromyography was indicative of a myopathy and computer-tomography of the thighs revealed muscular fatty infiltration. A muscle biopsy (from the gastrocnemius) showed excess lipid on Sudan black staining consistent with lipid myopathy (see Figure 2b). Treatment with a beta-blocker, ACE-inhibitor, Eplerenone and Warfarin was commenced and a defibrillator implanted.

We performed targeted exome sequence capture on peripheral blood DNA samples from the patient using the SureSelect Human All Exon v4 kit (Agilent). The Illumina HiSeq system was used to generate sequence data. The resultant paired end sequencing data were aligned against the human genome reference sequence 19 (hg19) using the Novoalign

software (Novocraft Technologies, Selangor, Malaysia). Duplicate reads, resulting from PCR clonality or optical duplicates, and reads mapping to multiple locations were excluded from downstream analysis. Depth and breadth of sequence coverage was calculated with custom scripts and the BedTools package [3]. Single nucleotide substitutions and small insertion deletions were identified and quality filtered within the SamTools software package [4] and in-house software tools. Variants were annotated with respect to genes and transcripts with the Annovar tool [5]. A total of 23,419 variants were identified. Variants were characterised as novel if they were previously unreported in the dbSNP137, 1KG data and 968 in-house reference exomes. Filtering resulted in 22 homozygous variants that were either non-synonymous or frameshift. Out of those, 11 were predicted to be damaging using online in-silico prediction tools (SIFT, Polyphen). A homozygous missense mutation c.497A>G (p.D166G) in *PNPLA2* encoding ATGL was deemed to be the causative mutation given the patient's phenotype (see Figure 2a).

Screening of the family confirmed his parents and wife to be carriers of the mutation. They were asymptomatic and physical examination, ECGs and echocardiograms were normal. The patient's wife was pregnant and unfortunately antenatal screening revealed that the child was homozygous for the mutation. The parents decided to terminate the pregnancy. After improvement of the patient's shortness of breath following initiation of medical therapy, his symptoms deteriorated and he underwent cardiac transplantation. A diagnostic endomyocardial biopsy prior to the transplantation and histological examination of the explant heart both showed extensive interstitial fibrosis (see Figure 2c), myocyte hypertrophy and fine cytoplasmic vacuolation (see Figure 2d). The patient currently remains well on follow up in NYHA class 1.

In summary, we report a 22 year old patient presenting with DCM secondary to a homozygous mutation c.497A>G (p.D166G) in the catalytic domain of *PNPLA2*. The majority of less severely affected patients with NLS-D-M harbour non-sense mutations in the C-terminal region which disrupt only the hydrophobic portion of the protein that binds to the lipid droplets [7]. The c.497A>G (p.D166G) mutation has previously been reported in a patient also presenting with DCM in his 20s. Biochemical investigation showed intact intracellular localization of ATGL and binding to the lipid droplets, but total loss of catalytic activity [1]. Studies of ATGL-knockout mice have suggested that the cardiomyopathy phenotype is a result of the inability of the cardiac muscle to utilize free fatty acids, which are taken up from the plasma and rather than directly used for β -oxidisation, are re-esterified to triglycerides. In the presence of ATGL deficiency these then accumulate in the cardiomyocytes and cause hypertrophy and subsequent cardiac failure [1, 8]. This may explain why mutations affecting the catalytic domain of the enzyme, leading to a total loss of function of ATGL as in the described case, lead to an earlier and more severe cardiac phenotype than mutations in the hydrophobic portion of the protein, which impair the binding to the lipid droplets but do not lead to a total loss of activity.

In a review of 37 published cases of NLS-D-M 44% had cardiomyopathy, of which 44% were dilated, 31% hypertrophic and 25% an undetermined form of cardiomyopathy [2]. In most patients cardiomyopathy manifests after the development of myopathy [9]. In one report heterozygous relatives of an affected individual exhibited muscle weakness and pain, varying degrees of neutral lipid storage in muscle and Jordan's anomaly [10]. Therefore carriers of mutations in *PNPLA2* should also carefully be evaluated. Our patient had raised ALT, AST and CK levels as a teenager and further investigations might have led to the diagnosis at an earlier stage. A prompt diagnosis will become even more important in the

future as promising therapeutic approaches have been reported. In one study overexpression of *PNPLA2* reduced the number and area of cellular lipid droplets in fibroblasts from affected patients. Enzyme replacement therapy may therefore become a viable treatment as it is in glycogen storage disease 2 and Fabry disease [11]. There is also evidence that β -agonists directly activate hormone-sensitive lipase (LIPE) which may compensate for the disturbed function of *PNPLA2*. In vitro studies on patients' myoblasts suggest partial rescue of TAG breakdown after supplementation with clenbuterol [9]. However, treatment with β -adrenergic agents in patients with cardiomyopathy would obviously have to be considered carefully. *ATGL*-deficient mice develop a lethal cardiomyopathy. They exhibit diminished cardiac peroxisome proliferator-activated receptor (PPAR) activity and mitochondrial function, and treatment with PPAR agonists restored cardiac function and prevented premature death in one study. In a human study bezafibrate (a PPAR- α agonist) treatment in an individual with NLS-D-M resulted in a marked lowering of cardiac and muscle fat content [12].

In conclusion, we describe NLS-D-M as a rare cause of DCM. The potential severity of disease progression and possible therapeutic options in the future illustrate the importance of establishing a diagnosis promptly. Our case illustrates the utility of whole exome sequence analysis in establishing a diagnosis and aiding management in rare cardiovascular disorders.

As sequencing costs are dropping rapidly and modern sequencers are able to produce large volumes of data, whole genome sequencing will also become increasingly useful in clinical application delivering a more comprehensive view of the entire genome and enabling the identification of disease causing variants outside the coding regions of the genomic DNA implicated in regulatory or promotor function. Last but not least, Cardiologists should keep lipid myopathies, muscular dystrophies and myotonic dystrophies in mind as a differential

diagnosis for cardiomyopathies in the presence of raised CK levels and skeletal muscle weakness.

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Acknowledgements: This study was funded by St. George's Charitable Foundation and the research was supported by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Guy's and St Thomas' NHS Foundation Trust and King's College London.

Disclosures: There are no disclosures

Figure legend:

Fig.1

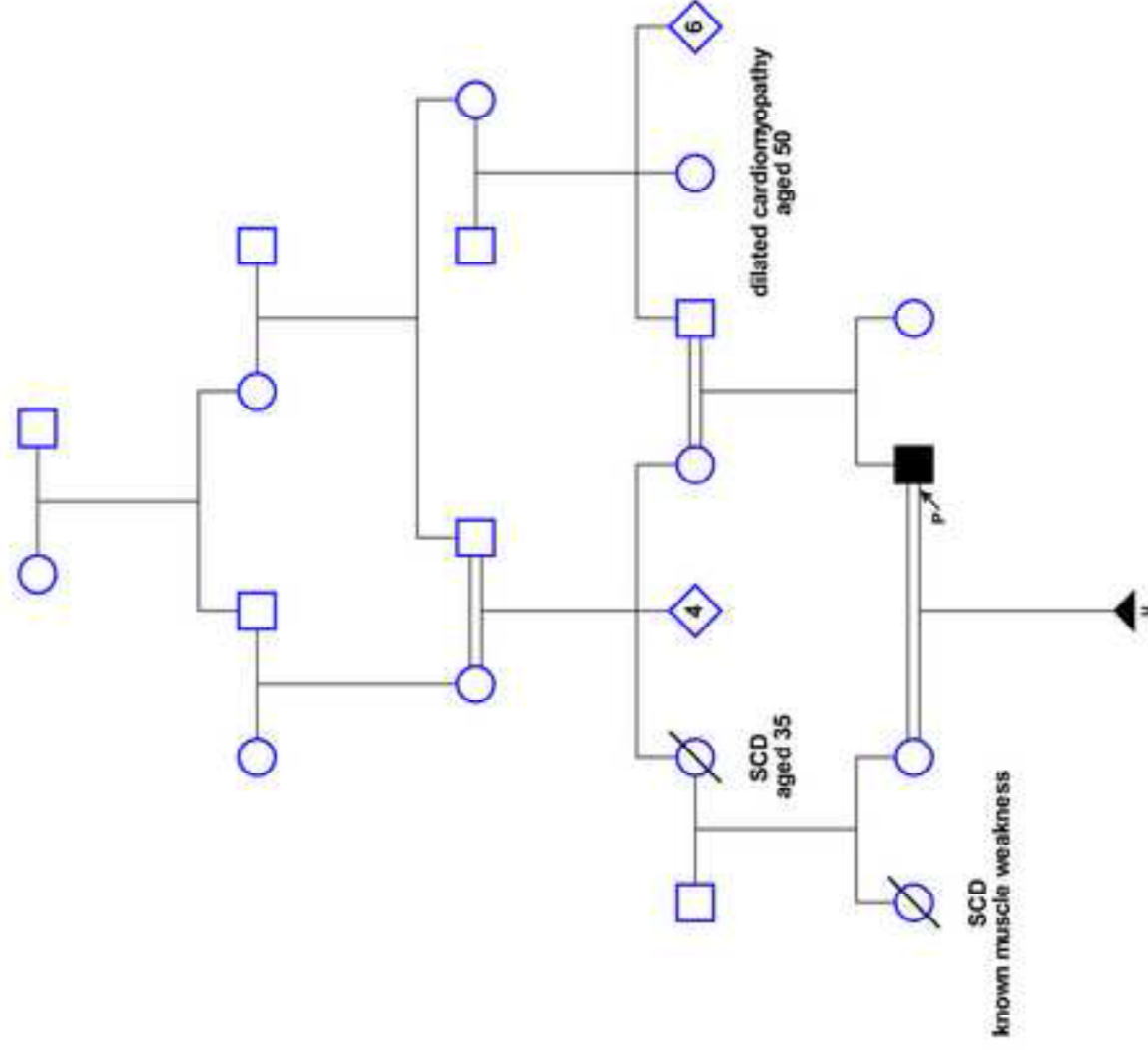
A) Pedigree of the family. Affected individuals are represented by black symbols. B) Parasternal long-axis and B') parasternal short-axis view on transthoracic echocardiography showing dilated left and right ventricles; note the presence of the defibrillator lead in the right ventricle . C) Cardiac MRI horizontal long axis view showing late Gadolinium enhancement in the lateral wall.

Fig.2

A) Sanger sequencing validation of *PNPLA2* (NM_020376:c.497A>G; p.Asp166Gly) homozygous variant (upper panel) identified by whole exome sequencing. B) Muscle biopsy cryosection stained for lipid. Note large lipid globules in a sub-sarcolemmal location. Sudan black, objective lens magnification 40x, scale bar = 50 microns. C) Section of the left ventricle from explant heart showing extensive interstitial fibrosis. Haematoxylin and eosin stain. D) Section of the explant heart showing myocyte hypertrophy and fine cytoplasmic vacuolation. Haematoxylin and eosin stain.

A

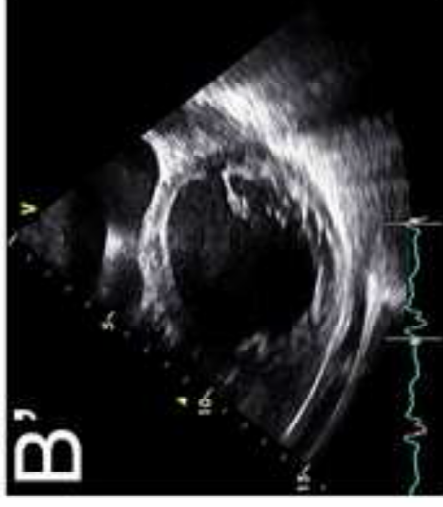
Family PNPLA2



B



B'



C

