Challenges in molecular diagnosis of Wilson disease

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A HOLISTIC APPROACH IS STILL REQUIRED FOR THE DIAGNOSIS OF WILSON'S DISEASE

The study by Poon *et al*[1] outlines the issues associated with the use of current molecular diagnostic methodologies to rule in or rule out a diagnosis of Wilson's disease (WD) or primary copper excess.[1] WD is an autosomal recessive condition caused by pathogenic variants in the *ATP7B* gene, which encodes P-type ATPase protein/enzyme. Loss-of-function of the protein prevents incorporation of copper into caeruloplasmin, with reduced biliary copper excretion and concomitant copper deposition in hepatic parenchymal cells, the brain, kidneys and cornea.[2] Individual approaches to WD diagnosis have poor diagnostic accuracy. Clinical symptoms (*e.g.*, neurological decline) are ambiguous, clinical signs (*e.g.*, Kayser-Fleischer rings) take time to manifest, appropriate laboratory tests (*i.e.*, serum copper and caeruloplasmin, and urinary copper excretion) may be incomplete or inconclusive and genetic testing may miss rare variants. However, when these approaches are used synergistically for WD diagnosis, accuracy improves.

In this study, clinical information was available for 27 of the 51 patients who had WD genetic testing. Eight of these 27 patients had a clinical diagnosis of WD based on clinical presentation, Kayser-Fleischer rings and/or laboratory testing. In the remaining cases genetic testing was performed to exclude the condition. Of the eight patients with WD, three had no genetic diagnosis. Interpretation of genetic variation in the *ATP7B* gene and the clinical significance of variants identified is fraught with difficulty. It is well established that genetic variation does not always impact protein functionality. Interpretation of genetic variants necessitates the consistent use of guidelines and Poon *et al* have used those of the American College of Medical Genetics & Genomics/Association of Molecular Pathology (ACMG/ACP) which are generally accepted. Building on these, international expert clinical groups, such as the various ClinGen Variant Classification Expert Panels (VCEP), are evolving, which are able to 'fine tune' and adjust the basic ACMG/AMP guidelines to specific disease-gene pairs. Also as Poon *et al* point out, this necessarily relies heavily on comprehensive up-to-date variant databases, contributed to and curated in perpetuity by the clinical and laboratory community (*e.g.*, https://databases.lovd.nl/shared/genes/ATP7B, which, it is noted, is currently lacking a curator).[3] Unfortunately, at the date of writing, there is no VCEP or equivalent for WD, and while many variants are reported to ClinVar, in the absence of a VCEP this resource is not curated and thus of limited utility. As the authors highlight, interpretation of putative missense and splicing variants is particularly difficult. Indeed, in many diseases a large proportion of putative missenses are actually pathogenic because they cause aberrant splicing. While supporting evidence for these types of variant may come from *in vitro* functional studies of both protein and mRNA splicing, these are not always translatable to the *in vivo* setting.

The absence of a genetic diagnosis in the cases outlined could, therefore, be due to a number of reasons, both biological and technical: for example, that the genetic variant was undetectable with the techniques used; was missed by the bioinformatic analysis of the raw sequencing data; that somatic mosaicism rendered the cellular content insufficient to detect a genetic variant; something more complex.[4] Alternatively, and perhaps most likely, that the initial clinical diagnosis of WD was simply incorrect.[5] Ultimately, failure to achieve a genetic diagnosis does not exclude a diagnosis and may be evidence of genetic heterogeneity, that is, that pathogenic variants in another gene or genes may also be responsible for WD. Familial hypercholesterolaemia is a prime example of genetic heterogeneity: pathogenic variants in *APOB*, *LDLR*, *LDLRAP1* and *PCSK9* can all be causative, and some patients may have variants in more than one gene, often leading to more severe and intractable disease.

In addition to the complexities of genotypic diagnosis, biochemical assessment of phenotype, that is, copper status can be problematic, and uncertainty about the clinical diagnosis makes interpretation of a genotype that much more difficult. Unless the patient is severely deficient, serum copper measurement can be inadequate as a biomarker of copper status.[6] Measurement of the activity of copper-containing enzymes may prove more telling or adjustment of copper for caeruloplasmin could more accurately represent copper status in WD.[7–10] Aside from copper excess or deficiency, circulating copper/caeruloplasmin concentration can also be affected by age, gender, oestrogen, the acute phase and protein losing conditions, among others.[2 11] Serum caeruloplasmin measurement also has confounders. Serum caeruloplasmin concentrations overlap in heterozygous WD carriers, homozygous WD patients and healthy control subjects.[11] Poor commutability of caeruloplasmin reference materials has led to a lack of method standardisation.[12] Consequently, interlaboratory differences in reference intervals and clinical decision limits used for interpretation of caeruloplasmin and copper-caeruloplasmin associated indices are evident.[6 13 14] Immunoassays, routinely used for serum caeruloplasmin measurement, detect not only the copper-bound enzymatically active caeruloplasmin but also the less important apo-caeruloplasmin or copper free-caeruloplasmin. To avoid this and for the diagnosis and/or management of WD patients, it has been recommended that caeruloplasmin is measured using only its oxidase activity,[11] however, this is still not practiced (recent in-house review of United Kingdom National External Quality Assessment Scheme (UKNEQAS) External Quality Assessment/Proficiency testing returns) probably because there may not be a CE marked or equivalent assay available. Routine clinical laboratory tests are being evaluated to assess non-caeruloplasmin copper to improve the diagnosis of copper toxicity but are yet to become mainstream.[15]Elevated urinary copper excretion and liver biopsy will be more useful in establishing the diagnosis but are more cumbersome and invasive, respectively.

Genetic testing using current technologies in combination with information gleaned from gene databases, but in the absence of a VCEP or equivalent, may identify only a fraction of loss-of-function *ATP7B* variants with rarer ones undoubtedly missed. The authors demonstrate that while certain variants are common to their own population (as, genetically, a population is defined by the frequency of its alleles), targeting of these variants

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alone, even locally, would be insufficient for a genetic diagnosis of WD. Poon *et al* comment on the hazards of only testing for so-called known variants. In other settings, such as pharmacogenetics, studies consistently show that around a third of pathogenic variants are rare or even unique, which necessitates comprehensive genetic analysis to make a meaningful test. Diagnostic yield is low for Sanger sequencing-based genetic testing given an inherent inability to detect large deletions, duplications or gene rearrangements. Sanger sequencing is also relatively expensive and time consuming. The authors use of multiplex ligation-dependent probe amplification (MLPA) in addition to Sanger is a means to mitigate some of these challenges, as MLPA identifies exon deletions and duplications. However, when used together in patients previously determined to have no or only one pathogenic WD variant, large deletions and large duplications were shown to be infrequent in the *ATP7B* gene. Although there are still gaps in our knowledge this finding may make the use of MPLA redundant in the context of WD. In addition, next generation sequencing with specialist bioinformatic analysis to detect large deletions, duplications and rearrangements is rapidly replacing Sanger sequencing and becoming the norm in genetic laboratory practice (https://www.england.nhs.uk/genomics/nhs-genomic-med-service/).

The presentation of WD is known to be highly variable with no individual laboratory test capable of its diagnosis.[11] Therefore, we would advocate use of a clinical scoring system, such as the Leipzig scoring system[16] advocated by the European Association for the Study of the Liver,[17] but with method-dependent population-validated cut-offs,[18] as well as the setting up of a VCEP, or equivalent, for WD.

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