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# KCNQ1 p.L353L affects splicing and modifies the phenotype in a founder population with long QT syndrome type 1

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## ABSTRACT

**Background** Variable expressivity and incomplete penetrance between individuals with identical long QT syndrome (LQTS) causative mutations largely remain unexplained. Founder populations provide a unique opportunity to explore modifying genetic effects. We examined the role of a novel synonymous *KCNQ1* p.L353L variant on the splicing of exon 8 and on heart rate corrected QT interval (QTc) in a population known to have a pathogenic LQTS type 1 (LQTS1) causative mutation, p.V205M, in *KCNQ1*-encoded Kv7.1.

**Methods** 419 adults were genotyped for p.V205M, p.L353L and a previously described QTc modifier (*KCNH2*-p.K897T). Adjusted linear regression determined the effect of each variant on QTc, alone and in combination. In addition, peripheral blood RNA was extracted from three controls and three p.L353L-positive individuals. The mutant transcript levels were assessed via qPCR and normalised to overall *KCNQ1* transcript levels to assess the effect on splicing.

**Results** For women and men, respectively, p.L353L alone conferred a 10.0 ( $p=0.064$ ) ms and 14.0 ( $p=0.014$ ) ms increase in QTc and in men only a significant interaction effect in combination with the p.V205M (34.6 ms,  $p=0.003$ ) resulting in a QTc of ~500 ms. The mechanism of p.L353L's effect was attributed to approximately threefold increase in exon 8 exclusion resulting in ~25% mutant transcripts of the total *KCNQ1* transcript levels.

**Conclusions** Our results provide the first evidence that synonymous variants outside the canonical splice sites in *KCNQ1* can alter splicing and clinically impact phenotype. Through this mechanism, we identified that p.L353L can precipitate QT prolongation by itself and produce a clinically relevant interactive effect in conjunction with other LQTS variants.

## BACKGROUND

Long QT syndrome (LQTS) is characterised by a prolongation of the heart rate corrected QT interval (QTc) on 12-lead ECG and can predispose to syncope, seizures and sudden death if its trademark arrhythmia of torsades de pointes occurs.<sup>1</sup> Tragically, sudden death may be the first manifestation. Approximately 80% of congenital LQTS is caused by mutations in at least 17 LQTS susceptibility genes which predominantly encode for either pore-forming ion channel subunits or

channel-interacting proteins.<sup>2</sup> *KCNQ1* encodes for the  $\alpha$ -subunit of the slow delayed rectifier potassium channel,  $I_{Ks}$  (Kv7.1), and is the gene responsible for LQTS type 1 (LQTS1).<sup>3</sup> To date, hundreds of *KCNQ1* mutations have been identified, including missense, nonsense, canonical splice site, in-frame insertions/deletions and frameshift mutations. Incomplete penetrance and variable expressivity are well recognised within LQTS1 families.<sup>4–7</sup> The genetic underpinnings for the heterogeneity in expressivity and penetrance largely remain unexplained.<sup>4–8</sup>

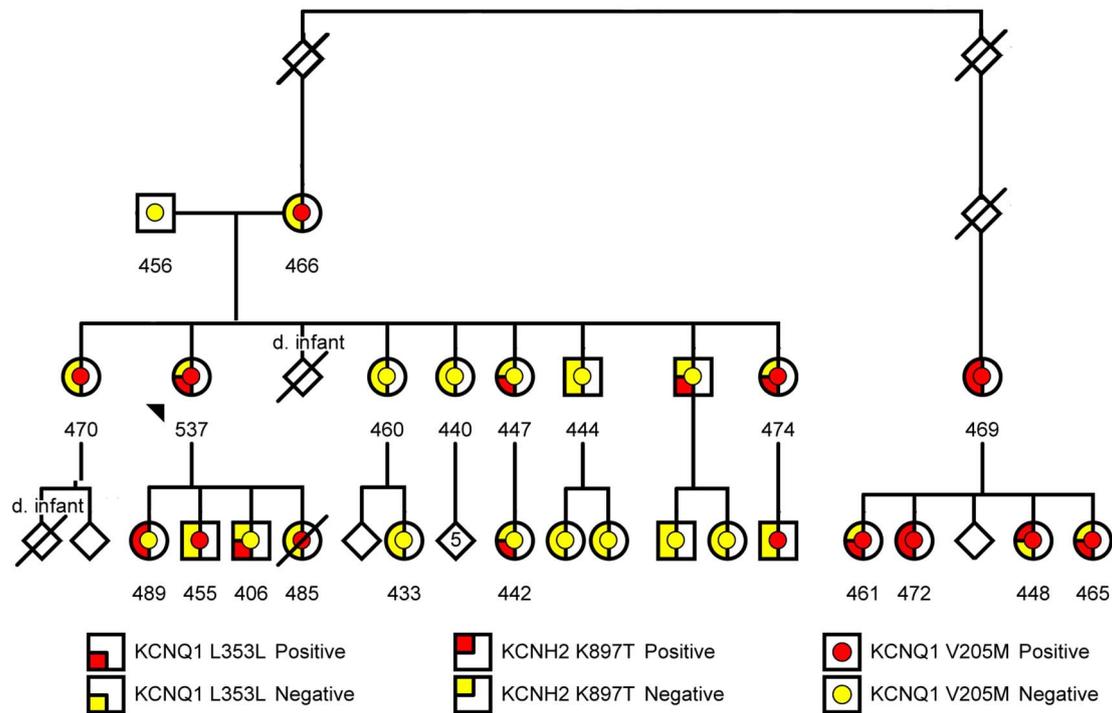
Although LQTS is relatively rare (1:2000),<sup>9</sup> it is highly prevalent (~1:125)<sup>10</sup> in a remote Canadian First Nations community of 5500 people in northern British Columbia (the Gitksan). Through community initiation, those diagnosed with LQTS and their relatives (~800) have participated in our research which identified a novel c.613 G>A missense variant in *KCNQ1*. As previously described, this mutation results in a valine to methionine substitution at position 205 (p.V205M) in Kv7.1's S3 transmembrane region and negatively impacts  $I_{Ks}$  by slowing activation and accelerating deactivation.<sup>11–12</sup> Besides the functional evidence for loss-of-function, a prolonged QTc segregates with p.V205M-positive status compared with variant-negative relatives and associated sudden cardiac death (SCD) further supports its designation as a LQTS1 pathogenic variant.<sup>10–11</sup> Typical for LQTS, the phenotype is variable in this population, with some presenting earlier and more severely than others and sex differences in presentation have been noted. Although inherited LQTS traditionally has been considered to be a single gene disorder, evidence supports that the presentation is determined by additional genes and interactions with exogenous triggers, such as QT-prolonging drugs, physical activity and emotion. Indeed, LQTS is considered a model for complex inheritance.<sup>13</sup> Therefore, this founder population provides a unique opportunity to identify genetic or environmental modifiers of disease in LQTS.

In 2004, sequencing of the most common genes causing LQTS in a First Nations index case (see figure 1) detected the p.V205M variant,<sup>11</sup> along with a novel synonymous variant, a c.1059 G>A substitution in the *KCNQ1* gene that maintains the original amino acid (Leucine) at position 353



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**Figure 1** Pedigree of Family 1 harbouring the *KCNQ1* p.V205M, p.L353L and *KCNH2* p.K897T variants. The arrow indicates the proband. Note symbols representing the variant status and corrected QT (QTc) values (ms) below each participant.

(p. L353L). The significance of p.L353L was not clear, but it emerged again within the population when further molecular studies of the variable phenotype were initiated. Through pedigree analysis, p.L353L was confirmed to be inherited in *trans* to the p.V205M mutation. Population studies have confirmed the variant to be prevalent in the community, and to date, we have identified more than 70 adults with the p.L353L variant. This variant is not documented in Ensembl, dbSNP and ExAC.<sup>14</sup> We sought to determine the underlying role of the p.L353L variant on the splicing of *KCNQ1*'s exon 8, its potential QT-prolonging effect alone and as a genetic modifier in patients with p.V205M-mediated LQTS1.

## METHODS

### Enrolment

As part of a larger study and in keeping with participatory methods,<sup>11</sup> participants were invited to enrol if they had clinical features of LQTS or were related to an individual with a diagnosis of LQTS. Referrals for study entry were through affected family members and physicians. Community level approval was obtained as well as individual informed consent from all participants. Health information was documented upon enrolment through questionnaire and medical records to confirm clinical diagnosis or suspicion of LQTS. Chronic diseases such as cardiovascular disease, diabetes, autoimmune disease, medications, alcohol use and street drug use were also recorded when possible.

### Genetic analysis

Blood or saliva samples were collected and DNA was extracted and stored by standard methods and in keeping with DNA on Loan agreement with the community.<sup>15</sup> Genotyping was carried out for the known *KCNQ1* c.613 G>A (p.V205M) LQTS1-causing variant as previously described<sup>11</sup> and for a second *KCNQ1* known disease-causing variant (p.R591H) documented in an adjacent First Nations community<sup>10</sup> in the BC Provincial Health Services Authority (PHSA) lab of the BC

Children's Hospital, Vancouver. Expanded LQTS gene sequencing was carried out initially on five participants with a diagnosis of p.V205M-mediated LQTS1 and as well for another 37 participants with a borderline or increased QTc without the p.V205M mutation. The *KCNQ1* p.L353L variant was observed in several of those who had expanded sequencing in this initial study, and the previously published and controversial *KCNH2* p.K897T variant<sup>16–18</sup> was also noted frequently. Genotyping for the *KCNQ1* p.V205M, p.R591H, p.L353L and *KCNH2* p.K897T variants was then carried out on all participants over the age of 16.

### QTc determination

At enrolment, an ECG was performed and all other available 12-lead ECGs were collected from medical files and reviewed. Heart rate QTc measurements were assessed blinded to variant and clinical status on all available 12-lead ECGs. QT intervals were determined in all leads by the tangent method<sup>19</sup> and the longest QT interval in any lead for an individual in any available ECG recording, corrected for rate, was considered the *peak* QTc.<sup>20–21</sup> The peak QTc for each participant was recorded and used for analysis. Twelve hundred and sixty-nine manually read ECGs (mQTc) accounted for 61% of the eligible study population (257 persons). For those without mQTcs (162 persons), the Stata 13-IC program was used for the linear interpolation of mQTc on cQTcs (ECG computer calculated QTc) for the missing values of the mQTcs.

### In silico splicing predictions

Four in silico predictive algorithms (Sroogle<sup>22</sup> (<http://sroogle.tau.ac.il/>), ESEfinder<sup>23</sup> ([http://rulai.cshl.edu/cgi-bin/tools/ESE3/ese\\_finder.cgi?process=home](http://rulai.cshl.edu/cgi-bin/tools/ESE3/ese_finder.cgi?process=home)), RESCUE-ESE<sup>24</sup> (<http://genes.mit.edu/burgelab/rescue-ese/>) and Human Splicing Finder<sup>25</sup> (<http://www.umd.be/HSF3/>)) were used to predict the impact of the p.L353L on splicing regulatory motifs. All tools were used with default settings.

### RNA extraction and RT-PCR

Total blood RNA was obtained with the PAXgene System (Qiagen, Hilden, Germany) from three p.L353L-positive and two p.L353L-negative individuals from the First Nations population and one control from outside the First Nations population. cDNA was generated using the iScript Select cDNA Synthesis Kit (Bio-Rad Laboratories, Munich, Germany). Previously published primers<sup>26</sup> were used to PCR amplify the *KCNQ1* cDNA from exons 5 (5F: 5'-GGGCATCCGCTTCC TGCAGA forward) to 10 (10R: 5'-CATTGTCTTTGTCCAGC TTGAAC reverse) for gel electrophoresis. The relative levels of total *KCNQ1* transcript were compared between p.L353L-positive and p.L353L-negative individuals using the comparative 2<sup>-ΔΔC<sub>t</sub></sup> method where the samples were normalised to the total *GAPDH* levels using PrimeTime qPCR primers for *GAPDH* (Integrated DNA Technologies) and an exon 9 forward primer (9F: 5'CGCATGGAGGTGCTATGCT) and the exon 10 reverse primer for *KCNQ1* levels. The previously validated forward primer spanning exon 7 and exon 9 boundaries (7.9F: 5'-CTTTGCGCTCCCAGCG/ACCG, 'p' indicates the exon boundaries) and exon 10 reverse primer was used for the selective amplification of the mutant transcript (Exon 7.9). A forward primer spanning the exon 8 and exon 9 boundaries (e8.9F: 5'CAGCCTCACTCATTACG/ACCG) was used to assess the wild-type (WT) exon 8 splicing levels. Absolute transcript levels were assessed via a standard curve for each primer pair obtained using serial dilutions of a recombinant plasmid-containing cDNA. All RT-PCR experiments were run on an ABI PRISM 7900HT System (Applied Biosystems) using the QuantiTect SYBR Green PCR Kit (Qiagen). All samples were tested in triplicate. The transcript values were normalised to the total *KCNQ1* transcript levels and were reported as mean ± SD.

### Statistical analysis

Unadjusted column statistics for average QTc were carried out separately for men and women in each mutation/variant category and for all those negative. Following preliminary descriptive analyses, linear regression (Ordinary Least Squares, OLS) was used to explore the relationship of the genetic variants on QTc. Bivariate and multivariate analyses were carried out considering the *KCNQ1* p.V205M, *KCNQ1* p.L353L, *KCNH2* p.K897T variants independently, and in combination, adjusting for age, chronic disease (cardiovascular and autoimmune), past and current alcohol abuse and prescribed medications known to prolong the QT interval. An indicator variable was included to account for the potential influence of the interpolated QTc (1.96 and 3.26 ms in the female-adjusted and male-adjusted models, respectively). Akaike's information criterion was used in model selection for best fit regarding the addition of the confounding factors. The analyses were run separately for men and women. Robust SEs (Huber-White sandwich estimate of variance) were applied to account for the borderline heteroscedasticity present in the model residuals.<sup>27</sup> Bonferroni correction was performed on multiple pairwise testing adjusted for up to 28 comparisons (table 3). *Stata 13-IC* was used for all statistical analyses.

Incorporation of monomers encoded by full-length or mutant Kv7.1 into tetramers was modelled statistically using binomial distribution as previously described.<sup>28</sup>

## RESULTS

### First Nations participants

Four hundred and forty-two adults above age 16 years (302 women and 140 men) were eligible for analysis. Four women

homozygous for the p.V205M mutation and subjects of a previous publication were excluded.<sup>21</sup> One man was excluded in all analyses as an outlier. This man was positive for both the *KCNQ1* p.L353L variant and the *KCNH2* p.K897T variant, but had been treated with arsenic trioxide, a chemotherapeutic agent which is also a potent QT-prolonging drug.<sup>29</sup> To ensure the validity of our data, participants with other variants which might affect the phenotype, including the *KCNQ1* p.R591H variant present in a nearby community,<sup>10</sup> were omitted from our analysis. See table 1 for details of the genotypes of remaining participants (n=419) included in this study. There were 61 individuals positive for the *KCNQ1* p.V205M mutation alone, 55 positive for the p.L353L variant alone and 23 positive for the *KCNH2* p.K897T variant. In total, 12 persons were positive for the p.V205M mutation and the p.L353L variant, and 8 others had various combinations of the three genetic variants. Ninety-one men and 169 women were negative for all three variants.

### QTc comparisons

Unadjusted mean QTc values with 95% CIs for those negative for all variants, positive for p.V205M, p.L353L, p.K897T each alone and combined p.V205M\*p.L353L, p.L353L\*p.K897T and p.V205M\*p.L353L\*p.K897T are shown in table 1.

### Regression analyses

See table 2: For men (n=137), the reference (baseline) QTc was 418.4 ms when adjusted for age, alcohol use, QT-prolonging drug use and cardiovascular disease. The p.L353L variant alone increased the QTc by 14.0 ms (p=0.014, see table 2A), whereas the p.V205M mutation alone increased the QTc by 29.3 ms (p<0.001) above baseline. However, when the p.V205M\*p.L353L variant combination was inherited, the QTc was predicted to increase by another 34.6 ms (p=0.003) above that dictated by each variant alone. When holding all variants at their mean QTc and controlling for the above covariates (also held at their mean), the predicted adjusted means demonstrate that those men with the combination of p.V205M\*p.L353L variants will have a QTc approaching 500 ms (see figure 2A). This result was significantly different than for p.L353L or p.V205M variants alone (p<0.014, p<0.001, respectively) and for those with no documented variants. These results remained significant after Bonferroni correction for multiple testing (see table 3A).

For women, after adjustment for age and other covariates, the increase in QTc based on the p.L353L variant alone was

**Table 1** Variant status of participants included in the regression analyses and their unadjusted average QTc

| Variant status of participants (n=419) | Men (n=137) |                    | Women (n=282) |                    |
|--|-------------|--------------------|---------------|--------------------|
|  | N           | QTc (ms) Mean (SD) | N             | QTc (ms) Mean (SD) |
| Negative for all variants              | 91          | 425.9 (29.3)       | 169           | 447.8 (26.2)       |
| V205M only                             | 21          | 457.0 (17.6)       | 40            | 477.8 (30.3)       |
| L353L only                             | 15          | 435.3 (19.6)       | 40            | 461.7 (33.7)       |
| K897T only                             | 5           | 430.9 (38.6)       | 18            | 441.7 (24.7)       |
| V205M*L353L                            | 5           | 520.1 (64.7)       | 7             | 476.3 (31.3)       |
| V205M*K897T                            | 0           | –                  | 3             | 451.0 (12.8)       |
| L353L*K897T                            | 0           | –                  | 3             | 486.3 (5.5)        |
| V205M*L353L*K897T                      | 0           | –                  | 2             | 470.5 (2.1)        |

QTc, corrected QT.

**Table 2** Adjusted regression analyses of all participants by sex

| Variable   | Beta coefficient† | 95% CI‡        | p Value |
|--|-------------------|----------------|---------|
| (A) Men, interaction model, n=137, intercept=418.4 ms, adjusted R <sup>2</sup> =0.54           |                   |                |         |
| V205M  | 29.3              | 19.2 to 39.4   | <0.001  |
| L353L  | 14.0              | 2.9 to 25.1    | 0.014   |
| K897T  | 5.7               | -19.3 to 30.7  | 0.651   |
| V205M*L353L  | 34.6              | 12.0 to 57.2   | 0.003   |
| L353L*K897T  | -                 |                |         |
| V205M*K897T  | -                 |                |         |
| V205M*L353L*K897T  | -                 |                |         |
| (B) Women, interaction model, n=282, intercept=444.3 ms, adjusted R <sup>2</sup> =0.28         |                   |                |         |
| V205M  | 31.5              | 20.1 to 42.9   | <0.001  |
| L353L  | 10.0              | -0.6 to 20.6   | 0.064   |
| K897T  | -4.2              | -16.1 to 7.6   | 0.481   |
| V205M*L353L  | -14.5             | -42.6 to 13.7  | 0.312   |
| V205M*K897T  | -27.2             | -43.1 to -11.3 | 0.001   |
| L353L*K897T  | 29.9              | 4.7 to 55.0    | 0.020   |
| V205M*L353L*K897T  | -14.0             | -51.3 to 23.3  | 0.461   |
| (C) Men and women, interaction model, n=419, intercept=422.3 ms, adjusted R <sup>2</sup> =0.40 |                   |                |         |
| V205M  | 30.0              | 21.8 to 38.3   | <0.001  |
| L353L  | 11.2              | 3.1 to 19.3    | 0.007   |
| K897T  | -0.6              | -11.7 to 10.5  | 0.914   |
| V205M*L353L  | 7.2               | -17.4 to 31.5  | 0.558   |
| V205M*K897T  | -26.7             | -40.5 to -12.8 | <0.001  |
| L353L*K897T  | 19.3              | -6.5 to 45.1   | 0.142   |
| V205M*L353L*K897T  | -25.9             | -60.0 to 8.2   | 0.136   |

†Beta coefficients from OLS linear regression representing the baseline (intercept) and change in QTc (ms). Model was adjusted for age, past and current alcohol abuse, QT-prolonging drug use, cardiovascular disease and indicator variable for interpolated QTc.

‡95% CI using robust SEs.

QTc, corrected QT.

10.0 ms ( $p=0.064$ ) and 31.5 ms ( $p<0.001$ ) for the p.V205M mutation. In contrast to men, however, interaction of p.V205M\*p.L353L did not show a statistically significant increase in QTc (see [table 2B](#)). However, the combination p.L353L\*p.K897T conferred a positive effect ( $p=0.02$ ) resulting in an increase of 29.9 ms from that expected with the p.L353L or the p.K897T alone. The predicted QTc associated with p.L353L\*p.K897T at 483.2 ms ([tables 2B](#) and [3B](#)) was significantly higher compared with those women without any documented genetic variants after Bonferroni correction for multiple testing (28 comparisons). All individuals with the p.L353L\*p.K897T variant combination underwent expanded LQTS gene sequencing (standard clinical LQTS sequencing<sup>30</sup>) with no other LQTS pathogenic variants detected. [Table 2C](#) presents the results when sexes were combined ( $n=419$ ). Although the QTc effect of the p.V205M and p.L353L remains significant when inherited alone ( $p<0.001$  and 0.007, respectively), there is no longer evidence of statistical significance for the p.V205M\*p.L353L ( $p=0.558$ ) as was seen with the male-only analysis. See Supplemental data for figure of predicted margins (QTc) for all variant combinations.

For family-based association of QTc with variants alone and in combination, see [figures 1](#) and [3](#). Note in particular the p.V205M\*L353L genotype in three men in [figure 3](#) and the associated QTc in comparison with family members.

### In silico analysis

To determine if p.L353L could impact splicing, four in silico tools were used to determine if the variant may impact any predicted splicing regulatory elements. In support of the role of

this variant on splicing, all four tools predicted exon splicing enhancer (ESE) motifs directly at the p.L353L position, and these sites were predicted to be disrupted by the synonymous nucleotide substitution (see [table 4](#)). Additionally, many of the tools predicted a change in the type of ESE from an alternative splicing factor /splicing factor 2 (ASF/SF2) ESE to a SRp40-binding site.

### Assessment of mutant transcript levels

In order to identify alternatively spliced products, the lymphocyte mRNA-derived cDNA from the proband (see [figure 1](#)) was amplified using primers in exon 5 (5F) and exon 10 (10R). Two bands were identified, which correlated with full-length sequence and an alternatively spliced product that skipped exon 8 (annotated hereon as  $\Delta 8$ ), which were assessed by direct sequencing ([figure 4A](#)). The contribution of the  $\Delta 8$  transcript to the overall *KCNQ1* transcript levels was assessed in three p.L353L-positive and two negative individuals from the First Nations population and one p.L353L-negative control from outside the First Nations. While the total relative *KCNQ1* levels were indistinguishable between p.L353L-positive and p.L353L-negative individuals ( $p=0.69$ ), the p.L353L-negative individuals (WT:  $82.9\pm 5.8\%$ ;  $\Delta 8$ :  $9.2\pm 0.6\%$ ) had significantly less skipping of exon 8 than p.L353L-positive individuals (WT:  $68.9\pm 4.2\%$ ,  $p=0.03$ ;  $\Delta 8$ :  $25.3\pm 2.1\%$ ,  $p=0.003$ , [figure 4C](#)).

This  $\Delta 8$  transcript has been identified previously and shown to generate non-functional channels which exert a dominant-negative effect on WT channels.<sup>26</sup> Given the increase in the  $\Delta 8$  transcripts, statistical modelling fitted to a binomial distribution was used to predict the likelihood of functional Kv7.1 tetramer formation. The increase in  $\Delta 8$  transcripts from 9.2% to 25.3% results in a 2.3-fold reduction in the likelihood of functional protein tetramer formation from 65.6% to 28.6% ([figure 4D](#)).

### DISCUSSION

Our study suggests that the *KCNQ1* p.L353L synonymous variant impacts splicing efficiency resulting in the generation of alternatively spliced transcripts and ultimately decreasing functional tetramer formation. While it has a moderate independent effect on the QTc, it is unlikely to increase the QTc sufficiently to result in a LQTS phenotype alone. However, our results suggest a potential modifying and sometimes synergistic effect on the QTc when inherited with other variants.

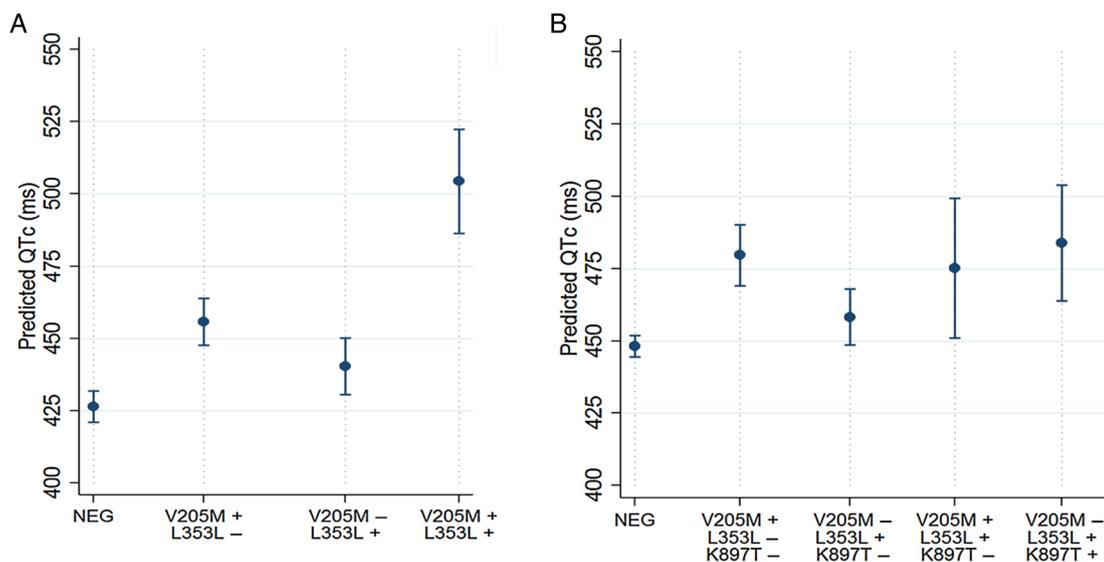
### LQTS as a complex condition

A prolonged QTc interval on 12-lead ECG ( $\geq 450$  for men and  $\geq 460$  for women) is a marker of increased risk for arrhythmia and SCD<sup>10 31</sup> and may be a result of genetic and/or non-genetic factors. Since the time the first LQTS gene (*LQTS1*) was mapped to 11p in 1992 (subsequently confirmed as *KCNQ1*), the variability in the phenotype has been highlighted.<sup>32</sup> At that time, Vincent *et al* compared QTc values of those linked to the chromosome 11p15 locus (carriers) and their unlinked relatives (non-carriers), demonstrating that carriers had a significantly higher QTc. However, they also showed that there was an overlap in QTc values between carriers (range 410–590 ms) and non-carriers (380–470 ms) and not all carriers were symptomatic. Ongoing evaluation of LQTS cohorts and families has yielded consistent trends of variable clinical presentation.<sup>4</sup> It is well established that a combination of mutations confers a more severe phenotype, with greater risk of SCD<sup>33 34</sup> and common variants combined with mutations can modulate the overall effect on phenotype and the channel or channels involved.<sup>18 35–37</sup> For example, the LQTS1 phenotype in a South

**Table 3** Adjusted predicted margins and multiple pairwise comparisons

| Variant status of participants     | Sample size | Margin (QTc) | Delta-method SE | Unadjusted groups | Bonferroni groups |
|------------------------------------|-------------|--------------|-----------------|-------------------|-------------------|
| <b>(A) Men†</b>                    |             |              |                 |                   |                   |
| Negative for all variants          | 91          | 426.4        | 2.6             | A                 | A                 |
| L353L                              | 15          | 440.4        | 4.9             | B                 | AB                |
| V205M                              | 21          | 455.8        | 4.1             | C                 | B                 |
| V205M*L353L                        | 5           | 504.4        | 9.1             | D                 | C                 |
| <b>(B) Women‡</b>                  |             |              |                 |                   |                   |
| Negative for all variants          | 169         | 448.4        | 1.9             | A                 | A                 |
| K897T                              | 18          | 444.0        | 5.7             | A                 | A                 |
| L353L                              | 40          | 458.1        | 4.9             | AB                | AB                |
| L353L*K897T                        | 3           | 483.2        | 10.0            | C                 | B                 |
| V205M                              | 40          | 479.2        | 5.1             | C                 | B                 |
| V205M*K897T                        | 3           | 447.5        | 2.6             | A                 | A                 |
| V205M*L353L                        | 7           | 474.5        | 12.2            | BC                | AB                |
| V205M*L353L*K897T                  | 2           | 459.0        | 4.0             | B                 | AB                |
| <b>(C) Men and women combined‡</b> |             |              |                 |                   |                   |
| Negative for all variants          | 260         | 441.2        | 1.5             | A                 | A                 |
| K897T                              | 23          | 440.4        | 5.4             | AB                | AB                |
| L353L                              | 55          | 452.1        | 3.7             | BC                | AB                |
| L353L*K897T                        | 3           | 470.4        | 11.0            | CD                | ABC               |
| V205M                              | 61          | 470.5        | 3.6             | D                 | C                 |
| V205M*K897T                        | 3           | 443.1        | 2.9             | AB                | AB                |
| V205M*L353L                        | 12          | 488.8        | 11.0            | D                 | C                 |
| V205M*L353L*K897T                  | 2           | 455.0        | 3.8             | C                 | BC                |

Margins sharing a letter in the group label are not significantly different at the 5% level.  
 \*Adjusted for six comparisons† should be preceded by only 1 symbol (as seen above for men)  
 †Adjusted for six comparisons.  
 ‡Adjusted for 28 comparisons.  
 QTc, corrected QT.

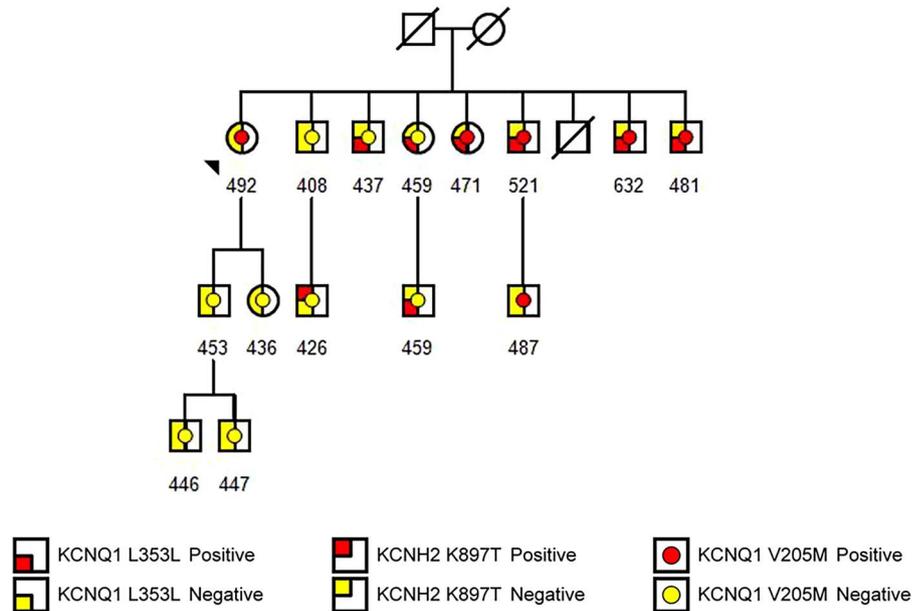


**Figure 2** (A) Adjusted predicted corrected QT (QTc) in interaction regression model in Men. Predicted effects of expected QTc in men with p.V205M and p.L353L variants above baseline QTc of 418.4 ms. (B) Adjusted predicted QTc in interaction regression model in women. Predicted effects of expected QTc in women with p.V205M, p.L353L and p.K897T above 444.3 ms. Both models were adjusted conditionally for age, cardiovascular diseases, past and current alcohol abuse and QT-prolonging drugs. The predicted QTc is shown on the Y-axis in ms and variant status on the X-axis.

African Founder population with the *KCNQ1* p.A341V mutation was influenced by common variants in *NOS1AP*, a regulator of neuronal nitric oxide synthase.<sup>38</sup> Since then, the effects of these *NOS1AP* variants on the QTc interval and clinical risk have been confirmed in a large heterogeneous LQTS cohort.<sup>39</sup>

Furthermore, numerous additional genomic regions (16q21 near *NDRG4* and *GINS3*, 6q22 near *PLN*, 1p36 near *RNF207*, 16p13 near *LITAF* and 17q12 near *LIG3* and *RFFL*), containing genes not previously recognised to be associated with LQTS, contribute to a prolonged QT interval in population-based

**Figure 3** Pedigree of Family 2 harbouring the *KCNQ1* p.V205M, p.L353L and *KCNH2* p.K897T variants. Note three male siblings with p.V205M\*p.L353L and high corrected QT (QTc). The arrow indicates the proband. QTc values (ms) are listed below each participant.



**Table 4** In silico analysis of the *KCNQ1* c. 1059G>A (p.L353L) variant

| Algorithm tool used   | Prediction performed by the tool  | Interpretation of the <i>KCNQ1</i> gene >ENST00000155840 transcript >exon number: 8, c.1059G>A variation                                |
|-----------------------|---|---|
| ESEfinder 3.0         | ESE finder for SRp40, SC35, SF2/ASF and SRp55 proteins  | Identifies CTGAAGG as ESE. Predicts the G>A change in this motif to result in splicing regulatory protein binding from SF2/ASF to SRp40 |
| RESCUE-ESE 1.0        | ESE Hexamer finder  | Identifies TGAAGG as ESE and predicts the G>A change resulting in enhancer motif sequence disruption                                    |
| Human Splicing Finder | Combines 12 different algorithms to identify and predict mutations' effect on splicing motifs | Predicts the alteration of an exonic ESE site leading to potential alteration of splicing   |
| Sroogle               | Splicing regulatory sequences identifier  | Predicts ESE motifs at the c.1059 position and predicts a loss of ESE site with the G>A change  |

ESE, exon splicing enhancer.

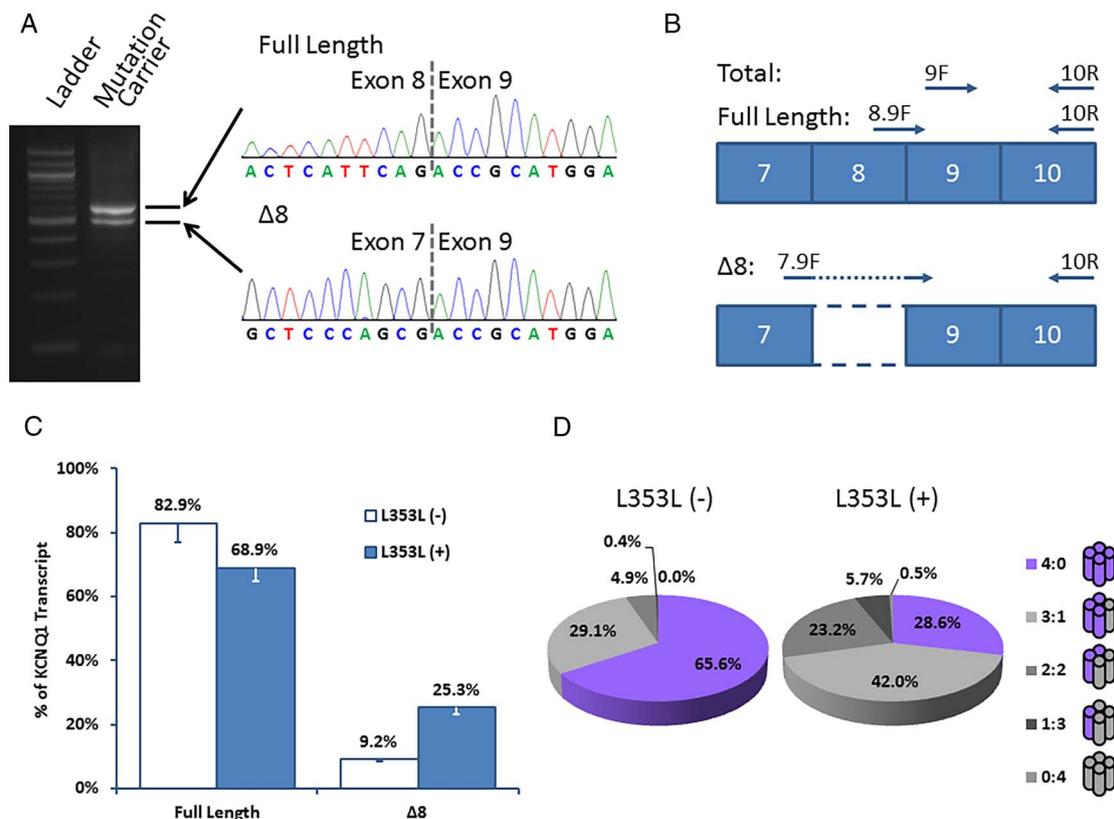
cohort studies,<sup>40 41</sup> each of which could impact on the QTc in conjunction with pathogenic variants. Little exploration, however, has been carried out on the potential impact of such variants on splicing, and in particular synonymous variants may be ignored, considered unlikely to be functional.

### Splicing in disease

While synonymous variants are often dismissed as unlikely contributors to phenotype, increasingly their role in the pathogenesis of disease is being recognised.<sup>42</sup> Even within LQTS, synonymous mutations have been recognised to cause disease. The hot spot mutation *KCNQ1* A344A is the result of a c.1032G>A transition involving the terminal codon in exon 7.<sup>43</sup> This synonymous mutation alters the 5' splice site of intron 7

and leads to the skipping of exons 7 and 8.<sup>26 44</sup> Several other splice site mutations situated in exon-intron junctions have been associated with LQTS with varying degrees of severity, including *KCNQ1* c.477+1 G>A, inherited homozygously in a German family with LQTS and profound hearing loss,<sup>45</sup> *KCNQ1* c.1032+3 A>G resulting in skipping of exon 7 and a mild LQTS phenotype<sup>44</sup> and *KCNQ1* c.1251+1 G>A causing exon 9 skipping and a mild LQTS phenotype that triggered ventricular tachyarrhythmias during periods of hypokalaemia in a Japanese cohort.<sup>46</sup> An intron-1 mutation c.387-5 T>A in *KCNQ1* resulted in incomplete skipping of exon 2 with 10% of WT mRNA still expressed and homozygous individuals had a severe cardiac phenotype but no hearing loss.<sup>47</sup> These mutations highlight the potential role of splicing in the pathogenesis of LQTS.

However, these LQTS1 causative mutations have all occurred at either the first or the last nucleotide of the exon or within the intron's canonical 5' or 3' splice sites. In contrast, this study has identified the first synonymous variant localising outside the terminal positions of the exon that exerts a role in disease pathogenesis. As p.L353L (c.1059G/A) is 27 nucleotides downstream of the start of the exon and 70 nucleotides upstream of the end of the exon, this is far outside of the critical 5' and 3' splice site motifs. The evidence presented within this study suggests that the p.L353L (c.1059G/A) nevertheless results in decreased recognition of exon 8 leading to increased dropping of this exon from the mature mRNA. Based on the in silico evidence, this likely is due to the disruption of the ASF/SF2 ESE or conversion of ASF/SF2 to a SRp40 ESE motif. There is evidence that while SRp40 acts primarily as an ESE, this is location dependent and thus SRp40 can also act as a strong to mild silencer of the splice site activity.<sup>48</sup> In this previous study, the SRp40-binding site was moved along an exon of the *ADAR2* gene, which has suboptimal splice efficiency, and at approximately the position SRp40 appears in the p.L353L mutant of *KCNQ1*, splice efficiency dropped. While we see that p.L353L disrupts splicing incompletely (by 24%), previous studies have also correlated a similar level of splicing disruption with a mild QT phenotype, suggesting that even small splice disruptions may contribute to disease.<sup>44</sup>



**Figure 4** Assessment of p.L353L mutant *KCNQ1* transcript levels. (A) Gel electrophoresis (amplified using 5F and 10R primers) showing the full-length sequence and alternatively spliced product skipping exon 8 ( $\Delta 8$ ). (B) Schematic of primers designed for the selective amplification of the  $\Delta 8$  transcript (7.9F and 10R), the full-length transcript (8.9F and 10R) and total *KCNQ1* transcript (9F and 10R). (C) Graph showing the percentage of *KCNQ1* transcripts in p.L353L-positive versus p.L353L-negative individuals. (D) Chart and schematic of the statistical model fitted to a binomial distribution used to predict the likelihood of functional Kv7.1 tetramer formation. WT, wild-type.

While splicing can be tissue specific, blood RNA has been used as a surrogate in the majority of the splicing mutations previously assessed in LQTS and was used in our experiments. The difference between p.L353L-positive and p.L353L-negative individuals suggests that the p.L353L variant has an impact on splicing, an effect likely recapitulated in the heart and shown clinically with an increase in QTc.

### Sex differences

Women and men are well known to present differently in LQTS. Several mechanisms have been proposed to explain the sex difference, such as sex hormones which are believed to have both genomic and non-genomic effects on the QT interval. Oestrogen reduces repolarisation reserve in women and is therefore believed to be responsible for higher premenopausal susceptibility to drug-induced QT prolongation.<sup>49</sup> Although our sample size of those with the combination p.V205M\*p.L353L genotype is small (seven women and five men), the consistently higher increase in QTc in men, above that seen with p.V205M alone is not evident, on average, in the women with the same combination genotype. This finding is of interest in that mechanisms that affect the QTc in men disproportionately are rarely speculated on.<sup>50</sup> However, male-specific effect has been observed in studies of modifying variants in LQTS1 populations, such as in the study by Lahtinen *et al*,<sup>51</sup> where the presence of the variant p.D85N in *KCNE1* was shown to modify the QT interval in men with LQTS1, but not in women. More recently, this phenomenon was observed in two additional Swedish founder populations with LQTS1 where QTc

prolongation with *NOS1AP* variants was seen in men but not in women.<sup>52</sup> Our results further support these observations that in certain circumstances men may be at higher risk, although the underlying mechanism is unclear.

### Does the p.L353L destabilise the protective effect of *KCNH2* p.K897T?

The effect of the *KCNH2* p.K897T variant remains controversial in that it has been shown to confer both risk for<sup>18 36</sup> and protection from<sup>17</sup> a prolonged QTc. Our regression analysis in women suggests that the p.K897T variant likely lowers the QTc overall, consistent with other published reports.<sup>17 53</sup> Also consistent with published reports suggesting the p.K897T may impair repolarisation if inherited with other variants,<sup>18</sup> the combination of the p.L353L\*p.K897T was associated with an increase in QTc compared with those negative for all variants. The resultant QTc effect clinically was indistinguishable from those with the pathogenic p.V205M variant alone (see figure 1 and table 3B). We were unable to assess this in men. More study is required to confirm this finding.

### CONCLUSION

Our study provides the first evidence that synonymous variants outside the canonical splice sites in *KCNQ1* can alter splicing and reduce WT transcript levels. The First Nations population which hosts a LQTS1 founder mutation (p.V205M) provided the unique opportunity to explore the effect of this distinctive splice variant on the QTc and the LQTS phenotype. We identified that p.L353L can affect the QT interval alone and may

produce a synergistic effect in conjunction with other variants in certain circumstances. Sex-specific effects were observed, an emerging phenomenon reported in other LQTS1 Founder population studies.

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