

REVIEW

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# Functional evaluation of gene mutations in Long QT Syndrome: strength of evidence from in vitro assays for deciphering variants of uncertain significance

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## Abstract

**Background:** Genetic screening is now commonplace for patients suspected of having inherited cardiac conditions. Variants of uncertain significance (VUS) in disease-associated genes pose problems for the diagnostician and reliable methods for evaluating VUS function are required. Although function is difficult to interrogate for some genes, heritable channelopathies have established mechanisms that should be amenable to well-validated evaluation techniques.

The cellular electrophysiology techniques of 'voltage-' and 'patch-' clamp have a long history of successful use and have been central to identifying both the roles of genes involved in different forms of congenital Long QT Syndrome (LQTS) and the mechanisms by which mutations lead to aberrant ion channel function underlying clinical phenotypes. This is particularly evident for *KCNQ1*, *KCNH2* and *SCN5A*, mutations in which underlie > 90% of genotyped LQTS cases (the LQT1-LQT3 subtypes). Recent studies utilizing high throughput (HT) planar patch-clamp recording have shown it to discriminate effectively between rare benign and pathological variants, studied through heterologous expression of recombinant channels. In combination with biochemical methods for evaluating channel trafficking and supported by biophysical modelling, patch clamp also provides detailed mechanistic insight into the functional consequences of identified mutations. Whilst potentially powerful, patient-specific stem-cell derived cardiomyocytes and genetically modified animal models are currently not well-suited to high throughput VUS study.

**Conclusion:** The widely adopted 2015 American College of Medical Genetics (ACMG) and Association for Molecular Pathology (AMP) guidelines for the interpretation of sequence variants include the PS3 criterion for consideration of evidence from well-established in vitro or in vivo assays. The wealth of information on underlying mechanisms of LQT1-LQT3 and recent HT patch clamp data support consideration of patch clamp data together (for LQT1 and LQT2) with information from biochemical trafficking assays as meeting the PS3 criterion of well established assays, able to provide 'strong' evidence for functional pathogenicity of identified VUS.

**Keywords:** Arrhythmia, Channelopathy, hERG, KCNH2, KCNQ1, Long QT syndrome, Variant of uncertain significance

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## Introduction

The now commonplace use of genetic screening for inherited cardiac conditions can be of high value in identifying the genetic substrate that underlies an observed clinical phenotype. However, such testing is not without problems: the term “genetic purgatory” has been used to describe the situation in which the genetic test-requesting physician, patients and their relatives are “left stuck” when a genetic variant is identified, the significance of which is unknown (‘variant of uncertain significance’, VUS) [1]. A VUS designation is made when there is insufficient information to distinguish between benign and pathological nucleotide changes [2]. Recognizing the increased complexity of genetic information consequent to the widespread adoption of next generation sequencing, the American College of Medical Genetics (ACMG) and Association for Molecular Pathology (AMP) published in 2015 standards and guidelines for the interpretation of sequence variants [3]. These guidelines contain a series of complementary criteria that can be used in combination to classify variants into one of five categories: ‘pathogenic’, ‘likely pathogenic’, ‘uncertain significance’, ‘likely benign’ and ‘benign’ [3]. According to this framework, variants designated as of uncertain significance are not to be used for clinical decision making [3]. These guidelines have also been adopted outside of the United States; for example, the UK Association for Clinical Genomic Science (ACGS) has adopted the 2015 ACMG/AMP guidelines and has itself provided best practice guidelines for variant classification that detail how ACMG/AMP evidence criteria should be addressed [4, 5].

Within the ACMG/AMP framework, the “PS3” criterion is used to consider results of “well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect on the gene or gene product” [1]. With ~20,000 genes in the human genome, many genes will produce gene products for which (if present in the heart) the function is not well known, or for which there are not well-established functional tests. For proteins for which secondary/tertiary structures are available, either through high resolution microscopy or *in silico* homology modelling, it may be possible to predict likely changes to protein structure due to nucleotide coding changes. However, the ACMG/AMP and ACGS guidelines make clear that *in silico* studies, including protein modelling, cannot be used as functional evidence under the PS3 evidence criterion (they may instead be eligible for consideration under the phenotype specificity criterion PM1) [3, 4]. By contrast, the function of some gene products is very well established, through extensively validated, precise methods of investigating protein function. Ion channels are open to such interrogation. This article aims to consider recent evidence supporting the

notion that cellular electrophysiological approaches can reliably distinguish between benign and pathological variants, and thus provide PS3 ‘strong’ functional evidence for elucidating VUS in cardiac ion channelopathies.

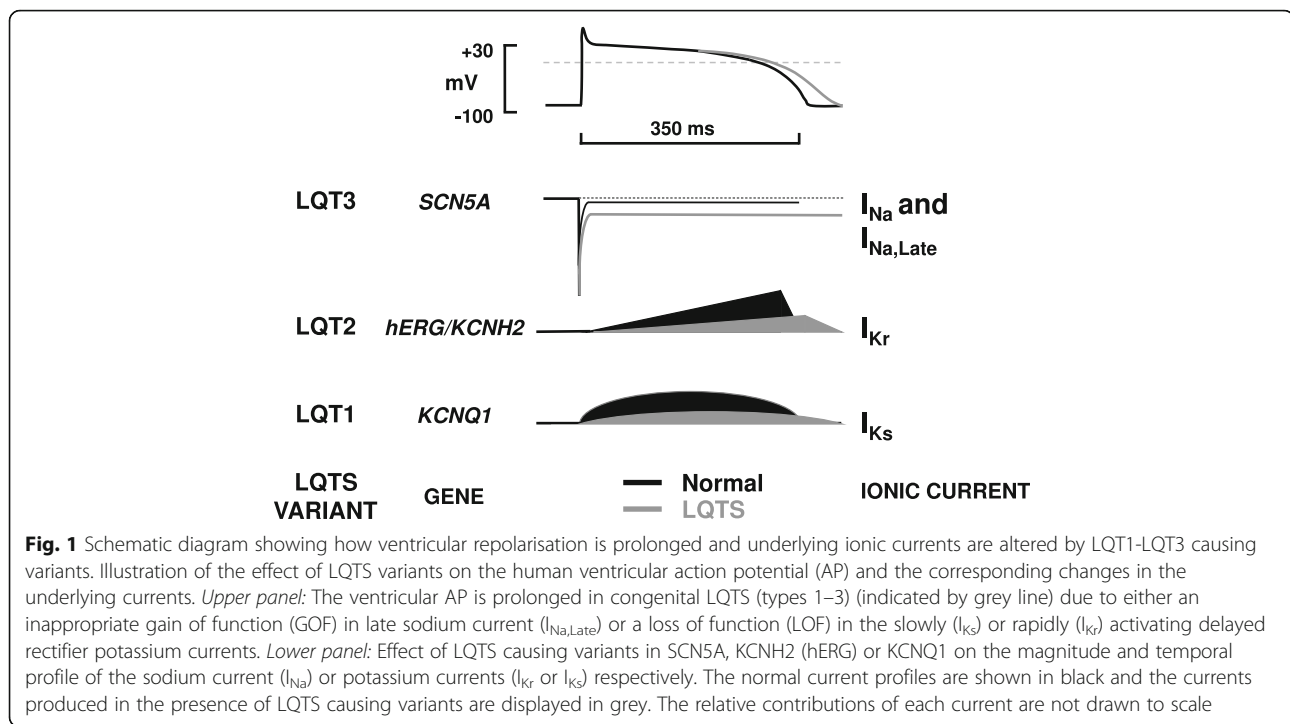
## Channelopathies

The term channelopathy encompasses a wide range of disorders that share in common a basis due to an acquired or genetic dysfunction in an ion channel. Channelopathies have been identified in the nervous system, cardiovascular system, skeletal muscle, urinary system and respiratory system [6]. Cardiac channelopathies include Long QT Syndrome (LQTS), Short QT Syndrome (SQTS), Brugada Syndrome (BrS), Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT), familial atrial fibrillation (fAF) and some forms of sick sinus syndrome (SSS) [6, 7]. The two most widely studied channelopathies are probably cystic fibrosis (CF) and congenital forms of LQTS (cLQTS). Electrophysiological measurements of ion channel (dys) function have been central to the development of current understanding of both conditions. Although 17 forms of cLQTS have been identified, ~95% of genotype positive cases involve mutations to 3 genes, *KCNQ1*, *KCNH2* and *SCN5A*, which are considered to be definitively causally linked to LQT1-LQT3 ([8, 9] and see Fig. 1). A reliable approach to functional interrogation of the products of these genes should therefore be of considerable value to deciphering VUS in cLQTS.

## Measurement of ion channel current- a brief historical perspective

The quantitative measurement of ion channel currents through the voltage clamp technique is long-established, dating back well over 60 years [10–12] and for patch clamp over 40 years [13, 14]. These techniques enable control of transmembrane voltage and direct measurement of transmembrane ion flux as electrical current, permitting accurate measurement of the amplitude, time-course and voltage dependence of current flow across cell membranes.

These methods were crucial for the early functional identification of the LQT1–3 subtypes of cLQTS. *KCNH2* and *KCNQ1* encode proteins that form the pore-forming subunits of the ‘rapid’ and ‘slow’ delayed rectifier potassium currents ( $I_{Kr}$  and  $I_{Ks}$ ) that are important for normal ventricular repolarization ([15] and see Fig. 1). Native  $I_{Kr}$  and  $I_{Ks}$  were first pharmacologically resolved and characterized in ventricular cardiomyocytes using whole-cell patch clamp [16, 17]. Whole-cell voltage clamp recording was subsequently used to identify *KCNQ1* (alternative nomenclature KvLQT1, Kv7.1) as the pore-forming subunit of  $I_{Ks}$  channels and the target of mutations causing the LQT1 variant of cLQTS [18, 19]. It was also used to



determine that the *KCNH2*-encoded hERG protein (alternative nomenclature Kv11.1) underlies the pore of the  $I_{Kr}$  channel and is the target for LQTS2 mutations [20–23]. Voltage clamp of *SCN5A* encoded Nav1.5 channels was important for the demonstration of the role of *SCN5A* gain-of-function mutations in LQTS3 [24–26] and, later, of the role of *SCN5A* in BrS [27]. It is notable that approaches to the interrogation of the propensity for novel drugs to produce an *acquired* LQTS and *Torsades de Pointes* arrhythmia also involve patch clamp assays [28–30].

Manual patch clamp has been used in much of the academic/preclinical research that underpins current understanding of LQTS and other channelopathies, though a detailed coverage of the numerous studies involving manual patch clamp is outside the intended scope of the present article. However, taking *KCNH2*-mediated LQTS2 as an illustrative example, a combination of hERG protein measurement (using Western blotting) and manual patch clamp was used to demonstrate that the majority (28/34) of missense mutations exhibit a class 2 (impairment of the intracellular processing and trafficking of channels to the cell membrane [31]) phenotype and demonstrate the potential for pharmacological correction. It also showed that a small number of missense mutations do not exhibit a loss-of-function phenotype, thus highlighting the necessity for functional studies to interpret the effects of individual variants [32]. A subsequent large scale analysis of 167 LQTS2-linked hERG mutations used biochemical analysis supported by manual

patch clamp of selected variants to demonstrate that a class 2 mechanism mediates the effects of most (close to 90%) mutations in 3 of 4 major structural domains of the hERG channel (the exception being the distal C-terminus) [33]. At the time it was conducted this study was the largest mutational study for any inherited disease [33].

#### Deciphering VUS using automated patch clamp

Despite the pivotal value of cellular electrophysiology in understanding ion channel function, such methods are not routinely used to study genotyped variants identified in channelopathies. This is because, as highlighted recently by others [2], the gold standard approach of manual patch clamp is both labour- and time-intensive. A potential solution is to employ automated, planar patch clamp technology. Automated patch clamp approaches have been largely driven by the need to screen large numbers of compounds for ion channel effects during drug development [28]. Automated patch clamp has its own technical challenges including incorporation of temperature control and obtaining and maintaining high electrical resistance seals necessary to secure reliable voltage clamp; however, in principle, it has the potential to offer a means of interrogating channel variants in volume, significantly faster than is possible with conventional, manual patch clamp. This is supported by recent work with *KCNQ1*, *KCNH2*, and *SCN5A* (summarised in Table 1).

There are over 600 disease-associated *KCNQ1* variants [37], but only a small proportion of these have been

**Table 1** Automated patch clamp technology enables the phenotyping of *KCNQ1*, *KCNH2* and *SCN5A* variants to be performed in a high-throughput manner

Study:	Expression system:	Automated patch clamp platform:	No of variants studied:		Classification:			
			Homozygous state:	Heterozygous State:	P or LP:	B or LB:	GOF:	VUS:
<i>KCNQ1</i> [34]	CHO-K1	Syncropatch 768 PE - Recording at RT	78 (30 training + 48 test variants)	22	44	16	3	15
<i>KCNH2</i> [35]	HEK-293	Syncropatch 384 PE - Recording at ~ 25 °C	23	30	26 <sup>a</sup>	2 <sup>a</sup>	0 <sup>a</sup>	2 <sup>a</sup>
<i>SCN5A</i> [36]	HEK-293 T	Syncropatch 384 PE - Recording at RT	83 (73 previously unstudied)	N/A	54	17	0	12

P Pathogenic, LP Likely Pathogenic, B Benign, LB Likely Benign, GOF Gain of Function, VUS Variant of Uncertain Significance. *KCNQ1* variant classification is inferred from in text information and data presented in Supplementary Tables S4a and S4b [34]. *KCNH2* variant classification is as reported in [35]. <sup>a</sup> Indicates that pathogenicity was classified in the heterozygous state to mimic the patient phenotype [35]. *SCN5A* variant classification is taken from Supplementary Table S1 [36]. The Syncropatch 384 and 768 PE automated patch clamp platforms are developed by Nanion Technologies

functionally characterized. In order to evaluate the utility of automated patch clamp for elucidating the pathogenicity of VUS, Vanoye et al studied 78 known *KCNQ1* variants, including 30 training and 48 test variants [34] (Table 1). Automated and manual patch clamp recordings were made at room temperature from mammalian (CHO-K1) cells transfected with wild-type (WT) or mutant *KCNQ1* (together with its known  $\beta$  subunit, *KCNE1*). Where comparative data on mutant effects on current amplitudes and kinetics were available for both manual and automated methods, there was generally a strong concordance between the two [34]. As most cases of LQTS are heterozygous (autosomal dominant or de novo), and functional  $I_{Ks}$  channels are tetrameric, the authors also studied 29 variants as WT/variant heteromultimers through WT:mutant co-transfection (7 variants could not be analysed due to confounding expression effects, leaving 22 for analysis). Of these, 17 showed current densities similar to those carried by the mutant expressed alone, indicative of dominant negative pathogenicity. Interestingly, two variants failed to show significant differences from WT current when studied in co-expression experiments (as also seen reported for some *KCNQ1* variants in [38]), highlighting the importance of performing experiments to mimic heterozygous conditions [34]. This approach enabled approximately two-thirds of the variants studied to be reclassified based on functional evidence (Table 1).

There are over 1000 *KCNH2* variants in public databases and, similar to the situation for *KCNQ1*, only a relatively small fraction of the known variants has been subject to functional evaluation. There appear to be differences in severity of the effects of mutations in different regions of the hERG channel, with mutations in the pore region associated overall with higher risk than mutations in non-pore regions [39, 40]. A recent study has employed automated patch clamp recording to determine whether this approach may distinguish pathological from benign non-synonymous variants [35] (Table 1) and twinned this with a high-throughput

channel trafficking assay for comparison. hERG channels were expressed in mammalian (HEK-293) cells and through use of a bicistronic expression vector, wild-type and mutant channels could be co-expressed to mimic heterozygous heritability. Of the 30 variants studied in the heterozygous state, 20 had designations of pathogenicity or otherwise in ClinVar on the basis of segregation/bioinformatic predictions (16 as pathogenic, 1 benign and 3 VUS). For these 20 variants the predictions of the patch clamp experiments demonstrated very high concordance with the ClinVar classifications [35]. For the ten variants that had not received classification in ClinVar, eight of these showed changes consistent with clear pathogenicity and two displayed defects that were less marked but consistent with likely pathogenic [35]. Overall, the results of this study highlight the value of patch clamp in distinguishing pathogenic changes to hERG channel function.

A 2018 comprehensive literature review showed a clear correlation between electrophysiological parameters of *SCN5A*-encoded Nav1.5 channel current and BrS and LQT3 [41]. Three hundred fifty-six variants were found for which electrophysiological parameters were available: peak current, late current, voltage dependent activation and inactivation and recovery from inactivation. The authors also quantified carriers presenting with/without disease. Peak current and late current respectively correlated with BrS and LQT3 penetrance [41]. Half-maximal activation voltage ( $V_{0.5}$ ) and recovery from inactivation respectively associated with BrS and LQT3 penetrance [41]. More recently, automated patch clamp has been applied to 83 *SCN5A* variants: 10 control variants that had been studied previously; 10 suspected benign variants and 63 suspected BrS associated variants [36]. Variants showing < 50% peak current density of WT or with a > + 10 mV shift in voltage-dependent activation were considered to have significant loss of function (and thereby to meet the ACMG/AMP PS3 criterion) [36]. Twenty-two of the sixty-three suspected BrS loss of function mutations had < 10% peak current;



another 23 had partial loss of function (10–50% peak current). Of the 73 previously unstudied variants, 60 were classified in Clin Var as VUS (with 11 likely pathogenic and 2 likely benign) prior to patch clamp analysis. After patch clamp had been performed, 47 were classified as pathogenic/likely pathogenic, 16 were benign/likely benign and 10 were VUS [36]. Additional use of structural modelling suggested potential mechanisms for loss of function with particular mutations [36]. Overall, as for *KCNQ1* and *KCNH2*, *SCN5A* VUS appear amenable to elucidation through high throughput patch clamp approaches.

The above studies highlight the feasibility and comparative speed of using automated patch clamp technology to predict variant pathogenicity in a high-throughput manner. For example, in their study Vanoye and colleagues estimated that 12 *KCNQ1* variants could be generated and evaluated within the very rapid time-frame of 2 weeks [34], though presumably this estimate involves some further streamlining, as the study of 48 homomeric variants occupied ~12 weeks. A key contribution of these studies [34–36] is to demonstrate the feasibility of functional electrophysiology data-driven variant classification and the associated ability to ‘decrypt’ VUS. Indeed, with reference to the ACMG/AMP guidelines, the authors of the Vanoye et al study commented “Among the criteria weighted as strongly supportive of classifying variants as pathogenic is the availability of data from in vitro or in vivo functional studies demonstrating a damaging effect on the gene or gene product (PS3 criterion)”, continuing “Our approach to high throughput functional evaluation of *KCNQ1* variants offers a rich new data source that satisfies the PS3 criteria and can aid variant classification” [34].

#### **Heterologous cell-based patch-clamp analysis- still a gold standard?**

It should be clear from the foregoing text that patch clamp investigation of recombinant channels is likely the most direct approach to provide insight into functional consequences of mutations to *KCNQ1*, *KCNH2* and *SCN5A* and, by extension, to other genes (e.g. *RYR2* [42]) that encode pore forming subunits of cardiac ion channels. The functional outcomes are particularly enhanced when patch-clamp is combined with complementary biochemical approaches that can interrogate trafficking status. However, heterologous expression removes the channel from the normal cardiomyocyte environment that may contain other channel accessory proteins and signalling pathways or express genetic modifiers of disease severity (e.g. [43]) that are absent in a heterologous expression system. An important question that therefore arises is whether or not there are

superior methods for investigating channel function to inform rare variant classification?

A potential approach to address the cellular context of variants is to use a genetically modified animal model in which the target mutation can be engineered, either by modification of the orthologous gene in that species, or through transgenic expression of the human gene. Unfortunately, where *KCNH2* and *KCNQ1* are concerned, this is not straightforward. The most commonly used mammalian species for genetic modification is the mouse and mice possess abbreviated ventricular action potentials that differ markedly in profile from those in humans and that do not rely on  $I_{Kr}$  or  $I_{Ks}$  for repolarization [44]. For this reason, mice are precluded from use in cardiac safety testing of new drugs [45] and they are similarly unsuitable for studying *KCNH2* and *KCNQ1* mutations. Transgenic rabbit models of LQT1 and LQT2, as well as *KCNH2*-linked short QT syndrome have been made and used to interrogate mechanisms of arrhythmia in these syndromes (e.g. [46–49]). However, the manufacture of such models is expensive and time-consuming and, at present, impractical for routine functional investigation of rare variants. Zebrafish possess an orthologue of hERG and the zERG break-dance trafficking mutant exhibits a prolonged QT interval [50–52]. Also, knockdown of endogenous zERG channels in embryonic and larval zebrafish and expression of wild-type and variant hERG has been suggested to provide a method for in vivo study of *KCNH2* mutations and polymorphisms [53, 54]. *KCNQ1* mediated channels are expressed in zebrafish heart, but the  $I_{Ks}$  channel  $\beta$  subunit, KCNE1 is expressed at only low levels [55, 56]. A transgenic *SCN5A* zebrafish model manifested bradycardia and conduction system abnormalities with a mutant but not wild-type human *SCN5A* construct [57]. Zebrafish therefore have potential for interrogating LQT1-LQT3 mutations and the production of zebrafish models is likely to be much less expensive than of transgenic rabbits. The ability to undertake detailed interrogation of mutation consequences in adult zebrafish hearts is likely to be mutation-dependent, however, and not to be high throughput.

A promising alternative is the use of patient derived induced pluripotent stem-cell (iPSC) derived cardiomyocytes (hiPSC-CMs) and/or to use genetic modification approaches (CRISPR-Cas9) to introduce a mutation against an otherwise isogenic iPSC background (e.g. [58–68]). However, this approach still involves considerable lead-in time to produce viable myocytes, which show an immature phenotype, including a lack or paucity of the inwardly rectifying potassium current,  $I_{K1}$ , that sets the resting potential in adult cardiomyocytes and spontaneous activity (and corresponding immature action potential morphology) that is absent in adult

cardiomyocytes [68–70]. The variable morphology and duration of action potentials produced by individual hiPSC-CMs [71] (due to developmental immaturity and ‘sub-type specification’) also make it challenging to determine whether observed differences are attributable to effects of the variant being studied or to sampling considerations. Additionally, the comparatively small size of endogenous  $I_{Kr}$  and  $I_{Ks}$  and need to pharmacologically isolate them from overlapping currents makes precise characterization of kinetic changes more difficult than in heterologous systems. Thus, whilst genetically-modified animals and hiPSC-CMs may have utility for studying disease mechanism and potential patient-specific treatment approaches, they cannot yet supplant the study of heterologously expressed channels.

### Biophysical and computational modelling

It is worth here highlighting the additional utility of biophysical modelling [72] to contextualize results obtained with recombinant channel electrophysiology. Correctly used, biophysical modelling is qualitatively distinct from computational modelling of protein structure or the use of bioinformatic tools that aim to predict pathogenicity, neither of which can provide direct *functional* insights. A number of human ventricular action potential models exist that are based on experimental data (e.g. [73–76]). Modification of the formulations that describe specific ionic currents, based on experimental data, can accurately reproduce changes to current density and kinetics observed in recombinant mutant channel recordings. The consequences of these changes for overall electrophysiological behaviour can then be interrogated. Computational cardiology approaches range from single cell to organ level simulations [72]. A recent example of the utility of combining electrophysiological, biochemical and modelling approaches comes from a study of *KCNH2* variants identified from genetic testing of 292 cases of sudden infant death syndrome (SIDS). Voltage clamp and Western blot data on 4 identified variants showed similar functional expression levels as WT channels, but accelerated activation and deactivation gating in two mutants. Incorporation of these changes into a ventricular action potential model showed that the modest kinetic changes observed did not result in significant action potential changes, suggesting that the mutations found may not have been causally linked to SIDS [77]. Thus, whilst biophysical modelling cannot reasonably be used in isolation for functional evaluation of variant effects, when it is used to evaluate consequences of altered currents established in heterologous expression experiments, it is a valuable component of the overall functional evaluation.

In the absence of biophysical modelling, conventional voltage clamp can usefully be combined with the action

potential voltage clamp technique (“AP clamp”), in which conventional rectangular waveforms are replaced with cardiac action potential waveforms as voltage commands (e.g. [78–80]). This technique takes into account membrane potential “history” [81] and thus elicits currents with their normal physiological time-course and voltage-dependence. AP clamp can therefore be used to obtain a “snapshot” of how a mutation influences the contribution of a current during repolarisation. By way of illustration, Fig. 2a and b show examples of  $I_{hERG}$  carried by WT hERG and WT hERG co-expressed with a recently reported loss-of-function variant (T634S), showing reduced current under conventional voltage clamp (Fig. 2a) and under AP clamp (Fig. 2b). In this example, the further incorporation of the observed extent of current into a human ventricular AP model was used to demonstrate the repolarisation delay consequent to  $I_{hERG}/I_{Kr}$  reduction (Fig. 2c). A file with example AP waveforms that can be downloaded and imported into voltage clamp acquisition software to conduct AP clamp experiments is freely available as an online supplementary appendix to [83].

### Validated approaches to meet the ACMG/PS3 “strong” criteria?

The inherent challenge in interpreting what represents “well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect on the gene or gene product” [1] to match the PS3 ‘strong evidence’ code (and its benign counterpart, BS3) has recently led the Clinical Genome Resource (ClinGen) Sequence Variant Interpretation (SVI) Working Group to propose recommendations for evaluators of the clinical validity of functional data [84]. The steps include: (i) define disease mechanism; (ii) evaluate the applicability of general classes of assays used in the field; (iii) evaluate the validity of specific instances of assays and (iv) apply evidence to individual variant interpretation [84]. In broad terms these steps seem reasonable and in the case of LQT1–3, at least, it should be clear that the nature of the channel subunit dysfunction is amenable to elucidation through both manual and automated patch clamp. However, the recommendations also consider benchmarking through inclusion of known pathogenic and benign variants, suggesting inclusion of a total of 11 pathological and benign variants to arrive at a PS3 moderate evaluation [84]. The validity of patch clamp for the study of *KCNH2*, *KCNQ1* and *SCN5A* mutations has already been demonstrated on well over this number of variants for LQT1–3 and, in principle, the inclusion of 11 control variants in automated patch clamp would not represent an excessive additional workload. That said, relatively few centres outside the pharmaceutical industry possess automated patch clamp platforms and for manual patch clamp

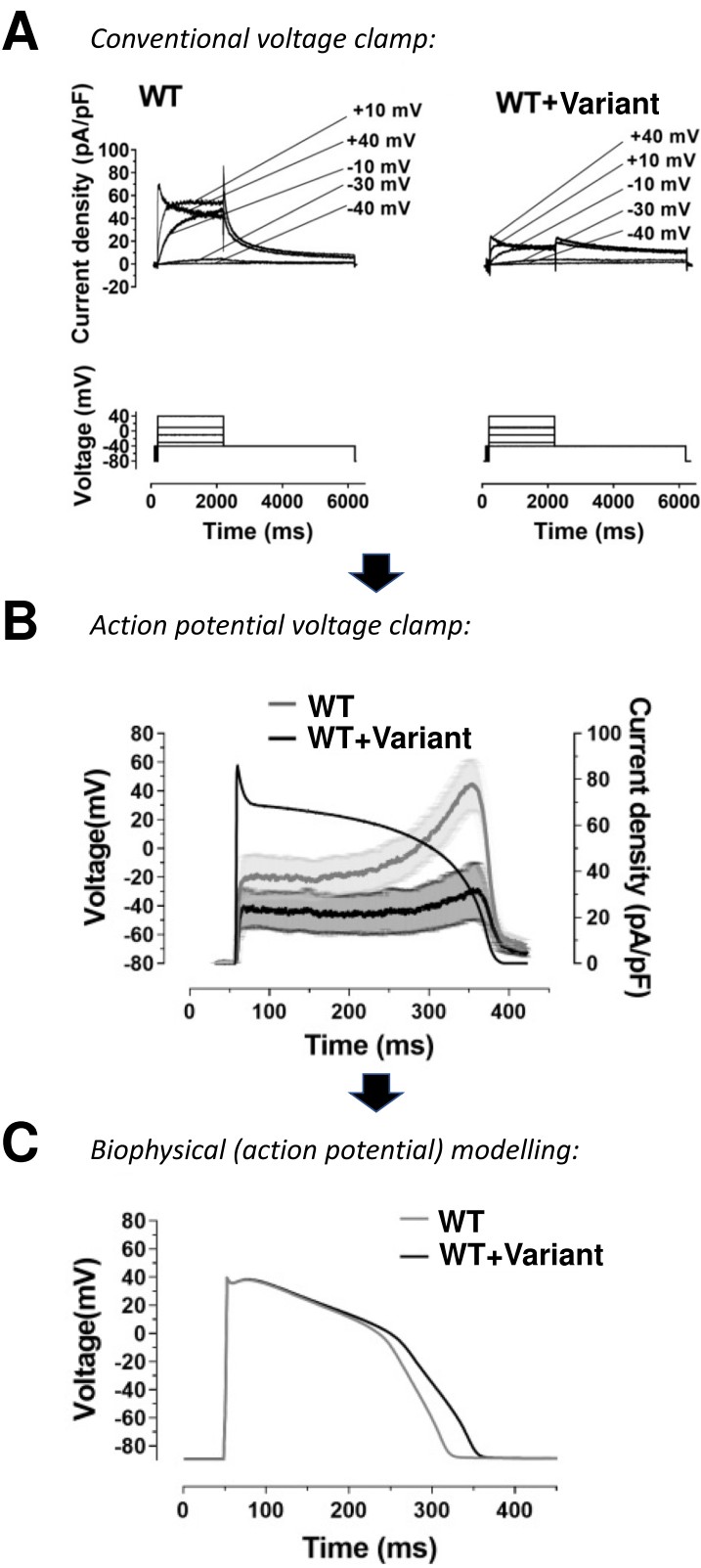


Fig. 2 (See legend on next page.)

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**Fig. 2** Conventional voltage and action potential (AP) clamp combined with biophysical AP modelling. **a** Shows families of hERG currents ( $I_{hERG}$ ; upper panels) elicited by a series of depolarising voltage commands to a range of test potentials (lower panels) under conventional voltage clamp. Numbers next to ionic current records denote voltage command potential. Left hand side shows WT  $I_{hERG}$  data, whilst right hand side shows data for co-expression of WT channel with a loss-of-function variant (T634S [82]). Currents under the two conditions are shown on the same scale, indicating a marked loss-of-function effect of the missense mutation studied. **b** Shows profile of WT  $I_{hERG}$  compared with that of WT + Variant (T634S, as per **a**)  $I_{hERG}$ , elicited by a ventricular AP command (shown superimposed over current records). The plotted currents show mean  $\pm$  SEM data from 5 experiments under each condition. The method allows direct visualization of the effect of this variant – producing a reduction in current throughout the AP plateau and repolarisation phase. **c** Shows the consequences of incorporation of the T634S hERG variant effect on ventricular AP repolarisation, using a human ventricular AP model.  $I_{Kr}$  was scaled to reflect the extent of current reduction found in voltage clamp experiments to mimic heterozygous variant expression conditions. This led to delayed repolarisation and AP prolongation. For further details see [82]

characterization of a new variant, the inclusion of an additional 11 control variants would represent both an unrealistic and entirely unnecessary burden. Our view is that the experimental approach is sufficiently well validated for the pathologies in question that this is not required. A more realistic scenario for a novel channel variant is comparison with the WT channel under homozygous expression conditions and under WT-variant (heterozygous) co-expression conditions, with inclusion of a well-established pathological mutation as a comparator. This is consistent with independent comments on the use of heterologous expression for variant classification that “Mammalian transient transfection studies are widely used as functional studies for missense variants and are generally considered strong evidence provided that the experiments include appropriate controls such as a positive wild-type control, a negative vector alone, a correction for transfection efficiency with multiple replicate experiments, and ideally, known pathogenic and benign variants. When evidence suggests a dominant negative mechanism, the experimental set up should include a 50:50 heterozygous mix to explore multimer disruption” [85].

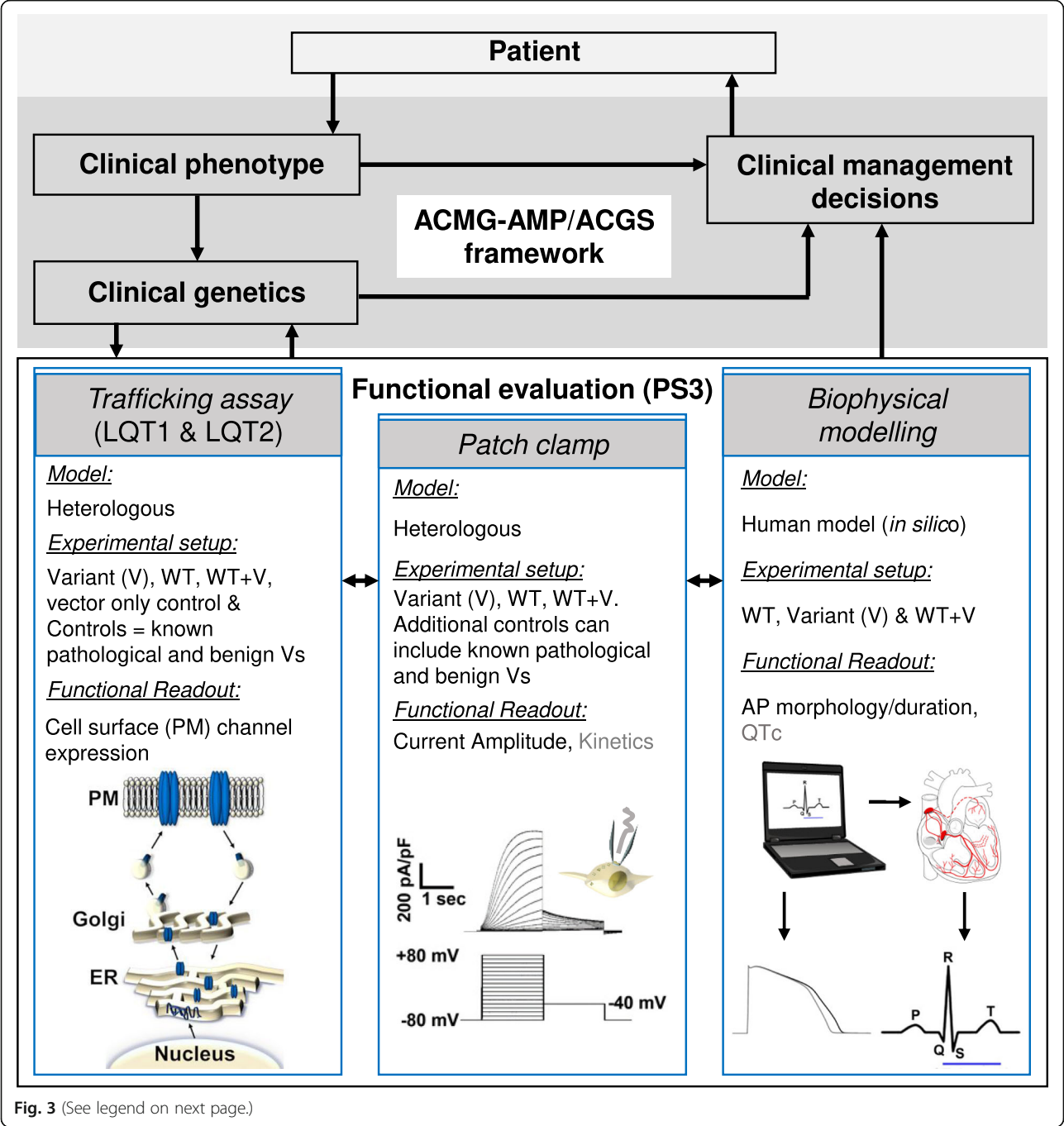
The working group’s recent guidance on ‘stacking evidence’ is conservative: where multiple functional assay results are available for a single variant, the evaluator is advised to apply evidence from the assay that is most well validated [84]. The committee was unable to reach consensus on whether results from different types of functional assay could be combined [84]. In the case of an ion channel gene mutation that leads to a trafficking defect, biochemical assays can show whether or not cell surface expression of the protein is reduced (and also whether the total amount of the protein in the cell is changed or not), whilst patch clamp shows the extent to which net ionic current through the channels in the membrane is reduced. These are complementary measures that are (a) mutually supportive and (b) combine to provide mechanistic insight into the nature of channel dysfunction present. It would thus be erroneous to evaluate the combination of evidence from the combination of the two approaches as equivalent to the strength

of one of them alone. Furthermore, once functional consequences have been further explored through biophysical modelling, a clear result from the combined approach should exceed any reasonable threshold for ‘strong’ evidence. Indeed, in recent work on a *KCNH2* variant found on molecular autopsy, the combination of manual patch clamp characterization and surface expression without modelling was considered to meet the PS3 ‘strong’ criterion [86]. Fig. 3 summarizes an integrated approach to the functional interrogation of suspected LQT1–3 variants, utilizing recombinant channel approaches. This scheme does not preclude additional work with hiPSC-CMs and/or genetically modified animals, where these are available; rather it focuses on utilizing recombinant channel data to best effect.

## Conclusions

Vanoye and George have recently highlighted that whilst reclassification of ion channel variants using data from functional assays conforms with the ACMG guidelines, and classification of a *KCNH2* variant on the basis of dominant negative loss of function behaviour is reasonable, some caveats are necessary [2]. In particular, a failure to find a functional or biochemical change in a heterologous expression assay does not necessarily entirely exclude pathogenicity [2]. For example, dysfunction might only become evident under particular stressors such as adrenergic drive [2], or in the presence of additional genetic modifiers [87]. Moreover, functional evidence is only one part of the overall picture and however strong it may be, it does not directly address issues of penetrance or expressivity. We have focused on *KCNQ1*, *KCNH2* and *SCN5A* as the function of these genes is fairly well-understood. The issue of what constitutes definitive evidence may be more difficult for other LQTS subtypes where the causal links between gene mutation and disease phenotype are less well-established [9]. Functional studies also have to be considered with available information on penetrance/expressivity. For example, in a recent study of *KCNE1*-linked LQT5, ECG penetrance of heterozygotic family members was only ~20% [88], so many mutation





(See figure on previous page.)

**Fig. 3** Schematic diagram of integrated approach to VUS evaluation, incorporating functional evaluation of recombinant channels. Clinical evaluation, establishing a clear clinical phenotype is the key driver of clinical management. Where the clinical phenotype suggests LQTS, interrogation of known LQTS genes proceeds through clinical genetics. The flow chart assumes that results of such analysis yields a VUS in *KCNQ1*, *KCNH2*, or *SCN5A*. For all 3 genes, previously uncharacterized variants can be investigated using whole-cell patch clamp measurements of current carried by recombinant channels heterologously expressed in a mammalian expression system. At the minimum, experiments should involve multiple replicate recordings from WT and variant (V) channels separately and with equal co-expression of WT + V to mimic the heterozygous state. Key measurements are of current size (which can be normalized to cell surface area, as cell capacitance, to facilitate pooling of data from the different experiments) and time-course. Differing conventional square-wave voltage clamp protocols can be used to enable quantification of amplitude and kinetics. Action potential voltage clamp will give a direct 'snapshot' of effect of the variant on current profile during a physiological waveform. For *KCNQ1* and *KCNH2* variants, a complementary trafficking assay should be performed allowing quantification of expressed mature/surface channel protein. Such a trafficking assay should use the same range of WT and variant expression conditions as patch clamp, ideally also including vector only control and known LQTS variants as controls. Biophysical modelling may not be required where variants produce a large dominant negative effect on channel expression, but it is useful in all cases and necessary where the predominant effects under patch clamp are on current kinetics. At the minimum, such modelling should investigate variant effects on AP duration in a human ventricle model. More sophisticated multicellular modelling can investigate effects on simulated QT interval. Modelling should include conditions to mimic heterozygous inheritance. The integrated results from functional evaluation can inform overall variant classification of pathogenicity and inform clinical management

carriers may be unaffected. This underscores the key role for clinical phenotype in diagnosis [1]. Additionally, whilst KCNE1 is well-accepted to be the  $\beta$ -subunit of the  $I_{Ks}$  channel and LQT5 mutations are likely to manifest in studies of *KCNQ1* and *KCNE1* [89, 90], *KCNE1* can also co-assemble with the hERG channel and *KCNE1* mutations may rarely adversely affect  $I_{Kr}$  function [91–94]. The situation is potentially more complex for *KCNE2*, as this can produce promiscuous interactions with multiple different ion channels [95, 96] and require co-expression with multiple different ion channel partners to identify/exclude a pathogenic functional effect.

Therefore, both the design and interpretation of strength of evidence from in vitro functional experiments must consider fully what is known about the role of the protein in question. Ultimately, it may be difficult to arrive at a single adapted PS3 criterion that applies equally to all heritable arrhythmias, or indeed to all genes associated with a single arrhythmia syndrome. Disease-specific adaptation of the ACMG/AMP guidelines has been undertaken for *MYH7*-associated cardiomyopathies, including adaptation of the PS3 criterion [97]. Such adaptation is likely to be useful for heritable LQTS and other inherited arrhythmia syndromes. Incorporation of specialist knowledge of the available, established functional/biochemical assays is required to optimise disease-specific guidance for the PS3 criterion application. This would ensure that the strength of functional/biochemical evidence is assessed using best-in-practice, realistic methods and enable accurate dissemination of the weight of supporting evidence to direct variant classification and therefore the overall decision-making by clinicians. The price of failing to correctly identify a VUS as benign is the incorrect attribution of causality, whilst that of failing to classify a pathological

VUS as 'pathological' or 'likely pathological' is the omission of information that may have utility for clinical management.

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Figure 2 is reproduced and modified from [82] (doi: <https://doi.org/10.1016/j.bbrc.2020.04.003>) under a Creative Commons Attribution 4.0 (CC BY 4.0) license.

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JCH and SCH conceived and drafted the article. AGS helped draft and revise the article critically for important intellectual content. All authors approved the final version of the article.

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