SCN1A variants from bench to bedside – improved clinical prediction from functional characterization

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Abstract

Variants in the *SCN1A* gene are associated with a wide range of disorders including familial hemiplegic migraine (FHM), genetic epilepsy with febrile seizures plus (GEFS+) and the severe childhood epilepsy Dravet syndrome (DS). Predicting disease outcomes based on variant type remains challenging. Despite thousands of *SCN1A* variants being reported, only a minority has been functionally assessed.

We review the functional *SCN1A* work performed to date, critically appraise electrophysiological measurements, compare this to *in silico* predictions, and relate our findings to the clinical phenotype.

Our results show, regardless of the underlying phenotype, that conventional *in silico* software correctly predicted benign from pathogenic variants in nearly 90%, however was unable to differentiate within the disease spectrum (DS vs GEFS+ vs FHM). In contrast, patch-clamp data from mammalian expression systems revealed functional differences among missense variants allowing discrimination between disease severities. Those presenting with milder phenotypes retained a degree of channel function measured as residual whole-cell current, whereas those without any whole cell current were often associated with Dravet syndrome (p=0.024).

These findings demonstrate that electrophysiological data from mammalian expression systems can serve as useful disease biomarker when evaluating *SCN1A* variants, particularly in view of new and emerging treatment options in Dravet syndrome.

Keywords: *SCN1A*, Dravet syndrome, GEFS+, Familial hemiplegic migraine, patch-clamp, electrophysiology, functional testing

Introduction

Variants in the *SCN1A* gene are associated with a wide spectrum of diseases ranging from genetic epilepsy with febrile seizures plus (GEFS+) and familial hemiplegic migraine (FHM) to the severe childhood epilepsy Dravet syndrome (DS) (Claes et al., 2001, Dravet et al., 2005, Brunklaus et al., 2012). Phenotype prediction based on interpretation of genetic findings remains challenging and functional analysis of variants *in-vitro* is considered the gold standard to determine pathogenicity.

Truncating variants are known to lead to loss of protein function and affected individuals are likely to present with DS. However, the functional effects of a missense variant can be variable with phenotypes ranging from milder presentations such as GEFS+ to severe phenotypes such as DS. The proposed molecular pathology in epilepsy had been loss-of-function (LoF) (Yu et al., 2006; Ogiwara et al., 2007) whereas familial hemiplegic migraine cases are associated with gain-of-function (GoF) variants (Mantegazza et al., 2018). Recently a subgroup of *SCN1A* missense variants associated with severe epileptic encephalopathy and movement disorder has been described due to presumed GoF effects (Sadleir et al., 2017, Berecki et al., 2019). Ishii et al. (2017) showed that truncating *SCN1A* variants appear to be associated with a greater rate of cognitive decline compared to missense variants, suggesting that the variant type is predictive of cognitive function.

Whilst standard *in silico* prediction tools are useful when distinguishing disease from non-disease, they do not allow severity prediction within the disease spectrum (Zuberi et al., 2011; Holland et al., 2017). Furthermore, the pathogenicity of many variants remains undetermined. Interrogation of *ClinVar* reveals that according to American College of Medical Genetic criteria, 40% of *SCN1A* reports are still classified as being *"variants of uncertain significance"* (http://www.ncbi.nlm.nih.gov/clinvar/, accessed February 2019) illustrating the need for better characterisation of missense variants. Functional data are often not available to complement variant interpretation as these are labour intensive and costly to obtain. Although thousands of *SCN1A* variants are reported, only a small fraction have been functionally assessed.

We have reviewed the functional *SCN1A* work performed to date as a resource to aid variant interpretation. We critically appraise the original electrophysiological measurements, evaluate pathogenicity, compare this to *in silico* predictions, and relate our findings to the described phenotype. This comprehensive review of very well characterised *SCN1A* variants offers a useful tool for clinicians, geneticists and genetic scientists when evaluating different *SCN1A* variants. It also allows scientists to select variants of particular interest for future research.

Methods

Review of *SCN1A* functional missense variants studied by whole-cell patch-clamp experiments: to collect functionally tested missense variants, we performed a PubMed (up to February 2019) search using terms "clamp" and "*SCN1A*" using R package RISmed 2.17. We included *SCN1A* missense variants, which have been functionally tested by whole-cell patch-clamp experiments and summarized the relevant patient phenotypes in supplementary table 1. Variants observed in the general population, thus present in the Genome Aggregation Database (gnomAD, http://gnomad.broadinstitute.org/) were annotated as such. We only included variants characterized in mammalian cell lines to improve biophysical comparisons. Variants were then categorized either as gain-of-function (GoF), loss-of-function (LoF) or 'mixed' function regarding their biophysical properties. We assessed the effect of different voltage properties such as whole-cell current, peak current density, persistent current, activation, fast inactivation, recovery from fast inactivation, slow inactivation, and recovery from slow inactivation on channel function and related this to the phenotype. We define any biophysical change entailing an increase in the Na+ permeability as GoF, and the opposite for LoF. A few cases showed a paradoxical change i.e. decrease in the peak current and increase in the persistent current. Whenever there is an increase in the persistent current, we

define the variation as GoF. In contrast where there was a gain in peak current accompanied by a depolarising shift of inactivation this would be considered 'mixed'.

***In silico* prediction:** variants were classified according to ACMG guidelines (Richards et al., 2015). We scored variant pathogenicity for these *SCN1A* variants using a range of commonly used *in silico* prediction tools including "Align GVGD version 2007", "SIFT version 6.2.0", "Mutation Taster version 2013", "Polyphen-2 v2.2.2r398" and "Mutation Assessor". We further considered "CADD" and "REVEL" scores. We predicted the variant effect on splicing with SSF, MaxEntScan, NNSPlice (v0.9), GeneSplicer. We compared these *in silico* predictions with the functional measurements obtained via *in-vitro* patch-clamp work.

Clinical phenotypes: in order to distinguish *SCN1A* variants by phenotype we took a pragmatic approach and (1) firstly grouped all those variants together associated with a phenotype of *DS only*; (2) secondly all those associated with more than one phenotype including *DS/GEFS+/FS+*, and (3) thirdly all those associated with a phenotype of familial hemiplegic migraine (*FHM)*.

Data analysis: patients with missing data were excluded from the relevant analyses. Chi-square statistics were used to determine categorical differences between groups. Statistical analysis was performed using SPSS version 23.0.

Results

We identified 58 missense *SCN1A* variants in the literature that were functionally characterised by whole-cell patch-clamp experiments between 2002 and 2019. Of these, 26 (45%) were associated with a phenotype of *DS only*, 21 (36%) were associated with a range of phenotypes including *DS/GEFS+/FS+* and five (9%) were associated with *FHM* (Figure 1; table 1; supplementary table 1).

Four variants were present in gnomAD: one showed mixed function and had very high gnomAD frequency (c.3521C>G, p.Thr1174Ser; C=0.001730/479 - including 3 homozygotes) and was assumed not disease causing; one showed normal function and was assumed not disease causing (c.4723C>T, p.Arg1575Cys). One demonstrated LoF effects on functional analysis (c.4096G>A, p.Val1366Ile) and one mixed effects (c.2576G>A, p.Arg859His). Both were considered pathogenic and included in the analysis; the two polymorphisms were not. There was one variant associated with a phenotype of non-DS early infantile epileptic encephalopathy (c.677C>T, p.Thr226Met) that was also not included. The remaining three cases had either insufficient data available (n=2) or the effect could not be determined (n=1). See table 1 and supplementary table 1.

The majority of functional effects were LoF (71%, 37/52), followed by 'mixed' (21%, 11/52) and GoF (8%, 4/52). There was no difference in the number/percentage of LoF variants found according to the phenotype: In the *DS only* group 77% had LoF variants (20/26) compared with 76% (16/21) in the *DS/GEFS+/FS+* group with the remaining variants classified as 'mixed' function. All four GoF variants were observed in phenotypes with *FHM*.

More detailed analysis of the electrophysiological data across all variants revealed a complete loss-of-function with no measurable whole cell current in 44% of variants (23/52) with the remaining 56% of variants (29/52) showing some measurable current. There was a significantly greater proportion of complete loss-of-function with no measurable whole cell current in the *DS only* group (62%, 16/26) compared with the *DS/GEFS+/FS+* phenotype group (29%, 6/21, $\chi 2 = 5.07$, df = 1, p=0.024) in which a larger proportion of measurable current was observed (Figure 2).

Examining whether or not the functional data changed the ACMG classification of any of the missense variants revealed that in those 47 out of 58 variants that were not already categorised as ACMG class 5, the addition of functional data resulted in a change of ACMG classification in 94% (44/47) of variants (table 1). This led to an up classification showing increased variant pathogenicity in 43 cases, of which 28 from class 4 to class 5, 14 from class 3 to class 4 and one from class 2 to class 3. In one case the classification went down from class 3 to class 2 indicated less pathogenicity.

In silico prediction did not allow discrimination between phenotypes: using all five conventional prediction tools the *in silico* analysis estimated the variants to be pathogenic in 89% of cases regardless of the underlying phenotype (*DS only*, *DS/GEFS+/FS+* or *FHM*; Figure 3). Application of the emerging diagnostic tool REVEL correctly identified the two polymorphisms as the two lowest scores of 0.4 and 0.5 with all other disease variants showing values of >0.7 regardless of the phenotype (*DS/GEFS+/FS+* or *FHM*). Similarly, CADD scores correctly identified one of the 2 polymorphisms as having a low score of 16 with all other disease variants showing values over 20 regardless of the phenotype.

We observed five variants with identical amino acid (AA) positions but different AA substitutions. Those presenting with AA substitutions of greater physico-chemical difference due to introduction of the amino acid cysteine were associated with more severe phenotypes: c.2576G>A, p.Arg859His (DIIS4, GS = 29) was reported in GEFS+ with mixed functional effects, whereas c.2575C>T, p.Arg859Cys (GS = 180) was seen with the more severe phenotype DS with loss-of-function effects. c.2837G>A, p.Arg946His (DIIS5-S6, GS = 29) was associated with both mild and severe phenotypes compared to c.2836C>T, p.Arg946Cys (GS = 180) which was reported in association with the severe DS phenotype only. c.4943G>A, p.Arg1648His (DIVS4, GS = 29) was seen with both mild and severe phenotypes whereas c.4942C>T, p.Arg1648Cys (GS = 180) was only associated with the severe phenotype. c.5054C>T, p.Ala1685Val (DIVS5, GS = 64) was reported in association with a partial epilepsy with antecedent febrile seizures (PEFS+) phenotype compared with c.5054C>A, p.Ala1685Asp (GS = 126) which was associated with DS. c.530G>C, p.Gly177Ala (D1S2-S3, GS = 60) and c.530G>A, p.Gly177Glu (GS = 98) were both seen in DS with loss-of-function.

Discussion

Dravet syndrome with an incidence of 1:15,000 is considered a model disease for the study of genetic epilepsies (Wu et al., 2015). Although children present in infancy, the full clinical phenotype only emerges after the second and third year of life (Dravet et al., 2005, Brunklaus et al., 2012). Hence, when an *SCN1A* mutation is found we cannot predict how a child will progress in the future, with no evidence that a particular mutation type can inform developmental outcome or medication choice. As new therapeutic strategies for DS are emerging, early diagnosis and treatment are paramount. Comparing clinical with functional and *in silico* data we were able to show that patch-clamp recordings aided discrimination between disease phenotypes, whereas bioinformatic tools estimated between disease and non-disease but not within disease.

How functional characterisation can aid clinical prediction

In silico prediction tools are widely used by genetic laboratories to estimate the pathogenicity of any given variant. The majority are based on bioinformatic algorithms considering conservation across species, amino acid characteristics, and previous reports, with the aim to differentiate pathogenic from benign variants. A common problem is that most computational algorithms have high sensitivity but low specificity and therefore overestimate disease pathogenicity (Holland et al., 2017). With *in-vitro* functional characterisation used as gold standard comparator, our results show that conventional

pathogenicity software classified nearly 90% of the here examined variants correctly as disease causing, regardless of the underlying phenotype. This figure increased to nearly 100% when using novel tools such as CADD and REVEL. *In silico* tools are therefore useful when predicting disease from non-disease; however, they are not able to differentiate within a disease spectrum, for example between DS, GEFS+ and FS+, highlighting their use as measure of amino acid conservation but not function.

Considering ACMG criteria we found that functional measurements were clearly helpful in the delineation of variant pathogenicity resulting in up-classification towards increased pathogenicity in nearly all cases. Such a change may have direct clinical implications and adds to its clinical utility, confirming that functional data represent a useful biomarker for *SCN1A* related disease.

Further, we were able to show that functional measurements are better at discriminating between disease severity. As expected, review of the electrophysiology revealed that the majority of Dravet syndrome and GEFS+ associated variants have LoF effects (Yu et al., 2006; Ogiwara et al., 2007) whereas those seen in FHM have GoF effects (Mantegazza et al., 2018). Truncation variants are expected to lead to complete abolition of protein function and LoF. Whilst our data show that the vast majority of missense variants associated with DS/GEFS+/FS+ also lead to LoF effects, there are subtle differences among missense variants. We found that those presenting with milder phenotypes frequently retained a degree of channel function, measured as residual whole-cell current. In contrast, those without any detectable whole cell current were often associated with Dravet syndrome only. This is in line with recent work by Nissenkorn et al. (2019) who examined four *SCN1A* variants regarding their functional properties and developmental phenotypes. Three variants had no detectable sodium current and were associated with classical DS, whereas the one variant with detectable current was associated with a milder phenotype with lower seizure burden and better cognitive function. Even sophisticated bioinformatic tools, used to assess protein misfolding [SWISS-MODEL], were not able to distinguish between different *SCN1A* variants.

Functional variation leads to heterogeneous phenotypes

Review of the patch-clamp data revealed that not all variants fall neatly into gain or loss-of-function categories. There appears to be a significant degree of functional variation resulting in heterogeneous phenotypes. Several mutations have mixed effects including some which may be considered to increase sodium currents. We re-examined these to determine whether on balance any mutation linked to epilepsy could be described as a pure gain-of-function. Several mutations with mixed functions (c.4982T>C, p.Phe1661Ser, Rhodes et al., 2004; c.5422T>C, p.Phe1808Leu, Rhodes et al., 2005; c.5726C>T, p.Thr1909Ile, Ohmori et al., 2006) do show reduced current density in reporter cells, which is consistent with an overall loss in sodium current. Several additional mutations show normal current densities when cells are held at highly hyperpolarised potentials (-120 mV), but these mutations also demonstrated shifts in voltage dependence of steady state inactivation (c.2422A>T, p.Thr808Ser, Rhodes et al., 2005; c.4942C>T, p.Arg1648Cys, Rhodes et al., 2004) and are likely to produce vastly reduced currents when in neurons which rest closer to -70 mV, where a larger proportion of the channels will be inactivated. Similarly, although c.3610T>C, p.Trp1204Arg was originally not associated with a clear loss-of-function, follow up studies revealed that p.Trp1204Arg produced a 40% loss in current density using slightly less hyperpolarised potentials of -100 mV (Becchi et al., 2015).

One of the more interesting mutations is c.2593C>G, p.Arg865Gly. This mutation has consistent gain-of-function effects in heterologous systems (Volkers et al., 2011), but its location suggests a new insight into possible mechanisms of *SCN1A* epilepsies. p.Arg865 is one of the voltage sensing arginines in the S4 of the second domain of the channel. Its position is equivalent to p.Arg875 in *SCN4A* where it causes a loss-of-function due to an increase in slow inactivation (linked to a periodic paralysis

phenotype; Wu et al., 2014), and p.Arg814 in *SCN5A* where it is associated with Brugada syndrome, a classic loss-of-function disorder (Loussouarn et al., 2016). None of the studies have been probed explicitly for gating pore leaks, but the position of the amino acid and the prevalence of gating pore leaks introduced by loss of these S4 arginines, indicates a potential novel mechanism for disease: loss of interneuronal viability due to proton leaks. Further work will be needed to verify this mechanism, but it may also be potentially applicable to other mutations affecting S4 arginines identified in *SCN1A*. These leak currents are small and difficult to study, and thus far have been mainly confined to recordings from the robustly expressing muscle channel *SCN4A* in large xenopus oocytes, where sufficient expression can be obtained to measure the protons leaking through the S4 gating pore, but potential studies producing the equivalent of c.2593C>G, p.Arg865Gly and even c.4943G>A, p.Arg1648His in *SCN4A* might indicate whether these changes are likely to introduce gating pore leaks in the closely related *SCN1A* sequence.

These observations are consistent with recent findings of an *SCN1A* mutation (c.677C>T, p.Thr226Met) that has been described in individuals presenting with a severe early epileptic encephalopathy different from DS (Sadleir et al., 2017, Berecki et al., 2019). This variant has been shown to have some gain-of-function effects, including a hyperpolarising shift in activation, however this is accompanied by a hyperpolarising shift in inactivation. When modelled, the net consequence in rapid firing cells was entry into depolarising block, where cells were no longer able to fire action potentials due to accumulation of channels in inactivated states. Consequently, although in some conditions the currents can be larger, the overall effect is a loss of neuronal activity.

The variant c.3521C>G, p.Thr1174Ser has been seen in many different clinical phenotypes including both epilepsy and familial hemiplegic migraine (FHM). Analysis revealed that p.Thr1174Ser could in some conditions induce overall loss-of-function, and in others gain-of-function, representing a switch between overall gain and loss-of-function, in keeping with both, pro-migraine and epileptogenic effects (Cestèle et al., 2013a). However, this particular variant is found with very high frequency in gnomAD (C=0.001730/479 - including 3 homozygotes) and *in silico* tools describe it as a benign variant suggesting that on balance this is likely a polymorphism.

It is worth highlighting the limitations of functional *in-vitro* work, as mammalian patch-clamp expression systems do not always reflect the mutational effects *in-vivo*. We were not able to systematically assess whether the functional read-outs match *in-vivo* work from mouse models. It is also likely that a few variants and proteins will never arrive at the cell membrane because their cellular trafficking is altered. Thus, a Gof missense variant *in-vitro* could act as LoF variant *in-vivo*. The converse may also be true. For example, the c.4946T>A, p.Leu1649Gln FHM mutant is non-functional when expressed in a human cell line because of impaired plasma membrane expression (Kahlig et al., 2008; Cestèle et al., 2013b); however, is rescued when co-expressed with interacting proteins *in-vivo*, resulting in GoF which is in keeping with other FHM variants (Cestèle et al., 2013b).

Differences in amino acid composition are reflected in the phenotype

Several variants were observed at identical amino acid (AA) position with different AA substitution. The grantham score (GS) as measure of physico-chemical difference indicates that in variants at identical location, those with greater GS are associated with more severe phenotypes, as previously shown across a number of voltage gated sodium channel disorders (Grantham 1974; Brunklaus et al., 2014). The variant c.4943G>A, p.Arg1648His (GS = 29) has been associated with mild and severe phenotypes and represents one of the voltage sensing arginines in DIVS4 (Lossin et al., 2002). It is therefore a very strong case for the gating pore leak hypothesis - alternative to a 'gain-of-function' mechanism. In contrast, the c.4942C>T, p.Arg1648Cys variant (GS = 180) which is mainly found in severe phenotypes demonstrated shifts in voltage dependence of steady state inactivation (Rhodes et al., 2005, Rhodes et al., 2004), that are likely to produce vastly reduced currents when in neurons

which rest closer to -70 mV where a larger proportion of the channels will be inactivated. Interestingly, the same p.Arg1648 residue which hyperpolarises voltage dependence of steady state inactivation when changed to a cysteine, has very subtle effects when changed to a histidine. A detailed study demonstrated that in fact c.4943G>A, p.Arg1648His does produce reduced currents in transfected cells, but this is due to accumulation of slow inactivated states. The effect is elegantly matched by failure to initiate action potentials in interneurons of knock-in-mice, suggesting a mechanism consistent with loss-of-function (Hedrich et al., 2014). The c.2576G>A, p.Arg859His variant (GS = 29) reported in GEFS+ is equally one of the crucial voltage sensing arginines in DIIS4 (Volkers et al., 2011). The change of a histidine for a cysteine c.2575C>T, p.Arg859Cys (GS = 180) results in more severe DS phenotype with loss-of-function, again strengthening the case for the gating pore leak hypothesis (Bechi et al., 2015). Holland et al. (2018) recently proposed that variant location might help guide treatment of sodium channelopathies. Whilst there is good evidence that pathogenic missense variants are clustered in functionally important areas of the SCN1A protein, our observations illustrate how closely the phenotype is linked particularly to changes in amino acid composition (Zuberi et al., 2011; Meng et al., 2015; Ishii et al., 2017). Recently Gonsales et al. (2019) proposed a multimodal analysis of *SCN1A* missense variants considering characteristics such as mode of inheritance, population frequencies and protein localization to improve the interpretation of clinically relevant variants.

Clinical implications

Both, *in silico* prediction and *in vitro* functional data are important for variant classification as indicated by the ACMG guidelines including criteria PS3 - for functional data and criteria PP3 – for concordant computational predictions (Richards et al. 2015). In addition, our findings illustrate that detailed electrophysiological knowledge has the potential to impact on clinical management. Being able to predict the disease phenotype from functional data may facilitate swift appropriate treatment of epilepsies that are predicted to be severe. This may involve use of medications that have been shown beneficial in DS and avoiding those that are known to lead to exacerbation of seizures such as sodium channel blockers (Guerrini et al., 1998). Yet, it is not understood why certain individuals with DS respond well to lamotrigine (Dalic et al., 2015). A possible explanation might be that the increased selectivity of lamotrigine for persistent currents means that individuals who have mutations that generate increased persistent currents benefit from this increased selectivity. Further examples to show that effective treatment may be determined by the underlying sodium channel defect comes from FHM individuals with gain-of-function *SCN1A* variants who respond well to the sodium channel blocker carbamazepine (Castro et al., 2009). Naturally, these 'variant/treatment' associations are influenced by many additional factors such as modifier genes or post-translational modifications, explaining the often seen intrafamilial differences.

A precedent for using functional data to change management in the epilepsy field was recently reported in GLUT1 disease (Zaman et al., 2018). Using a rapid functional assay measuring the rate of glucose transport between control and disease groups, disease severity could be partly explained by the extent of GLUT1 dysfunction, highlighting how genetic and clinical assessments can be complemented by a simple functional assay. To date the generation of similar data for *SCN1A* are mainly available via research laboratories and compounded by technical challenges and low throughput.

New efficacious treatments have recently become available for DS and gene therapy approaches are on the horizon promising to not only treat seizures but to address all aspects of the developmental and epileptic encephalopathy/channelopathy (Ceulemans et al., 2016, Devinski et al., 2017, Brunklaus & Zuberi, 2014). Early identification of severe phenotypes and prompt initiation of treatment may be crucial. To this end functional data may serve as important biomarker, particularly for missense

variants which are not targeting known functional sites of the channels, highlighting the need for automated tools allowing easy and early access to patch-clamp analyses. P~~Such p~~latforms offering rapid and high throughput SCN1A electrophysiological assays, such as automated patch clamp setups, ~~(e.g. dynamic clamp)~~ are still being developed, ~~have so far been developed with varying success~~ and challenges remain due to the variability of channel expression, and modulation of the channels by the cells chosen for expression. However, these platforms will be important ~~but are required~~ in the future to aid risk stratification based on clinical and genetic data allowing early detection of severe disease (Li et a., 2017). In line with recent ILAE guidelines early *SCN1A* testing is recommended after the first prolonged seizure and will facilitate appropriate treatment as soon as possible.

Conclusion

Evaluation of over 50 functionally characterised *SCN1A* variants reveals that electrophysiological testing of channel function contributes to the prediction of disease severity, whereas *in silico* tools are useful in distinguishing benign from pathogenic variants. As new therapeutic strategies for Dravet syndrome are emerging early diagnosis and treatment are paramount. Patch-clamp data from mammalian expression systems can serve as useful disease biomarker for clinicians, geneticists and genetic scientists when evaluating different *SCN1A* variants.

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Figure legends

Figure 1 | Missense variant locations and phenotypes across SCN1A protein
Figure 1 illustrates the missense variant locations and phenotypes across a 2D schematic view of the SCN1A protein ($Na_V1.2$). We did not include polymorphisms or variants with undefined functional properties (Table 1 and supplementary table 1). DS = Dravet syndrome, GEFS+ = Genetic Epilepsy with Febrile Seizures Plus, FS+ = Febrile seizures Plus, FHM = Familial Hemiplegic Migraine.

Figure 2 | Whole-cell current as a marker of function
Figure 2 illustrates whether a whole-cell current was measurable in variants of the 2 phenotype categories: *Dravet syndrome (DS) only* (n=26) or *DS/GEFS+/FS+* (n=21). GEFS+ = Genetic Epilepsy with Febrile Seizures Plus, FS+ = Febrile seizures Plus. The 2 groups are compared using a $\chi 2$-test of association (df=1).

Figure 3 | *In silico* tools predicting pathogenicity according to phenotype
Figure 3 illustrates whether all 5 *in silico* tools predicted the variant to be pathogenic according to the phenotypic categories: *Dravet syndrome (DS) only* (n=26), *DS/GEFS+/FS+* (n=21) and *FHM* (n=5) (p = NS). GEFS+ = Genetic Epilepsy with Febrile Seizures Plus, FS+ = Febrile seizures Plus, FHM = Familial Hemiplegic Migraine.

Conflict of Interest Statement: no conflict of interest to declare

Data Availability Statement: Data sharing is not applicable to this article as no new data were created or analyzed in this study.