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Addendum to the report dated October 18th, 2022 Report on analysis of the Folbigg CALM2 G114R mutation and its impact on calmodulin protein function

– prepared in connection with the 2022 Folbigg Inquiry

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Date: November 12th, 2022

Addendum to the report dated October 18th, 2022

**Report on analysis of the Folbigg *CALM2* G114R mutation and its impact
on calmodulin protein function**

– prepared in connection with the 2022 Folbigg Inquiry

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Statements for the Inquiry:

Aalborg November 12, 2022

To whom it may concern:

I, Michael Toft Overgaard, acknowledge for the purpose of Rule 31.23 of the Uniform Civil Procedure Rules 2005 that I have read the Expert Witness Code of Conduct in Schedule 7 to the said rules and agree to be bound by it.

The opinions within this report are wholly or substantially based on my specialised knowledge as a professor in protein chemistry, and my experience within characterising the effect of genetic mutations at the protein level.

I have no prior experience with legal proceedings.

I am legally married to and live with the co-author of this report, Mette Nyegaard. We have two children (age 22 and 24). We have a long history of scientific collaboration.

Yours sincerely,

Michael Toft Overgaard

Professor

Head of Department

.....

Aalborg November 10, 2022

To whom it may concern:

I, Mette Nyegaard, acknowledge for the purpose of Rule 31.23 of the Uniform Civil Procedure Rules 2005 that I have read the Expert Witness Code of Conduct in Schedule 7 to the said rules and agree to be bound by it.

The opinions within this report are wholly or substantially based on my specialised knowledge as a professor in personalised medicine, and experienced researcher within human genetics and linking variation in genes to diseases.

I have no prior experience with legal proceedings.

I am legally married to and live with the co-author of this report, Michael Toft Overgaard. We have two children (age 22 and 24). We have a long history of scientific collaboration.

Yours sincerely,

Mette Nyegaard

Professor

Addendum- Report on analysis of the Folbigg *CALM2* G114R mutation and its impact on calmodulin protein function

Statements for the Inquiry:

Aalborg November 10, 2022

To whom it may concern:

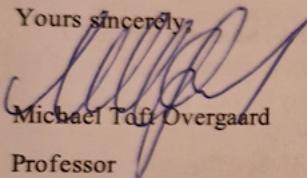
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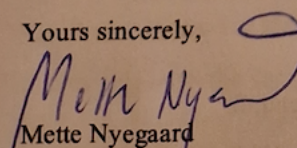
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Mette Nyegaard

Professor

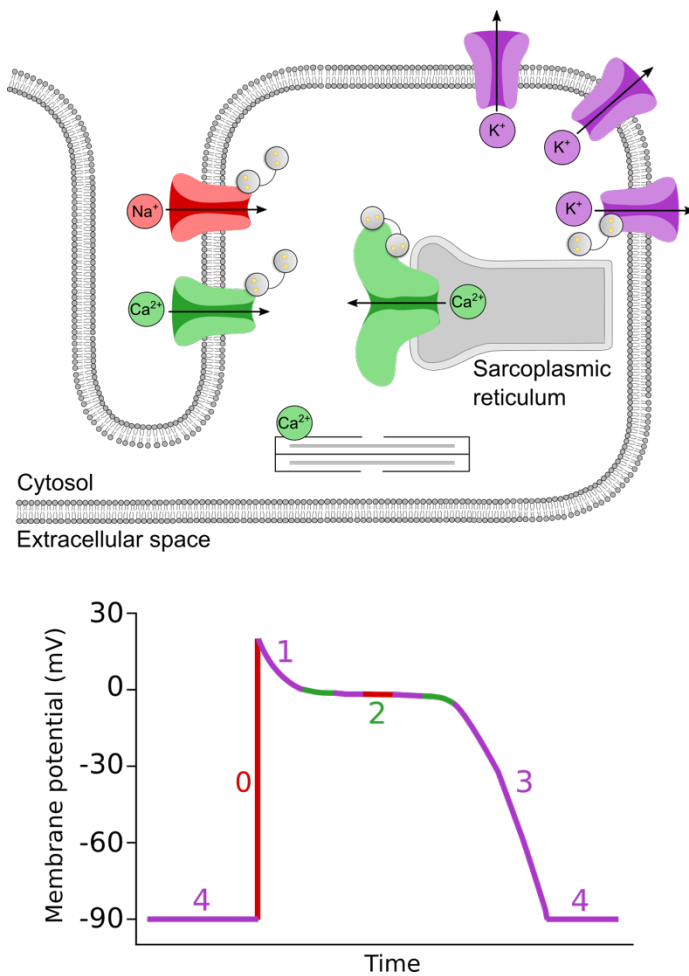
We wish to add several considerations to our initial report submitted on October 18.

This additional report is presented in 4 parts. In **Part A**) we give a short background overview of the cardiac excitation contraction coupling, and the role of calmodulin in this process (i.e., explaining the role of calmodulin in regulating heartbeats). In **Part B**) we address the concern raised in the report by Prof Skinner and Prof Kirk (hereafter referred to as ‘the Skinner report’) and the report by Prof Wilde (hereafter referred to as ‘the Wilde report’) regarding: **(B1)** the apparent mismatch between the functional effects identified for the *CALM2* G114R mutation and the phenotypic presentation of the two Folbigg daughters, where we present novel data from binding assays of calmodulin variants to the voltage gated sodium channel, Nav1.5 (protein product of the *SNC5A* gene), **(B2)** the possibility of the functional assays to produce ‘false positives’, and **(B3)** the results from the functional assays not translating directly into clinical phenotypes. In **Part C**) we raise concerns regarding the use of the ACMG system to place a deterministic judgement of the Folbigg mutation, and in **Part D**) we provide our comments on the finding of a single *CALM3* G114R carrier in the Regeneron dataset.

Part A. The role of calmodulin in cardiac muscle cell contraction (heart beats)

Here we aim to 1) provide an overview of the main ion-channels whose function is driving the cardiac contraction cycle, and 2) illustrate in which of these channels the functions are modulated by calmodulin. In principle, all the calmodulin regulated ion-channels may be implicated in the pathogenic molecular mechanism linking calmodulin mutations to cardiac arrhythmia and sudden cardiac death. A more detailed explanation of the role of calmodulin in the cardiac excitation contraction cycle is presented in the review paper by Sorensen in the *Febs Journal* (Sorensen, 2013).

In a short, simplified version, contraction of cardiac muscle cells is tightly controlled by timely opening and closing of specific ion-channels, producing action potentials that lead to an increase in calcium concentration, which in turn drives contractile elements in the cardiac muscle cells to contract. As illustrated in the figure below, four main types of ion-channels are involved. When a heart cell is in a relaxed resting state, a large negative cell membrane potential is established by an open potassium channel, Kir2.1, resulting in a high outward flow of potassium ions (‘polarized state’) (Phase 4). Following a small electrical signal originating in the heart sinus node, the cardiac voltage gated sodium channel Nav1.5 (product of the *SCN5A* gene) opens, resulting in a fast inward flow of sodium ions which creates a rapid depolarization of the cell membrane (Phase 0). This depolarization event results in partial inactivation of Nav1.5, opening of Kv4.3 (Phase 1), opening of Cav1.2 and Kv11.1 (Phase 2) and then inactivation of Cav1.2 and opening of Kv7.1 (Phase 3), resetting the cell membrane to a polarized state (Phase 4). In phase 2, when Cav1.2 opens, a small inflow of calcium ions activates physically close and opposing sarcoplasmic calcium release channels, RyR2, allowing a large inflow of calcium that triggers muscle contraction. Importantly, calmodulin binds to both Nav1.5, Cav1.2, RyR2, and Kv1.7, enabling these channels to regulate their activity in a calcium dependent manner, a key ingredient for keeping the correct timing of ion-channel opening, inactivation and closing to produce regular heart beats.



Several heart cell ion channels are regulated by calmodulin. Top panel: Schematic illustration of a cardiomyocyte (heart muscle cell) with major ion-channels; the sodium channel is shown in red (Nav1.5), calcium channels in green (left: Cav1.2 [cell membrane] and right: RyR2 [sarcoplasmic reticulum]), and potassium channels in magenta (Kv4.3, Kv11.1, Kv7.1). Calmodulin is shown as gray dumbbell structures attached to the calmodulin regulated ion channels (Nav1.5, Cav1.2, RyR2, Kv7.1). Bottom panel: Illustration of the cardiac action potential phases, with the important ion-currents indicated with colors: depolarizing sodium current in red, depolarizing calcium ion current in green, and repolarizing potassium currents in magenta.

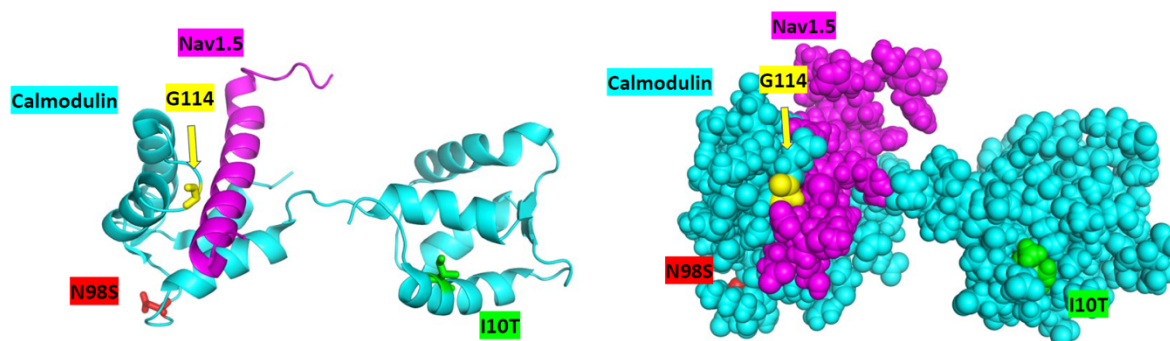
Part B. Concerns raised in the Skinner report

B1. Concerns that the results from the functional assays do not fit with the clinical manifestation in the two girls

Both the Skinner and the Wilde reports raise the concern that given the effect we show for the novel Folbigg G114R mutation on Cav1.2 and RYR2 in the functional assays, the clinical manifestation in the two Folbigg girls (death before the age of two while asleep) “is remarkably unlikely”.

Further, the Skinner report raises concern about fever as a trigger of arrhythmic events for calmodulinopathies, as this has until now only been seen in Brugada Syndrome. They opine that this cannot be relevant for the Folbigg case, as Brugada syndrome is a sodium channel disease in children, caused by mutations in the *SNC5A* gene (Nav1.5). However, the Skinner report does not recognize that the Nav1.5 sodium channel is critically regulated by calmodulin, as evident from calmodulin's role in cardiac contraction (see Part A).

To address their concerns, we investigated existing molecular structures of Nav1.5 in complex with calmodulin, to ascertain where the mutated G114 residue in the Folbigg variant is located relative to the core binding site for calmodulin in the Nav1.5. This type of investigation takes advantage of known protein structures determined by global research groups and is an established method in the scientific community to understand 3-dimensional protein structure and visualize how proteins fit together and pack into large complexes. The 3-dimensional structure of calmodulin bound to the Nav1.5 anchoring peptide is shown in the figure below and reveals that the Calmodulin G114 residue (marked in yellow) is located *exactly at the binding interface* with the cardiac sodium channel.



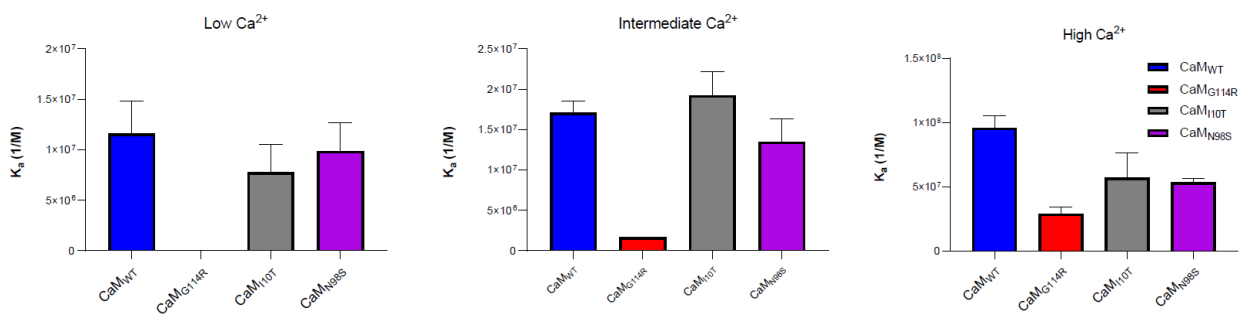
Calmodulin G114 is located in the binding interface with the cardiac sodium channel. Non mutated Calmodulin binding to a calmodulin binding fragment of the Nav1.5 channel (PDB 2L53) without the presence of calcium, shown as cartoon structures (left panel), and with atom space filling (right panel). Calmodulin is shown in cyan, the Nav1.5 fragment in magenta. Three calmodulin amino acid residues are highlighted in colors: G114 in yellow, N98 in red, and I10 in green.

The figure illustrates how the G114 residue is located in a *key position*, tucked in between calmodulin and the part of the Nav1.5 ion channel that constitutes the anchoring point for calmodulin binding to Nav1.5. The Folbigg *CALM2* G114R mutation causes a change in the G114 residue whereby the Glycine amino acid residue is changed to a Arginine amino acid residue. Based on the 3-dimensional protein structure, we predict that changing this small neutral Glycine (the smallest of the 20 amino acids) into a large and positively charged Arginine (one of the largest of the 20 amino acids), is highly likely to cause a disturbance of calmodulin binding to this important anchor point.

To test this, we performed a calmodulin-Nav1.5 binding assay, similar to the assays performed in the Brohus paper with Cav1,2 and RyR2 calmodulin binding domains. However, this time we tested binding properties of three different calmodulin variants, plus the non-mutated WT, to the Nav1.5 calmodulin binding domain.

We were able to perform this experiment within days, as we already had the different calmodulin variants produced and purified from our previous research. Because we had been planning and preparing for studying calmodulin variants binding to Nav1.5, we also had the Nav1.5 calmodulin binding domain available for immediate use.

For this assay we included normal non-mutated calmodulin (WT), G114R (the Folbigg variant), N98S (arrhythmogenic calmodulin variant with mixed phenotypes, CPVT, LQTS) and I10T (UK Biobank variant, no apparent arrhythmia phenotype). The N98S was included for comparison, because this mutation has a similar impact on calcium binding and calcium channel binding as the G114R variant (documented in the Brohus paper). The I10T was included because it is a variant found in GnomeAD, and later in the UK Biobank in a carrier reporting no apparent cardiac phenotype (from ICD10 data available in the UK Biobank through our approved project). Please note that we do not want to call any calmodulin missense variant absolutely benign, but at least we think this variant represents a likely benign variant with respect to a cardiac arrhythmia phenotype. The preliminary results are shown in the figure below, and clearly demonstrate that the Folbigg G114R calmodulin variant **impair binding to the cardiac sodium channel**.



G114R calmodulin variant impair binding to the cardiac sodium channel. Calmodulin variants binding to the anchor binding peptide of the Nav1.5 sodium channel. Binding is displayed as association constants, i.e., the larger the value, the stronger the binding between calmodulin and Nav1.5. WT calmodulin (in blue): non-mutated. G114R calmodulin (in red): Folbigg variant. I10T calmodulin (in grey): apparent non-arrhythmia variant. N98S calmodulin (in magenta): arrhythmogenic variant. Data presented as mean values of duplicate measurements +/- SD.

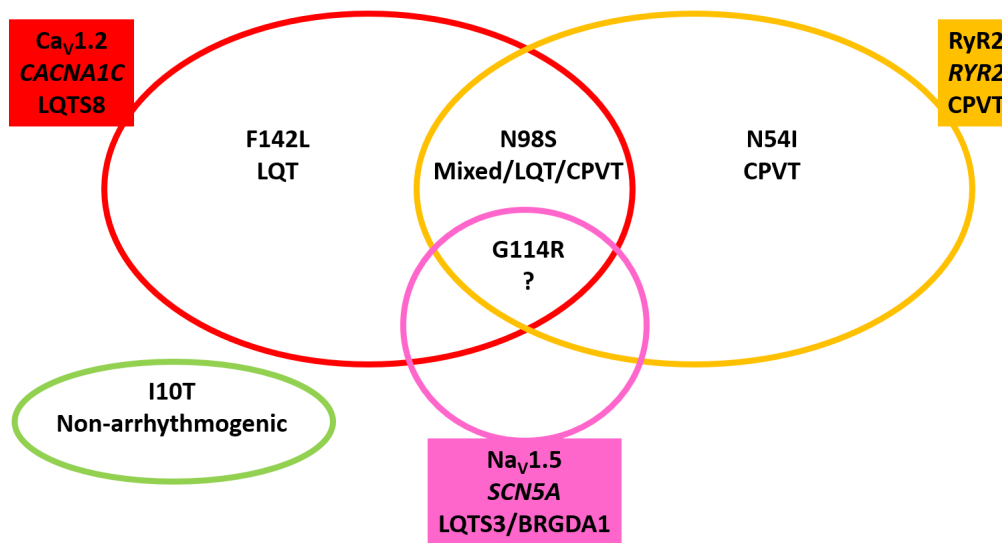
The data in the above figure show that the Folbigg mutation G114R exhibits a dramatic reduced binding to Nav1.5 at both low and immediate calcium levels, compared with both the non-mutated calmodulin WT, and the N98S and I10T variants. The G114R variant also displays a reduced binding to Nav1.5 even at high calcium levels. In other words, the data substantiate that the Folbigg mutation causes a change in the calmodulin protein shape exactly at the site where it binds to Nav1.5.

These novel data strongly suggest that the important Nav1.5 sodium channel can be added to the molecular machinery that contributes to the phenotypic spectrum for calmodulinopathies.

What are the implications:

These new data provide a novel compelling mechanistic explanation for an expansion of the phenotypic spectrum of calmodulinopathies. They suggest including the possibility of phenotypic effects of disturbing the Nav1.5 generated sodium currents. A visualization of this expansion is illustrated in the figure below.

Calmodulin mutation impact on ion-channels and associated phenotypes



The figure above visualizes how individual calmodulin variants affect the function of different cardiac ion-channels differentially, giving a molecular mechanistic foundation for the observed phenotypic spectrum for calmodulinopathies. For each ion-channel, the protein name (first line) and the gene name (second line), are given together with the assigned OMIM phenotype(s) (third line). Each individual calmodulin mutation is given by the amino acid substitution (first line) and the observed phenotype(s) (second line). Note that the combined functional data for the G114R Folbigg mutation places it in a unique new area of the potential impact of a calmodulin mutation.

The phenotypic spectrum and clinical presentation of carriers of *SCN5A* mutations (the Nav1.5 sodium channel) include both LQT syndrome (LQTS3), where mutations result in a *gain of function* effect, and Brugada syndrome (BRGDA1), where mutations result in a *loss of function* effect. The clinical presentation can also be a complex mix of the two phenotypes, and this is even seen within family members carrying the same mutation (Makita, 2008).

The reason for such complex behavior is likely the Nav1.5 double role during cardiac contraction (see Part A). Opening of the Nav1.5 is the depolarizing event that initiates the Action Potential, but following membrane depolarization, a sodium ‘late current’ still remains active during phase 1 and 2. Calmodulin is known to be a key regulator of both the activation and the inactivation of Nav1.5 (Kang, 2021). Therefore, by extension, a carrier of a calmodulin mutation that interferes with calmodulin-Nav1.5 binding, is at risk of showing phenotypic features similar to mutations in the Nav1.5 channel.

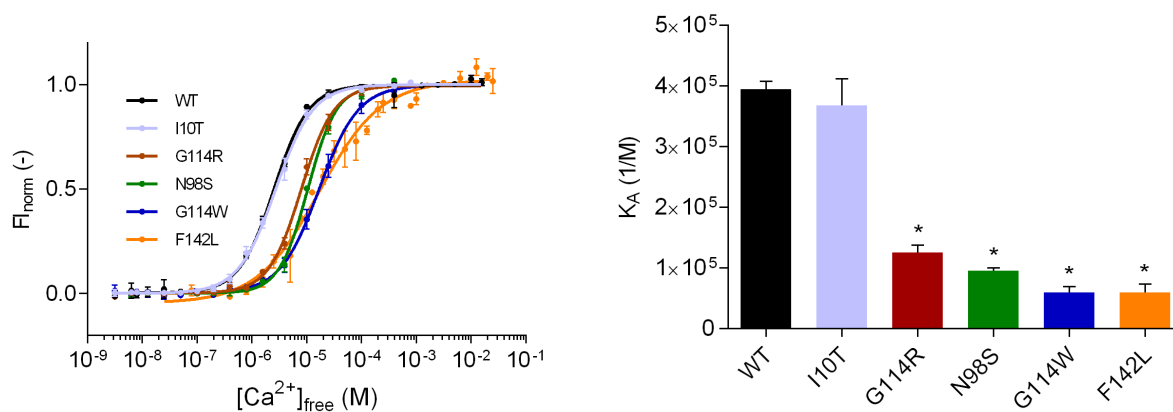
B2. Concerns raised that the functional data may produce false positive results.

In connection to the functional assays in the Brohus paper, the Skinner report raised concerns regarding false positives, i.e., that any genetic variant (also benign variants common in the population) may show the same functional effects as the variants claimed to be pathogenic.

We agree that it is informative to include common benign variants in functional assays, where the effect of a potential pathogenic variant is evaluated. However, common benign variants were not included in the Brohus paper, because 1) there are no known common variants in calmodulin, and 2) it is unclear if any benign calmodulin mutations exist at all, given the evolutionary constraint on all amino acid residues of this protein.

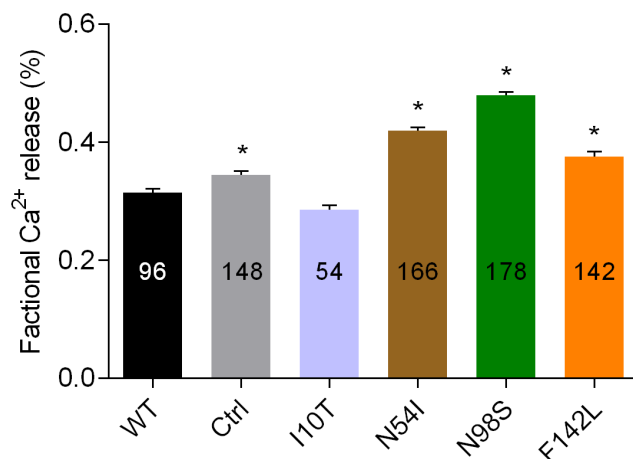
To address this concern, we have now included functional data from a calmodulin missense variant, I10T, identified in GnomeAD, and later observed in a carrier in the UK Biobank. The UK Biobank includes phenotypic and clinical information for the individuals included in the repository, with no ICD10 codes or any medication information indicating that this carrier has a cardiac arrhythmia phenotype. Functional data for the I10T variant is shown in the figures below for the assays measuring calcium binding strength (unpublished data from the Overgaard lab), and the assay determining functional effect on the RyR2 calcium release (unpublished data from the Chen lab). The figures demonstrate that the ‘non-arrhythmogenic’ I10T variant **does not significantly impact calcium binding** in contrast to the Folbigg G114R variant and the three established arrhythmogenic calmodulin variants. Similarly, the I10T variant **does not affect RyR2 fractional calcium release**.

Calcium binding assay



The I10T variant does not affect calmodulin binding. Calcium-binding curves (left) of the C-terminal domain of calmodulin variants I10T, N98S, G114R, G114W, and F142L showing reduced calcium affinity (right), for the Folbigg variant G114R and the arrhythmogenic variants N98S, G114W, and F142L (data from Brohus 2021 [WT, G114R, G114W and N98S] and Søndergaard 2015 [F142L]).

Store overload induced calcium release (SOICR) assay

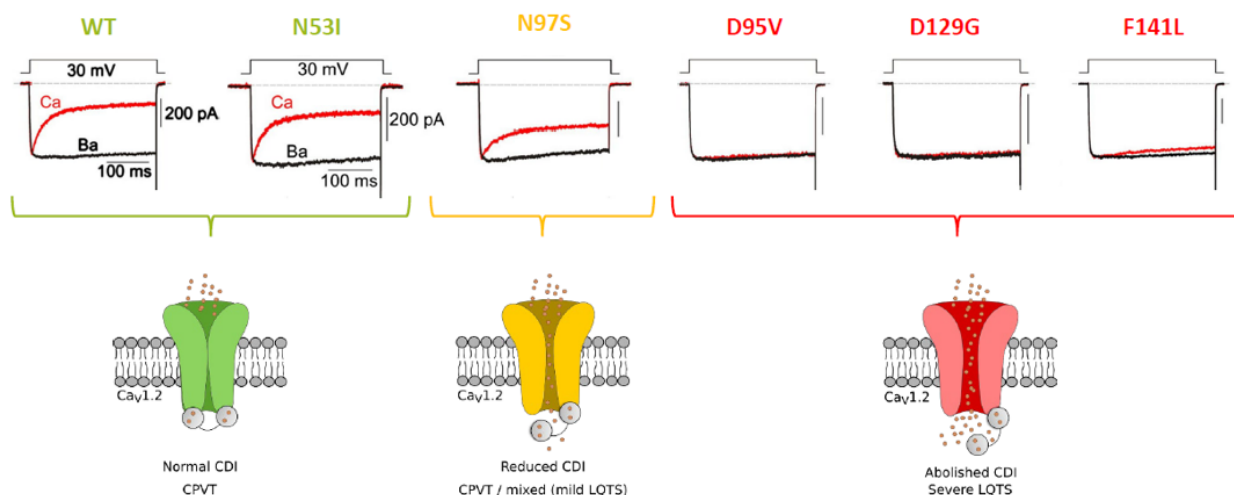


The I10T variant does not affect RyR2 fractional calcium release. Comparison of measured RyR2 fractional calcium release in the SOICR assay performed using HEK293 cells in the Chen lab. The fractional calcium release was calculated from 54-178 single cell traces (number for each variant indicated in the bars). Data for N54I, N98S, and F142L have been published (Søndergaard, 2015; Søndergaard, 2016). For the fractional calcium release, calmodulin variants - and the Ctrl - were compared to those with calmodulin-WT by one-way ANOVA with a Holm-Sidak post-hoc test. Asterisks indicate statistically significant differences ($p < 0.05$), and error bars show SEM.

The results demonstrate that the ‘non-arrhythmogenic’ calmodulin I10T variant effect cannot be distinguished from non-mutated calmodulin in the assay where we assess the impact on RyR2 function. This means that I10T does not appear to adversely affect the calmodulin-dependent inhibition of RyR2.

Single cell electrophysiological recordings of Cav1.2 function

For the single cell electrophysiology assay that determines the functional effect on the voltage gated calcium channel, Cav1.2, we did not have enough time to generate data for the I10T variant. However, we include below for reference a figure from the 2014 Limpitikul paper published in the Journal of Molecular Cell Cardiology, demonstrating that the CPVT (with no QTc elongation) calmodulin N54I variant does not produce an effect on the calcium dependent inactivation of the Cav1.2 channel in this assay. In contrast, the CDI is almost completely abolished for the severe LQTS linked D96V, D130G and F142L calmodulin variants, and partly diminished by the N98S variant displaying a mix of phenotypes (mild QTc, CPVT or a combination) (Limpitikul, 2014).



The non-LQT N54I variant does not affect Cav1.2 CDI. Figure from the 2014 Limpitikul paper (Limpitikul 2014), illustrating the absence of a functional impact for the CPVT (and non-LQTS) calmodulin variant N54I, a small impact for the N98S variant that display a mix of phenotypes including mild LQTS, and a full or almost full abolishment of the calmodulin mediated calcium dependent inactivation (CDI) effect for the D96V, D130G and F142L calmodulin variants, which are associated with a severe LQTS phenotypic presentation.

B3. Concerns raised that the functional assays do not translate directly into phenotype

We agree with the Skinner report that a functional change in a protein does not necessarily translate to a clinical phenotype. In other words, a functional change seen in the laboratory does not necessarily reveal what phenotype this would result in because the human body is highly complex in its nature. The only way to directly test if a mutation translates to a certain phenotype is to genetically alter a human being, i.e., insert a particular mutation into a person's DNA and observe if this produces the expected phenotype. As this approach is obviously highly unethical, instead, the mutation can be inserted into a mouse. However, mice are not humans, and this approach creates another problem, namely that it is not a given that the resulting phenotype would be identical – or even similar - in mice and humans.

As an alternative, mutations can be tested for properties that are *already known* to be part of a molecular mechanism for a disease. For example, when we found the first ever mutation in calmodulin in a family with CPVT, it was natural to test if that mutation led to altered binding to RyR2, because RyR2 was already an established disease gene for CPVT. Using a binding assay similar to the one used in the Brohus paper we showed that the mutation indeed caused RyR2 to become leaky; the exact same mechanism through which established RyR2 CPVT mutations were known to make RyR2 leaky (Søndergaard, 2015).

Just to be clear, the calcium binding, and the channel binding assays used in the Brohus paper are not cell based. These assays determine *inherent biophysical properties* of the isolated protein variants, and thus do not depend on any cell biological variability.

Another example where functional assays have been informative for explaining unusual clinical presentation of an arrhythmic calmodulin mutation is the Kato paper, included as evidence for this Inquiry (Kato, 2022). Here the authors make use of the same calcium binding assay as employed in the Brohus paper, and several single cell electrophysiology based functional assays for determining the N138K mutation functional effect on both Cav1.2 mediated calcium current, and the Kv7.1 mediated potassium current.

The authors in the Kato paper conclude that the N138K variant impairs the calcium-binding affinity and Cav1.2 inactivation (CDI), but unexpectedly potentiates the Kv7.1 mediated potassium ion current. This allowed them to conclude that the variably expressed phenotype of N183K, compared with previously published de novo LQTS-calmodulin variants, is likely explained by a milder impairment of Cav1.2 CDI combined with augmentation of the potassium current.

This means that *identical and similar functional assays*, as used in the Brohus paper, allowed the authors to identify a novel molecular mechanism for calmodulinopathies. This constitutes an example where single cell based, non-diagnostic laboratory assays are highly informative and expand our understanding of underlying molecular mechanisms for phenotypic effects that are otherwise difficult to investigate and explain. For establishing molecular disease mechanisms, the scientific community rarely uses assays developed for diagnostic purposes. This in no way implies that these assays cannot be of very high integrity and consistency.

Part C. Use of the ACMG system

The Skinner report advocates for the use of the ACMG guidelines and the ACMG classification system to evaluate and classify the Folbigg variant.

We respectfully disagree with using this system to make deterministic judgements regarding the arrhythmogenic potential for this novel variant. The ACMG guidelines were developed for use in *clinical settings* to determine appropriate *clinical action*. The classification system relies heavily on data generated *by the research community* and was never intended as a tool to investigate **novel** links between variants and phenotype.

The ACMG guidelines by design cannot appropriately take multiple lines of scientific data and scientific evidence into consideration. Consequently, scoring the *pathogenicity* of novel mutations using the ACMG classification system will more likely than not result in a classification as ‘Variant of Unknown Significance’ (VUS) - not because there is evidence *against* pathogenicity, but because evidence *for* pathogenicity relies heavily on already established evidence for pathogenicity, which does not exist for novel variants (as they are new to science).

It is of course ok to classify new variants according to the ACMG criteria, as one piece of the puzzle, but putting so strong emphasis on the exact ACMG category, and distinguishing so strongly between if the variant falls into the category “VUS” or “likely pathogenic” is, in our view, highly problematic for this case.

In summary, in our opinion, the use of ACMG guidelines to make deterministic judgements for the novel *CALM2* G114R Folbigg variant is highly problematic, because the ACMG classification

system was not developed for this purpose, was never intended to be used this way, and by design is not informative regarding pathogenicity of novel mutations.

Part D. Our comments on the finding of a single *CALM3* G114R carrier in the Regeneron dataset

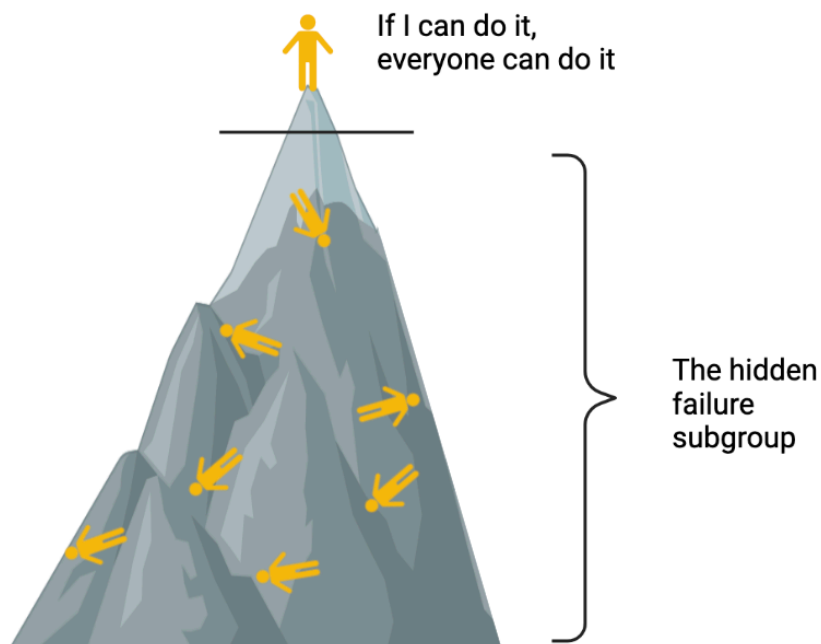
To the best of our knowledge, Kathleen is the only known carrier of a *CALM2* G114R variant in the world. The Skinner report (paragraph 2.3) notes that a carrier of a G114R variant (in *CALM3*) is present in a database of individuals sequenced by the company Regeneron (RGC million Exome database). We were not aware of this new resource at the time of our previous report, and we will therefore comment on it here.

As Regeneron sequenced a large proportion of the UK Biobank, and the carrier was of European ancestry, we searched the UK Biobank to find this individual. The biobank holds almost half a million sequenced individuals, with selected phenotype information made available for approved researchers. Within the UK Biobank we indeed found a single *CALM3* G114R carrier, which is most likely the same carrier as mentioned in the Skinner report. According to the information available to us in the UK Biobank, the individual appears to be alive, with no associated ICD10 codes (i.e., no diseases are listed) or indications of any remarkable phenotype.

Does the existence of a second carrier mean the variant is benign? A very important aspect of interpreting this observation is to be aware of potential bias associated with different types of data regarding the ‘effect size’ or ‘severity’ of a variant. Specifically, a dataset of sequenced patients suffering from heart arrhythmia will be enriched by people who cannot tolerate a calmodulin variant, and the data set will therefore likely *overestimate* the pathogenicity penetrance of the variant. On the contrary, large biobanks of people who are adults and have signed up themselves (such as the UK Biobank) is enriched by people who can tolerate being a carrier of damaging variants, and this dataset will therefore likely *underestimate* the variant penetrance.

The bias in the last scenario is well known in epidemiology and is called the ***survivorship bias***. Survivorship bias happens when a visible ‘successful subgroup’ is mistaken for the entire group due to the ‘failure subgroup’ *not being visible*.

This can be compared to a situation where it seems that an incident was not all that dangerous because the only people who were involved in the incident, and who could speak about it, were those who survived. In this example the real danger of the incident cannot be ascertained as the size of the ‘failed subgroup’ (those who did not survive) is unknown.



In the case of the G114R variant, the size of the hidden ‘failure subgroup’ is not known and cannot be inferred based on the visible ‘success subgroup’ of Kathleen and the European *CALM3* G114R carrier. What we do know, however, is that there is an unusually strong evolutionary selective pressure on the calmodulin coding regions of the human genome, as also noted in the Skinner report (paragraph 2.5 on page 56). Large datasets of people with different conditions (including early death) are required to better understand the selective forces are at play, however, at present it is clear that calmodulin is highly preserved across all vertebrates.

What are the implications

Kathleen is currently the only person in the world – known to us – with a *CALM2* G114R mutation. Claiming that because she is alive then anyone with this mutation should also be alive is an example of ‘survivorship bias’ and is an error of logic.

In this context, we consider the identified carrier in the UK Biobank of a G114R variant (albeit in another gene) an important piece of the picture:

- It shows that it is possible to survive with a G114R *CALM3* mutation– similar to that Kathleen Folbigg is a surviving carrier of the G114R *CALM2* variant.
- The existence of only a single carrier in the large UK Biobank demonstrates how **ultra-rare** these variants are in the population.
- The observation of a live carrier is not direct evidence the variant is benign, as it does not elucidate how many may not have survived – i.e., the observation must be interpreted with ‘survivorship bias’ in mind. To correctly estimate penetrance of the G114R very large

sample sizes are needed of patients suffering from arrhythmia or sudden cardiac death to understand who and how many did not survive.

- It underscores the crucial role of functional data at the protein level for determining potential pathogenicity of an ultra-rare variant, because it is impossible to do meaningful statistics on survivorship when variants are this rare and the genes are so constrained.

Conclusion

Our conclusion has not changed from our first report.

In our opinion, based on the updated research data and the current understanding of human genetics, the *CALM2* G114R mutation is sufficiently deleterious to have caused the death of the two Folbigg daughters.

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