EXHIBIT AY

12 April 2019

Comments by Professor Vinuesa and Professor Cook in response to the opinion offered by Dr Buckley and Professor Kirk dated 9 April 2019

Professor Carola Vinuesa and Professor Matthew Cook

We have two substantial disagreements with the reports, including the response to our report, of Dr Buckley and Professor Kirk's team. We would also like to correct the record regarding some specific remarks offered by Dr Buckley and Professor Kirk. The first of our outstanding disagreements is that we strongly disagree that the ACMG criteria should, or even can, be applied in this case. Our second point of disagreement relates to the interpretation of specific gene variants identified.

The ACMG criteria cannot and should not be applied in this case

We outlined caveats previously in both our original report and the supplementary report, and reiterate them again here. First, we have considered the possibility that our task is not a diagnostic exercise that aims to identify clinically-actionable mutations. Second, and irrespective of the first point, the fundamental limiting factor we face in reporting genomic variants here is uncertainty regarding the phenotypes we are attempting to explain. The logic of enquiry is therefore different from that of routine diagnostic genomics, because in this case, the phenotypic information is incomplete, absent or uncertain. The current exercise should lie somewhere between a research approach and a clinical approach.

To try and make this distinction clear it is necessary to explain why sequencing the Folbigg family is inherently different from routine clinical diagnostic genomics.

In diagnostic genomics, a phenotype is defined by clinical examination and investigations. Those examinations and investigations are done with a view to making differential diagnoses which could plausibly be caused by a single gene defect. DNA sequencing (WGS or WES) of the diseased individual and their family is then performed, and identified genetic variants are evaluated according to ACMG criteria. Only after this process can we conclude whether a genetic defect is causally related to the phenotype.

By contrast, in the Folbigg case there is a large degree of phenotypic uncertainty. Not only uncertainty with respect to the Folbigg children, but also uncertainty with respect to Ms Folbigg, and her wider family. This makes the issue of interpreting the genetics of the family much less routine than for ordinary diagnostic genomics. Here the main issue is how can one confidently exclude candidate variants given this phenotypic uncertainty? Keeping in mind again that in diagnostic genomics one only defines a variant as "pathogenic" if it is 99% likely to cause disease, and "likely pathogenic" if it is 90% likely to cause disease. This is a very high threshold and many disease-causing variants are invariably not classified as "likely pathogenic" or "pathogenic" given the lack of evidence, but in time will presumably be classified as such.

For the above reasons, we see the logic of enquiry as follows:

a. Assume multiple different possible phenotypes (cardiac, neurological etc);

b. Perform WGS /WES analysis on the assumption that such a phenotype exists or existed, and classify the variants revealed by this process.

In other words, the genetic investigations have been performed under a series of phenotypic postulates. This logic is implicit in the approach to candidate gene list compilation acknowledged by all parties. If the phenotypic uncertainty is dismissed, and therefore all plausible significant pathology is considered unlikely, then the value of genetic investigation is negligible. On the other hand, if we agree on the logic of the enquiry, and indeed the purposes of genetic sequencing in this Inquiry, then the limitations of strict application of the ACMG criteria become obvious.

Disagreement relating to interpretation of specific gene variants identified

We cannot exclude rare variants such as those found in *CALM2*, *MYH6*, *IDS* and *NLRP1* as potentially contributing to the deaths of the Folbigg children based on available information.

The specific corrections to the record regarding specific remarks offered by Dr Buckley and Professor Kirk are detailed below:

CALM2

1. Phenotypic uncertainty

    c. ECG examination has found no positive evidence of long QTc in KF although this cannot be excluded in the absence of additional tests including an exercise ECG. This conclusion is even less certain in the offspring because of the quality of the ECGs (or absence of ECGs).

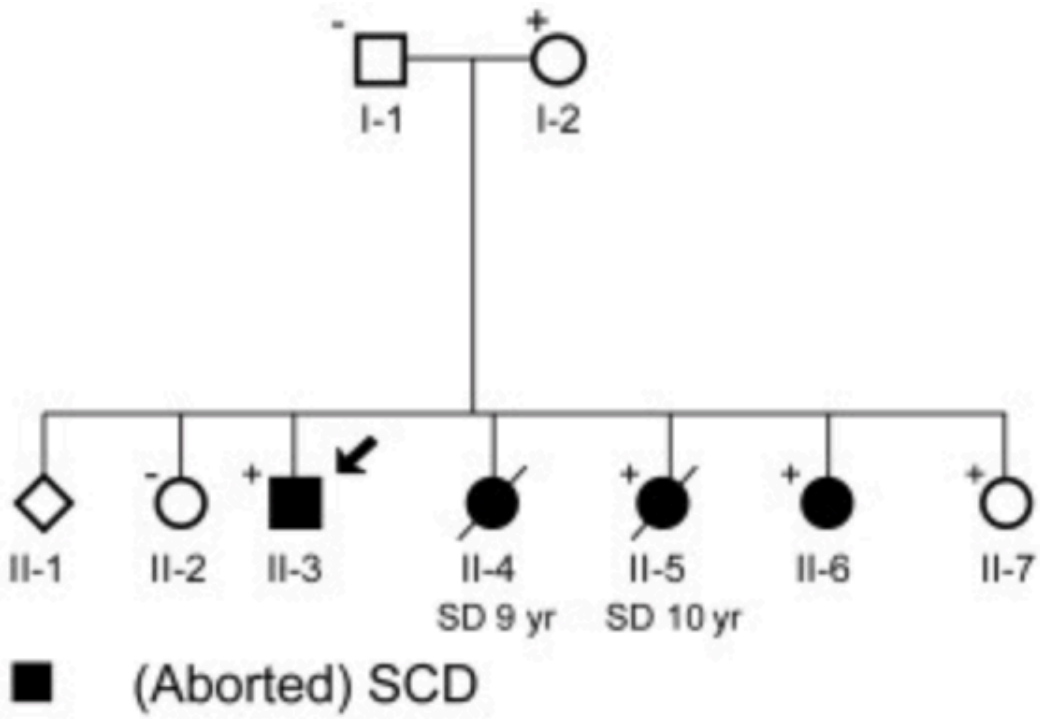
    d. Although Professor Kirk and Dr Buckley focus exclusively in the phenotype of Long QT Syndrome (**LQTS**), this is only one of the phenotypes conferred by mutations in *CALM2*. Others include catecholaminergic polymorphic ventricular tachycardia (**CPVT**) and idiopathic ventricular tachycardia (**IVT**), which are **not** excluded by normal QTc when measured at rest, and are important causes of syncope and sudden death (1).

2. CALM2 structure

    a. We disagree with the functional inferences of Professor Kirk and Dr Buckley based on the amino acid residue affected by the identified *CALM2* mutation. Calmodulin (CaM) proteins interact with a very large number of proteins, including ryanodine receptors, which regulate calcium release from sarcoplasmic reticulum, and L-type voltage-gated calcium channels. The latter undergo calcium-dependent inactivation but the former can be regulated by conformational changes in CaM. Different mutations have been shown to affect different channels, and to some extent, confer different phenotypes (i.e. LQTS, CPVT, IVT and SUD). Indeed, there are mutations located in the EF hand domain, as the one in this kindred, that do

not encode calcium-coordinating residues (2) but still cause CPVT, IVT and sudden death.

b. Specifically, two of the three inherited (i.e. not *de novo*) publishedpathogenic *CALM1/2* mutations (*CALM1* and *CALM2* encode proteins with identical amino acid sequence) are not calcium-binding residues and have been shown to cause CPVT, idiopathic ventricular fibrillation (IVF) and SUD. For example, a CALM2-F90L mutation was identified in a 5-year-old boy who died while playing, with a negative autopsy (10). Furthermore, a CALM1-F90L mutation was described in a Moroccan family with episodes of idiopathic ventricular fibrillation (IVF) within the first 2 decades of life and mild QT prolongation only in the recovery phase after exercise (9). This mutation was present in the healthy mother and 4 sequenced siblings; only 2 siblings suffered sudden death (pedigree below: "+" indicates mutation present in sequenced individual; "-" indicates mutation absent in sequenced individual).



The published pathogenic CaM mutations not occurring in calcium-binding/coordinating residues are:

CALM1-p.N54I – 4 index cases CPVT
CALM1-p.F90L – 1 case IVF
CALM2-p.F90L – 2 cases SUD

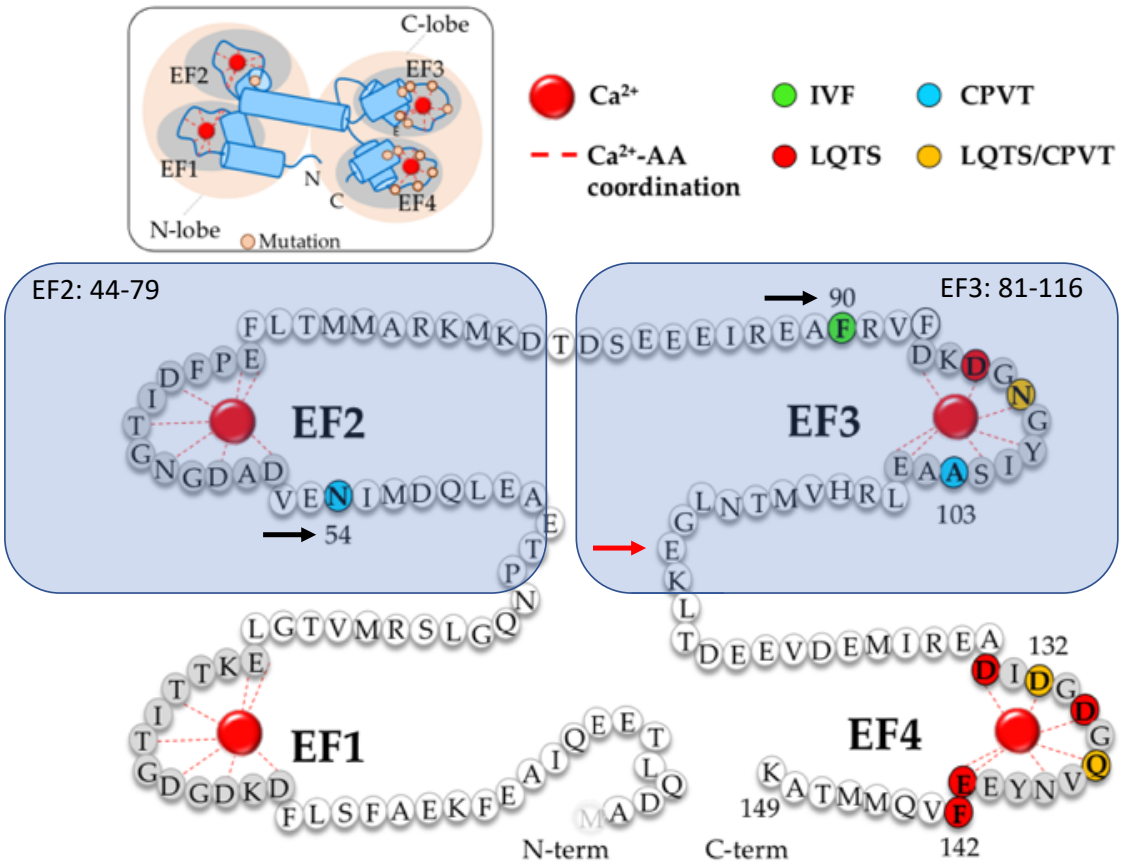


Figure from References 3 and 4 (red arrow, mutation present in KF family)

3. Variant desert in exons 1-4 of CALM2

   Calmodulin is one of the most highly conserved proteins in nature. Three genes encode the same protein, and still there is a very low rate of mutations in CaM. There are various measures of genetic constraint (ie intolerance to loss of function or missense mutation). Gnomad provides a single statistic for the entire gene, but more recently, analysis has been made by domains on the assumption that absence of genetic variation on one or more exons ascertained from large human cohorts implies strong purifying selection (5). The substantive point to be made is that there are no known benign missense or LoF mutations within exons 1-4 of *CALM2* (G114 is encoded in exon 2) which encodes the C-lobe.

4. Exclusion of candidate variant on the basis of KF being unaffected.

   Even in KF does not have a cardiac phenotype after complete cardiac screening, gene variants shared by her and her offspring cannot be excluded completely on this basis. It is widely accepted that AD genetic diseases can be incompletely penetrant for different reasons including for example protective alleles or milder alleles requiring a second mutation in an interacting partner. The latter is a possible scenario given the burden of VUS's in cardiac genes identified in the family and their potential interaction with other genes, environment and other factors.

MYH6

With regards to the comment that there is no evidence of any cardiac phenotype which has been associated with *MYH6* variants in any family member, we want to draw the Inquiry's attention to published reports that are available to support: cardiac arrhythmias arising from mutations in this gene, incomplete penetrance, and variable penetrance, referenced in our initial submission.

Pathogenic variants in genes known to cause cardiomyopathies and cardiac arrhythmias have been found in autopsy-negative sudden unexplained deaths. An expert in this field (Ackerman) states:

> *A standard forensic autopsy may fail to recognize subtle features of a cardiomyopathy or that sudden death may occur with subclinical disease. This supports the evaluation of cardiomyopathic genes in addition to channelopathy genes even in the setting of seemingly autopsy-negative SUD. As evidenced in our cohort, half of the exertion-related SUDY cases have a VUS in one of the channelopathic or cardiomyopathic genes that remains stuck in genetic purgatory currently* (10).

Where Ackerman refers in that last sentence to "genetic purgatory" he is referring to the fact that these seemingly disease-causing variants are unable to be classified as pathogenic or benign given the absence of other evidence at this time. Additional work will clarify the causal association between the genetic variant and the phenotype. Put another way, as this field progresses, the costs of sequencing continue to drop and more research is conducted these VUS will likely end up being categorised as pathogenic or benign. Until this functional analysis is done, we can make no further comment.

IDS

GAG in urine may not be the definitive diagnosis as per review below from an authoritative source (6).

> "*The current approach to the diagnosis of MPS II depends on demonstrating an absence or reduced iduronate 2-sulfatase (I2S) enzyme activity in white cells, fibroblasts, or plasma. Most affected males have no detectable I2S activity using the artificial substrate. Identification of a hemizygous IDS pathogenic variant by molecular genetic testing confirms the diagnosis of MPS II in a male proband and may be useful in persons with an unusual phenotype or a phenotype that does not match the results of GAG analysis.*"

We would defer to a metabolic disease specialist on this matter.

NLRP1

Several lines of evidence suggest that mutations in *NLRP1* can cause myocarditis. First, the NLRP1 inflammasome is expressed in cardiac myocytes. Second the mouse model of the syndrome in question (engineered *Nlrp1* mutation) was characterised by neutrophilic myocarditis in 25% of animals, and this pathology was considered a likely cause of death (7).

The statement by Professor Kirk and Dr Buckley that there are no reports of cardiac disease in this rare syndrome is not correct. A cardiac conduction defect and left ventricular dysfunction has been identified in a patient with NLRP1-associated autoinflammation (8).

Professor Carola G. Vinuesa
LMS (MBBS), PhD, FFSc (RCPA), FAA

Email:carola.vinuesa@anu.edu.au

Professor Matthew Cook
MBBS, PhD, FRACP, FRCPA, FFSc (RCPA)

Email: matthew.cook@anu.edu.au

References

1. Napolitano C., Priori S.G. Diagnosis and treatment of catecholaminergic polymorphic ventricular tachycardia. Heart Rhythm. 2007;4:675–678.

2. Wang K, Holt C, Lu J, Brohus M, Larsen KT, Overgaard MT, Wimmer R, Van Petegem F. Arrhythmia mutations in calmodulin cause conformational changes that affect interactions with the cardiac voltage-gated calcium channel. Proc Natl Acad Sci U S A. 2018 Nov 6;115(45):E10556-E10565. doi: 10.1073/pnas.1808733115.

3. Urrutia J, Aguado A, Muguruza-Montero A, Núñez E, Malo C, Casis O, Villarroel A. The Crossroad of Ion Channels and Calmodulin in Disease. Int J Mol Sci. 2019 Jan 18;20(2). pii: E400. doi: 10.3390/ijms20020400.

4. Jensen, H.H.; Brohus, M.; Nyegaard, M.; Overgaard, M.T. Human Calmodulin Mutations. Front. Mol. Neurosci. 2018, 11, 396.

5. Havrilla, JM, Pedersen BS, Layer RM, Quinlan AR. A map of constrained coding regions in the human genome. Nature Genetics, 51, 88–95 (2019).

6. Scarpa M. Mucopolysaccharidosis Type II, In: GeneReviews®. Editors: Adam MP, Ardinger HH, Pagon RA, et al., editors. Seattle (WA): University of Washington, Initial Posting: November 6, 2007; Last Update: October 4, 2018.

7. Masters SL, Gerlic M, Metcalf D, Preston S, Pellegrini M, O'Donnell JA, McArthur K, Baldwin TM, Chevrier S, Nowell CJ, Cengia LH, Henley KJ, Collinge JE, Kastner DL, Feigenbaum L, Hilton DJ, Alexander WS, Kile BT, Croker BA. NLRP1 inflammasome activation induces pyroptosis of hematopoietic progenitor cells. Immunity. 2012 Dec 14;37(6):1009-23. doi: 10.1016/j.immuni.2012.08.027.

8. Garrelfs MR, Hoppenreijs E, Tanke RB. Transient left bundle branch block and left ventricular dysfunction in a patient with NLRP1-associated autoinflammation with arthritis and dyskeratosis syndrome. Cardiol Young. 2019 Mar;29(3):435-438. doi: 10.1017/S1047951118002342.

9. Marsman RF, Barc J, Beekman L, Alders M, Dooijes D, van den Wijngaard A, et al.. A mutation in CALM1 encoding calmodulin in familial idiopathic ventricular fibrillation in childhood and adolescence.J Am Coll Cardiol. 2014; 63:259–266. doi: 10.1016/j.jacc.2013.07.091.

10. Anderson JH, Tester DJ, Will ML, Ackerman MJ. Circ Cardiovasc Genet. 2016 Jun Whole-Exome Molecular Autopsy After Exertion-Related Sudden Unexplained Death in the Young. 9(3):259-65. doi: 10.1161/CIRCGENETICS.115.001370. Epub 2016 Apr 25.