SPECIAL INQUIRY

THE HONOURABLE THOMAS BATHURST AC KC

SEVENTH DAY: FRIDAY 17 FEBRUARY 2023

INQUIRY INTO THE CONVICTIONS OF KATHLEEN MEGAN FOLBIGG

AUDIO VISUAL LINK COMMENCED AT 10.06AM

<CALUM ARCHIBALD MACRAE, AFFIRMED(10.00AM)

<EXAMINATION BY MS ROY

Q. Professor, can you provide your full name for the record?
A. My full name is Calum Archibald MacRae.

Q. And your place of work?
A. My place of work is at Brigham and Women's Hospital in Harvard Medical School.

Q. You are a medical doctor?
A. I am, yes.

Q. You obtained your medical degree from the University of Edinburgh in 1985?
A. That is correct.

Q. You also had a PhD in Human Molecular Genetics from the University in London that was conferred in 2004?
A. Correct.

Q. And you are currently a Professor of Medicine and Cardiology at Harvard Medical School?
A. That is also correct.

Q. You are an Associate Member of the Broad Institute of Harvard and the Massachusetts Institute of Technology?
A. The Broad Institute, yes, of Harvard and MIT.

Q. What is the Broad Institute?
A. It's a large institute that was founded over a decade ago to investigate the genomics, particularly human genomics, but the genomics of health and disease.

Q. You are a Principal Faculty Member of the Harvard Stem Cell Institute?
A. That is correct.

Q. At our request, you provided a copy of your CV to the Inquiry; for the record, that is Exhibit 34-03, red page 51. That CV was prepared 30 January 2023, I take it it's still up to date?
A. There have been three or four publications since then, but the majority of it is completely current.

Q. I couldn't go to every entry but relevantly, you hold active appointments at a number of hospitals and other research institutes in and around Boston?
A. I do.

Q. You already mentioned Brigham and Women's Hospital?
A. Yes.

Q. And also at Massachusetts General Hospital?
A. That's true.

Q. Can you please tell us in your own words what are the areas of specialised knowledge and expertise that you draw upon to express the opinions you have in your report?
A. So I have worked in the genetics of human cardiac disease for almost 30 years. I have worked across a fairly broad range of conditions including inherited cardiomyopathies, inherited arrhythmias and inherited vascular disease. I have worked in multiple different areas including originally in London in the National Inherited Heart Disease Centre. Subsequently, when I came to Harvard about 30 years ago, I began working in the fundamental science; the genetics and the basic biology of heart muscle disease and arrhythmias, as well as more recently, on similar areas in vascular disease. Also, I'm a - I founded the Inherited Heart Disease Clinic at Massachusetts General in 2002. Subsequently, when I moved back to Brigham and Women's Hospital to become the Chief of Cardiology at Brigham and Women's Hospital, I joined the Cardiovascular Genetics Group in - at Brigham and Women's Hospital, which is the oldest cardiovascular genetics group in the world and includes my prior mentors who are still practicing actually, and I continue to practice there on a regular basis; seeing something in the range of 350 to 500 new families with inherited heart disease every year. And then, the final area of expertise that I draw on is I also am one of the clinical leads in the Undiagnosed Diseases Network and Undiagnosed Diseases Centre in Harvard, which integrates both Mass General, Brigham and Women's and Children's Hospital in Boston, and is devoted to trying to understand the mechanisms of diseases that have not been characterised properly or fully using genomics and a whole host of other tools, including human studies and in vitro studies in animal model studies, to try and understand gene function at an individual patient level. So those are all components of what I do on a day-to-day basis.

Q. Would it be fair to say that your expertise spans the range from clinical diagnostics to novel, experimental laboratory research?
A. Absolutely.

Q. In that sense, you've also - I should clarify, you don't focus on a specific

inherited cardiac disease, but you span the range of human inherited cardiac diseases?
A. Exactly and I, you know, one of the elements that I think this case highlights is that there is overlap in some settings and people have discussed it in lots of different parts of the evidence that has been presented, between the difference and so I think it's sometimes important to understand what those differences are, and so as a result of that, I've tried not to restrict myself to a single subgroup, but to look at cardiovascular disease at large.

Q. Would it be fair to describe you as an expert generalist in the field of cardiogenetics?
A. I think that's a reasonable description. I certainly have spent the last 30 years working in exactly that space.

Q. What about calmodulin genes in particular, do you have specific expertise with respect to calmodulin genes?
A. I have looked after patients with calmodulin variants. I have studied the biology of calcium entry into the heart and the pathways that regulate it, from early development right the way through to adulthood, and I've studied its role, as well as many of the proteins that it interacts with in the genesis of arrhythmias both in early life and in later adult life.

Q. Have you published specifically with respect to calmodulin?
A. I don't think there are any papers that have calmodulin in subjects as the main focus but much of the work we've done in understanding how the heart forms involves calcium entry into the cell and how different points of entry into the cell are modulated by the proteins that interact with the calcium once it arrives in the cell, and in that setting, we have looked at calmodulin function and published on it in a variety of different settings, but not - it's been part of what we study rather than a sole focus in a particular arrhythmia or a particular clinical case.

Q. You may have said this already, but have you personally been involved in any calmodulinopathy cases?
A. Yes, I have looked after - I looked at my records a couple of weeks ago after I was asked to participate in this Inquiry, and I have seen at least three patients who've had calmodulin variants. I don't think any of them to date have been definitively confirmed to have calmodulin as the cause of their underlying arrhythmia.

Q. Would those patients have been submitted to the ICalmR, the Calmodulin Registry?
A. No, I would not have submitted to the Registry because I would not yet be convinced that their disease was associated in a causal way with the underlying variant. I think there's quite a large difference between seeing something - a variant in a patient - and knowing that that variant truly is part of their underlying arrhythmia. And so, I would typically have been very careful when I submit variants, to make sure that we actually have objective evidence supporting the pathogenicity, not just in vitro, but also in the individual patients that we're looking at, if at all possible.

Q. Coming to your involvement with this Inquiry. You were first contacted by members of the team assisting this Inquiry in January of this year?
A. That is correct.

Q. Shortly thereafter, on 13 January of this year, you were retained to provide an expert opinion in the form of a report?
A. That is correct.

Q. At our request you did that quite quickly. You provided a report just two weeks later, on 27 January?
A. Yes, that's true.

Q. For the record, that report appears at Exhibit 34 at tab 03. For the purposes of preparing that report you were provided with a very large volume of material?
A. I was, yes.

Q. This included reports from other geneticists and cardiologists who are assisting the Inquiry?
A. Correct.

Q. Subsequent to providing your report to the Inquiry, did you receive another round of expert reports, including a number of reports that responded to your report?
A. Yes, I did see all of those reports and read them in full.

Q. Prior to being contacted by the solicitor team, were you aware of what I'll call the Folbigg case?
A. I had only heard about as much as was presented in US media, and I believe I did read one article on it in - I think it was either the Economist or the Financial Times, but I don't remember. One of the English periodicals that I read did mention it on one occasion, I think.

Q. Were you otherwise aware of the G114R variant as was reported in the Brohus article?
A. I had been aware of it only - as much as I read the literature, I had read that article in detail only after I had been retained for this Inquiry. I had studied as part of the work that we've done in variant effect mapping the whole range of the calmodulin genotypes, and that was obviously one of those genotypes.

Q. Is it correct you were also approached by Professor Jonathan Skinner at the Sydney Children's Hospital about the Inquiry?
A. Doctor Skinner had asked me shortly before you had contacted me, or your team had contacted me, if I would be willing to give evidence in a broader general way, yes.

Q. Prior to being retained as an expert in this Inquiry, you mentioned you had not read the Brohus article in detail. Do I take it from that that you had not formed a view either way as to the pathogenicity of the G114R variant?
A. At that point I had not formed any opinion at all about that particular variant,

yes.

Q. I'm going to spend some time with you this morning, your evening, with different aspects of your report, but I'd like to begin with your conclusion. The final page of your report, page 23 of your pagination, red page 50 of ours - this is Exhibit 34, tab 3 at red page 50. You say:

> "In summary, [this is at the final paragraph of that page] it is my opinion that there is no genetic, basic science or clinical evidence presented that would support the identified variants as causal in the death of the Folbigg children."

A. Yes, that's true.

Q. You've put that quite unequivocally, "there is no evidence".
A. No extant evidence that would support that. There are - there's evidence that the calmodulin gene is important in different forms of sudden death in young children. There's evidence that the calmodulin variants that were present in two of the children have some in vitro effects. But none of that links the - none of that in vitro pathogenicity is linked to any clinical pathogenicity. And so, at present there is no evidence that would support its pathogenicity one way or the other that has been reviewed. I think - and I can - I'm happy to go into more detail--

Q. I'll take you through it in a staged way. Can we start back, then, towards the beginning, at red page 32, which is - this is, again, still in Exhibit 34-3 - the first page of the substance of your opinion.
A. Yes.

Q. At 1.2 you're addressing the role calmodulin protein plays in regulating the effects of calcium on the heart. In that context, in the final paragraph on that page, you say:

> "One of the ways in which scientists understand the particular roles proteins play is to see what happens when the protein is not present in the cell… [which] can be helpful for recessive diseases… For autosomal dominant diseases, where there is only one copy of the variant inherited, it is important to understand the function of each variant separately as functions may be gained as well as lost."

Pausing first. G114R in the Folbigg family is autosomal dominant?
A. That would be the model that one would have to postulate if you were trying to assume or prove pathogenicity, because there's only a single copy, and it came from the maternal lineage, yes.

Q. You continue - this is over the page now; I think we're onto page red 33.

> "In the heart, as in all other cells, the precise final effects remain difficult to predict for an individual variant and so investigators have begun to try to map the functional effects of every single variant in

> key cardiac proteins to allow a granular picture of the role of variation in the final function of each gene and protein-so called variant effect mapping."

And there's a reference there to Exhibit 61, which is an article that we'll come to in a minute. But first, can you help the Inquiry by explaining what you mean by "variant effect mapping"?
A. Yes, of course. So, one of the biggest problems when you look at genetics is you see a lot of variation. Each of us differs in many, many different ways, probably by as many as a million different variants, not all of which are pathogenic or even thought to be pathogenic, but are the things that make us each different. And so, one of the important aspects of trying to relate a genetic variant to disease is to anchor the disease to the gene in some rigorous and meaningful way. And the way we normally do that is by looking to see if a particular set of variants in a gene, when transmitted to the next generation again and again, is sufficient and necessary to cause the disease. And that would be what is generally called segregation analysis. That's what we see when diseases pass through families. There are other types of genetic variation which are less clearly transmitted, or they are transmitted but they are less clearly associated with disease. And then there are variants in the middle where you need multiple defects in order to actually see the disease. These would be resescents, you need both copies to be disturbed or inactivated or perturbed in some way resulting in the disease.

And so, you can imagine that this is something that takes a lot of time and energy on a per patient basis, because very many of the variants, particularly in conditions that are cardiac and are associated with risks of sudden death and therefore may not be easily passed on to multiple generations, we are often in a position where we're looking at new variants or variants that are only present in a few individuals, and we have to try and work out, is this causing the same abnormality in this particular family that was seen in the original families that proved conclusively that the disease and the gene were related? And so, that, as you might imagine - and you can see the extent of the evidence that can't be assembled - is time consuming on many, many levels at an individual patient and family. And so, one thing investigators including ourselves have begun to do is to say, can we take representative assays, measurements of the function of a protein, and look in a dish at not just one or two variants at a time but all of the variants in the entire sequence of that gene and rank them in terms of their likelihood for causing a particular functional abnormality?

And so, that really is variant effect mapping. You - the goal would be ultimately that you would, instead of having to study each variant separately yourself, you would be able to go back and look at the in vitro evidence in multiple different assays for that variant causing any effect on any of the biology of the relevant protein. Now, obviously this is in a very nascent stage at the moment, so typically we just have one or two assays for each gene, and we still have to, in many instances, make the connection between the things we measure in a dish and the actual abnormalities in patients who have diseases. And it's worth remembering for this part of the discussion that a

single gene can often cause multiple different diseases depending on the variant. And this is particularly the case for autosomal dominant disorders, where typically - but not always, but typically - the mechanism is not loss of the native function of the gene as much as it is gain of another function, a new function, that leads to a perturbation of the physiology of the part of the heart or the vascular system. And so, what's important--

Q. Can I interrupt you for a minute, Professor?
A. Yeah, of course.

Q. Just to expand on that; I was going to come to it anyway. The difference between loss of function and gain of function.
A. Yeah. So, loss of function is basically when all of the activities of the gene are just ablated. So, what you would do is say, I eliminated the protein from the cell, what happens when that is done? And as you might imagine, because individual variants are very typically - not all of them are, but the majority of variants that we see in human disease are single base pair changes; the chances of that one base pair knocking out all of the functions of the gene are relatively modest. Although, it does happen for sure. So, typically what we find is that the types of variants that we see associated with different diseases either are affecting one very small and precise function of the protein - because most proteins have many functions - or they're actually adding a new function that makes the protein go to the wrong place or makes it interact differently with another protein; or potentially it actually pulls even the normal copy of the protein out of the cell by either making it be precipitated within the cell, so complex in a way that stops it working, or by causing it to be degraded or wrapped.

So, those are all new functions. These are things that the gene or the protein normally does not do, and suddenly this single base pair change is enough to make it do those things. And so, that's one of the difficulties in deciding the assays to build in a dish as most of what we know and easily know about a protein is just what happens when you eliminate it completely. It's much more difficult to understand what the potential gains-of-function might be. That's one of the reasons that variant effect mapping obviously is going to take a long time to get to the point where we understand, this particular assay not only gives us index of pathogenicity in a dish but it also translates to a particular function in a human and therefore to a particular disease. There are very few genes and proteins for which that has been systematically done in human disease. The majority of them are enzymes where you can actually measure the whole of the protein's function in a dish in either direction, but for the majority of proteins that's not the case because they are doing very different things in every different cell in their body.

Q. Is deep mutational scanning a subset of variant effect mapping or just another term for the same thing?
A. It's largely another term for the same thing. You're essentially systematically changing every single residue in the protein and you're looking at a single assay at a time to say - and really just scoring every residue for its effect on that assay. That's really the basis of deep mutational scanning and

variant effect mapping.

Q. Is it right that you are currently the recipient of a US National Institute of Health grant to pursue work in this area?
A. That is correct.

Q. Can you tell us about that work?
A. Yeah, I was just going to say that is a - I'm a co-investigator, actually who is working on a very particular piece of this grant which is the reconstitution of variants in an actual whole organism in a way that replicates the genetics of humans in order to really act as the final arbiter in terms of understanding how each of these assays relates to a cardiac phenotype. We're working with a group at Stanford, a group at Vanderbilt and a group at Toronto who are doing deep mutational scanning of genes that cause vascular disease, genes that cause arrhythmias or genes that cause heart muscle disease and my expertise is in following up in animal models at very high-throughput. Each of these disease areas in the Zebrafish model, which is a very efficient way of looking at heart function in hundreds if not thousands of ..(not transcribable)..

Q. The article that's referred to or that's cited in the first paragraph we were just looking at, you've referred to it as Exhibit 61, it's the I think a preprint, we might pull that up. It's Exhibit 15, tab 17. This was the preprint, the lead author was Floyd. You're a co-author of this article? Let me stop for a minute, can you see what's on the screen, can you see the article on the screen?
A. I can actually, yes.

Q. I should call it the paper. You were a co-author of this paper?
A. Well, this is the preprint, I was a co-author of this preprint, yes, and of the paper as finally published also.

Q. Is this part of the work that you were describing pursuant to the grant or related to it?
A. This is directly related to it, this was actually preliminary work that we had done before or as the grant was being submitted for evaluation.

Q. What was your role in relation to this paper?
A. My role at this stage was really trying to identify the key variants that we would go on and then model in the Zebrafish, in a rigorous way to show whether or not any of the results of the variant effect mapping done in cells in a homozygous form, ie, all of the protein in the cell was mutant, or all of it was wild-type. But we were going to model and are still in the process of modelling these in a heterozygous form which was how they're inherited in humans.

Q. I might have misunderstood, this preprint, does this publish the results of a modelling in Zebrafish?
A. No, not yet. I was involved really as we were evaluating the variants with respect to understanding which are the ones that are extremes or essentially in the middle. What we're trying to do is pick the variants that we would then go on and model in an animal model because they would best represent the spectrum of what is happening in calmodulin.

Q. In that answer you've indicated this includes calmodulin, variants in the calmodulin genes?
A. Absolutely. All - I mean essentially as has been discussed by several of the witnesses, the calmodulin genes have essentially identical proteins, all three genes in humans. They have probably quite different functions, although they I'm sure overlap in many ways for certain functions, but the reality is at a variant effect mapping level you can study the function of all three genes with a single set of experiments.

Q. That, as you said, this was a preprint, so that's prior to peer review?
A. This was prior to peer review, yes. As is now quite common, as people try and share the results of early scientific work, we pre-published it on a preprint server and that's also quite useful because it allows you to cite it in a grant application as well.

Q. That would be helpful. As of February 2023 it's correct that the article is now available in the publication Circulation: Genomic and Precision Medicine?
A. Yes, that's correct.

Q. That's post peer review?
A. That is after peer review at that journal, yes.

Q. I'll ask the Inquiry to go to it, it's Exhibit 15, tab 257. Between the preprint and this version, there were a number of changes made, is that right?
A. Sorry, you broke up a little bit just then.

Q. Between the preprint version and this February 2023 version there were a number of changes made, is that right?
A. Yes. As is typical for a preprint moving to publication, yes.

Q. The final version, it would be fair to say, represents changes that might have been made as a result of peer review?
A. There are - there were changes before it was submitted, there were, include additional data, revision of the text to represent that new data precisely and then there would be additional modifications based on input from both peer reviewers and potentially editors, yes.

Q. It is the final published version that reflects the concluded views of the authors?
A. Precisely. Yes.

Q. This article in the final form that we're looking at at the minute can be taken to reflect your views as well?
A. Absolutely, yes.

Q. It'd be helpful if you could explain, or perhaps you've already done it, some aspects of this article. In the first paragraph it says:

> "Genetic testing is becoming the mainstream in the management of cardiovascular disease. However, up to 80% of variants reported

> on genetic testing are classified as variants of uncertain significance (VUS), which cannot be acted upon with high confidence in the clinic."

I won't read the balance of the paragraph but I'll read the final sentence:

> "We show that prospective determination of the functions of large numbers of variants in calmodulin genes (*CALM1, CALM2,* and *CALM3*) allows rapid integration of functional data in clinical decision-making in paediatric cardiac arrest cases, illustrating the promise of this strategy in overcoming the VUS barrier toward widespread implementation of genomic medicine."

So if I understand correctly, you are reporting on actual clinical cases in which the variant mapping that you describe has been used in the context of clinical decision making, is that right?
A. The majority of the data was collected diagnostic to the clinical cases of course, but they were used as part of the evaluation of the these particular cases, yes, for sure.

Q. In the next paragraph, you say in three - and I wonder if we might zoom in just a little bit to make it easier to see, thank you - in three paediatric cardiac arrest cases, I'm sorry, Professor, I'm asking the Court to zoom in if possible, thank you.

> "In 3 paediatric cardiac arrest cases with VUS in 1 of the 3 genes encoding the CALM (calmodulin) protein, we incorporated data from a previously published proactive variant effect map based on calmodulin yeast fitness complementarity."

First of all, are any of those three paediatric cardiac arrest cases your patients?
A. No, none of these are my patients.

Q. The reference there to the previously published proactive variant effect map, is that the map originally published in a 2017 paper authored by - I'm going to butcher this name - Jochen Weile?
A. It is - it's related to that. There is, ah, substantial additional data incorporated because obviously as we are evaluating a lot of these types of maps, we're recognising you need to replicate and repeat experiments so that you're minimising individual experiment variation. Because you're testing a very large number of conditions, and so under new settings the more information you have the more reproducible and the more reliable your results will be.

Q. I appreciate and I'll ask you to expand on that in minute, that you've added to that data, but this is in a sense a starting point for the map that you report in the--
A. Yes. Absolutely.

Q. I ask the Inquiry to go to that article, the Jochen Weile article, which is Exhibit 15-173. Excuse me as I locate my own copy. If we could look at this page that's on the screen which is red page 2711, in the second column, bottom half of that column, beginning “Deep mutational scanning”, the final - thank you - sentence of that paragraph beginning on that page, "Such maps can accurately identify functionality of a clinical variant in advance of that variant's first clinical presentation." Do you agree--
A. Yes.

Q. --with that? That's what you've been describing to us?
A. I think that - I think that's a reasonable assertion. Obviously, you know, this is the first phase in understanding that relationship, but yes, you're right, in general that is one of the things that you are hoping to accomplish by this type of analysis. Obviously, the most important thing to understand is: is the assay that you're using representative of the function that's important in the disease that you're studying? And that can vary because many, as I said earlier, many, many genes have, in fact the vast majority of genes have multiple functions and so you need to be certain that you are understanding the particular assay. Let's say in this instance, yeast viability of growth, fitness essentially, and a particular disease entity, which may not be fully represented by just simply the growth of a cell. So for example, you know, there are lots of variants in some genes that will cause a particular disease of one site of - one half of the protein is variable and will cause a totally different outcome if the other half of the protein is variable, just because the functions are different. In addition to that, there's also the problem that you're looking at a different context and at a very different cell type. So there's a lot of steps in understanding the relationship between "pathogenicity" in a dish and the clinical effects in a patient. I think that's one of the things--

Q. I'm sorry, Professor, can I just interrupt you?
A. Yeah, of course, please.

Q. Just to see if I can clarify, what I was asking you about was - such maps can accurately identify functionality of a clinical variant in advance of that variant's first clinical presentation. Do I take you to be agreeing with that in principle, but acknowledging that there are limitations?
A. I'm - I am agreeing with it in principle absolutely and acknowledging that at the moment, it's - there are very few cases where that can be said to have been done in a systematic way; largely because of the lack of clinical information and also, the lack of patient-mechanistic information. They would let you take a variant in a dish with a particular effect and relate that effect to a patient. So it's basically making the distinction between pathogenicity in a scientific sense in an assay in yeast or in any other model system, and clinical pathogenicity where you're essentially demonstrating that that function that you assayed in yeast is relevant in the human, by independently showing that the variant has an effect in humans. So those are two separate things and to make the correlation that we propose in this - or was proposed, this was actually a manuscript from one of my colleagues and collaborators, but I was not involved in this particular work - the important thing is to connect the function in a dish with that particular function in a patient, and that's obviously

quite a way off for most, if not all, diseases.

Q. Can I take you then to red page 2715 of that article, which is still the Weile 2017 article, it's page 5 of that article, and the second column, the bottom half where it begins "Variant impact maps for five additional disease-implicated genes"?
A. Yes.

Q. It says, "Having validated the framework, we sought to map functional variation for disease-relevant genes", passing over the higher-throughtput TileSeq approach and coming down to where it says, "and CALM1, CALM2, and CALM3, associated with cardiac arrhythmias (Long QT Syndrome (Crotti et al, 2015) and Catecholaminergic Polymorphic Ventricular Tachycardia (Nyegaard et al, 2012)). Because the three calmodulin genes encode the same polypeptide sequence, performing DMS for CALM1 also provided maps for CALM2 and CALM3." I want to step through that piece by piece. First of all, do you agree that CALM1, 2 and 3 are associated with Long QT Syndrome?
A. There are definitely data that would implicate a series of *de novo* mutations in at least two of those genes with the Long QT Syndrome. The data for all three are not completely perfect, but I think it's not an unreasonable supposition. But probably the best data are for CALM2 and CALM3, yes.

Q. I won't ask you to expand on this - we will come back to it - but do you agree that the CALM1, 2 and 3 genes are associated with CPVT?
A. I think the case there is much more tenuous. I think there is some early evidence that there may be, and this is one of the advantages of efforts like the Calmodulin Registry is you begin to understand - here's a list of variants and you begin to build the case for whether or not they are associated with other diseases, but I would argue that - in fact, I wouldn't argue, it's not a point of argument; it's actually a point of fact - there are not sufficient independent observations suggesting in a rigorous way that Catecholaminergic Polymorphic VT is associated with transmission of the calmodulin genes to be definitive about that, but it's certainly - it is certainly possible and I think there are obviously lots of activities ongoing to try and improve our understanding of it.

Q. I'm sorry to interrupt you, Professor, but I might come back to that, I'm conscious of both your and my time - well, mine and the Court's time, and that we are keeping you late, but I will come back to that.
A. Of course.

Q. The second aspect of that passage that I wanted to ask you about is what you have already touched on, that “because the three calmodulin genes encode the same polypeptide sequence, performing DMS for CALM1 also provided maps for CALM2 and CALM3." Before I ask you about that, I acknowledge in your report you describe that there are different outcomes for deleting CALM1, 2 and 3 in mice. That is total loss-of-function modelling; is that right?
A. Absolutely.

Q. In your report - I can't recall if I have asked this already, but are you aware of any loss-of-function CALM variants identified in humans?
A. It's not obvious whether the mutations, the variants, the pathogenic variants described in humans are loss of function or gain of function. There are pathological variants that I think have been quite well described in the calmodulin genes to be associated with the emergence of a phenotype. There are multiple *de novo* variants that are in the same residue that lead to the same phenotype, and that was very well described in - and rigorously described in the original Crotti manuscript. Their - it's not obvious what their functional effects are. There are, separately in the same paper, data showing that these variants, these relatively small number of *de novo* variants, are associated with loss of or attenuation of calcium affinity and that has been associated with the disease. But there's no evidence in humans or in animal models, where there are multiple copies of calmodulin, some with the variant and some without, to show that that's actually what happens in a whole organism. So at the moment, it is correlative, but I do believe the human genetics supports the causality for *de novo* variants in the calmodulin 2 gene and a very penetrant and early form of Long QT Syndrome associated with severe ventricular arrhythmias, yes.

Q. Coming to - if I understand correctly what you have said, the study in mice had to do with total loss of function.
A. Yep.

Q. As in, the calmodulin genes just don't function at all, they don't.
A. No, I understand, all I'm really - the only point I was making there is that the functions of the calmodulin genes do not perfectly overlap and there are many potential reasons for that, so it's a - essentially a logical fallacy to make assumptions when there's direct evidence to the contrary that they actually all behave the same. They behave the same in a map for yeast function, but then we have to take that yeast function and understand how it relates to biology of calmodulin 1, 2 and 3 separately in humans. That's - that's the real crux of the logical gap I think in some of the inferences that have been made in - in evidence given in the Inquiry, as it is assumed that loss of calcium affinity is somehow the only potential assay that could reflect on the human condition. Even if that were the case, even if that were the case, it has only been studied in isolation – i.e. all variant or all normal - and the reality is that nobody has ever been found with two copies of the abnormal gene, and so it's very difficult to make that leap without a lot of additional work, and so that's partly why - well, that plus a long history of similar inappropriate conclusions in this field makes me very confident that it is - there's still a long way to go between showing a simple and very rigorous in vitro assay of calcium binding actually as of relevance when you have multiple proteins, some of which are normal; some of which are mutant in a human heart cell. That's a very different proposition.

Q. Can I take you, Professor, to page 45 of your report, Exhibit 34-03?
A. Yep.

Q. In the middle of the page, the first paragraph under point 1 - just to get

some clarity around what you've just said--
A. Of course.

Q. Where you say, "As noted, the three calmodulin genes are highly conserved at the protein level, yet appear to have distinctive phenotypes when their function is eliminated and do not appear to be fully interchangeable." That is referring back to the mice studies for total loss of function. You say there "Importantly, gain of function of a specific sequence in the same residue might be interchangeable despite these loss of function data."
A. Yep.

Q. Are you just merely acknowledging that possibility?
A. Absolutely, yes, that it's a definitive possibility because it is conceivable that even though the genes don't have the same function when they're knocked out, that there are variants that lead to them gaining the same function when they're present. So this is one of the - the - I think the most important features of human genetics is that it's one of the reasons why it is so difficult to evaluate variants except in the clinical setting in which they occurred, because there are so many potential biological activities, that simple experiments in a dish, whether I do them or anybody does them, are unlikely to reflect the complexity of being a human.

Q. Professor, I think we well-appreciate what you say about the translation from the Petri dish to the human. Since completing your report, have you had the opportunity to consider the reports of Professors Toft Overgaard and Nyegaard which responded to your report?
A. Yes, I read that in full.

Q. Could we bring it up? It is Exhibit 6-06, p 1114 of that report, red p 117.

In the middle of that page there is an observation that the “Number (and %) of CPVT probands by CALM gene in the 2023 Calmodulin Registry” shows roughly an equal distribution between CALM1, 2 and 3.
A. Yep.

Q. I won't ask you to comment on that immediately, but I'll take you then to evidence that we received on Monday of this week from Professor Nyegaard. This is in Exhibit 6-07 red page 137, and Professor Nyegaard explained that this was demonstrating the similar missense variants - and I appreciate this is probably the first time that you are seeing this, so I'll give you a moment with it. But this demonstrates that similar missense variants in the different calmodulin genes occasion substantially similar phenotypes across the different CALM genes. Take a moment if you need it, but I wanted to ask if that data is capable of supporting the assertion that genes can be treated as interchangeable for the purpose of variant analysis.
A. So, the weak link in the chain here is the rigour with which these phenotypes were shown to be related to the gene in all the particular genotype in humans. And so, I think I mentioned in my report that one of the problems in this field has been for a long time - it's the same in all parts of human

genetics, it's not unique to sudden death genetics. It's that there's initially great rigour in showing that a particular gene is associated with a particular phenotype, and then once that happens, people will literally just start looking at the variants in individual people and not looking at whether they're transmitted with the disease, whether they are rigorously associated in a *de novo* sighting with the disease, whether there is experimental evidence in animal models that recapitulate the human genotype and show that that individual variant is causal. What they start to do is really just build registries where they list the variants that were found in particular conditions, and this has been shown time and time again to lead to misassociation between individual variants and disease types. And there are lots of reasons why that might be the case biologically, but practically, what it does is it ends up inflating the likelihood of a particular gene causing different disorders. And so, if I'd seen the--

Q. Professor, sorry to interrupt you. Can I clarify, are you referring to the weak link being the evidence connecting the CALM variants to the CPVT phenotype?
A. So, again, this is - one of the things that I think is important is remembering not just - there's not just a general association. It is actually, you need to be able to show that a particular set of residues is consistently seen with a particular phenotype. Then - and that has been done, I think very eloquently, by Crotti and Colley.

Q. Professor, I'm sorry to interrupt you. You continue.
A. No, it's okay. I'm just trying to explain that that is done often at the start very rigorously, but then afterwards, we - I mean, for example, I've looked at the Calmodulin Registry, and I could not discern from the data that were available for most of the variants that were seen after the original descriptions, whether just those genes were sequenced, whether all of the known arrhythmia genes were sequenced, whether the entire genome was sequenced. And that has a substantial effect on the likelihood of association. But then in addition to that, there's also the need for real rigour in showing that a particular variant is associated with a particular disease, and I've been through this many, many times in my career. We had variants and the original genes that we identified for cardiomyopathy that we were certain were the cause, but they were nothing to do with the cause, we found the real variants in the same copy five/six years later. So, it's not--

Q. Can I clarify, Professor? In this case, by reference to what you've just said - and you were referring to some inadequacies in the Registry data - you've otherwise accepted that Long QT Syndrome is causally linked to calmodulin variants?
A. I think there's very good data that would pass any set threshold of rigour that calmodulin causes, *de novo* mutations in calmodulin, cause an early onset severe Long QT Syndrome.

Q. You draw a distinction between the rigour of that evidence and what you've seen - and we'll come to it - of the evidence linking CPVT to calmodulin variants. Is that right?
A. Absolutely. There's no comparison.

Q. We will come back to that.

JUDICIAL OFFICER

Q. Professor, you were careful to say that it was shown in *de novo*. We're not dealing with a case of a *de novo*--
A. That's exactly right. And so, that's - again, I think you make an excellent point that typically if variants are associated with a phenotype, and it's a *de novo* phenotype that may be lethal in early life, it's highly unlikely that that will ever be seen to be transmitted to the next generation. And what we often find is that these variants are recurring and you never see them in families; you only see them as *de novo*. And so, you can to some extent predict the likelihoods of those types of relationships - ie, *de novo* events - based on severity of the clinical phenotype. And if you don't see that severe clinical phenotype and you don't see *de novo* variants, you're immediately starting to think to yourself, there's uncertainty on both sides. There's uncertainty both in terms of the genetic transmission of variants in the family, as well as uncertainty with respect to the phenotype that you're seeing with those variants. And so, I think that's one of the points and I think you picked it up and defined it very precisely. It is very important to understand these things independently before you put them together to make a causal chain.

Q. Leaving that question aside for a moment, or making the assumption that they are *de novo*, would you accept that a variant in any one of the three CALM genes - a *de novo* variant in one of any CALM genes - is capable of causing or resulting in the patient having LQTS?
A. I would say I think that's a reasonable assumption. I think it varies residue by residue. So, there are some residues where there'd be multiple cases of *de novo* variants at that particular location associated with the phenotype. And so there, the probability of a chance association is dramatically reduced, if you have independently occurring rare events that coincide. If what you have, on the other hand, is just an association of one variant with one clinical report, that unfortunately does not meet the threshold for a rigorous demonstration of pathogenicity. It is a great start, and I laud the Calmodulin Registry and all other registries for building these types of databases, because the only way that you can really build that case is to build a scenario where there are multiple independent variants at the same residue with the same discrete conditions; and that takes time. But at present, the evidence is constrained for a relatively small subset of residues in the calmodulin genes.

ROY

Q. Professor, I was going to do it later, but let's come now to deal with the CPVT evidence. Can I have Exhibit 15 at tab 31. For the benefit of the Court's record, I'm on page 11 of my notes. This is the article - the lead author is Professor Nyegaard - from 2012, reporting of the title "Mutations in Calmodulin Cause Ventricular Tachycardia and Sudden Cardiac Death". In your report at red page 35 - again, Exhibit 34, tab 03, red page 35 - in the second from the bottom paragraph on that page, you said, "The specific example cited for CALM and CPVT is the original family reported by Nyegaard et al,

2012." That's this article, is it?
A. That's correct.

Q. You say:

> "In this family, the primary unbiased linkage analysis reveals two equally likely genetic locations on different chromosomes for the potential casual variant in this family, which as the authors note has a quite different phenotype than virtually all other calmodulin variants reported."

A. Exactly, yes.

Q. This was the first reported case of a calmodulin variant, as I understand it. So, I take it you're not referring to the authors of this article as having noted that it's quite a different phenotype than all others?
A. That's right. It's just simply, it did not - it had never previously been associated with CPVT until this manuscript, yeah.

Q. What's said there is, "which as the authors note has a quite different phenotype than virtually all other calmodulin variants reported".
A. Yes.

Q. But at the time of this paper, no calmodulin variants--
A. So, they - I don't know where they mention it, but they note that this is - CPVT has not been reported. That was one of the reasons that they, I'm imagining, submitted it to the American Journal of Human Genetics, since it would be a - the first attempt to connect this particular gene with this particular phenotype.

Q. But are you aware that this is the first time a calmodulin variant had ever been reported in a human?
A. Yes. But there are other - there are other phenotypes in other organisms, that's all. So, it's just a range of calmodulin phenotypes.

Q. At the time of this article there was already established a phenotype for calmodulin variants?
A. In other - in the - most experiments that I mentioned were done long before then.

Q. This is referring to in other species?
A. Other species and in others. I mean, there are fly experiments from 1993. So, just that the functions of calmodulin had not been thought because, remember, as the authors allude, this is a very highly conserved gene. It's in every cell practically in your body, in the heart. It's actually relatively lowly expressed in the heart compared with many other tissues. And so, it had such a fundamental phenotype in other species that it would be surprising that there was enough of a buffering in the organism to allow it to get to the point where it could cause an arrhythmia. But that was the supposition, and it was certainly a reasonable attempt to try and demonstrate that. As I mentioned, I think the

authors themselves suggest that it was - it was not perfectly possible. But again, I don't think they overstated their claims; they just simply said it looked as if this was reasonable evidence to support it. But typically for - in human genetics you would require two independent families, each of which would statistically be sufficient to demonstrate that. And I would argue that that's definitely the case with a variant that is causing a phenotype that's so unusual - so unusual compared with the spectrum of other variation in human and other model organisms. And then the authors - I don't see it on this page, but in their - in their own description of the work point out the fact that there was another locus in another chromosome that had the same statistical likelihood--

Q. Professor, I'll stop you there. I'll come back to that.
A. Yes.

Q. Can I just ask you first; you used the expression "buffering". You've used the expression "genetic buffering" elsewhere in your report. What does that mean?
A. Just means that oftentimes when there are variants, they can, because of the other variation in a natural individual's genome, or because of the need for some environmental challenge to result in the gene expressing itself, you can often end up in a situation where a variant may not cause the expected phenotype in an individual. But that's, again, another reason why we - even if something looks like it's pathogenic in one family, you have to re-evaluate it completely in a second family. There are many, many - or in any other families, there are many examples, particularly in the cardiac literature, of variants that have highly penetrant sudden death in one family and no evidence of sudden death in the next. And that's, again, just emphasising the fact that each of these - at the moment, because of the lack of available data to do otherwise, in almost every instance you have to evaluate each case on its own merits.

Q. Could we please have red page 550, in the same document you were just in? Exhibit 15-31, which is page 704 of the article. If it's possible to zoom into the pedigree at the top of the page. This is a pedigree of the family that's described in this article and that describes four generations if I'm reading that correctly?
A. I can see it, yes, and you used the terminology perfectly.

Q. The black indicates the presence of the phenotype?
A. That is correct.

Q. I'm on a roll. There would be 12 individuals if you count up all the black dots?
A. Yes.

Q. On the next page of that article, which is red page 551, the article page 705, there is a description initially in the first column of the phenotypes of the affected individuals that was described and it's summarised at the bottom of the first paragraph in the first column. "the phenotypic picture of the family

is characterised by CPVT-like features with symptoms including frequent syncope and three cases of sudden death or cardiac arrest." Do you see that?
A. Yes, I do.

Q. The next paragraph and over to the next column describes the genetic investigations that were performed. There is a reference to initially there being 12 subjects, 9 affected, 3 unaffected, and continuing in the right-hand column about a quarter of the way down the page it says, "Two possible linked loci were identified [on two different chromosomes], each with a maximum LOD score of 3.01". Is that what you're referring to when you say there are two equally possible loci identified?
A. That is exactly right.

Q. Then it goes on and says, "A follow-up analysis with microsatellite markers... in these two regions was performed and included an additional six subjects from the family (one affected… two unaffected… and three healthy married-in individuals)", so presumably almost as a control, "In total, 18 individuals with the microsatellite markers were genotyped. Primer sequences were retrieved from the NCBI UniSTS database", I'm not going to pretend to know what that means. "The microsatellite analysis excluded the locus on chromosome 6 and mapped the disease locus to chromosome 14". So, can you explain why notwithstanding that further analysis you say that the two different chromosomal locations remain equally likely?
A. So, it's really a matter of relative likelihood. You like to have odds of about 1000:1 in favour of a particular locus over the next most likely locus. By their own data they point out that there's only really about barely a one log order difference between the 14 locus and the chromosome 6 locus and none of the additional information is presented. The number of individuals that they suggest they add to the pedigree, you would have anticipated a LOD score closer to 6. So the fact that the LOD score is only 3.9 just makes me wonder what exactly the recombinant events are that would result in this. It doesn't meant to say that they're wrong, it just means to say there's no data to allow me to assess how likely it is that their conclusion is correct or not, that's all. And to be completely fair to the authors, it's not unreasonable to publish this because on its own it's not sufficient to make the relationship between the gene and the disease. You would anticipate another independent family would be necessary with similar levels of likelihood, and so one of the ways in the literature that's accepted to do this is to present things, warts and all, and then have others try and replicate it and see if they can find additional families that map to the same locus, and the only point I would make is that since 2012 when this was published, that has not been the case.

Q. You don't weigh the fact of *de novo* variants arising in unrelated individuals in other variants with similar phenotypes as in any way confirming that data?
A. No, actually again I think his Honour made the precise observation which is *de novo* variants associated with neonatal lethal or very early onset arrhythmias are quite distinct from a four-generation pedigree transmitting a phenotype that in some individuals didn't present until they were quite late in adulthood. So it's a different phenotype, it's a different type of genetics, it's a transmitted genotype, so you'd just like more rigorous data before you accept

it. I'm not saying it's impossible, I'm just saying there is no supportive evidence that would meet the standard thresholds that we've adhered to for 30 years, but this is a definitive locus, that's all.

JUDICIAL OFFICER

Q. Professor, could I ask you this? You don't dispute as I understand it the methodology used in this particular article, and - but you--
A. No. Not at all, yeah.

Q. --what you say in effect is that although the conclusions may be correct, to properly verify them a fair degree of further information would be needed?
A. Exactly. That's exactly it.

Q. But having said that, in the absence of any further evidence, would you accept that the conclusion is at least a possibility which may or may not be verified at some future time?
A. Yes, absolutely.

ROY

Q. Further to that, Professor, when you refer to there being no evidence, on that occasion I heard a qualifier, that would meet the thresholds that you've come to use over 30 years. I previously understood you to say there was no evidence and now I'm understanding you to say there is no evidence that would meet the thresholds. Are those the same things?
A. Essentially yes, because of the history of human genetics. I mean there are, you know, for example there are many examples, particularly in this field where the precision of relationship between phenotype and genotype can be blurred. That there have been things that have been identified as causing a human disease. They've been "established in the literature" and only decades later have been shown not to have any effect at all, and most of the examples, if not all of the examples, have fit this precise pattern. The initial linkage was either in a single family or was equivocal. I mean, for example, some LOD scores are, you know, 10 to the minus 12, that's how large a definitive LOD score might be. This is at the margins, and particularly when you know there's another locus that has a LOD score of three in a completely different chromosome, you're always a little bit concerned. But the reality is this is a problem that has - it's not a problem, it's a lesson that we learn in the Long QT Syndrome, we learnt it in other disorders where genes were implicated for many years and tested in many patients and people recorded associations between other variants and the final phenotype. But then when we looked at population studies, it was clear that many of the variants that had been reported as disease causing were actually quite common in the general normal population and there was no evidence that the genes themselves were associated at a population level with the disease in question. And so all I'm saying is--

Q. Professor, can I stop you there?
A. Sorry.

Q. The experience that you were describing, I think almost every other expert to this Inquiry has described, this sort of auto - the cleaning house of genetic variants that had been thought to be pathogenic through using methods like this. Does that mean that the evidence, that experience, does that mean that that evidence is of no value and worthless or is it some evidence, it's just no longer sufficient evidence?
A. It was never sufficient evidence but it is valuable evidence, yes.

JUDICIAL OFFICER

Q. It is evidence which put together with other material may lead to a particular conclusion, is that a fair summation?
A. That's a very fair summation.

ROY

Q. I'll go back. Can I take you back to the Weile paper - is it Veal or Weile?
A. I think it's “Weile” actually.

Q. Thank you, the Weile paper. Have you had a chance to review the report of Professors Vinuesa, Arsov and Cook, in response to the evidence of Toft Overgaard and Nyegaard in which they cite the Weile paper, and that's at Exhibit 5-07, I might pull it up, at red page 195, where they extract the calmodulin functional map from the Weile paper?
A. Yes.

Q. If we can look at red page 2718 and if possible zoom in, I think you were there. Sorry, can we stay on one - yes, thank you. This is red page 195. If we zoom in to the map at the top of the page, if possible thank you, yes, the blue, and what Professors Vinuesa, Arsov and Cook have said they've done here is to extract the map from the Weile paper and then they have pulled out what it reported in respect of these G114, and they describe this, it's on the previous page but you don't need to go to it, they say this is only one of two residues in calmodulin, the other one being leucine at 106 that does not tolerate any substitutions. If I'm reading this correctly, and please tell me if I'm not, in effect the fact that the column that corresponds to position 114 is all blue with no white, other than the wild-type which is indicated in yellow, shows that there is no variant that is predicted by this yeast model to not have a deleterious effect. Is that a fair summary?
A. Deleterious effect in this yeast growth assay?

Q. In yeast, in yeast assays, yes?
A. That's very reasonable, yes.

Q. If we can come back to your - to the Floyd paper at Exhibit 15-257, at red page 3592, and similarly zoom into the top of the page, it'll be there. Yes, 3590, excuse me, at the top of the page and on the middle, sort of, there's the "Log-likelihood ratio" and you're there using green to be "benign", white to be "ambiguous" and blue to be "pathogenic or likely pathogenic"?
A. Yes.

ROY: If we could also try and zoom in to the far upper right-hand corner. Are we at our limits of zooming? You should see the numbers along the X axis at the very top and if I could ask the Court to draw the cursor across to 115?

Q. If you could perhaps take it from me, accept that that shows a mixture of blue, white and green along G114 in your model?
A. Yes.

Q. That suggests that, according to this model, there is a prediction of variation between likely benign and likely pathogenic in respect of variants at that position?
A. For this particular yeast assay, yes, that's the case and as a consequence of--

Q. Can I stop you there, Professor?
A. Of course.

Q. What I had taken you to before, I understood, was the yeast assay. This, I had understood, is the report of the variant effect map that uses additional information that you have reported in the 2023 paper?
A. That's exactly what I was going to say. That was a - the initial paper was a first-pass, this is a multiple replicates, integrating all of the information across the entire protein for many, many different - for all the residues that were - that could be assayed, and averaging the effects over multiple experiments.

Q. Just to be clear what this is showing is not merely additional inputs, the machine learning; this is actual further functional assays in relation to yeast being reproduced, and incorporating that new data?
A. Absolutely, yes.

Q. Nevertheless, in relation to G114R specifically - which I'm going to give up any hope of isolating the square on this model in this copy - Professors Toft Overgaard and Nyegaard, I think you are aware, plotted out the data to demonstrate that G114R is still predicted to be likely pathogenic in this model?
A. I think my recollection is it's in the likely pathogenic range, yes, I have to tell you at this magnification, it's difficult for me to do it from anything other than recollection.

Q. Let me take you to Exhibit 6-07, red page 122; can you see the "Yeast fitness scores vs. Cardiac Pathogenicity Log-likelihood Scores from Floyd et al."?
A. I see that, yes.

Q. Do you recognise this as a reproduction of what appeared in the report from Professors Toft Overgaard and Nyegaard that was responding to your report?
A. I do.

Q. Have you had a chance to assess whether this accurately plots the Floyd paper data?

A. I think it's a reasonable representation. It's tough to - from a colour grid to transpose but I think they did a very good job, yeah.

Q. Both G114R and G114W appear high in both axes, essentially - well, mid on the yeast score and then high on the LLR Y axis?
A. Yes, although as I said before, the, you know, the question is what are the clinical data that are being used there but you're right, that's perfectly reasonable, yeah.

Q. The red dots are otherwise plotting ClinVar identified pathogenic variants?
A. Yes.

Q. And the green represent gnomAD variants?
A. Yes.

Q. Professors Toft Overgaard and Nyegaard, if I understood correctly, gave evidence to suggest that this model would suggest that the variant effect map reported in the Floyd paper might be a decent predictor of - "decent" is my word - might be a good predictor of pathogenicity but not necessarily a good predictor of whether or not a variant is benign. Would you accept that characterisation?
A. I think that's a very reasonable characterisation and actually is shown very clearly in their plot that they submitted of how the calcium affinity correlates with Long QT. There is essentially - there's a whole range, in fact, the vast majority of variants are where there's a reduction in up to, I think, 15 or 20%, I don't remember the exact calibration on the axis, are associated with a range of QTs from right in the middle of normal, all the way up to 550/600 milliseconds and that--

Q. Just to stop you there, Professor, you are referring there to other data that Professors Toft Overgaard and Nyegaard have reported in relation to their calcium assays?
A. Exactly, so in that setting, the calcium--

Q. But can I stop you? What's the relevance to what is shown on this slide?
A. Because it's relevant to what you were saying about the ability to - it's very difficult to associate these with both sensitivity and specificity because of the lack of information content in the assays and the lack of clinical correlation. So I was just going to show you this is essentially showing the same problem with the yeast fitness data that exists for calcium affinity, which is that there is a wide range of clinical phenotypes associated with a wide range of calcium affinities, and so there's no doubt that the residues are associated with abnormalities of calcium binding or yeast fitness. The difficulty is relating that function, that one function of many functions of calmodulin, to an actual clinical output, an arrhythmia or a benign effect. Neither of those are well characterised by these assays because the rate-limiting step is the information content in this assay and the clinical phenotype. That's all. So they're both - they are just simply reflections of the same issues with in vitro assays and clinical outputs, and it's not surprising because in both the yeast and in the in vitro calcium affinity assays, we're only studying the mutant with respect to

the wild-type. The mutant never exists except together with the wild-type and any clinical family in which it's been described. So it's almost as if we're - it's true true and possibly related.

JUDICIAL OFFICER

Q. Professor, would you prefer to have a break or not?
A. I'm very comfortable doing whatever is easiest for all.

DISCUSSION AS TO SCHEDULING OF MATTER

SHORT ADJOURNMENT

JUDICIAL OFFICER: Professor, before I hand you back to Ms Roy, there are a couple of things that are troubling me and are probably just out of ignorance but I'd just like some clarification.

Q. You'd agree that this area of science is, to say the least, a very fast-moving one?
A. Yes.

Q. In that context, there is always an area for a significant degree of disagreement among persons specialising in the area?
A. I think that's generally true, yes.

Q. There's good examples in this case between what you say, for example, and what's come from Professor Toft Overgaard, Professor Nyegaard and Professor Schwartz. On the other hand you've got Professor Wilde whose evidence is a little bit closer to yours, and Professor Watkins saying it's all a matter of judgment. Now, what I want to ask you is this. As I read your evidence, you take no issue at all with the technical work done by Professors Toft Overgaard and Nyegaard or for that matter by Professor Vinuesa and Professor Cook. The area we're debating is not an area of technical work, is that right, is that correct?
A. So it - first of all I agree with you, I think the analysis of the genome and the analysis of the phenotype of the physiology of the variants in vitro is not in dispute. The difficulty is there are technical ways of relating variant information with clinical information and that's essentially what I do every day, and--

Q. I understand that.
A. --the difficulty is that the technically correct in vitro information does not inform at all the - in its current state, although it may do in the future, does not inform at all the clinical evaluation of these variants. So people are confounding in vitro pathogenicity for a calcium assay or for a yeast fitness assay with clinical pathogenicity for a specific phenotype.

Q. I understand the difficulty of translating, if I can use that word, the in vitro to the clinical situation. My task is to consider whether as a matter of possibility these girls could have died as a result of the variant, so I'm looking in effect at possibilities.

A. Totally understand.

Q. Now, in that context what appears to me to be the issue, and I may be wrong, is that you say that the evidence is not enough firstly to say with certainty that the girls died from a disease associated with those genetic mutations, is that correct?
A. That is correct.

Q. Other people have said, no one has said it's a matter of certainty, but other experts, including Professor Toft Overgaard, Professor Schwartz for example, have said that it is a possibility. Professor Schwartz quite strongly I should add. Now, in that context, this isn't a Court, it's an Inquiry which is why I can ask you in this way, in that context could you accept that having regard to the technical work, if I can call it that without any disrespect, which is being done, there is at the present time, because that's when I've got to decide this issue, a possibility that these girls died as a result of a disease associated with this mutation?
A. So, I was asked the question originally, did I think it was a reasonable possibility, and I would simply say, I think it's a possibility but I don't think any of the evidence suggests that it's a reasonable possibility.

Q. When you say you don't think it's a reasonable possibility, is what you're really saying that in your opinion the material so far presented can't rise to that level? It may in the future rise to that level, it may rise to a probability, but where the difference appears to me, which I've got to resolve ultimately, is that a number of highly distinguished experts, indeed it's embarrassing to have to make this decision in those circumstances, who are taking a different view as a matter of judgment, and that's what Professor Watkins, who I think you've read his paper, haven't you?
A. I have, yes.

Q. Professor Watkins I thought drew the distinction very well, there's a matter of interpretation of the data, but there is a matter of judgment ultimately about what you draw from it, and - I don't want to - I'm not trying to force you to change your views but what I am saying is, do you accept that as a matter of judgment people with expertise in this field could form a view that the evidence provides a little more certainty than it does in your opinion? That's really I think the nub of it.
A. So I understand completely the conundrum, and I'm trying to convey with the right level of nuance exactly what the basis of my opinions are.

Q. I understand that.
A. So, if you'll bear with me for a couple of minutes, but--

Q. Yes, sure.
A. --the fundamental problem is the in vitro data as presented are technically outstanding but there is no relationship whatsoever, I'm being quite concrete about that, none whatsoever to the clinical situation, or even to the in vivo situation where you have a copy of the variant in association with maybe five copies of the normal wild-type sequence. And so it's actually an incredibly

interesting problem to understand how those could relate, but we know already that going from a single copy of the variant to a half variant, half normal in a cell typically is associated with dramatic changes in the outcomes, and this has been seen many, many times. So that basically says I believe all the work that has been done, I think it may prove in the future - for example, were these assays shown in a heterozygous form to predict a particular set of phenotypes in multiple instances in an animal model - it is completely conceivable that there could be a type correlation. But at the moment, there is no evidence one way or the other.

All it tells me is calmodulin variants, almost all, almost every one that has ever been reported, whether it's in fly, yeast or human, affects calcium affinity and in fact, I mentioned this in my original report: There's - there are beautiful maps of calcium affinity going all the way along the calmodulin molecule and you move one amino acid in one direction or another and you have completely different phenotypes that are unrelated to the calcium affinity or to the - potentially to the yeast fitness, although we don't have any data there. So you're left in a situation where you have, as I said earlier, a set of very elegant in vivo data that are not connected in a rigorous or meaningful way, not through anything other than the absence of these experiments having been completed - I'm not besmirching in any way the work that's been done, I'm just saying that past experience has shown that modelling a single allele, particularly for ion channels or their related proteins, is a very difficult thing to do without understanding how the entire complex fits together in that in vitro setting and you know, this has been - there were 23 mouse models made of the Long QT Syndrome and there were people who gave evidence to this Inquiry who said it was impossible for the mouse to develop Long QT. That was the only possible explanation, and then finally, an investigator made two copies, two different mutations where they replicated exactly the human state, and both of them spontaneously developed Long QT Syndrome. So all of the--

Q. But doesn't all that show that in this area, which is developing very quickly, firstly, that there is a real degree of uncertainty as to what can be derived from any primary material?
A. I agree but all I'm really trying to emphasise is the fact--

Q. In the present case, what I am confronted with in respect of Sarah and Laura Folbigg is two girls who died from unexplained causes, and when one is talking about a natural cause of death, the only thing at the moment that can be linked to their death is the genetic mutation. Now, that is what I'm confronted with, and what I am asking you is, again, in those circumstances, taken in that particular factual position, do you accept, I think, that it is a possibility that the deaths were caused as a result of that mutation? I think you've already told me that, but would you accept--
A. Absolutely.

Q. Sorry?
A. Absolutely, I agree it's a possibility, I'm just - all I'm trying to reinforce is the fact that you have an incomplete and non-contributory - not through all fault of the investigators--

Q. I understand that. It is that absence which leads you to reject that it is a reasonable possibility?
A. That and a couple of other facts that Professor Watkins alluded to in his report. One is - so you have essentially a phenotype which is not Long QT Syndrome, not CPVT, it's unfortunately sudden unexplained death at large, that's in four children. You have an uncertain relationship between the genes in general and that phenotype. You have at least two children who did not have or carry the genotype, whose deaths you still have to explain. So in a probabilistic manner, all I'm trying to do is say--

Q. Professor, part of my task is to have to weigh those factors, of the other two children.
A. Yes.

Q. That is my job. What I'm asking you about is looked at in isolation and assume - let's be blunt about it - a death from natural causes. Do you accept - I think you accept, as you said, it's a possibility that the mutation caused the death - but do you accept, looking at it in isolation and in the absence of any other explanation, it would be open to a person of skill and experience to form the view that there was a reasonable possibility that the deaths were caused as a result of that genetic mutation?
A. I think it is certainly possible that somebody could make that conclusion. They would have to subsume a weak phenotypic gene - gene-phenotype relationship, an incomplete in vitro to clinical-type relationship, and the relatively low probability - even assuming that both of the girls died from a single variant - that two other children would have died of a related - in a related way phenotypically in the same family. So I'm not saying there's no rationale probabilistically for a genetic cause; I'm not saying there is no rationale probabilistically for an environmental or extrinsic cause. What I am saying is it is very difficult coming into this *a priori* to suddenly make the case, having never seen any evidence to that effect before, that these two variants in this family with a relatively uniform set of unfortunate deaths in the children, this is the explanation for two of them but the other two have died from a completely unrelated phenomenon. It is just probabilistically unlikely because of the way that these things are - tend to occur in families. So the fundamental premise of genetics is that because things occur in the same family, it is likely - because unusual events occur in the same family, it is likely that they share an aetiology and so the basic plausibility of this way upstream of whether or not this particular calmodulin variant can or may ever or has done, cause a sudden death in a young child. There are multiple stats upstream of that that have to be analysed before you get to the point where you're starting to make a partial association with incomplete penetrance plus in vitro data that do not necessarily reflect the clinical outcome suddenly changing that probability. That's all I'm really saying. I am not saying that there's--

Q. That was very helpful to me. I think I understand clearly where you are now; thank you.

JUDICIAL OFFICER: Ms Roy?

Q. Sorry, Professor, I think I have disturbed the even flow of events.
A. No, not at all and I apologise - I apologise for running on; I'm just trying to make sure I capture the entire scope. I think Professor Watkins--

Q. No, that's fine.

ROY

Q. Professor, can I take you to your report at red page 42 and the conclusion you expressed there at the bottom of the page, which is what we've just been discussing?
A. Yes.

Q. "There does not appear to be a reasonable possibility that the CALM2 G114R variant caused Sarah and Laura's deaths"?
A. Yes.

Q. "Indeed, as noted, even if both Sarah and Laura's deaths were the result of a single shared genetic event, the overall likelihood of this occurrence in addition to two other causally independent deaths in the same family remains several orders of magnitude less likely than the probability of a single shared genetic or external factor." I think his Honour asked this but I wanted to be clear about the answer: If you assume that the girls had no brothers and were the only two children to die in this family, would that change your assessment?
A. Then it would be quite difficult to be completely sure one way or the other, but it would certainly - from the human genetic standpoint, it would certainly be a more reasonable possibility, yes. Although it still requires you to create a new phenotype associated with this particular gene and to make pathogenicity inferences that are not yet fully developed, but it's totally reasonable.

Q. What is the new phenotype that would need to be associated with this gene?
A. Sudden death with no antecedent or associated phenotypes including in the mother who bears the same allele.

Q. Do you consider that the possibility of a phenotype in Kathleen Folbigg has been positively excluded?
A. No, not completely but again, I'm - I'm just saying this is a completely new association with calmodulin in a broad sense. I mean, there are a couple of other similar cases, but it's a - this is a very clear phenotype. It's actually - and I thought the pathologist dealt with it very elegantly and completely. These fulfil the classic features, both in terms of timing and circumstances, of the Sudden Unexpected Death Syndrome, the Sudden Infant Death Syndrome; there are lots of labels. But the reality is that it's a very discrete phenotype from the arrhythmic phenotypes that are typically seen in children in this setting and in fact, when you look at the overall spectrum of children that die like this, the incidence of cardiac mutations is unfortunately quite low otherwise we would be screening quite extensively for them. It is, in some reports, as low as 10%, but it is somewhere between 10 and 20%, so the majority of this phenotype is traditionally caused by some other mechanism, and so that

immediately changes the pre-test probabilities as you go into evaluating even the two daughters and the mother. But I still agree with you that it's a possibility; as I did with his Honour. These are - it's - what you're trying to look at is the relative probabilities of different sets of outcomes and remember, we are doing this on a routine basis; to try and understand how to help families deal with their remaining children. So this is a very common occurrence and so the real question is, if I saw those two daughters with their mother having passed away, if I was involved in evaluating that family, would I be able to predict on the basis of genotype whether the - another child was at risk or not? I would argue there is no way that I could predict that. I would have to treat all of the other children as if they were affected until proven - or vulnerable until proven otherwise. So that's at the core of what I'm trying to tell you.

Q. Can I ask it this way: If this family came to see you in clinic and they were looking to make reproductive decisions as to having a fifth child, would you encourage them to screen for the G114R variant?
A. I would not. I do not think there is enough evidence to support that.

JUDICIAL OFFICER

Q. That's because I think, Professor, isn't it, that two of the children in Ms Roy's hypothesis, hypothetical, died as a result of other - without having the variant?
A. I'm sorry, I don't fully understand the question.

Q. The reason you said - the reason you answered Ms Roy's question in the negative was because she asked you to assume a fifth child, in circumstances where two of them had died without the variant.
A. I thought she had asked me if there were - the question prior, that if there were only the two children who had passed away, and the mother--

Q. I'm sorry, I must have misheard it.
A. Yeah. We should clarify that, because I think you're right, your Honour, it is quite important.

Q. Let me ask you on that hypothetical, the two children had died having the variant, but for no other explained cause. Would you give some advice to the parents if they were proposing to have another child?
A. Yeah, so, I would find it very difficult to give advice in one direction or another, other than to suggest that when the children were born, we would genotype them and follow them so that we could build a case potentially for future family members when the information was sufficient to make a definitive conclusion one way or the other.

Q. You'd warn them this was something that needed to be monitored?
A. Yes, exactly.

Q. The reason you do that is because of the existence of the mutation and sudden death and no explained cause?

A. No, I would - I'm sorry, I misunderstood the question. I would say that the child would be genotyped and monitored irrespective of the genotype, so that you would be building a correlation between transmitted genotype and an outcome, and that would help you build the case for using the variant alone. But it's very difficult to use the variant alone. I mean, we literally discuss this probably once a week because it's a remarkably common occurrence to find a small nuclear pedigree like this where there's a new variant, and there's just not enough information to make definitive binary decisions. There's a very significant liability if you make the wrong recommendation. And so, if you don't know enough to know that with potentially one in a thousand chance - over one in a thousand over chance that this is likely to be the cause, you would be very conservative in your advice to the mother about subsequent children. This is a very common occurrence in families with sudden death.

ROY

Q. Can I ask about that probability that you've used, one in a thousand chance? My understanding is if you were to apply the ACMG criteria, you would need a classification of likely pathogenic in order to make clinical decisions. That's a probability of 90%. One in a thousand is 99.9%. Are you saying it's not valid to make clinical decisions on the basis of a 90% probability?

A. It depends what the clinical decisions are. So, if you were potentially trying to decide on whether to follow somebody in one particular way or another, whether to give them a particular type of imaging or not, you might change it based on that probability. But if you're going to essentially - and this comes up not infrequently - make a decision about the viability of a foetus or make a decision about following a patient to look for risk, you would definitely not do it for 90%; that's a--

Q. Are those equivalent though, terminating a foetus and advising a patient to look for risk? Do both of those require the same degree of certainty?

A. Well, absolutely, because you're making - you're making an important negative assumption. If I were to say to a mother, "you did not have this variant; your next child did not have this variant", and then basically never do any further testing on that child, I would be--

Q. That would be to positively exclude the possibility, but what I understood you to say - and I must have misunderstood - was that without a 99.9% probability of pathogenicity, you wouldn't even tell a family to look out for risks associated with calmodulinopathies?

A. No - no, I'm not saying that. What I'm saying is, the risks are evident from the unfortunate deaths already, so the calmodulin variants would not change that risk in an individual; that in the next child or in another family member, I would be working very diligently with the family to collect more information, if it were feasible, to try and sure up the case. But I mean, this is not a subtle choice; there are many, many - I think I mentioned it in my report - there are many, many instances of likely pathogenic variants becoming benign over time. In fact, the ratio of that occurring as opposed to things that are classified

as benign becoming pathogenic is about somewhere in the range of 5:1. So, the general tendency that we have is to over classify as pathogenic variants--

Q. Has there ever been a calmodulin variant downgraded in terms of pathogenicity?
A. I'm not aware of it but it's - as we've said, there are very few variants in existence. So, it's difficult not only in a clinical setting but even in the in vitro setting to make negative correlations because the information content at the moment is lost.

Q. Professor, you've told us you see between - I think you said 300 and 500 patients a year. Is that right? Or families a year.
A. Families, yes, roughly in that range, yeah.

Q. You've been practising for approximately 30 years, is that right?
A. I've been - not continuously; some of the time I was doing quite a lot of administrative work. But certainly for 25 of those years I've had a fairly active practice, and when I was in London before I came to the States, even, you know, higher volumes, almost every case in the UK that had inherited heart disease came through to the single centre that I worked in.

Q. In that time have you encountered cases with multiple genetic variants in a single family that are associated with severe phenotypes?
A. I have seen - I mean, obviously that - there are a couple of qualifiers there. I've certainly seen two or three families where that has occurred. But typically, as I said, for it to occur you require the parents with - bearing individual variants to have had children that end up with both variants, or you required a much larger and more diffuse *de novo* event that results in multiple pathogenic variants occurring by chance alone. And so, those are very, very infrequent and typically associated with less severe phenotypes than we see in this setting. It would be very unusual to see a family where there are - you can estimate probabilities - to see a family where there are, four out of four children died suddenly, and that is something that is the result of two transmitted variants. So, it would be unusual. Is it possible? Absolutely possible, yeah, absolutely.

Q. Can I take you to page 47 of your report?
A. Sure.

Q. Exhibit 34-03 at page 47. At the bottom of that page, the second paragraph from the bottom, you detail the initial abnormal phenotypes around which the Inquiry is based: unexpected death in four full siblings, a single agonal electrocardiogram from one sibling, acquired bilateral hypoxic/anoxic brain injury in one sibling, and histological evidence of myocarditis in one sibling, and that these have been summarised by several of the prior witnesses. The acquired bilateral hypoxic/anoxic brain injury in one sibling, you're referring to Patrick?
A. That's correct.

Q. You're basing your opinion of that injury on the basis of reports of other

experts that have been provided to you?
A. Yes. I've read the reports of the imaging data and the autopsy data.

Q. Then you go on to say:

> "In this setting, a general estimate of the likelihood of all four siblings having died from independent causes is so unlikely as to be virtually impossible… Simple probability estimates thus suggests that the events are unlikely to be independent and infers either a shared genetic cause or a shared extrinsic (or environmental) cause."

You referred to "probabilities" otherwise than there, but how is that a meaningful assessment of probabilities in this case, that the separate independent causes for four individual deaths--
A. I'm sorry, I didn't fully understand your question.

Q. In this case, on the hypothesis that G114R was responsible for the deaths of two of the children, that wouldn't be, then, a case of comparing the probabilities of four completely independent causes of death; you would have at least one cause of death that was linked.
A. Yeah. Absolutely, yep.

Q. So, it's not the case that the relative comparator is four independently occasioned causes of death?
A. No, it's effectively three independent events because the mutation is a single event and was present in both of the children who had passed away. Yes, that's correct.

Q. That similarly hypothesises or presumes separate causes for the deaths of the two boys as against each other?
A. Exactly. And so, the relative likelihood of that is really quite low compared with either a shared genetic event - which we have not yet detected despite whole genome analysis, which is certainly possible - or a shared environmental event, which would also, you know, require independent evidence.

Q. You haven't accounted for the possibility of two connected causes: a connected cause between the girls and a connected cause between the boys?
A. Again, I'm sorry, I don't fully--

Q. You said it would be three independent causes: two connected with the girls and then each of the boys being independent.
A. Yes.

Q. But that would seem not to allow for the possibility that the cause of the death of the boys was connected.
A. No. Again, I was just treating one particular set of conditions. There's a separate set of probabilities for the two - if the two boys were connected and the two girls were connected. But again, the relative likelihood of these rare events occurring, clustering separately in pairs of children in the same family,

is, as I said before, very, very low. And so, you're starting to hypothesise events that are, you know, difficult to estimate in the best of times.

Q. Professor, are you familiar with what's colloquially known as Meadow's Law?
A. I am, yes.

Q. Can you distinguish the reasoning that you're bringing to bear in this case from that reasoning?
A. There's no difference in the reasoning, it's simply that probabilities are probabilities. You're - the event is always - the *de novo* risk is always the same of a rare event, and so rare events will occur - cluster very infrequently. And it's certainly possible that completely independent rare events will occur in an individual, the pre-test probability of the next event is unchanged. But the overall probability of seeing all four in a single family suggests a shared influence - it doesn't exclude other possibilities. You're just looking at relative likelihoods, that's all. And as I mentioned in my report, those shared likelihoods are most commonly - most likely there are very few other options. There are theoretical additional options, but the core options are a shared genetic mechanism or a shared environmental mechanism.

Q. While we're on probabilities, Professor Hugh Watkins - which is at Exhibit 14, tab 10, which I might bring up briefly. Red page 322. It says - and it'll appear on your screen in a minute - in the second black dot point:

> "The genome sequencing was done [he's referring to the genome sequencing in this case] to look for a possible genetic explanation for sudden death in the Folbigg infants. I believe that the *a priori* chance that a pathogenic or likely pathogenic variant would be identified in one of the small number of genes associated with sudden death under the age of two is low. In a routine clinical setting, I would absolutely conclude that a variant in this gene with these characteristics was the likely explanation following a young sudden with a negative autopsy."

Do you agree with that?
A. I think that's a perfectly valid conclusion for a single young sudden death, absolutely.

Q. It's not your conclusion?
A. Well, we're not talking about one, we're talking about multiple.

Q. I'm not asking you about this case. Would you agree with that conclusion in that hypothetical?
A. I - I think, you know, you can estimate the likelihood, it's basically, in this particular syndrome it is in the range of 20%, let's say we'll give you the upper bound of the data that are available, so you've got about a one in five chance that a sudden infant death would be associated with a pathogenic or likely pathogenic variant, and so, it would be a likely explanation and you would investigate it as such, yes.

Q. Do those numbers account for the variant being in calmodulin?
A. Again, they would account for, as I said, if you notice Professor Watkins very clearly says in one of the small number of genes associated with sudden death under the age of two.

Q. Yes.
A. Up until this particular set of investigations, it was not clear that that type of autopsy negative sudden death was associated with calmodulin, and so that's essentially all I'm saying is that, with a weak association between the gene and the disease, with a weak association between the in vitro phenotypes and the clinical syndromes, with a totally different phenotype in four related children it's very difficult to come up with a high likelihood that this is the explanation for the events in the family. Is it possible? Absolutely. Absolutely possible. It is more likely that it is some other unrelated shared cardiac, shared genetic variant or some shared environmental variant, that's just a simple statement of probability.

Q. Professor, I'll see if I can shorten it. In two further matters I'm going to take a sideways step then we may not have to come back to it. Can we go to please Exhibit 6, tab 7, page 123 - this is just dealing with some other matters that have come up in the exchange of reports. This was provided in the evidence of Professor Nyegaard earlier this week and I think it's expanding on something that she's put in her report that you would've seen to the effect that the Floyd paper was overestimating the number of missense variants in CALM2 from the gnomAD database?
A. Yeah, I think that's not an unreasonable assumption. I mean it's very difficult to know which genes - which variants are expressed in which tissues, but that also makes the assumption that we know which tissue is important for the variant potentially causing the phenotype, and it could be that adreneuronal or adrenergic - adrenal expression is more important than cardiac, so all I would say is this just highlights the difficulties in moving from the--

Q. Can I just clarify there before you move on--
A. Yeah, of course.

Q. --to - I just want to nail down this point. In terms of that referring to different tissues, do you understand that this is referring to whether it's expressed in different tissues or whether it's expressed at all?
A. I mean the bottom line is it's not obvious from raw genomic data without experimentation which parts of a gene are necessarily expressed or not. That's - one thing is that there are uncertainties at each level and without experimentation--

Q. If I may stop you there, Professor, I'm sorry, just in the interests of time.
A. Of course.

Q. Page 126 please. Would you consider - I'll give you a moment I'm sorry. This is part of Professor Nyegaard's evidence again and she's pulled out the CALM2 list from the gnomAD database and identifies the relevant

PEXT score, P-E-X-T score, which is identified in red on the right of the screen?
A. Yes.

Q. And that one needs a threshold greater than 0.1 to consider the missense variant described there to be expressed in humans. Are you able to comment on - is it within your expertise to comment on that?
A. Yeah, absolutely it's within my expertise. This - these are theoretical data, not objective experimental data, so they're perfectly reasonable to use as an estimate, but all I'm saying is this is part of why in vitro data are so difficult to use in the clinic. There are so many unknown unknowns and uncertainties that it's not easy to go from the first experiment to a prediction of clinical pathogenicity--

Q. Professor, can you take it that we do understand what you say about those translations. Can I ask about specific industry practice, would you dispute that it's well-established that if not the PEXT score, that it's well-established that only certain transcripts, the appearance of a missense variant in only certain transcripts is expressed in humans? I butchered that terminology I'm sure.
A. Yeah, I'm not certain I understand that. So, there are a number of problems that exist in each of these assertions. They're perfectly reasonable as an approximation when you have no other data, and that's how they're used in human genetics, but all I'm saying is that these are typically used in settings where there's a very-well established relationship between the gene and the phenotype, and you're trying to evaluate individual variants.

Q. Can I put it this way--
A. That's very different - very different, the setting.

Q. Yes. Can I put it this way, Professor? If the other experts in this, that have given evidence to this Inquiry who have published in this area, and the other papers that have been considered by this Inquiry have confined their use of gnomAD missense variants for the purposes of having a benign baseline have confined them to those with a PEXT score over one, is that a legitimate approach?
A. It's perfectly reasonable, yeah, absolutely.

Q. Would it concern you that the paper - the Weile paper and also the Floyd paper have not done that?
A. Not at all, we're trying to create an unbiased map and so we don't want to make assumptions that we can't validate experimentally, so it's - again this is, remember, we're not trying to build a clinical tool with the variant effect map, we're trying to build a set of technologies that may ultimately lead to a clinical tool, and so you need to understand all of the uncertainties, all of the limitations, all of the pros and cons of different approaches, and so that's essentially why we took an agnostic view of this particular index because it's completely conceivable for example that variants have effects at a RNA level and not a protein level which you would never detect if you excluded them from your analysis. So that's all, it's not a - it's just a scientific approach that tries to eliminate bias, and that's very different from a clinical approach where

you're trying to make an active decision with somebody in front of you.

Q. It may be the last thing I do, can we go back to the Floyd review article, Exhibit 15-257, the special report?
A. Yes. Of course.

Q. Thank you. I just wanted to take you to some of the details that are reported here in the three paediatric cardiac arrest cases. The first is in the second column at the bottom on that page - it says “Case 1 was an otherwise healthy 11-year-old boy who presented with aborted ventricular fibrillation arrest preceded by anxiety. Postarrest EKG [over to red page 3590] prolonged QTc interval of 525 milliseconds, and exercise testing provoked ventricular [I can't pronounce that] bigeminy”?
A. Bigeminy.

Q. Bigeminy, thank you. “Clinical genetic testing identified an allele with 2 suspicious VUSs in cis in CALM2 [and then they're named there, 248 and 83], neither of which were present in population databases.” Just pausing there - is that, there's two novel missense variants in *CALM2* identified in that case, is that unusual?
A. That would be very unusual, yes, given the population data that you showed even on the previous slide, yeah.

Q. The “Parental genotypes were unknown. Both *CALM2* variants had high LLRs in the CALM variant effect map”, that's the map that's reported in this paper?
A. Yes.

Q. “indicating a high likelihood of pathogenicity for each variant”, and then it says, “Based on the likelihood of detrimental functional effects of both variants in the variant effect map, this allele was deemed to contribute to the patient's phenotype.” That's a case--
A. Absolutely, yeah.

Q. --of using the variant effect map clinically? Is that right?
A. Yeah. I did the - absolutely. So the - if you note the patient had had extensive genetic testing and it doesn't say that we used the allele to make any clinical decisions, it just simply says we felt that the likelihood of two very rare variants in a gene that not long before had been associated with a very similar phenotype, severe prolonged QT in a young child or in a child, that makes sense, that's essentially, you know, you've got a known disease gene, disease to gene association that's been validated independently in multiple *de novo* variants, and you have two very unusual variants in the same - on the same allele in calmodulin that makes quite a lot of sense to say this looks like all of the prior calmodulin-associated diseases, it makes sense to make that association. It doesn't mean that we would have sort of ignored any other information, it just says this looks like we found the most likely variant because this has been described many times before.

Q. The next sentence says, "Prior to perspective functional variant effect

mapping, it would have taken months to test the functional effects of these variants on calmodulin function." Can I take it from that that prior to - and in other cases where you don't have a variant effect map - you would ordinarily seek to validate with functional assays?
A. Almost never, we wouldn't. Certainly not with most genes in clinical practice because the functional assays are not really known for most genes.

Q. What does this refer to then when it says if you didn't have the variant effect map it would've taken months to test the functional effects?
A. Yeah, we - it would've taken months had we wished to try and understand how - I mean the reality is this would be such an unusual occurrence, are you having two missense variants in a very short gene where there's almost no variation in the background population, you want to really understand what happened, how it would affect function and whether there was some specific set of circumstances at a genetic level and an RNA level or at a protein level that might have led to this outcome. So we would probably do a lot of functional studies in this setting, but the vast majority of clinical sudden death cases were not able to do functional testing except in a very restricted research environment and so this is a statement to say, now with other scalable functional assays, we can see a future where it's possible that everything you would ever identify would have already have been tested, which would be a huge advance in many ways.

Q. Professor, in this case, as I read this, there is QTc - Long QT phenotype, a clear Long QT phenotype?
A. Yeah.

Q. There are two variants of uncertain significance in CALM2?
A. Yep.

Q. There's no parental genotype so you don't know if they're *de novo* or inherited and then you have the variant effect map, and that together, the variant effect map predicting pathogenicity in both of those, that was enough to conclude in this case that that was responsible for the patient's phenotype?
A. No, if you read the wording, it is quite careful, it was “deemed to contribute to the patient's phenotype".

Q. "Deemed to contribute", okay, so it had a pathogenic effect relative to the patient's phenotype?
A. It seemed - it seemed as if it was likely that that was the case based on, you know, as I said, the pre-test probability - because you've seen the gene and phenotype together before - but is it definitive? No, it can't be.

Q. Then in case 2, it was "an 8-year-old female patient with a ventricular fibrillation arrest after falling from monkey bars”; again, a phenotype consistent with Long QT with a QTc of 547 milliseconds. There was a “heterozygous variant of VUS in *CALM3*” identified that “had not been observed in population databases [and] the variant was upgraded to pathogenic only after parental testing confirmed its *de novo* status". With all of those things taken together, it met the criteria of pathogenic, so that's 99% certainty under the ACMG

guidelines; is that correct?
A. That's correct.

Q. And then "The CALM variant effect map shows an LLR consistent with pathogenicity…which would have facilitated variant interpretation in real time without the delay of obtaining parental testing results." Can I take it from that that you're saying that the variant effect map would be sufficient to show pathogenicity under the Guidelines even without parental testing, without the factor of it being *de novo*?
A. No, that - I think that's an overstatement, based even on what we said there but also, for sure based on the facts too.

Q. Can you explain that?
A. So again, there is - as I've said probably too many times for you all - no data looking at a heterozygous calmodulin variants in any of the assays that we have discussed in this evening's proceedings or have been discussed in prior proceedings or reports. So it's very difficult to know what the functional assay that predicts clinical pathogenicity really would be.

Q. Professor, can I maybe rephrase the question? That last sentence says, "The CALM variant effect map shows an LLR consistent with pathogenicity"
A. Yep.

Q. Then it says, "which would have facilitated variant interpretation in real time without the delay of obtaining parental testing results." Now, as I read that, it's suggesting that the variant effect map predictions would be a suitable substitute for parental testing. Is that not right?
A. That - that is the hypothesis that this report is suggesting, yeah.

Q. This is your report.
A. But - I know, exactly, but I don't think anybody is suggesting - I mean, remember, I've worked for 25 years on multiple genes; some genes we have 20-plus assays in vitro and we still don't know which ones predict the clinical phenotype so you have to - all I'm saying is you have to be circumspect and all we're suggesting here is that--

Q. I understand what you're saying. I'm sorry, I cut you off, you were just coming to say what you were suggesting.
A. No, don't worry, I'm just saying we're suggesting that this is a potential methodology that would accelerate our ability to correlate functional information across the whole gene with individual variants. But, you know, for example, even, even, even the sentence that you quote says quite clearly "an LLR consistent with pathogenicity", but remember, if you go elsewhere in the literature, that LLR - and in this paper also - that LLR is dependent on the clinical associations, and we've already said that a significant proportion of the clinical associations are still not fully proven. So you're - everything is a work in progress, that's I think the most important takeaway here.

Q. Accepting that, Professor - I'll try one more time and then I'll leave it - nevertheless, is it wrong to read that sentence as suggesting that the

variant effect map in this case - just the facts of this case, case 2 here - would have been sufficient to essentially - could be given the same weight as evidence of *de novo* variant status?
A. I don't think the sentence says that but it would certainly contribute to the evaluation of pathogenicity in a useful way and it would do it efficiently and that's really what - that's what the sentence says, so it will have facilitated variant interpretation; it doesn't mean it would have defined it, it just means it would have facilitated it. So I think there's a reason that there are multiple criteria in the ACMG, it's - as I've mentioned to you before, there are - there are variants where I would 100%, 100%, I'd - I've done the work myself, would 100% would have said they are definitely the cause of the disease in family A, and exactly the same variant has no phenotype over in the next family over. So a lot of this is taking the information in these in vitro assays and putting it into a clinical context and that's really what this is saying, is the availability of prior functional information accelerates but does not replace the clinical evaluation. That's all.

Q. Can I ask you then about case 3?
A. Yes.

Q. Which was:

> "a 17-year-old male patient with sudden death during sleep, with cardiac fibrosis on autopsy histology. Molecular autopsy revealed a VUS in *CALM1* [which is numbered] which was not observed in ClinVar or population databases. No other candidate variants were identified. The family history was significant for hypertrophic cardiomyopathy in a paternal uncle and sudden death in a paternal grandmother… The variant interpretation was upgraded to 'athogenic by the laboratory upon demonstration of *de novo* status."

Just pausing there and just to confirm this: That is not Long QTS phenotype, I take it?
A. Absolutely not.

Q. Okay. "However, the variant effect map shows an LLR near 0, in a range where there is an equal prevalence of pathogenic and benign variants. In the setting of the patient's cardiac fibrosis, which is not a typical feature of CALM-related disease" - just pausing there, has it ever been identified to your knowledge in a CALM-related disease?
A. There are very structured reports. I would - off the top of my head, I'd - I don't think it has but I would be - I would be summarising information that I don't fully recall. So I think it's reasonable to assume from this statement, I think we did look at the literature at that stage quite extensively and it was certainly not typical at that stage. But again, because of the associations that are being reported, it's possible somebody has made the assertion, but whether it's valid or not, I would hesitate to state.

Q. Reading that sentence again, "In the setting of the patient's cardiac fibrosis, which is not a typical feature of CALM-related disease, and the family history of

cardiomyopathy and sudden death, these results serve to underscore the possibility that this *de novo* variant is not solely responsible for this patient's sudden cardiac death. Together, these data suggest that another cause of cardiomyopathy may coexist in family members, who should be clinically screened accordingly." Now that, as I read it, is suggesting notwithstanding that there was a pathogenic classification by the laboratory, which occurred without a Long QTS or CPVT phenotype, that essentially the unique features of that case, those distinctions in phenotype, together with this variant effect map, suggest that this family should not assume that the CALM variant is causal in this case, is solely causal; is that right?
A. Exactly. So this is a great example of exactly what I was just saying - here is a *de novo* variant in calmodulin in a family with a different disease. It's not present in two other affected individuals and the phenotype is actually much more like the cardiomyopathy in the paternal uncle and the paternal grandmother than it is to any calmodulin-related phenotype - yet, there is a calmodulin variant that has a strong signal of pathogenicity in this assay. So again, all it's really reflecting is the point that our paper was really designed to make which is, there's a huge gap between pathogenicity in the lab and clinical pathogenicity, and only by filling out those gaps systematically and not just one at a time based on clinical association or potential but doing it across multiple assays in multiple models across the whole genome--

Q. Your point is well made, Professor--
A. You will eventually build a picture of what the best way forward really is, but it is certainly not the case at the moment.

Q. Can I ask you very specifically about this case and what is written in this article, in your paper?
A. Yeah.

Q. It says, "these results serve to underscore the possibility that this *de novo* variant is not solely responsible for the patient's sudden cardiac death". So you don't consider that the results have eliminated, certainly not eliminated the possibility and the reasonable possibility of a role in this patient's sudden cardiac death. Is that fair?
A. I think that's - I mean, again, I prefer not to use, sort of, generic terms. I mean, the probabilities if you look at this family, the likelihood that this individual has the variant, whatever the variant is that caused the hypertrophic cardiomyopathy in his uncle and grandmother, is close to 25%. Whereas the chances of seeing a *de novo* variant in calmodulin is actually - as you've shown me many times - closer to about 10 to the minus 6, 10 to the minus 9, depending on where the variant is. So it's highly likely that it's actually the cardiomyopathy that caused the sudden death rather than the variant in calmodulin. But, the data that are available in the assay allow you to not make any assumptions but go on and as exactly as it's said here, another cause of cardiomyopathy may co-exist. We should be clinically screened accordingly. So there's just not enough information in this family with three generations and a pathogenic variant in the assay to make any clinical decisions based on either the family history or the assay - showing you that the clinical decision-making is completely independent of these in vitro assays at

the moment.

Q. Nevertheless, notwithstanding all of that, there was - this variant was initially found to be pathogenic, notwithstanding there is no Long QT phenotype or CPVT phenotype, that's right?
A. It was upgraded to pathogenic in the laboratory based on the *de novo* status in the absence of any phenotypic - remember, we're again - this is part of the conundrum - that you need to come up with a probability for the genetics in isolation for the phenotype and then look at both together. Anything else, anything other than that is bias. And so the reality is, the last thing you want to do with a patient in front of you or a family in front of you is to base it on assertion or supposition rather than evidence from the family itself. That's all.

Q. Applying that to this case, wouldn't that suggest, having regard to the family history and the mismatch in phenotype, that the way I understand you to apply this criteria is that there shouldn't be a consideration of this variant in this case as causally related to the phenotype?
A. No, not at all. What I'm saying is there's huge uncertainties. So if you look, this was a patient who had died suddenly at the age of 17. The other family members had died at discrete ages. This phenotype is quite consistent with hypertrophic cardiomyopathy; not consistent with known calmodulin. Yet we've imagined that it would be worth testing the pathogenicity of this for good reason because it was a *de novo* variant. That gave an independent assessment of laboratory pathogenicity, but when it came to the clinical pathogenicity, which, as I have said many times, is independent, we did not feel that we could change any of our management based on either of those findings. So all it says to you is that the majority of the laboratory information is really just creating a primary interpretation of the variants. That then has to be placed on an individual family basis in a clinical context and that can lead to very different conclusions and that is what I think you've heard me say all through the evening - that we've tended to have the pathogenicity in the lab and the pathogenicity in the clinic confounded, and they're actually quite discrete probabilities.

Q. I'll ask you one final thing, Professor. Turning again back to the case at hand and the variant at hand, G114R. Applying the variant effect map reported in this paper shows pathogenicities consistent with pathogenicity. If you focus only on information available for Laura Folbigg, the youngest of the four children—

JUDICIAL OFFICER: The eldest of the four children.

ROY: She lived the longest, she wasn't the eldest of the four children.

JUDICIAL OFFICER: Oh, yeah, yeah, yeah.

ROY: --focusing solely on that case and therefore excluding consideration of the other children, would you deem the allele in this case to contribute to the patient's phenotype in a similar manner to the case we just looked at?

A. I think it's a good question. I think that is a more reasonable possibility than that it caused the actual deaths themselves. It could have contributed, but I think that's about as strong as the evidence would support. But I think it's a reasonable statement, but I would just simply qualify the distinction between causality and a contribution.

Q. I'm sorry to keep telling you I'm done when I'm not. In your view is there a possibility that the CALM variant in Laura contributed to or occasioned myocarditis?

A. That's an excellent question. I don't think there are any data to support that at the moment but there's certainly evidence, for example, from some of the - most experiments, that calmodulin can affect immune function. There are all sorts of possibilities. But again, all I'm trying to say is these are uncertainties for which we have no real data. And so, I just - I prefer not to be speculative but simply to take the facts as they are and try and come up with the most useful, probabilistic interpretation of the data available to help guide either the clinical decisions or, hopefully, the Inquiry.

Q. Just to be clear, where other experts have potentially speculated on the role of the interaction between calmodulin to G114R and myocarditis in the direction of the myocarditis triggering an arrhythmia, you were - and I accept you say at a base level of speculation - at least raising a bare possibility that the variant might in fact have caused the structural abnormalities and the myocarditis?

A. It's entirely feasible, that's how little we know about calmodulin function at this residue. So, you know, I've said to you before, you can literally march up the calmodulin gene, and every residue that you change has an effect on calcium affinity but yet the phenotypes can be in different tissues, different organs. It's very, very complicated. And so, all I'm saying is there are no data in either direction, but the possibility remains that either of those things could have happened - that the myocarditis could have interacted with a contributing variant in calmodulin to cause an arrhythmia, or it could have been that the calmodulin variant caused the myocarditis. That's how little we know about this particular variant and its phenotypic expression. As I've said before, we're still talking about obviously very little likelihoods of each of these, and I had understood that you were trying to really make some general estimates about the probabilities of this being a causal variant in these particular cases. But that's - those are all reasonable assertions.

JUDICIAL OFFICER

Q. Can I just ask you, Professor, a couple of things about what you say of the probabilities at page 47 and 48? That's the last paragraph of 47 going onto 48. The probabilities you're measuring is the likelihood of the children dying from a single unifying cause as distinct from discrete causes. Is that correct?

A. That's correct.

Q. Underpinning that is a number of assumptions. I'm just talking generally, not necessarily by reference to this case. If you have two children with a particular condition which the other two children don't have, that markedly

impacts the probabilities. Just a matter of logic it does, doesn't it?
A. Yes, absolutely. It reduces them by a factor of about 10 to the minus 4, yeah.

Q. If you add to that - and I'm just asking you to assume this - two children have one condition, another child has a separate and distinct condition and dies from something that can be identified, that lessens the probabilities even further, does it not?
A. Absolutely, yes.

Q. The probabilities could also be affected by the different age the children died, and I'm talking in particular now in relation to one child dying totally in infancy, one dying with a condition after eight months, and the other two dying later. That affects the validity of the probability theory, doesn't it?
A. It does to some extent. I mean, if you look at the population data which the initial probabilities are based, there's definitely a tail in that probability curve after the age of 12 to 16 months. And then it drops off more steeply after the age of ten. But you're 100% right, yes.

Q. That, I want to suggest to you, is the difficulty with probability theory. It's used - and I say this with respect - really on this basis: you can't say with a degree of certainty that you're satisfied that any of these deaths were caused by an identifiable cause. Then you go back to the probability theory and say, it must be a unifying cause. That's where I have some difficulty, Professor, I'm afraid.
A. No, I'm actually not saying that it must be, I'm just saying that the relative probabilities favoured - I'm not saying there's no chance at all. Quite the opposite, I'm agreeing with you quite clearly that there are nuances to the probabilistic analysis, but they can be estimated. And the - if you think about the relative probabilities of a shared genetic cause, 1 in 16 is the estimate for a shared genetic cause. And the estimate for any of the others, just even *a priori* single sudden death unexplained is, from Australian data, about 1 in 3,133 hundred. So, the relative probabilities start to become really quite significant, but I agree with you completely, the absolutes are very difficult to estimate.

Q. I'd like to discuss it further with you but I don't think we've got the time.
A. Sorry.

JUDICIAL OFFICER: Yes, Dr Woods.

WOODS: Your Honour, in light of the compendious and extremely able examination by Ms Roy, I have no questions.

NO EXAMINATION BY DR WOODS

<EXAMINATION BY MR JORDAN

Q. Professor MacRae, can you hear me all right?
A. I can, yes, thank you.

Q. Professor MacRae, there's only one area of evidence that I would like to give you an opportunity to consider, and that is: I think you've already given evidence that you were provided with a report prepared only very recently by Professors Toft Overgaard and Nyegaard. That's the report that comments directly on your own report.
A. Yes, I have read it, yeah.

Q. Do you have access to that report, or can we provide access please? It's tab 6-06. Do you see there, Professor, this is the report by the two eminent Danish professors? It's dated February 10, 2023. The heading is "Provision of additional material including comments to evidence of Professor MacRae". Do you see that?
A. I do see it, yes, thank you.

Q. What I would like to do, Professor, is to give you an opportunity to respond as you see fit to any of the comments made against you in that report.
A. As I said, I respect Professor Toft Overgaard and Professor Nyegaard's expertise. I respect the data that they've generated. I'm just simply saying that the facts of an allele in isolation on calcium affinity have never been shown to result in a clear predictive utility for a given disease. In fact, if you scroll through this report, I honestly don't remember exactly where it is but there's a very nice diagram that Professors Toft Overgaard and Nyegaard provide of the relationship between Long QT and calcium affinity. If you could go to that, it sort of nicely illustrates my whole point, which is not that their data are wrong - there we go. So, it's not the diagram. I'm not sure. Can you go up one frame? Maybe there's a different one. There we are.

Q. Just for the record, that's red page 113.
A. Okay. So, if you look at this diagram, you know, there are variants that have quite low but nevertheless significant changes in calcium affinity; so C-domain calcium affinity reduction that have QT intervals that are in the normal range. And then there are others to the left of them at the top of that green bar where something with lower calcium affinity has a QT that's 650 milliseconds. So, the one thing that you can be sure of is that there's not a strict correlation between calcium affinity of this assay and the clinical phenotype. Now, I would also suggest that the numbers of variants are small. You need lots of independent variants to be able to make a more rigorous case. And I'm not saying that this assay is not relevant; I'm just saying there is no data that would allow you to say that a calcium affinity, let's say of 5%, had a definitive effect on QT prolongation. It's just not - there's no correlation whatsoever. In fact, you can see there's a beautiful vertical - there are a wide range of QTs that are associated with the same calcium affinity reduction.

So, again, this is great work. We're trying to build a set of assays, as is Professor Toft Overgaard and Professor Nyegaard, that will ultimately be able to predict the relationship between in vitro effects of variants and their clinical effects. But I would argue - I mean, one of the things I would say is, I would not dismiss these data until they were tested in a heterozygous form. It may be that there's very clear correlation, it's just not been tested. This is a - this is

just an early stage experiment which was beautifully conducted but has substantial limitations when you're trying to translate it into a situation where, at the very best, there's a one to one relationship between the mutant and the wild-type, and at the other extreme it may be one to five depending on where, which compartment in the cell, or which particular channel complex you're talking about. So, there's a vast number of unknowns that they still have to address; and I'm confident, knowing their work, that they're likely to be pursuing this. But at this stage there's no information content, with respect, to predicting clinical outcomes. I'm sorry to say that but it's totally true.

Q. Is there any other specific part of this report that you would now like an opportunity to respond to?
A. I think the one other thing they did mention was I think they argued that their original linkage analysis had shown a definitive locus, and I - this was brought up in Ms Roy's questioning. I would just say I think that the original genetic information is reasonable but still not definitive. An independent confirmation in another family would have clinched it for me, but in the ten years since this paper was published there has never been another calmodulin-associated extended pedigree with CPVT sufficient to make the statistical argument. That would be my only point, but again, I said in response to earlier questions I think this is elegant work, I've done very similar work myself, it's exactly what I would've done. I would've published it in the hope that somebody else would replicate it independently and that may still happen, but at the moment as his Honour asked me to give evidence in the current setting, these data do not adjust my assessment of the likelihood of the G114R causing a clinical phenotype that is distinct in two children.

Q. Thank you so much for your patience, Professor.
A. You're welcome, thank you.

NO EXAMINATION BY MS HORVATH, MS LOVE, MR HASTINGS AND DR WATERHOUSE

ROY: Thank you, Professor.

JUDICIAL OFFICER: Thank you very much, Professor. We're all very grateful for you to have been able to make the time at a probably somewhat ungodly hour on what's still probably a fairly cold evening. Thank you very much indeed.

WITNESS: Well, thank you very much for your time and for your really expert questioning from all, thank you.

<THE WITNESS WITHDREW

AUDIO VISUAL LINK CONCLUDED AT 1.19PM

ROY: Your Honour, we have a matter of housekeeping. Our next witness is not scheduled until 10am on Tuesday and so - and this is just confirming for the benefit of everyone in the room - we don't propose to call anyone on

Monday or have any need to assemble.

JUDICIAL OFFICER: Well, we'll adjourn until 10am on Tuesday.

ADJOURNED PART HEARD TO TUESDAY 21 FEBRUARY 2023