SPECIAL INQUIRY

THE HONOURABLE THOMAS BATHURST AC KC

SECOND DAY: TUESDAY 15 NOVEMBER 2022

INQUIRY INTO THE CONVICTIONS OF KATHLEEN MEGAN FOLBIGG

CALLAN: Your Honour, this morning we'll hear evidence from Professors Toft Overgaard and Nyegaard. Can I deal with the tender of some further material which includes an addendum report that they have produced on the weekend? To that end, can I hand up a bundle which comprises what is described as an Exhibit list as at 15 November 2022. Shown in red in that document are updates to briefing material which is correspondence sent to each of the experts. As your Honour would have observed, we sought to include in the tendered material all relevant communication with the experts and also what we propose would be marked exhibit 6-03, being the addendum report of Professors Toft Overgaard and Nyegaard dated 12 November 2022. Your Honour, something I neglected to do yesterday, which was to tender the two PowerPoint slides displayed to Associate Professor Raju.

JUDICIAL OFFICER: I was going to ask you about that, yes.

CALLAN: Yes. It's suggested that they be marked Exhibit 10-03 in this index and the material which accompanies it, which I confirm has been served on the parties.

JUDICIAL OFFICER: You can take it they're admitted.

MFI #3 INDEX SHOWING EXHIBITS TENDERED TO INQUIRY AS AT 15/11/22

<MICHAEL TOFT OVERGAARD AND METTE NYEGAARD,
AFFIRMED(10.33AM)

ROY: Starting with you, Professor Toft Overgaard, can you please tell the Inquiry your full name and your qualifications?

WITNESS TOFT OVERGAARD: Yes, it's Michael Toft Overgaard. I am Head of the Department of Chemistry and Bioscience at Aalborg University. I'm a professor in protein science, and I've been working - studying mutations in calmodulin and their effects for a number of years.

ROY: In terms of your academic qualifications, your degrees?

WITNESS TOFT OVERGAARD: I have a PhD in protein science from Aarhus University, and a Master of Science in Chemistry and Biotechnology, also from Aarhus University.

ROY: You're also a post-doctoral fellow in the Department of Structural Biology at Stanford University Medical School?

WITNESS TOFT OVERGAARD: That is correct, yes.

ROY: Thank you for travelling so far to assist us today, both of you. Can you tell us also about your specific area of expertise in relation to calmodulin?

WITNESS TOFT OVERGAARD: Yes, so, I was part of the team who identified and characterised the first human calmodulin mutation that was found in and published in 2012. And since then, my laboratory has been - had an interest in characterising new mutations - human mutations of calmodulin.

ROY: Is it right that you've co-authored 15 scientific papers describing calmodulin mutations?

WITNESS TOFT OVERGAARD: Yes, so far we have been published - 15 publications, yeah.

ROY: It would be correct to characterise you as a research scientist?

WITNESS TOFT OVERGAARD: Yes, indeed.

ROY: Professor Nyegaard, can you tell us your full name and qualifications?

WITNESS NYEGAARD: Yes, so, my name is Mette Nyegaard. So, I have a PhD in human genetics, and I'm a professor in personalised medicine at Aalborg University. So, my research area is within genetic diversity linking variation in our genome to phenotype. I also teach medical students in the genetic architecture of different diseases ranging all the way from monogenic diseases to complex multifactorial diseases.

ROY: Just for completeness, you are also a post-doctoral fellow at Stanford

Medical School?

WITNESS NYEGAARD: That's correct.

ROY: It would also be correct to describe you as a research scientist?

WITNESS NYEGAARD: Correct.

ROY: The two of you together have prepared two reports for this Inquiry; is that correct?

WITNESS TOFT OVERGAARD: Yes.

ROY: The first was prepared at the request of legal representatives for Ms Folbigg?

WITNESS TOFT OVERGAARD: That's correct.

ROY: It's the report titled "Report on analysis of the Folbigg CALM2-G114R mutation and its impact on calmodulin protein function" dated 18 October 2022?

WITNESS TOFT OVERGAARD: That's correct.

ROY: For the record, that report is found in the tender bundle at tab 6-02. Following circulation of your report to other experts in the Inquiry and your review of their comments on your report, you conducted some further research and laboratory analyses in the last week?

WITNESS TOFT OVERGAARD: That is correct.

ROY: I'm not going to ask you to tell us about that just yet. We'll come back to it. You considered that you've made some new discoveries?

WITNESS TOFT OVERGAARD: That is correct--

ROY: Or a new discovery?

WITNESS TOFT OVERGAARD: Yes.

ROY: You've put the details of that research into an addendum to your report?

WITNESS TOFT OVERGAARD: Correct.

ROY: You've also responded to some of the comments of other experts in that addendum?

WITNESS TOFT OVERGAARD: That is correct, yeah.

ROY: That document is titled "Addendum to the Report dated October 18,

2022" and the addendum itself is dated 12 November 2022?

WITNESS TOFT OVERGAARD: Correct.

ROY: That was tendered in the bundle this morning, your Honour. I also understand you wanted to make some minor corrections to that addendum report?

WITNESS TOFT OVERGAARD: That is correct. There was a few errors.

ROY: Those corrections were circulated to the parties by correspondence which has been included in the tender bundle, but I also have a short Errata which can be attached to the report. I'll hand it up. Your Honour, if that could be tendered to and appear at the end of the tender bundle at 6-03.

EXHIBIT #6-03 SUPPLEMENTED BY THE ADDITION OF ERRATA TO THE ADDENDUM DATED 12/11/22, ADMITTED WITHOUT OBJECTION

I take it those are in the nature of typographical errors, a missing reference, and don't substantively change the conclusions in your report?

WITNESS TOFT OVERGAARD: That is completely correct.

ROY: I won't then take you to them. Do you both have copies of both of your reports there with you?

WITNESS NYEGAARD: Yes.

WITNESS TOFT OVERGAARD: Yes.

JUDICIAL OFFICER: Professors, feel free to use any material that you wish to during the course of giving evidence.

WITNESS TOFT OVERGAARD: Thank you.

ROY: You were also both co-authors of the 2021 article, "Infanticide vs inherited cardiac arrhythmias" published in the Europace Journal?

WITNESS TOFT OVERGAARD: That's correct.

ROY: Commonly referred to in this Inquiry as the Brohus article?

WITNESS TOFT OVERGAARD: Yes.

ROY: Again, for the record, that article appears at tab 15-02 in vol 2 of the tender bundle. Finally, in preparation for the hearing today, it's correct that you've both prepared a number of PowerPoint slides to assist in communicating your evidence?

WITNESS TOFT OVERGAARD: That is correct.

ROY: This is a highly technical area of genetics. I think we'll be assisted by that. You provided those slides to those assisting the Inquiry so that we can refer to them today. Your Honour, what I propose to do with those slides, they were provided yesterday; I've combined them with slides that I prepared for this evidence. Some of those slides actually include new information that's not otherwise in the experts' reports. What we're going to do is I've prepared a printed numbered guide to the slides that should enable both myself and the professors to navigate to different slides as appropriate in response to their evidence today and in February when they're cross-examined. Because it contains new information, I will tender those slides as well as the guide at the conclusion of their evidence-in-chief.

JUDICIAL OFFICER: There'll be a mechanism to identify which ones were prepared by you and which were prepared by--

ROY: Yes. I can indicate on the record now that slides 1 through to 12 are prepared by me, and the remainder of the slides were prepared by the experts. We will also endeavour to give the professors access to a mouse to allow them to point and indicate parts of the slide. Before we cover your specific discoveries in connection with the variant in the Folbigg family, it would assist the Inquiry to first establish some of the more foundational concepts with respect to calmodulin. Can I ask you to first tell us about your role in the discovery of the first calmodulin variant?

WITNESS NYEGAARD: Yes, so, that was a study that I led. So, it was done in a Swedish family where there was a clear segregation pattern of some sort of CPVT-like phenotype. So, the way it was done back then was that we first identified the segment in the genome that's inherited together with the phenotype, and then you sequence that segment of DNA, and then we identified the first variant in calmodulin really ever seen. Then what you do, because anything - any variant that is basically sitting on this segment could be the causative variant. So, we looked in, you could say, an independent cohort of individuals or patients with different types of arrhythmia where there were no really genetic reason identified, and then we found another variant in the same gene in a patient with the same phenotype, and this variant was *de novo*. And this is a really strong--

ROY: I'm going to slow you down.

WITNESS NYEGAARD: Yep.

ROY: When you found the first variant - incidentally, what's the first variant called?

WITNESS NYEGAARD: It depends if you do genetic or protein numbering, yeah. N97. And, yeah, the first one in the Swedish family, N54I.

ROY: N54I. That was in a large Swedish family?

WITNESS NYEGAARD: Yes.

ROY: You said in your first report that it was extremely surprising to find any variant in a CALM gene.

WITNESS NYEGAARD: Yes, that was the reaction from the calcium field, that they had a hard time believing that we really identified a person or a family with a variant.

ROY: I'm going to ask you to explain what was so extraordinary about finding any variant in a CALM gene. I understand you may have some slides you want to refer to.

WITNESS NYEGAARD: Yes.

ROY: You have the numbered guide?

WITNESS NYEGAARD: Yes.

ROY: I'll ask you to use that and we'll test the technology. Only when you want to go to a slide, you can indicate it. But can you explain why it was extraordinary to find any variant, or considered extraordinary to find any variant in a CALM gene?

WITNESS NYEGAARD: Yes, so, that would be - if we could go through those three reasons in slide, that would be 14, 15 and 16. Yes, so the calmodulin is 149 amino acid protein. It's a calcium sensor, so it binds and regulates hundreds of enzymes, pumps and ion channels, and that in itself does not make it so special. What is really special is that there are three genes in the human genome encoding the exact same protein. So when a gene is encoded it first makes something we call an RNA transcript. So those will be different. And then it's translated into the same protein. But what is most, I think if you could advance one slide, it's that calmodulin is so evolutionary conserved, so what is shown on this slide is the amino acid sequence in the calmodulin protein. So the first upper line is the human sequence and then underneath is mouse; worm; drosophila, which is kind of a fruit fly; arabidopsis, which is a plant; and, then yeast at the bottom. And what you can really see is the extraordinary conservation through evolution of this protein.

ROY: What does it mean to say that there's conservation in a gene?

WITNESS NYEGAARD: So conservation means that the amino acid sequence is exactly the same.

ROY: And the amino acid sequence refers to the segment of DNA that is the gene?

WITNESS NYEGAARD: It refers to the amino acid sequence in the protein. So even though the genes are different and the transcripts are different they are translated into the same protein.

JUDICIAL OFFICER: So that's from the RNA transcript; is that right?

WITNESS NYEGAARD: So this is the protein sequence where each letter stands for an amino acid. Yeah. And then as you can see the yeast, no the worm, there is only really three amino acids difference between human and worm in that the calmodulin that they're using in the body. And one of the differences are here, you can see there is a difference here and then there are - there is a difference down, where is it, here and here.

ROY: Is the effect of what you're showing--

JUDICIAL OFFICER: Ms Roy, one difficulty that is going to emerge with this, is whilst it's very helpful to see what Professor Nyegaard is doing on the slides, is there any way we could have a record of this if we want to--

ROY: Of where it's being indicated?

JUDICIAL OFFICER: Where it's being indicated.

ROY: Yes, your Honour.

JUDICIAL OFFICER: There is a recording being taken of this material, isn't there?

ROY: I don't believe there is, no, your Honour, it's being live-streamed. That maybe something we can investigate, but it's not--

JUDICIAL OFFICER: And there's no way that we can retrieve the live-stream as part of the record, is there?

ROY: Not the way it's currently set up. We can make some fairly urgent enquiries. I withdraw that, it's being recorded. I gather it wasn't intended to be retained, but I'm sure we can make that amendment.

JUDICIAL OFFICER: Have any of the parties got any, and you too Professors, have got any objection to this recording being retained. I think I'd like to look at it when it comes around to my report. My memory is not bad, but it's not that good. So if you have no objection.

WITNESS TOFT OVERGAARD: No objections.

WITNESS NYEGAARD: No objections.

ROY: Thank you.

WITNESS NYEGAARD: Yes, so I think also one of the points by showing this slide is if you go one - advance one slide, is that this position, 114, is really extraordinary. Even more, it's one of the residues that are conserved all across even down to yeast. Yeah.

ROY: Your Honour, I'm sorry to interrupt, can I ask for a quick adjournment, a five minute adjournment, please, your Honour.

JUDICIAL OFFICER: Yes.

SHORT ADJOURNMENT

Ms Callan, you're going to take us--

CALLAN: Yes, your Honour. Professor Nyegaard, we were just dealing with the three slides by which you were explaining why calmodulin is so, I think you used the word "well-preserved"?

WITNESS NYEGAARD: Yes.

CALLAN: You indicated by reference to slides 14, 15 and 16 what you mean by that. In particular by reference to what appears on the screen now, slide 16, you were telling the Inquiry the discovery you had made and its significance because of how well-preserved these amino acids are. The middle line, can I just understand, is that a reference to the CALM2?

WITNESS NYEGAARD: It's all calmodulin coming from either calmodulin 1, 2 or 3, because they encode identical protein.

CALLAN: You were indicating some discrete areas where there is, if I can describe it this way, difference within the codes when you compare the human with mouse and others in that list, one of those areas is, you were describing, I just want to make it clear for the record what you were pointing out on the mouse.

WITNESS NYEGAARD: Yes. So it's conserved so calmodulin has the same amino acid sequence across all vertebrates. And then I added these other organisms to put it in context how well-preserved it is. And preservation is something that you see when there is no genetic variants or mutations. They happen all the time. It's kind of raining down on our genome and so there is something in nature that there is selective pressure against changes in calmodulin, which we can see here.

CALLAN: In blunt terms, how does that selective pressure manifest itself?

WITNESS NYEGAARD: Yes, that is what we don't know. Yeah, it can be many things, but we don't know the full spectrum.

CALLAN: You used the term *de novo* a moment ago in your evidence?

WITNESS NYEGAARD: Yes.

CALLAN: Is that a relevant term when we are considering this genome?

WITNESS NYEGAARD: So--

WITNESS TOFT OVERGAARD: Can I answer?

WITNESS NYEGAARD: Yes.

WITNESS TOFT OVERGAARD: Perhaps I can assist. So I think *de novo* is a relevant term when we look at inheritance in the human setting. But for this particular slide it's not that relevant. So these particular slides and the points that Professor Nyegaard was making is that even if we look evolutionary as far back as worm and plant and yeast, calmodulin is extraordinarily conserved. And the three changes she pointed out, that is the only three changes comparing human to worm calmodulin.

JUDICIAL OFFICER: Which means throughout the evolutionary process there's been no variation in that area?

WITNESS TOFT OVERGAARD: That is correct.

CALLAN: Until 2012?

WITNESS TOFT OVERGAARD: Until 2012, yes.

CALLAN: Just in that respect, sorry, before completing that topic of your discovery in 2012, this slide on the screen, slide 16, you have specifically drawn attention to the 114 location; is that correct?

WITNESS NYEGAARD: Correct.

CALLAN: According to this slide, what it shows is that that location has a consistency across all of those organisms?

WITNESS NYEGAARD: Yes.

CALLAN: What does that indicate?

WITNESS NYEGAARD: From a population genetics perspective, it says that this precision, it's very important that there is this particular amino acid sitting in this position because if it didn't matter it would not have been so conserved.

CALLAN: That finding that you made of a calmodulin variant, you were explaining your starting point was looking at one Swedish family. Could you explain for his Honour's assistance what you did to better understand that by looking at another cohort?

WITNESS NYEGAARD: Yes. So when you identify a variant that is segregating with disease because our genome exist in blocks, so to be - can you repeat the question please?

CALLAN: You were speaking about how you looked at another cohort.

WITNESS NYEGAARD: Yes.

CALLAN: Yes, to assist you to understand what you were seeing.

WITNESS NYEGAARD: Yes. It is when you find a rare variant segregating with disease in a family, that you need some kind of proof to make your research really rigid, that you actually do have the right variant, and you can do that by looking in other patients with the same or somewhat similar phenotype, and if you find another variant in the same gene it's really strong genetic evidence that you have identified, and this is important when you want to link new genes to disease, yes.

JUDICIAL OFFICER: Let me just try and understand that. You look for other people with similar phenotypes and you see if there's a variant in the calmodulin area so far as they're concerned; is that correct?

WITNESS TOFT OVERGAARD: Yes.

JUDICIAL OFFICER: Does it have to be the same variant; is that also right?

WITNESS TOFT OVERGAARD: That's correct.

WITNESS NYEGAARD: No, it's better if it's another one.

JUDICIAL OFFICER: Because that shows, as I would understand it, that any variation in that particular area - to put it loosely - can be disease causing; is that a fair summary of it?

WITNESS NYEGAARD: Yes.

JUDICIAL OFFICER: Thank you.

CALLAN: Your Honour, there may be some concurrent adducing of evidence today but Ms Roy understands--

JUDICIAL OFFICER: You've got concurrent witnesses, so I suppose it makes it understandable.

CALLAN: I understand Ms Roy is able to proceed and I'm grateful for that.

JUDICIAL OFFICER: Thank you.

ROY: Thank you, your Honour. I'm sorry for the interruption. Is there anything about the function of calmodulin in the body, the role it plays in the human body, that would explain why it may have been conserved?

WITNESS TOFT OVERGAARD: Can I answer?

ROY: Please.

WITNESS TOFT OVERGAARD: I think yes, indeed. So the function of calmodulin is that of binding to calcium. Calcium is an incredibly critical ion for all or many basic cell functions. So calcium is a signalling molecule. So calmodulin is basically able to bind to calcium, and I don't know if we actually

have slides here on this, so what calmodulin, in a sense, does - it is sensing the calcium concentration, changes in--

ROY: Before you move to the finer details of the interaction there, let me put it this way: at what point, after an egg is fertilised and a new person is beginning, does calmodulin become relevant to the function of that new being?

WITNESS TOFT OVERGAARD: In one perspective you can say the very first thing that happens when an egg is fertilised is a calcium signal, and that is what calmodulin does, it decodes calcium signals and translates to other proteins inside the cell.

ROY: So from the very beginning of human life, and also probably many different--

WITNESS TOFT OVERGAARD: Each life.

ROY: --from each life - from the beginning of life calmodulin is expected to play a critical role?

WITNESS TOFT OVERGAARD: Yes, so that's why it was regarded in the scientific community, up until 2012, that mutations or variants in calmodulin was incompatible with life.

ROY: Incompatible with life?

WITNESS TOFT OVERGAARD: Yes.

ROY: Professor Nyegaard, I think you have some other slides for us that relate to the conservation of calmodulin?

WITNESS NYEGAARD: So that would be slide 17. Yes, so every time a new person is born there are new mutations. It really depends, but on average, 70 new mutations are - and nature is producing all - we could say that genetic variants are raining down on our genome. So what we saw before is a result of evolution. What we can also do is take a snapshot into the human population as it is now and obviously that would be people who are alive right now and see - kind of look at what genetic variants are in calmodulin in such a snapshot in to the human population, and this is what I've indicated here, that we can now do this as a relatively new thing because sequencing cost has come down, so we can now sequence hundreds of thousands of individuals. So we are using this as a very valuable resource both to see what variants do we see and also what variants do we not see.

ROY: You describe it as a snapshot of the human population. What are you referring to?

WITNESS NYEGAARD: If you collect half a million people that are alive right now, this would be a snapshot of those people living right now.

ROY: Are there databases that do this?

WITNESS NYEGAARD: Yes, there are several really large initiatives in the world sequencing a lot of individuals, millions of individuals, and one really important one was the gnomAD or is the gnomAD. It's really a very used resource because they have put together different cohorts and then they started the sequencing, and what they have done is that they have made it available as an open resource.

ROY: What is a genome?

WITNESS NYEGAARD: A genome is the full set of DNA that is within each cell.

ROY: To take the gnomAD database, which correct me if I'm wrong, is spelt G-N-O-M-A-D?

WITNESS NYEGAARD: Yes.

ROY: To take that database, for example, what is the size of the data set? How many people's genomes are included in that data set?

WITNESS NYEGAARD: It is growing, so a lot of the measures that we're using - I think we will talk about them in a little while, those constraint scores, try to quantify our constraint of each gene in the genome, so that resource is based on 150,000 individuals, but--

JUDICIAL OFFICER: Is there any difference if they come from different ethnic origins?

WITNESS NYEGAARD: They are, yes.

JUDICIAL OFFICER: Yes, that's what I thought. Do you need to do a differentiation as far as that is concerned for the purpose of your research or at least for relevant purposes, does it matter?

WITNESS NYEGAARD: So the constraint scores, they may vary somewhat from population to population, but it's kind of a universal - if you have a really strong constraint gene it is across population.

ROY: How would you expect constraint to be revealed by analysing a data set like the gnomAD?

WITNESS NYEGAARD: Slide 23. So that's the gnomAD. So the disadvantage is that there are no phenotypes, so you cannot look up one genetic variant in one carrier and see if they have any disease.

ROY: Can you slow down?

WITNESS NYEGAARD: Yes, sorry.

ROY: When you say there are no phenotypes, there are no phenotypes attached, as in you don't get the information about the phenotypes of the genomes that are sequenced or people with the phenotypes are excluded from the database?

WITNESS NYEGAARD: So they have removed severe paediatric diseases but otherwise it's people with their different diseases. There are lots of diseases. It's really difficult to find 100,000 people that do not have any disease at all, so it's kind of cohorts.

ROY: And do you get the information - I'm sorry, I spoke over you. Can you repeat what you said?

JUDICIAL OFFICER: Kind of cohorts.

WITNESS NYEGAARD: Yes. So if you advance--

WITNESS TOFT OVERGAARD: Can I assist?

WITNESS NYEGAARD: So when I say there are no phenotypes it means that they are - there are, of course, these people who have phenotypes, or some of them, but it's not made available freely because of research ethical reasons.

JUDICIAL OFFICER: Is that the reason why severe paediatric diseases are removed as well? In the slide you note that--

WITNESS NYEGAARD: Yes, so that's what they write, that they have attempted to remove really severe diseases.

JUDICIAL OFFICER: Is that for ethical reasons?

WITNESS NYEGAARD: That is because if you want to use this resource as a filtering resource, filtering out variants, you - yes.

JUDICIAL OFFICER: I follow.

ROY: What does a search of the gnomAD database tell you about CALM variants?

WITNESS NYEGAARD: Yes, so if you advance one slide please.

JUDICIAL OFFICER: We're on to slide 24.

ROY: Yes, slide 24. Thank you, your Honour.

WITNESS NYEGAARD: Here you can look up different genes and if you look up calmodulin 2 you can see that they have listed the number of expected variants in three different categories.

ROY: What does it mean to say an expected?

WITNESS NYEGAARD: Expected is if there were no selective pressure, so what you would expect by chance.

ROY: By chance, you would expect variants to be dispersed roughly evenly or?

WITNESS NYEGAARD: Yes, that's correct.

ROY: Is that right, by roughly evenly, you would expect variants to be dispersed evenly?

WITNESS NYEGAARD: Yes, and so there's a little bit with the sequence, some sequences or stretches of sequences are more prone to genetic variation but more or less, yes, so they have divided into three different categories and synonymous means a genetic variant at the DNA level that does not change any amino acid. And missense variant means it changes at the DNA that changes the amino acid.

ROY: That's what we're concerned with--

WITNESS NYEGAARD: Yes.

ROY: --in your report and in this Inquiry?

WITNESS NYEGAARD: Yes. And then this "pLoF" means loss of function. There are also some variants that can disrupt the gene so that the protein from that particular gene doesn't exist. So, they're completely disruptive, the loss of function. And so, they are listing the number of expected variants, and then next to it they're observed. And as you can see, the synonymous ones which we kind of think that there are no really constraint because it doesn't change anything in the protein. You can see you would expect around 22 or 23 in this size of population that are sequenced here, and you observe 21. So, you can see you observe and - the same - or more or less as you would expect. And then if you go down to the missense, you would expect 77 and you see only eight. Yeah? And the loss of function, you would expect to see eight and you do not see any.

ROY: In terms of the reduced number of missense variations and the lack of any in loss of function, what does that indicate to you?

WITNESS NYEGAARD: So, it indicates that there are less missense variation observed compared to what we would expect by chance, yeah?

ROY: What does that signify? That there's less than you would expect by chance.

WITNESS NYEGAARD: I have made some slides, yes. So, I would like to - it's an example that we often use when we want to illustrate this, and it starts on slide 19. And it's really to show the power of the - of unobserved - unseen observations. So, here is--

ROY: When you say the power of unseen observations, you mean the power of the absence of other variants--

WITNESS NYEGAARD: Yes.

ROY: --in the database--

WITNESS NYEGAARD: Yes.

ROY: --in the calmodulin gene?

WITNESS NYEGAARD: Yes. So, if you look at this example from the World War II, and here is an aircraft returning from gunfire, and the allies were - you know, they were losing a lot of aircrafts, so they were thinking about where to put the armour to make these aircraft stronger. And so, if you advance one - so, the most - by intuition--

ROY: I'll just interrupt. Can you advance one slide, please? Thank you. We're now looking at slide number 20.

WITNESS NYEGAARD: Yes. So, your first instinct would be to put them where the bullet holes are, right? But then there was a mathematician saying, "Hey, wait" - if you can advance one slide.

ROY: Now slide 21.

WITNESS NYEGAARD: So, he said, "Remember that it's only the"--

ROY: I think we're not quite looking at slide 21, I'm sorry to interrupt.

WITNESS NYEGAARD: Are we not?

WITNESS TOFT OVERGAARD: Yeah, there's something else coming in.

WITNESS NYEGAARD: Okay? Yep. So, the first intuition would be to put the armour where the bullet holes are, but you should think about that the bullet holes are equally distributed among these aircrafts. So, there was this statistician who said, "Yep, but remember those that are not coming back, those that are not making it. Those are the planes that you do not see here. So, actually, you should put the armours where the bullet holes are not."

ROY: The implication being--

WITNESS NYEGAARD: Yes, so if you advance one slide.

ROY: Slide 21.

WITNESS NYEGAARD: Yeah, so it basically shows that because this - you only see those aircrafts that kind of make it, the - where the missing holes are, those are really the - where you don't want a bullet hole. So, if you advance

one more slide.

JUDICIAL OFFICER: Putting it bluntly, you can be shot everywhere except in those particular places and you'll get away with it? To use an Australian vernacular. Take that aircraft.

WITNESS NYEGAARD: Yeah.

JUDICIAL OFFICER: The places where it hasn't been hit are the places you cannot survive, notwithstanding what happens to the other areas. That's broadly the--

WITNESS NYEGAARD: Yes, so, where you see the bullet holes, those are the aircrafts that come back. So, those are actually the places where you can survive with a bullet hole. Yeah, exactly. So, if you think about the human genome, it's exactly the same. You have all these genetic variants across the genome, so we can look for regions in the genome where there are much less genetic variation or, kind of, these empty.

ROY: For the record, we're on slide 22. That would suggest to you that a variant in the missing spaces is fatal or inconsistent with life? It doesn't appear in the databases?

WITNESS NYEGAARD: Yes. So, if you go to slide 25. So, this is an attempt - these constraint metrics. There are different constraint metrics, but the gnomAD has developed these constraint metrics, and so they - one of them is observed, so you take the number of observed genetic variants and divide it by the expected. And so, you can see here for calmodulin 2 that it's 0.1 because you only see 10% of the variants compared to what you would see by chance - expect by chance. And so, in the - in some of the other reports, there were - they mentioned the Z-score, yeah?

ROY: Can you tell us what that is? You said Z-score?

WITNESS NYEGAARD: Z-score. So, it's also a constraint measure.

JUDICIAL OFFICER: How's that calculated?

WITNESS NYEGAARD: Yes, so, I was looking into how that is exactly calculated. So, it is - it's a little bit - yeah. So, they - gnomAD suggest that people use the observed divided by expected because it's possibly to use a confidence interval, because some of these genes - if they are very small genes, the number of counts are small, so it's difficult to do statistics. So, they urge people to use the upper bound of the confidence interval to actually, you could say, estimate how constrained is a gene. And this bound is set to 0.35.

ROY: Can I stop you there and see if I understand?

WITNESS NYEGAARD: Yeah.

ROY: We're going to come to this; I think, Professor Toft Overgaard, you're going to help us in a little while with understanding the genes and proteins and their structure. But they vary in size from one to another, and they vary in the number of amino acids that they have from one to another; is that right?

WITNESS TOFT OVERGAARD: Not calmodulin but other genes, of course.

ROY: Other.

WITNESS NYEGAARD: Yeah.

ROY: Calmodulin to a non-calmodulin gene would have a different number of amino acids in it.

WITNESS TOFT OVERGAARD: Yes.

ROY: What you're describing is controlling for the fact that you have a different number of amino acids for different genes, and therefore the number of--

WITNESS NYEGAARD: Yes.

ROY: --predicted variants is going to vary in accordance with the size of the gene, the number of amino acids in the gene; is that correct?

WITNESS NYEGAARD: Yes. So, these are statistical measures, and it's easier to do statistics on larger numbers. So, if you have a really large gene, you have a lot of expected variants and you have - you would also have a larger number of observed variants, and it's easier to get more, you could say, accurate statistics. So, I have not myself used this Z-score so much because it is the - if it's falling below or above some kind of threshold, there are two things wrong with that. It's a small gene, so the Z-scores will become inaccurate, and the gene is really conserved.

ROY: Is calmodulin a small gene?

WITNESS NYEGAARD: Yes.

ROY: As well as being highly conserved?

WITNESS NYEGAARD: Yes, very small.

ROY: How does it compare to other genes in terms of its conservation?

WITNESS NYEGAARD: So.

WITNESS TOFT OVERGAARD: Advance two slides, I think.

ROY: I think your data is on slide 27.

WITNESS NYEGAARD: So, just to put these genes in context, we tried to say - so, the - yes, so, here we made a list of genes that are insensitive to loss of function, completely insensitive to loss of function. This is the suggested threshold for insensitivity to loss of function.

ROY: What does that mean, "insensitivity to loss of function"?

WITNESS NYEGAARD: It means that - no, sorry, sensitive to loss of function. Yes, thank you. Yes. So - and then after that, we sort by this expected divided by observed. Just to give you an idea of how does other gene look, and if you - on this particular way of filtering, calmodulin 1 and 2 and 3, they are really within the top 15 of the most constrained genes in our genome, on this particular way of filtering.

ROY: How many genes in our genome have we assessed like this?

WITNESS NYEGAARD: In - these are files downloaded from the gnomAD, so there are - these values are from 20,000 genes. Yeah. So, this is out of around 20,000.

ROY: Out of around 20,000 genes, all three of the CALM genes are in the top 15, and actually, by that number, the top 13 of the most conserved genes in the human genome?

WITNESS NYEGAARD: Yes. In this particular way of filtering. But as I said, there are many different ways of calculating constraint, but they are really constrained, all of them.

ROY: Just before we move on from the use of the databases, are there other major databases?

WITNESS NYEGAARD: Yes, there is also the UK Biobank. It's also a really, really nice resource. So, it's half a million people from the UK. They invited around eight million people, and then half a million said yes to be part of this really fantastic initiative where they've had their genome sequenced and they share all their medical records, their biochemistry. They have questionnaires and some of them have brain scanning and heart scans and - yeah.

ROY: There's significantly more information in the UK Biobank than in the gnomAD database?

WITNESS NYEGAARD: Yes.

JUDICIAL OFFICER: Professor, can I just ask something. I don't quite understand. I thought your evidence was early on that in 2012, the first discovery of a variant in the CALM gene was--

WITNESS NYEGAARD: Yes.

JUDICIAL OFFICER: But how does that match with the chart in the slide we're

presently looking at, which seems to suggest that there are - except it's constrained, but there are - that seems to suggests there are a number of missense. Is that correct or not?

WITNESS NYEGAARD: Yes, so, this is because back then there were not these big resources. So, it--

JUDICIAL OFFICER: I see. These resources post-dated your discovery?

WITNESS TOFT OVERGAARD: Yes.

JUDICIAL OFFICER: I understand.

WITNESS NYEGAARD: Yes, and then actually what is also very important is that those observed missense variants, they are - there are only one or two individuals. For most of them, there's only one carrier. It's not that there are hundreds of carriers of this, but one mutation, one carrier.

ROY: Following on from his Honour's question, would it be right to say that at the time of your discovery in 2012, and the wider scientific community's prediction that there would not be CALM variants, arose from the fundamental role it plays in multiple functions of the human body?

WITNESS TOFT OVERGAARD: Both that and the observation that it's completely unchanged in all vertebrates.

ROY: I suspect these are questions for you, Professor Toft Overgaard. Can we come now to the role that calmodulin plays specifically in heart function?

WITNESS TOFT OVERGAARD: Yes, and I suggest to use a couple of the slides we provided.

ROY: Please.

WITNESS TOFT OVERGAARD: I'll see if I can find the number. So I suggest going to slide 29. So I made a series of slides to try and explain this in a bit of detail. Of course it's simplified, but is it okay if I go through the slides?

ROY: Please do.

WITNESS TOFT OVERGAARD: Okay. It's a little bit tricky with the pointer so please excuse me. So to understand the role of calmodulin in cardiac contraction I'll try to explain a little bit of the currents or the electrical changes that we see in the heart and where they actually come from. So in this first slide it's just a depiction of the heart. And to coordinate most of contraction in the heart there's a small electrical signal that comes from the sinoatrial node. So if you can advance one. So the small electrical signal from this node, then travels throughout the heart and coordinates basically the muscle contraction in the different compartments. And that results in an electrical signal that you can measure on the outside of the body. I think as explained

yesterday, in the ECG. So basically if you put electrodes on you can measure an ECG as changes in the electrical currents in the heart. Okay, so if you advance one.

ROY: This is slide 30.

WITNESS TOFT OVERGAARD: So slide 30. This is to try and explain that we're not cardiologists, so what we do is to look inside the cells and try to explain you can say where do these currents come from and the mechanism of cardiac contraction. So what we do is we look basically over here to the right. So the electrical signal and currents that's generated by opening and closing of different ion channels in the cardiac cell.

ROY: Can I just stop you there to understand what we're looking at?

WITNESS TOFT OVERGAARD: Yes.

ROY: What might be described as train tracks around the outside?

WITNESS TOFT OVERGAARD: Yes.

ROY: So that's the cell wall; is that right?

WITNESS TOFT OVERGAARD: Yes, so this one?

ROY: Yes.

WITNESS TOFT OVERGAARD: So I don't like to correct you but it's called the cell membrane.

ROY: Cell membrane.

WITNESS TOFT OVERGAARD: Yes.

ROY: Please do correct me.

JUDICIAL OFFICER: Don't hesitate to correct, yes.

WITNESS TOFT OVERGAARD: Yes. Cell wall is in bacteria and plants.

ROY: Thank you.

WITNESS TOFT OVERGAARD: So the cell membrane which is impermeable to ions, so if you want to have ions moving across the cell membrane you need dedicated ion channels. And so these are proteins encoded by other genes that's - whose function is to sit in the cell membrane and upon certain signals open or close for particular ions to go across. Okay.

JUDICIAL OFFICER: And one is calcium and one is sodium on the chart?

WITNESS TOFT OVERGAARD: Yes. So we'll be taking these a little bit one at a time. The different colours here indicates which ions are being opened for - gated for the particular occurrence. Yes. So if you advance one, can you advance one slide, please? Yes. So just to understand if you open a hole in the cell membrane what's going to happen, then the flow of ions will be from a compartment where you have a high concentration to the compartment where you have a low concentration. So that's why here it's listed that the concentrations of these three ions, so the sodium on top, the potassium - sorry, potassium, sodium and calcium ions, there's a dramatic difference in concentration outside and inside the cell. So this is when you then open you can take an example of the potassium ions, we have a low concentration outside the cell and we have a high concentration inside. So if you open a potassium ion channel there will be positive potassium ions flowing out of the cell. And that basically establishes an electrical current because you have a charged particle moving. Okay. So and then you can see the difference here. A couple of differences that are interesting. So one is that potassium is high inside the cell and low outside. Calcium and sodium is high outside the cell and low inside the cell. And then calcium you can see is very special in that the difference in concentration is around 10,000 fold. For the two other ions it's around 35, 40 fold.

ROY: As in the difference between the inside and the outside of the cell?

WITNESS TOFT OVERGAARD: Yes. I can explain maybe a reason why.

ROY: Please.

WITNESS TOFT OVERGAARD: It's a little complicated. But inside the cell we may use another ion which is phosphate for signalling. But also if you think about the nucleotide that generates energy, ATP, that's also a phosphate based nucleotide. So all the DNA are phosphate based molecules. And calcium and phosphate do not mix. So if you mix those they will precipitate and generate some solid material. So it's really important to keep the calcium concentration inside the cells very low. Okay.

ROY: Can you explain each of the three, if it's natural to your progression, each of the three channels?

WITNESS TOFT OVERGAARD: Yes, so perhaps if it's okay I'll go through the so-called action potential. So if you advance one slide.

ROY: Slide 31.

WITNESS TOFT OVERGAARD: 31. So just to put in perspective, we have the whole heart, and if you advance one, where the sinoatrial node starts an electrical signal they coordinate contraction, which generates the electrocardiogram that you can measure from the outside. If you go one forward. If we look at it from the cell perspective, so the individual cardiac muscle cells, the small depolarisation or electrical signal will generate what's called an action potential. So this action potential down here, this is a

measure of the potential across the cell membrane. And that, when you get a small initiating electrical signal from the sinoatrial node and action potential is generated by, you can say really coordinated open and closing of these four different types of ion channels, three different types of ion channels.

ROY: Can you step us through that sequence?

WITNESS TOFT OVERGAARD: Yes. So then advance one slide, please.

ROY: Slide 32.

WITNESS TOFT OVERGAARD: Yes. So we have five phases of the action potential. The numbering is a little bit weird so it's not our doing, so excuse us for that. So it's called phase 4, that is the starting point. So this is the resting stage of the cardiac cell, muscle cell. So this is when the cell is relaxed. If you note here there's a large negative membrane potential. And then - and that's the reason for this membrane potential is that particular potassium ion channels that are open. So we call that potassium ions flow out of the cell and that generates this membrane potential. So if you advance one slide.

ROY: Slide 33.

WITNESS TOFT OVERGAARD: So cardiac contraction or the start of the action potential comes with a small electrical signal from the - generated by the sinoatrial node and progressing throughout the heart. And there's very small electrical change across the cell membrane that activates the sodium channel.

ROY: Which is indicated in pink?

WITNESS TOFT OVERGAARD: That's in the red on my screen, but pink, maybe yes. And so the voltage-gated sodium channel, it's called the NAV1.5, that opens and that generates a really fast depolarisation of the membrane. That's because the sodium ions are flowing into the cell. At the same time the potassium channel is closed. Okay. So if you advance one. This rapid depolarisation activates another potassium ion channel and then there's a flow of potassium going out of the cell again. So that will repolarise the cell a little bit. At the same time the sodium channels are fast or rapidly inactivated, which means that they are close. But there are some of the sodium ion channels that are still a little bit open. So it partly inactivates and that becomes important.

ROY: That's slide 34.

WITNESS TOFT OVERGAARD: Yes. So if you advance one slide. So the next phase of the action potential is called phase 2. It's also called the plateau phase, because there's no really change here in the membrane potential. This is due to the voltage-gated calcium ion channel is opening up, the Cav1.2.

ROY: Which one is the Cav1.2?

WITNESS TOFT OVERGAARD: So this is the one here that I'm trying to point to, the green on to the left.

ROY: So that goes to the extra cellular space?

WITNESS TOFT OVERGAARD: Yes. So that will let in some calcium ions and they are depicted in green here. At the same time there's another - other potassium ions opening that counter balances the flow of calcium ions inside. So that's why there's no development in electrical current, but it's sort of balanced out.

JUDICIAL OFFICER: Are there two calcium channels on that screen?

WITNESS TOFT OVERGAARD: That is correct. So the small inflow of calcium ions, and I think you can advance one, yes, so this small increase comes inside the cardiac cells, the voltage-gated calcium channel on the outer membrane, it's positioned just next to the ryanodine receptor calcium channel. So that's the other one in green. It's a really large calcium ion channel that sits in the sarcoplasmic reticulum. And the sarcoplasmic reticulum is an internal calcium store.

ROY: Inside the heart?

WITNESS TOFT OVERGAARD: Inside the cell. So it's confined in another set of membranes, but it's filled with calcium.

ROY: And this is slide 36.

WITNESS TOFT OVERGAARD: This is slide 36, yes. So the small inflow of calcium ions through the Cav1.2 activates the ryanodine receptor, which then opens the ryanodine receptor and there's a large inflow of calcium from this sarcoplasmic reticulum calcium store. And those--

ROY: Just stopping you there for a second

WITNESS TOFT OVERGAARD: Yes.

ROY: So of the two calcium channels, the one that moves to the extra cellular space, so that opens from the extra cellular space--

WITNESS TOFT OVERGAARD: Yes.

ROY: --is called--

WITNESS TOFT OVERGAARD: Cav1.2

ROY: Cav1.2. And the channel that opens from the internal--

WITNESS TOFT OVERGAARD: Calcium store, yes.

ROY: --it's called?

WITNESS TOFT OVERGAARD: RyR2.

ROY: Thank you.

WITNESS TOFT OVERGAARD: Because that's a cardiac version of this channel. Yep. So when that opens lots of calcium comes out and this is trying to depict here, goes to the myofilaments and it's the calcium that makes the myofilaments contract. So that's actually the muscle contracting.

ROY: So the heart muscle contracts--

WITNESS TOFT OVERGAARD: Yes.

ROY: --at the point at which that calcium is released into the--

WITNESS TOFT OVERGAARD: Yes. When the calcium is released that triggers the muscle to contract. Yes. The development of the calcium concentration is just depicted down here. So basically when we open one channel the voltage-gated calcium channel, the small inflow of calcium ions open the ryanodine receptor, lots of calcium inside the cell and the muscle contracts. Okay. So also what I should say, the release of the calcium coming into the cell also inhibits, so that actually feeds back and closes these two channels again.

ROY: Closes which channels?

WITNESS TOFT OVERGAARD: The Cav1.2 and the RyR2.

ROY: So the fact of the calcium release itself is sensed and those channels are then closed?

WITNESS TOFT OVERGAARD: Yes. So basically it's all self-regulating, and I'll come back to that. But that's mediated by calmodulin. Yeah. But at first you need to reset the cell for the next heart contraction. So if you advance one slide.

ROY: Slide 37.

WITNESS TOFT OVERGAARD: So now the next phase, phase 3, it's called the repolarisation. This is where the two, both the Cav1.2, also the sodium channel, they both close, but also the ryanodine receptor has a channel, so they will close. It's not depicted here but, of course, the calcium is removed from the cell, it's pumped out and into the SR.

JUDICIAL OFFICER: What, through the same channels?

WITNESS TOFT OVERGAARD: No, the channels can just open. They don't have an active - so there are some pumps. It's a little bit complicated so we

didn't depict the pumps here or the mechanisms for removing. So calcium is removed, and now another potassium ion channel takes over, it opens up and the flow of potassium ions without the counterflow of calcium and sodium, re-establishes the membrane potential. So if you advance one slide.

ROY: Slide 38.

WITNESS TOFT OVERGAARD: We're basically back to another, the Kir2.1 potassium channel reopens and we're back to the resting state.

ROY: I'm going to try and put what you've said to us in lay terms.

WITNESS TOFT OVERGAARD: Okay.

ROY: Correct me as necessary. Essentially for each contraction or beat of the heart muscle there is a complex chain of openings and closings of channels in to and out of the heart muscle cell?

WITNESS TOFT OVERGAARD: That's correct.

ROY: Specifically the passages of calcium, sodium and potassium through channels specific to those elements, to those ions?

WITNESS TOFT OVERGAARD: That's correct.

ROY: That’s occurring in a complex and precisely timed sequence responsible for various stages of the heart contracting?

WITNESS TOFT OVERGAARD: Correct.

ROY: Which together, from everything you've just stepped us through, produces a single heart beat?

WITNESS TOFT OVERGAARD: That's correct.

ROY: Is it right - and I expect you'll take us through it in a minute - that the opening and closing of each of those channels at the correct time is affected by the calmodulin protein?

WITNESS TOFT OVERGAARD: Yes. If you advance one slide please.

ROY: Slide 39.

WITNESS TOFT OVERGAARD: This is - maybe even advance one more. So you have just highlighted which of these channels are intimately regulated by calmodulin.

WOODS: Your Honour, may I, through you, ask the witness if he'd speak a little more slowly.

WITNESS TOFT OVERGAARD: Yes. Thank you. Each of these channels indicated here with a circle, but also with a small grey fellow, that's calmodulin.

JUDICIAL OFFICER: We're still on slide 39, aren't we?

WITNESS TOFT OVERGAARD: This is slide 39, yes. So this indicates that calmodulin is an integral part of regulating these ion flows and channels.

ROY: Can you expand on that? What does calmodulin do in relation to these channels?

WITNESS TOFT OVERGAARD: It depends on how long you have. This is extremely complex, but in general--

ROY: Can you give us the condensed version?

WITNESS TOFT OVERGAARD: Yes. In short, you can say calmodulin is employed as a sub-unit of these channels, and it sits there as a calcium sensor.

ROY: If I try to put this in, again, very lay terms. Calmodulin is essential to each of those channels that you have circled on slide 39?

WITNESS TOFT OVERGAARD: Yes. The ones highlighted here, though some of the potassium ions that are not, as we know, regulated by calmodulin, but, yes, then, some of them are not.

ROY: If something is wrong with how your body makes calmodulin proteins there may well be something wrong with how these channels open and close?

WITNESS TOFT OVERGAARD: Indeed, yes.

ROY: If there's something wrong with how these channels open and close there will be something wrong with your heart rhythm?

WITNESS TOFT OVERGAARD: That is correct, and perhaps if you advance one - it's the same slides but more information. Here I've just tried to depict each of these channels. They are known for when they have mutations they can precipitate in to or lead to certain--

ROY: When you describe those channels having mutations, are you describing the gene that codes to build those channels?

WITNESS TOFT OVERGAARD: Yes. So here I've not mentioned the gene names but only the protein names.

ROY: That's the protein built by the gene?

WITNESS TOFT OVERGAARD: That is the protein built, so I can take one example, so the first calmodulin mutations identified by Professor Nyegaard

was in a family and this additional individual with CPVT like phenotype. So, of course, if we want to understand the mechanism, how can calmodulin produce a CPVT like--

ROY: Could I ask you to speak up again.

WITNESS TOFT OVERGAARD: Yes. So how can calmodulin produce a CPVT like phenotype? We would go in to the laboratory and look for - does it disturb the interaction of the RyR2 ion channel? Because it's known that this ion channel mutation in this are linked to CPVT.

ROY: Again trying to put that in lay terms, you observed CPVT - Professor Nyegaard observed CPVT in a large Swedish family that did not have the ryanodine, the RyR2 variant, but you identified a calmodulin variant which, for the reasons we've already covered, was surprising?

WITNESS NYEGAARD: Yes.

ROY: Because of the role of a variant in the gene that codes for the ryanodine receptor, the RyR2, it occurred to you to look to how the variant in the calmodulin gene was going to interact with that receptor?

WITNESS TOFT OVERGAARD: Basically it's well known that the calmodulin regulates the ryanodine receptor, so what we then do is to try and see is that particular regulation disturbed by the mutation.

ROY: Because the ryanodine receptor controls the flow of calcium, that's also why the focus initially on the calmodulin variants was on its relationship with calcium?

WITNESS TOFT OVERGAARD: Yes. So because we, already from 2012 and several subsequent publications, we know that this was a critical interaction, potential critical interaction for calmodulin mutations, we always - can say, "always", but we will then look for does any new mutation in calmodulin affect the ryanodine receptor function?

ROY: So that's the ryanodine receptor?

WITNESS TOFT OVERGAARD: Yes.

ROY: Then if we move to the other calcium channel, what's indicated there?

WITNESS TOFT OVERGAARD: This is the Cav1.2 channel. It's also known that calmodulin is a critical part of this particular channel's closing mechanism. It's called the calcium dependent inactivation, and so it's known that calmodulin is basically responsible for the calcium dependent inactivation of this channel, and so when we--

ROY: When you say, "the calcium dependent inactivation of the channel," you're referring to the channel closing?

WITNESS TOFT OVERGAARD: Yes.

ROY: As in the sequence that forms the heart beat?

WITNESS TOFT OVERGAARD: Yes. As a historical note, I can say we also ask the people who understand and look for the function of the Cav1.2, if they wanted to characterise the initial N54I mutation, and they said, "Well, we can do it but it may not be relevant because Cav1.2 is a Long QT associated thing," and so if the family, the Swedish family, if there's no sign of Long QT this may not be relevant.

ROY: At that point you only had the one family. Their phenotype was CPVT and it was suggested that it would be potentially unproductive to look at the receptor that's associated with Long QT Syndrome?

WITNESS TOFT OVERGAARD: That is correct.

ROY: Was that work ultimately done, to look at the effect on that receptor?

WITNESS TOFT OVERGAARD: Yes, it was because the year after the first publication - and so in 2013 - Lia Crotti and colleagues identified three other calmodulin variants with a severe Long QT phenotype, and so in 2014 I co-authored a paper with the late David Yue's group and they studied some of these voltage-gated ion channels or some of these ion channels and they show that - I think we even have a slide for that; is that--

ROY: I might keep you here for now.

WITNESS TOFT OVERGAARD: Okay - basically that showed that the severe Long QT calmodulin mutations had really severe effects on this particular ion channel.

ROY: So that expanded what was known about the possible phenotypes associated with calmodulin variants?

WITNESS TOFT OVERGAARD: Yes.

ROY: You've also indicated on this slide the sodium channel and the potassium channel?

WITNESS TOFT OVERGAARD: Yes, and that's because it's also well known that calmodulin is extremely important for the function of these particular two ion channels.

ROY: Prior to - in the last week, had anyone looked at the functional impact of CALM variants on either of those two channels?

WITNESS TOFT OVERGAARD: Yes, there has been publications on - at least one publication looking for potential functional effects of the sodium ion channel.

ROY: That's the purple channel?

WITNESS TOFT OVERGAARD: That's the red/purple one, yes.

ROY: I don't think we need to turn up the--

JUDICIAL OFFICER: I think it's the red channel.

WITNESS TOFT OVERGAARD: Yes, red in my--

ROY: I apologise.

WITNESS TOFT OVERGAARD: Sorry, the red one.

ROY: Yes, sorry. Sodium. There was one that had looked at sodium?

WITNESS TOFT OVERGAARD: Yes, and they looked in particular at the three first severe Long QT mutations and they could really not show any functional effect except for one particular mutation, that's called the D130G mutation, and only when they investigated a fetal splice variant of this particular channel, so there are different splice variants of these channels and some of them are differently expressed over time.

ROY: What about the potassium channel?

WITNESS TOFT OVERGAARD: I'm thinking because to recollect if there were studies done. There may have been. I don't have that overview right now.

ROY: Was that something that was looked at in relation to the article published by Kato?

WITNESS TOFT OVERGAARD: Yes. So if we move to more recent times, so earlier this year, 2022, there's a publication that demonstrates that there is an effect of another, one particular calmodulin mutation that's called the N138K variant. That, as they mention in the paper, surprisingly has a potentiating effect for this channel. So basically it means that this calmodulin mutation increases the flow of potassium ions, and that's interesting for that family because it was sort of predicted to be a severe Long QT mutation but there was a really broad phenotypic expression in this large family where they found this mutation, and they offer - suggest that this can be explained by a counter effect from interaction with this voltage-gated potassium ion channel.

ROY: Again if I try to put that in lay terms, this particular variant was assessed for its impact on the calcium channels?

WITNESS TOFT OVERGAARD: Yes. I didn't mention that. The main Long QT phenotypic expression of calmodulin mutations has - so the accumulated evidence we have is that they can mainly be explained by disruption of the interaction and function of the voltage-gated calcium ion channel.

ROY: In this particular family that was assessed, the assessment predicted that they would have severe Long QT Syndrome?

WITNESS TOFT OVERGAARD: Yes.

ROY: So there was a mismatch between how they presented, which was with variable expressivity?

WITNESS TOFT OVERGAARD: Yes, and also the severity of the QT, yes.

ROY: And variable severity. With what the analysis only of the calcium channels would have predicted?

WITNESS TOFT OVERGAARD: That's correct.

ROY: It was the article we've just referred to authored by Kato et al, and I'll give your Honour a reference; it appears in--

WITNESS TOFT OVERGAARD: Slide 41.

ROY: --tab 15 of vol 2 - I don't need your Honour to turn it up - of the tender bundle, behind tab 6.

JUDICIAL OFFICER: It's also referred to in Professor Vinuesa and Professor Arsov's report.

ROY: It's referred to in, I believe, almost every report, your Honour. Knowing the capacity for calmodulin to interact with each of the channels, the authors of that article tested its impact on the potassium channel?

WITNESS TOFT OVERGAARD: That's correct.

ROY: They found that the effect on the potassium channel essentially mediated the harmful effects on the calcium channel?

WITNESS TOFT OVERGAARD: I would almost say mitigated.

ROY: Mitigated?

WITNESS TOFT OVERGAARD: Yes.

ROY: In this family, they would've had a slightly different heartbeat sequence but it functioned for them?

WITNESS TOFT OVERGAARD: Yes. So, basically, that's - you can say it's an example of one mechanism where you can have a detrimental - in terms of protein function - calmodulin protein function - so, a detrimental variant where other effects unexpectedly compensate, in this case, for the severity of the Long QT.

ROY: I'm not going to ask you to explain it right now, but is it right that in the last week you have also now tested the CALM-G114R variant, being the variant in issue in this case, for its impact on the sodium channel?

WITNESS TOFT OVERGAARD: That is correct.

ROY: Again, we'll come back to it, but you found there was a significant impact, in summary?

WITNESS TOFT OVERGAARD: That is correct. I'll say there's a significant impact in how calmodulin can bind to the channel.

ROY: That's a new discovery?

WITNESS TOFT OVERGAARD: That is correct.

ROY: We'll come back to that. To do that, we need to understand how calmodulin is made. Starting at the very beginning, a gene is a length - I tried this before and you corrected me so you'll have to do it again - of DNA that provides the code to build a particular protein?

WITNESS TOFT OVERGAARD: That's correct.

ROY: Essentially, a gene is a list of the order in which amino acids should be layered so as to build a particular protein?

WITNESS TOFT OVERGAARD: Correct.

ROY: Can we bring up slide 3, please? This is figure 2 from your report.

WITNESS TOFT OVERGAARD: Yes.

ROY: We saw this figure, in particular, yesterday in Senior Counsel Assisting's opening remarks. If we first just look at the bottom panel and the three lines. Ignoring for a moment the circles that are stacked above each of the lines, is it right that each line of letters - I think that'll be quite small for some of us, but there is a string of letters is what makes up each line - sets out the list of amino acids in the order in which they appear in a typical, normally-functioning calmodulin protein?

WITNESS TOFT OVERGAARD: That's correct.

ROY: Is it the case that each of the calmodulin genes 1, 2 and 3 is identical?

WITNESS TOFT OVERGAARD: That's correct.

ROY: You've said already, but there are 149 amino acids in this particular?

WITNESS TOFT OVERGAARD: That's correct.

ROY: Each letter indicates the amino acid that should ordinarily appear in the numbered position?

WITNESS TOFT OVERGAARD: Correct.

ROY: On this figure, the numbers are marked at 20, 40, 60, 80 and so on?

WITNESS TOFT OVERGAARD: Correct.

ROY: For example, if we look at the 120th position in the CALM3 gene--

WITNESS TOFT OVERGAARD: And I have to actually - I can see one - in CALM2, the 120 marker is one position off.

ROY: Okay. What's indicated on this slide - thank you for the correction - underneath 120, the letter E is indicated?

WITNESS TOFT OVERGAARD: That's correct.

ROY: What does that signify?

WITNESS TOFT OVERGAARD: It means in the position 120, the amino acid, glutamic acid, is present. It's abbreviated, yeah.

ROY: E is a code of the amino acid, glutamic acid?

WITNESS TOFT OVERGAARD: That's correct.

ROY: If we come to the circles that sit above each of the genes - sorry, is this a gene or the protein?

WITNESS TOFT OVERGAARD: This is a protein.

ROY: We come to the circles sitting above the protein. Each of those circles indicates a known pathogenic variant?

WITNESS TOFT OVERGAARD: Yes, and I believe this is from the editorial that Professor Nyegaard and I provided for European Heart Journal when the publication of this Calmodulinopathy Registry was produced. So, this is an overview of all the mutation in that Registry in 2019.

ROY: As of 2019?

WITNESS TOFT OVERGAARD: Yeah.

ROY: We've already heard, but there have been more discovered since that time?

WITNESS TOFT OVERGAARD: Yes.

ROY: The letter inside the bubble indicates the different amino acid which is found in that variant?

WITNESS TOFT OVERGAARD: That's correct.

ROY: For example, going back to the incorrect 120th marker, if we look down at the CALM3 protein, to the left there's a blue W sitting above the 114th position, by my count.

WITNESS TOFT OVERGAARD: That's correct.

ROY: That tells us that, in a normal person there would be a G, which is what's indicated underneath the W?

WITNESS TOFT OVERGAARD: That's correct.

ROY: What does a G stand for?

WITNESS TOFT OVERGAARD: That's a glycine.

ROY: Instead, in this person, in this variant, there's a W?

WITNESS TOFT OVERGAARD: Yes, correct.

ROY: What does the W stand for?

WITNESS TOFT OVERGAARD: W, that's tryptophan.

ROY: Therefore, this variant is then written as "G", to indicate what should appear. "114", the spot at which it should appear, "W", what actually appears?

WITNESS TOFT OVERGAARD: That's correct.

ROY: That's the naming convention for variants?

WITNESS TOFT OVERGAARD: In the one-letter code, yes.

ROY: The top panel of that slide is the schematic diagram of the calmodulin protein when it's bound to calcium?

WITNESS TOFT OVERGAARD: When it's bound to calcium and no other targets, yes.

ROY: And nothing else?

WITNESS TOFT OVERGAARD: Yep.

ROY: This is essentially a different way of depicting the protein that the CALM gene builds?

WITNESS TOFT OVERGAARD: That's correct.

ROY: What you're showing us in the top part of the figure, in the schematic, is the location of various amino acids on the actual protein?

WITNESS TOFT OVERGAARD: Correct.

ROY: In rendered three-dimension, essentially.

WITNESS TOFT OVERGAARD: Yes.

ROY: The colourful structures we can see, on the right-hand side a lot of red, some orange, there's some blues and some yellows; those are the variants?

WITNESS TOFT OVERGAARD: That's the variants and they depict the phenotype characterised in the Calmodulinopathy Registry.

ROY: The colour corresponds, as your code indicates, to Long QT Syndrome is red.

WITNESS TOFT OVERGAARD: Yep.

ROY: Long QT combined with CPVT is orange, CPVT is yellow, and IVF, sudden unexplained death or other atypical presentation is blue?

WITNESS TOFT OVERGAARD: Correct.

ROY: There's also, harder to see, but pink squares that indicate calcium binding residue. Those appear in the bottom schematic?

WITNESS TOFT OVERGAARD: They appear in - so, it's sort of, like, in this position. So, you see - if we can take them here. So, there are one, two, three, four, five, six pink positions. So, we are - so, right now we're seeing something else than you are seeing, I think.

ROY: We've just lost our slide. We were looking at slide 3.

WITNESS TOFT OVERGAARD: Okay. That's fine. So, you can see those six pink squares repeated four times in each calmodulin. That indicates the four binding sites for calcium ions. So, if you go on top, we can see that that would correspond to these binding to these four black circles.

ROY: You're indicating on the schematic on the top there are four black balls on each of the two depictions of the protein. Those four black balls are calcium?

WITNESS TOFT OVERGAARD: That's correct.

ROY: Does calcium always bind in the same location?

WITNESS TOFT OVERGAARD: Yeah, that's correct.

ROY: These are known as the calcium binding sites?

WITNESS TOFT OVERGAARD: So, these are the amino acid residues that directly binds to calcium - a calcium ion.

ROY: Just looking at that, it would appear that pathogenic variants for, certainly, Long QT Syndrome in red seem to cluster around two of those binding sites in particular on the right-hand side.

WITNESS TOFT OVERGAARD: Yeah, so they're typically found in the - in one half of the calmodulin molecule that's called the C-terminal domain, or the C-domain.

ROY: That's the right-hand side?

WITNESS TOFT OVERGAARD: That's the right-hand side. And they are typically clustering or found in direct calcium binding residues. So, changing one of the amino acids that bind directly to calcium. And so, in most cases, we can predict and we can then also measure that the calcium binding is compromised.

ROY: That's, as we were talking about, the important role of calmodulin in binding to calcium and the movement of calcium through the cell, that you would predict that variants in the point at which the calcium ion binds to the calmodulin protein are more likely to be pathogenic?

WITNESS TOFT OVERGAARD: So, understanding - and perhaps we can - I don't know if you have the - there's another slide that sort of demonstrates - our understanding is the more severe the effect on calcium binding is, the more likely it seems that it's a severe Long QT phenotype.

ROY: Okay. We might come back to that. On this schematic - again, from 2019 - there are, it seems, fewer CPVT associated variants - those are yellow - or mixed phenotype - the orange?

WITNESS TOFT OVERGAARD: That's correct.

ROY: They seem to be more dispersed across the gene.

WITNESS TOFT OVERGAARD: That's correct, yeah.

ROY: If we can turn to slide 4, this is a figure from an article already referred to by Crotti et al. That was published in the European Heart Journal in 2019. I'll have to turn up a reference for your Honour but we don't need to look at it. Lia Crotti was also a co-author of yours in the Brohus article; is that correct?

WITNESS TOFT OVERGAARD: I'm sorry, I actually don't recall.

ROY: We can come back to that. But you cite from that paper in the Brohus article?

WITNESS TOFT OVERGAARD: Yes.

ROY: This figure comes from that article. This is essentially a different way of presenting similar information; is that right?

WITNESS TOFT OVERGAARD: That is correct.

ROY: The colour scheme is different; it's not actually indicated on the slide, so I will just indicate red is Long QT Syndrome, green is CPVT and yellow is IVF or sudden unexplained death or atypical phenotype.

WITNESS TOFT OVERGAARD: That seems to match, yes.

ROY: The pink balls in this case are the calcium ions that are binding at the sites indicated by the light grey lines.

WITNESS TOFT OVERGAARD: That's correct.

ROY: The additional information included in this schematic are the grey boxes and circles. What do those indicate?

WITNESS TOFT OVERGAARD: These indicate mutations in calmodulin found in some of these large data repositories; so, the gnomAD, for instance.

ROY: You agree?

WITNESS NYEGAARD: Yes. They - yes.

ROY: Would it be correct to say that the variants that are identified there, which come from the gnomAD database, indicate benign variants?

WITNESS TOFT OVERGAARD: No.

ROY: Can you tell me why not?

WITNESS NYEGAARD: Yes, so, from the gnomAD it's really easy to see how many - we call them counts - how many times or how many carriers are observed. And I have the numbers here. So, they are, for most - the vast majority of these, there is a single carrier among around 150,000 individuals. So, if we keep the constraint in mind, this is - these are not necessarily benign. They are not - so.

ROY: Can I ask it this way?

WITNESS NYEGAARD: Yes.

ROY: We'll revisit what we said about the gnomAD and what you said about

the gnomAD data base. The gnomAD database doesn't tell you the phenotype of genome. It doesn't tell you much of anything at all about the person who carried the genome other than implicitly that they survived long enough to have their genome sequenced.

WITNESS TOFT OVERGAARD: That's correct.

ROY: So inherently it indicates that a person survived long enough to have their genome sequenced; that's correct.

WITNESS TOFT OVERGAARD: That's correct, yeah.

ROY: But beyond that it doesn't tell you much; is that right?

WITNESS TOFT OVERGAARD: That's correct.

WITNESS NYEGAARD: That's correct.

ROY: However, the presence of a variant in a number of individuals may start to build a picture that it was a survivable variant?

WITNESS NYEGAARD: Yes. So we - these - the gnomAD database is a very widely used resource for identifying variant that has reached a certain frequency in the population. And then we - most of them we say that are benign. But variants come in a spectrum, so some of them have a little bit less. It's not the - you could say that the genetic architecture of diseases that have a genetic component doesn't have to be either, you know, monogenic. So they could have an influence on other things. They - we know that there is a selective pressure but we don't know how nature is kind of removing these variants. So at this frequency I would not call them benign.

ROY: How many times would you expect a variant or would you - what is the threshold? I'll start again. How many times would you want to see a variant in the gnomAD database to consider that an indicator that it was likely benign?

WITNESS NYEGAARD: It is a very good question. And this is - so it's - so you could say benign for one condition could influence another condition. So we have a lot of variants in our genomes. Some of them have a large effect on disease and this is what we're talking about here, that the first variant that was identified in calmodulin, they seem to have a really strong effect size. And then it's possible that others have a less effect on cardiac phenotype, but they can have an effect on other things. So it is really a fully benign variant that is - we probably have a lot of them. But I would say we tend to say, I don't know, 1% or 10%.

ROY: So you would want 1% of the database, for example, to have the variant before you would think that's a pretty good indicator that it's benign?

WITNESS NYEGAARD: So when you have a monogenic disease and you want to - so it really depends on the condition you're looking at. So if it is a

very rare condition and you see that in the gnomAD, you have a candidate variant and you're not sure if this is causing disease or not. And then you look in the gnomAD and see that it has a frequency of 1% and then maybe the disease has a frequency of 0.0001% of the population. So you say that this variant cannot cause this disease. Do you see what--

ROY: I see. So it would depend also on the frequency of the disease type that you're looking at?

WITNESS NYEGAARD: Yes. Yes.

ROY: I see. And this slide is slide 4, and it comes from the Crotti article, which is exhibit 2-BU.

WITNESS NYEGAARD: Mm-hmm.

ROY: Which is page 7849 of the tender bundle that is Exhibit 2. Sorry, for the record. So what does it tell you - I'll withdraw that. You looked at the variants indicated here in grey, you looked at the numbers in the gnomAD database; is that right?

WITNESS NYEGAARD: Yes. So you could say that when these data repositories are growing we will see more and more carriers. And this is exactly what we are trying to figure out right now, what phenotype are they attached to. Or what phenotype are they linked to.

ROY: And have you been able to make that analysis in relation to the variants indicated on this slide?

WITNESS NYEGAARD: They are too rare.

WITNESS TOFT OVERGAARD: Perhaps I can suggest, so one of the variants here is down here, I10T. It's just because we--

ROY: So I10T?

WITNESS TOFT OVERGAARD: I10T. So that's isoleucine at position 10. That is--

JUDICIAL OFFICER: Whereabouts is it?

WITNESS TOFT OVERGAARD: Sorry, your Honour?

ROY: On the left of the left of the slide and near the circle of seven grey variants that's listed there.

JUDICIAL OFFICER: I see, yes, thank you.

WITNESS TOFT OVERGAARD: So isoleucine that's a change into a threonine residue. This particular variant we actually used in some of our extra

data that we submitted in the addendum report. It's - we've been looking at this for a number of years but we've never published anything because of the fact that we don't have the phenotype associated with this. But recently it came in as part of this UK Biobank repository where there's an extensive phenotypic description. So we've looked into this and seen it--

ROY: So you found the same variant in the UK Biobank?

WITNESS TOFT OVERGAARD: I think it's the same individual, probably, going as--

ROY: Is there a way to determine if it's the same actual individual?

WITNESS TOFT OVERGAARD: That's your--

WITNESS NYEGAARD: I think it's a different one.

WITNESS TOFT OVERGAARD: Okay, never mind.

WITNESS NYEGAARD: But it's not - it's difficult to know.

WITNESS TOFT OVERGAARD: But there we can look up or a medic can look up and see if there are any recorded cardiac phenotypes on medical records or something, and there's - there's no-one for this particular carrier. So that's why we in the addendum material included the analysis of this variant as a maybe cardiac benign variant. So not inducing cardiac arrhythmia.

ROY: And that's from the information that was available through the UK Biobank?

WITNESS TOFT OVERGAARD: That's correct.

ROY: Professor Nyegaard, is it right that you've looked at, and correct me if I'm wrong, you've looked at all of the individual identified variants on the gnomAD database to identify how many carriers?

WITNESS NYEGAARD: Yes.

ROY: Can you summarise the result of that investigation?

WITNESS NYEGAARD: Yes. So for calmodulin 2, there are eight variants identified and they represent eight different carriers. So one carrier for each variant. So it's really hard to do statistics when there is only-

WITNESS TOFT OVERGAARD: One of each.

WITNESS NYEGAARD: But it is true that--

ROY: Sorry, it's hard to do statistics when there's only a small?

WITNESS NYEGAARD: Number of carriers per variant. Yeah.

ROY: Did you make the same analysis for 1 and 3?

WITNESS NYEGAARD: Yes. So for CALM1, there are six variants recorded in this gnomAD, and they - and it is represented by seven carriers. So one of the variants are carried by two individuals. Yep. And for CALM3, the number of variants, nine. And it's 12 carriers in total.

ROY: Is that, in your view, a small number of carriers?

WITNESS NYEGAARD: Yes.

ROY: How would you characterise that, in your words?

WITNESS NYEGAARD: So we call them ultra-rare. Yep.

JUDICIAL OFFICER: Because of the similarities you indicated before, for statistical purposes at least can you aggregate the CALM1, CALM2 and CALM3 people?

WITNESS NYEGAARD: Yes. So when a gene has very few variants sometimes you can per gene aggregate the variant into one. But for calmodulin we really don't know if one variant is causing the same phenotype as another variant, because calmodulin has so many functions. And for aggregating across the three genes, because the genes have different transcripts we may or may not be able to aggregate. So for cardiac phenotypes until now, it seems that the calmodulin are interchangeable. So if you have a variant in calmodulin 1 or in calmodulin 2 or in calmodulin 3 they can display as the same phenotype. But that's not necessarily true if calmodulin has other phenotypic effect. It may be that it's only calmodulin 2 and 3 and 1 and 2, but not 3. It depends on where the transcripts are expressed and when.

ROY: What is a transcript?

WITNESS NYEGAARD: A transcript is when you have this stretch of DNA. First a strand of RNA is synthesised and then from that RNA the protein is made. So we call that intermediate strand a transcript.

WITNESS TOFT OVERGAARD: So it's called a messenger of RNA in the central dogma of genomics.

ROY: I might move onto the variant in this case, the CALM2-G114R variant that was identified. Now that was identified in Ms Folbigg and her two daughters prior to your involvement in this case; is that right?

WITNESS TOFT OVERGAARD: That's correct.

ROY: If we can turn to slide 5, please. Now this is the same as the slide from

your report that we've been looking at previously. But I have indicated with a large red arrow an “R” where the variant appears in this case. Is that accurately depicted?

WITNESS TOFT OVERGAARD: That's correct.

ROY: The red is a poor choice because I don't mean to indicate it's necessarily associated with Long QT Syndrome. But that indicates the location and where there should be a G, glycine, there is an R. What does the R stand for?

WITNESS TOFT OVERGAARD: So R is an arginine.

ROY: You describe the change in your report between glycine and arginine as "dramatic"; can you explain that?

WITNESS TOFT OVERGAARD: In a protein chemical sense that's a dramatic change. So with glycine it's the smallest amino acids of the 20 natural amino acids.

ROY: Of the 20?

WITNESS TOFT OVERGAARD: Natural amino acids.

ROY: There's 20 natural amino acids?

WITNESS TOFT OVERGAARD: So the 20 amino acids that’s build into proteins. It has no site chain. So basically it's only the backbone. I don't have the structure of an amino acid here, but we have it in one of the other slides. So it's--

ROY: If it would assist you can pull it up.

WITNESS TOFT OVERGAARD: That might be helpful. I'll just go to my slides. I think slide 43 would be helpful. So this is the same depiction of the calmodulin structure that's used in the Brohus article. So this is a calcium bound state. This is the position of the - you can advance one, thank you. Yeah. So this is position 114 and you can see the glycine residue here. So basically it consists of four atoms, if you don't count hydrogen. And then in this particular position where I'm pointing right now, for all other amino acids there would be an extension that constitutes the side chain. So the glycine here is changed into an arginine.

ROY: Can you go back and explain that? Of all of them glycine is unique in amino acid for what reason?

WITNESS TOFT OVERGAARD: Because there's no extension from this particular point out here.

ROY: Would that be a binding site?

WITNESS TOFT OVERGAARD: For some, if we can go back, I can maybe explain if we go up here, you can see here it's another amino acid, so this is just one of the amino acids that has been changed in one of the calmodulin carriers where we know there's early onset death while asleep. So this is a particular calcium binding residue and the part of the amino acid that binds to calcium, that's the side chain. So in this case it's an aspartic acid, I believe, so it has acidic side chains or a negatively charged side chain that can bind to the positively charged calcium ion. Glycine has really no side chain. What that generates for the glycine, an ability to actually turn more sharply than other amino acids, so you can say it's a structurally important amino acid because it's more flexible than any other amino acid.

ROY: It's able to form more flexible shapes than any other amino acid?

WITNESS TOFT OVERGAARD: Yes, so we typically see this glycine in this particular position compared to this - it's called an alpha helix here, so it's an helix terminating residue, so it's more often seen at the very end of a alpha helix compared to other amino acids.

ROY: What's the significance of it being replaced by an arginine?

WITNESS TOFT OVERGAARD: That means that in this particular position you would put in this, which is the second largest amino acid--

ROY: The second largest?

WITNESS TOFT OVERGAARD: Second of the 20, yes. So the largest one is tryptophan, which is found in this other individual at position 114.

ROY: That's the G114W, the blue circle that we saw in the schematic.

WITNESS TOFT OVERGAARD: That is correct. Arginine is a rather long side chain, and at the very end there's a positive charge. So that's why we characterise this as a dramatic change from having nothing to having a large side chain that is positively charged.

ROY: Prior to conducting any tests or functional assays, which we'll come to in a minute, the mere fact of that change, would it cause you to predict anything about the pathogenicity of that particular variant?

WITNESS TOFT OVERGAARD: It really depends on where, in what position of the protein you are. So if you note we're at the opposite end, you can say, of the calcium binding for this particular domain, so you cannot immediately say whether or not this would affect, say, calcium binding or binding to any of the proteins that calmodulin regulate.

ROY: If we go back to slide 5--

JUDICIAL OFFICER: That question though, you just said you can't immediately to identify, does that depend on what the glycine should do or

what the substitute might do?

WITNESS TOFT OVERGAARD: Both, I guess.

JUDICIAL OFFICER: Thanks.

WITNESS TOFT OVERGAARD: Maybe for clarification, we know that every single position in calmodulin is important, so that's the evolutionary constrain that we understand.

ROY: In terms of the fact that we see a clustering of variants around the calcium binding site, on the one hand it's been suggested that that indicates a greater chance of pathogenicity where a variant is located near a binding site?

WITNESS TOFT OVERGAARD: That's correct.

ROY: On the other hand, Professor Nyegaard, what you said earlier, does it feed in that we're looking at places on the aeroplane where there are no bullet holes, in terms of where we're not seeing variants?

WITNESS NYEGAARD: You could say that this is an effect of cardiologists having Long QT patients that are not carrying any of the other known mutations. So when this gene was identified they would go and look in those Long QT patients, so that is why these links pop up. So what you see is always an effect of where you look.

ROY: Is that sort of the other side of the coin of the selection bias you were describing in the gnomAD database?

WITNESS NYEGAARD: Yes.

ROY: So in the gnomAD database we have presumably adult humans for this cluster of pathogenic variants, by design they're identified through the pathogenic presentation of disease?

WITNESS NYEGAARD: Yes. So as soon as this link to Long QT was made, the genes went in to what we call gene panels so if there is a patient with Long QT they would automatically be sequenced for calmodulin and so this is the exact effect you see, that there are lots and lots of Long QT patients here.

ROY: So that would suggest that there's an association between Long QT and those binding sites but doesn't necessarily, if I follow, indicate that there is no pathogenicity outside of those binding sites?

WITNESS TOFT OVERGAARD: That's correct.

WITNESS NYEGAARD: Yes, but you could say that if we're looking for the places with missing bullet holes they are actually - there are lots of places still where you don't see anything at all.

WITNESS TOFT OVERGAARD: Yes, but if I can comment.

ROY: Yes, please.

WITNESS TOFT OVERGAARD: You are exactly correct. It is a positive selection bias, in effect, that here is the result of looking at cardiac patients only.

ROY: Calmodulin is not only relevant to heart function?

WITNESS TOFT OVERGAARD: No, that's correct.

ROY: It's relevant to multiple other functions of the body?

WITNESS TOFT OVERGAARD: Correct.

ROY: But through the history that you've told us about this morning, the focus has been on the heart and, until very recently, on calcium?

WITNESS TOFT OVERGAARD: That is correct.

ROY: But you would have reason to believe the phenotype might expand on further research?

WITNESS TOFT OVERGAARD: That's correct.

ROY: You are both co-authors of the Brohus article; we've established that.

WITNESS TOFT OVERGAARD: Correct.

ROY: What was your role in relation to that article? What was your contribution?

WITNESS NYEGAARD: Yes. So I was part of the Danish calmodulin research team, so I was looking in data repositories, if we could find other carries, and then I was discussing the results and reading the manuscript carefully and critically, yeah.

WITNESS TOFT OVERGAARD: I led the protein functional characterisations, so some of these assays were performed in my laboratory.

ROY: You used a couple of terms: functional characterisation?

WITNESS TOFT OVERGAARD: Yes, so that's the effect of the mutation on the calmodulin protein.

ROY: You described an assay. You said you used an assay. What is an assay?

WITNESS TOFT OVERGAARD: That's a tough question. Maybe I can

explain briefly what we do to look at the function of the protein. We put the - all we need is the site of the mutation or the variant and what the variant, in comparison in this case, at position 114. We put in arginine so we have made--

ROY: You say you put it in, where - how are you putting it in?

WITNESS TOFT OVERGAARD: That's done. We actually order the small synthetic gene. We put that piece of DNA into a bacteria. We have that bacteria produce the protein, and then we extract the protein and purify it, so we only have the purified calmodulin protein, and we make the different variants. So we have the non-mutated protein, the wild-type, and then we have the G114R and, for this particular Brohus paper, we also made the G114W and we made the N98S to use as reference.

ROY: Then, having made those, what do you do with them?

WITNESS TOFT OVERGAARD: In this particular paper there was four different types of assays.

ROY: I might get you to explain that by reference to the paper. Rather I'll do it by reference to your report because you've explained it more for our purposes.

WITNESS TOFT OVERGAARD: Okay.

ROY: If we can turn up your report, which is volume 1 of the tender bundle. I think you have copies there separately.

WITNESS TOFT OVERGAARD: I think so.

ROY: Behind tab 6-02. Do you have copies?

WITNESS TOFT OVERGAARD: Yes, but can we have a short break perhaps.

JUDICIAL OFFICER: I'm sorry. Yes.

ROY: Yes.

JUDICIAL OFFICER: We'll adjourn for 15 minutes.

LUNCHEON ADJOURNMENT

Yes, Ms Roy.

ROY: Before the luncheon adjournment I was going to take you to the Brohus article. I just wanted to go back to one thing that I'm not certain we covered in your earlier evidence. The calcium binding locations that we see on this slide, both in the top of the schematic with the four calcium ions. This is slide number 3. And the equivalent domains that we see with the pink shaded boxes on the bottom, do those have a technical name?

WITNESS TOFT OVERGAARD: Yes, so it's the calcium binding sites and the structure that supports them are called EF-hands.

ROY: EF-hands?

WITNESS TOFT OVERGAARD: Yes.

ROY: So if we hear EF-hand motif or EF-hand domain what does that refer to?

WITNESS TOFT OVERGAARD: It refers - maybe I can try and point. So the EF-hand, I can maybe do it up here, so the EF-hand is two so-called alpha helices formed like a thumb and pointing finger.

ROY: It forms like a hand making an L?

WITNESS TOFT OVERGAARD: Forms like a hand and then the calcium will bind in the middle. So the calcium binding residues are part of the EF-hand. The amino acids that points toward calcium and directly binds to calcium.

ROY: Just in terms for the purposes of terminology, because it looks something like a hand forming sort of an L-shape with the three fingers furthest from the thumb curled down, that is referred to as a hand domain or a hand motif?

WITNESS TOFT OVERGAARD: Yes, it's the thumb and the next finger.

ROY: The thumb and the index finger, the shape that that makes?

WITNESS TOFT OVERGAARD: Yeah.

ROY: So known as the EF-hand motif?

WITNESS TOFT OVERGAARD: That's correct.

ROY: We were going to come then to your report. And you were explaining to us how you manufactured a calmodulin protein in your lab.

WITNESS TOFT OVERGAARD: Correct.

ROY: With variants that you nominated. Can I ask you to tell us about that again, I just want to be sure we're clear on how that works?

WITNESS TOFT OVERGAARD: So it works in the sense that we actually order from a company a synthetic gene that encodes the calmodulin protein. And then we can order it with the variants that we're interested in studying. So we get a piece of DNA, the gene for the calmodulin or the calmodulin variants. We put these into bacteria and use the bacteria's system for producing proteins from genes to make the protein. And then we harvest or

purify the protein from this bacteria. So we end up with a pure calmodulin protein where we can study basically the impact on the protein of the--

ROY: What I might do is as we work through the four assays, through the article, I might come back to that and ask for some clarification on some of the functional--

WITNESS TOFT OVERGAARD: That's fine, because it's relevant for the two first assays. But we do it slightly differently for the second two assays.

ROY: I see. Why don't we have a look at your report to this Inquiry. Page 8 of your report.

JUDICIAL OFFICER: This is the first report, Ms Roy?

ROY: The first report, which is in volume 1 of the tender bundle behind tab 6-02. If we could also at the same time, just getting a bit ahead, put slide 49 - I think that's the right one. You will tell me if I'm wrong. So turning to page 8 of your report, which is page 15 of the tender bundle. Here you describe the tests that were employed for characterising calmodulin mutations.

WITNESS TOFT OVERGAARD: Yes.

ROY: Just before we get into the precise technical detail, am I right in imagining this is happening in a lab with lab coats and lab technicians and lab equipment?

WITNESS TOFT OVERGAARD: Yes.

ROY: And that image of a laboratory scientist is what is being described here?

WITNESS TOFT OVERGAARD: Yes. And I have to say that it's my post-doctorate and scientific assistant doing the work not me.

ROY: Yes.

WITNESS TOFT OVERGAARD: It's a few days since I - yeah.

JUDICIAL OFFICER: Well that's helpful.

ROY: Wouldn't know anything about seniority privilege at the bar.

WITNESS TOFT OVERGAARD: No.

ROY: Can you take us through, please, we'll start with the first of the assays. And I might just use the convention of referring to the first assay, the second assay, the third and the fourth to describe what you describe at 1, 2, 3, 4 in your report.

WITNESS TOFT OVERGAARD: That's fine.

ROY: I might try this again. Can you describe in broad terms what is a functional assay?

WITNESS TOFT OVERGAARD: So a functional assay, it's where we go into the laboratory. In this case we take the calmodulin - I'll do it with this example here. So we take the calmodulin protein. It's always in a liquid. And then we add, in this case, increasing amounts of calcium.

ROY: I'll stop you. Are you describing this functional assay?

WITNESS TOFT OVERGAARD: This particular functional assay.

ROY: Okay, we'll do it that way.

WITNESS TOFT OVERGAARD: Yes. It's just I think maybe it's easier to illustrate. So here we have the calmodulin protein. We make sure that there's no calcium present, which means that we have the protein in a state where it doesn't bind calcium.

ROY: So that would be what we've been looking in each case the schematics have shown calmodulin in the state of being bound to calcium?

WITNESS TOFT OVERGAARD: That is correct.

ROY: And you isolate it in a state where it's not bound to calcium?

WITNESS TOFT OVERGAARD: That is correct. And then we add in small amounts of calcium. And at some point calmodulin will bind to the calcium. That makes a change in how it looks in the confirmation. And that change we can monitor using a technique called fluorescence.

ROY: Can you explain that technique?

WITNESS TOFT OVERGAARD: Yes, indeed. So we will take light at a particular wavelength and put it to calmodulin and then there are certain amino acids that can absorb this light and discharge another light or another wavelength of light, that's called fluorescence, with another energy so that it will actually change the wavelength of the light. And those particular amino acids in the case of calmodulin those are the phenylalanines and the tyrosines.

ROY: So it's right that this is all happening at the molecular atomic level and it's not something you can see under a microscope, it's sub-microscopic?

WITNESS TOFT OVERGAARD: That is true. So what we can see is the light. So we can basically - what happens when we then add calcium, the structure changes, the orientation changes. It means that the environment for these particular amino acids, the phenylalanines and tyrosines, that will change and that will create a change in the fluorescence.

ROY: So you're inferring from the change in the fluorescence what was

happening at the molecular level?

WITNESS TOFT OVERGAARD: Yes. And if we can go to the figure--

ROY: Is that right, that you're--

WITNESS TOFT OVERGAARD: That is correct, yes. So if we look at this left-hand side of the video.

ROY: I should say this is slide 49 that contains the results of the first functional assay?

WITNESS TOFT OVERGAARD: That's correct. So this is the first assay, the calcium binding assay, present in the Brohus paper. So on the Y axis it's denominated FI, that's fluorescence intensity. So you see the - and then on the X axis here, that's how much calcium we put in. And so when we increase the amount of calcium we see a change in the fluorescence. So basically here there's no calcium bound. Around here it starts to bind calcium and the black one, that's the wild type non-mutated.

ROY: It looks like your testing four different genes here.

JUDICIAL OFFICER: What's WT preface?

ROY: Yes.

WITNESS TOFT OVERGAARD: WT is a standard naming for the wild type. So the non-affected, yeah.

JUDICIAL OFFICER: Yes.

ROY: And WT stands for wild type?

WITNESS TOFT OVERGAARD: Wild type, yes. So we see at around here wild type starts to bind calcium. And the around here it has been saturated with calcium.

ROY: So it's unable to make any further--

WITNESS TOFT OVERGAARD: Then it has bound all the calcium it can. It's a little complicated, so what we are actually monitoring here is the fluorescence from tyrosine residues. And if you recall the two ends - I can show it here - the two ends of calmodulin. So the end domain is up here. The C-domain, this was where most of the these arrhythmogenic mutations are present. It is also this domain that contains the tyrosine residues where we can see the fluorescence. So in particular this is the C-domain where we monitor when does it bind calcium. That's also why we see, in effect, of the mutations in the C-domain. So we included three different variants and mutations here. The one in blue - sorry, the one in red, that is the G114R. So that is the particular mutation in the Folbigg family. And then we included the

G114W, which is a mutation in the same side, but to a tryptophan.

ROY: And that is the one that appears on the CALM3?

WITNESS TOFT OVERGAARD: That's the one on CALM3. But when you go to protein that really doesn't matter because it's the same protein product of all three genes.

ROY: So this is because they're identical, this doesn't distinguish between CALM1 and CALM2?

WITNESS TOFT OVERGAARD: No. No, that's correct. Yes. And then we have this other relatively well characterised variant because this was the variant, or the second calmodulin mutation ever discovered, present in the first paper in 2012. So that's the N98S variant.

ROY: And that was the second family that you included in your first publication?

WITNESS TOFT OVERGAARD: Yeah, actually an individual *de novo* case, yep.

ROY: An individual, and that was a severe Long QT phenotype?

WITNESS TOFT OVERGAARD: No, not in that particular paper.

ROY: No, not at that measure.

WITNESS TOFT OVERGAARD: That was a CPVT phenotype.

ROY: So it was the second CPVT phenotype and then later there were other Long QT Syndrome phenotypes associated with the N98S variant?

WITNESS TOFT OVERGAARD: That is true. And also mixed phenotypes.

ROY: Mixed phenotypes.

WITNESS TOFT OVERGAARD: Yeah. So this is, yeah, so compared with these and what you gather here is this black line has been shifted to the right for all of the variants. This means that we need to add more calcium before it starts to bind. So that can translate to the binding capacity or efficiency has been lowered. So all of these three mutations will reduce the ability of calmodulin to bind calcium.

ROY: So it reduces the ability of the calmodulin to bind to calcium. Is that something that you can show us on the heart cell slides that we were looking at? Would it make sense to look at that?

WITNESS TOFT OVERGAARD: Basically the other assays will take us then one step further. Because this is the core function of calmodulin is to bind

calcium. So everything is downstream of that you can say, yep.

ROY: This tells you that there's something wrong at the basal level?

WITNESS TOFT OVERGAARD: The fundamental function of calmodulin is changed for these mutations.

ROY: Is it right that the Y-axis is logarithmic?

WITNESS TOFT OVERGAARD: Not in this case. Here it's the X-axis that's logarithmic so, yes.

ROY: Sorry.

WITNESS TOFT OVERGAARD: We'll get to that.

ROY: Yes. Continue please.

WITNESS TOFT OVERGAARD: So the X-axis is logarithmic and what that means is that we change the calcium concentration from 10 to the minus 8 to 10 to the minus 2 so that's a million-fold roughly in this assay.

ROY: So that would suggest a profound effect?

WITNESS TOFT OVERGAARD: It's just that we cover basically the physiological calcium concentration range but we also go beyond and study below and above physiological levels.

ROY: Is there more to explain on this slide?

WITNESS TOFT OVERGAARD: I think it's fine. I think this is a way - the second panel is a way of summarising instead of looking at the curves. So here we have a number we can assign to how strongly a protein binds to calcium or anything else, so that's the affinity constant. It's reflected in maybe halfway of the full binding, so that may be an easier way to compare, so the wild-type here as an affinity constant of around 4, and these variants have an affinity constant around 1. So their binding strength is reduced roughly fourfold for this range.

ROY: It would appear from that that the effect is slightly less in respect of the G114R variant than either the N98S or the G114W?

WITNESS TOFT OVERGAARD: That's correct.

ROY: Is it possible just from this assay to make a prediction about phenotype?

WITNESS TOFT OVERGAARD: No, but with the knowledge we have, which is sort of nicely demonstrated in one of the other overview slides, you can say we can have an educated guess in what the phenotype would be.

ROY: Is that other slide referrable specifically to this assay or to all four assays?

WITNESS TOFT OVERGAARD: This one is in particular to this assay.

ROY: Can we go to that slide?

WITNESS TOFT OVERGAARD: Yes. I think it's one of your slides actually. So you can do page 7, slide 7. Yes. So this is an illustration of the summary of information we had in 2019 when the Calmodulinopathy Registry was established. So this sort of contains the information we had on functional assays of a number of calmodulin variants and how the phenotypic expressions of the arrhythmia expression was from these, and the summary is depicted down here. So mild to strong - maybe you can see this, that denotes the mutation or the variant effects on calcium affinity and that's calcium binding. So what it does describe is the more severe the impact on calcium binding, so the lower affinity or the less well it binds to calcium, in particular in this C-domain end, it seems to correlate with the degree of Long QT Syndrome in particular, and this seems to correlate with how efficiently calmodulin can assist the Cav1.2 channel inactivating.

ROY: To remind us, the Cav1.2 channel is the blue channel depicted on that slide.

WITNESS TOFT OVERGAARD: That's the blue channel and to the right, yes.

ROY: Which is the channel that moves from the etracellular space in to the inside of the heart cell?

WITNESS TOFT OVERGAARD: Yes. So you can imagine if the calcium dependent inactivation is performed by calmodulin on this channel, if you need more calcium to bind and to make the change when the channel closes, it will close later than for the wild-type, so it's open for a longer time.

ROY: Is that predicting the outcome of the third assay?

WITNESS TOFT OVERGAARD: Yes.

ROY: We'll come to that then.

WITNESS TOFT OVERGAARD: Yes.

ROY: We'll do it in order. Was there anything from the previous slide in relation to the first assay that you wanted to cover before we move to the second assay? Slide 49.

WITNESS TOFT OVERGAARD: No, I think it's fine. I can say that the effects that we measure would be, in this figure - we would characterise that calcium binding effect to be in this range, which is in between the mild and the strong effect, which signifies that we could be milder Long QT or CPVT from the

knowledge we had at that time point.

ROY: Before I move to the second assay, can I ask you how is that you translate what's happening in a Petrie dish - probably not, something else - but in a lab with these manufactured cells, into what's happening in a living, breathing human person and all the other functions that would be going on at the same time?

WITNESS TOFT OVERGAARD: The way we do this is that we take, in this case, a clinical phenotype described to us by the cardiologist or the physicians, and then we measure the properties of the protein and then we try to correlate those two. So in our assays we basically measure the inherent properties of the protein, which can then in the further assays that we are looking at, how do they affect the different channels, and from that we can try and visualise or explain the molecular mechanism of how this translates into what you observed in the full body.

ROY: You would have received all of the reports of other experts that--

WITNESS TOFT OVERGAARD: That is correct.

ROY: --are to be giving evidence in relation to cardiogenetics, and specifically the report of Professor Skinner and Kirk dated 31 October 2022.

WITNESS TOFT OVERGAARD: Correct.

ROY: And for the benefit of the Inquiry that's at tender bundle behind tab 7-03. Professors Kirk and Skinner make some comments on your initial report, and you had the opportunity to provide an addendum report that responds to some of those comments and some criticisms?

WITNESS TOFT OVERGAARD: That's correct, except I think they were not commenting on our initial report. They were commenting on, you know, the Brohus paper.

ROY: Thank you for that clarification. Yes, they're commenting on the Brohus paper. To the extent that your report and the Brohus paper article cover similar topics--

WITNESS TOFT OVERGAARD: They do, yes.

ROY: And you were contributors to both, you responded in that respect. I might turn that up. At page 8 of the report - I ask that the witnesses be given a copy. This is behind tab 7-03 and page 8 of their report appears at page 54 of the Exhibit. While that's being turned up, I'll just make it clear that both Professor Skinner and Kirk are at pains to say, in their report, that they acknowledge your expertise and they are not experts in these types of functional assays and they make no criticism of the quality of the work that you reported or the standards of your lab tests, and they also say that they appear to have been performed to a very high standard, but what we'll come to is

distinctions that they draw between testing that would be suitable for research and testing that would be suitable for diagnostic purposes, that is, if you were intending to diagnose people coming in to a clinic, patients with symptoms or if you were attempting to help people make reproductive decisions, depending on variants they might carry, and they consider it would be necessary to subject functional assay results of the kind reported in the Brohus article to additional scrutiny, but more or less what they've--

WITNESS TOFT OVERGAARD: I think, if I understand their comments correctly, is that the assays that we perform are not to a clinical diagnostic standard, so they're not supposed to be used for diagnosis.

ROY: I think that starts on page 54 or page 8 of their report, about halfway down the page, the second sentence of the second paragraph under 2.1, “Functional Data”, they say:

> "It's important to note, however, that these are single cell assays and the relationship of findings in such an artificial system, so far from the complex physiological system of a complete human, must always be interpreted with caution and that in a diagnostic laboratory setting functional assays are treated with caution because they can produce false positive results."

Do you have a response to that observation?

WITNESS TOFT OVERGAARD: Yes.

ROY: Can you share it with us?

WITNESS TOFT OVERGAARD: There are several ways that - there's a concern about these assays and how they translate into clinical phenotype. So "false positive" can mean different things at different levels. Let's say how to do this in the right order. So I think the integrity of the assays is, beyond doubt, highly respected in the scientific community, so these are assays used for delineating the molecular mechanism that actually takes a particular variant or mutation and explains why you end up with a certain clinical phenotype.

ROY: When you say, "these assays", are you referring to all four that we're going to review or just the two that your lab connected?

WITNESS TOFT OVERGAARD: I think all four have been used, and if I can perhaps give an example.

ROY: Please.

WITNESS TOFT OVERGAARD: The recent paper by Kato et al, which is commenting on, and also in the Skinner report, is an example where they have a clinical phenotype they don't understand because suddenly you have a Long QT family, so calmodulinopathy or Long QT in a large family, with a high

degree of variable expressivity, so more or less Long QT in individual family members, and they go back to the lab, to the scientists, and actually they perform the exact same assay, the fluorescence calcium binding assay and this was Madeline Shea, so the one who developed the assay, that we then used in our lab, inspired by her work. So Madeline Shea did the work for the Kato paper, and they say, based on the effects on the C-domain calcium binding they would expect a more severe Long QT, then they also used one of the later assays, I think assay 3, whilst similar to that, so electrophysiology characterisation of the Cav1.2 channel and they see a disturbance there, and those two together would expect to have a more severe Long QT phenotype, but then they go back and analyse one of the other ion channels that calmodulin also modulates, so the potassium ion channel, and they unexpectedly see for their particular variant that this variant potentiates that channel, and that then could balance out because it's the ion flow in the other direction - that balances out some of the effects from the calcium ion channel.

ROY: So that's the case we were talking about earlier, reported in the Kato article?

WITNESS TOFT OVERGAARD: Yes.

ROY: Where they regulate the stages of the heartbeat and the opening and closing of channels, they're a bit off but they're off in two places that somewhat counter each other?

WITNESS TOFT OVERGAARD: They counter each other. Why I bring it up here is because they used the exact same functional assays, both protein isolated assays and single cell based assays, to try and explain why they see this phenotypic pattern that they don't understand from the original single boxes of phenotypes that they are acquainted with.

ROY: The reason that that appeared unusual, this family that was described, is because, to the extent there have been calmodulin variant cases up to this point, they have mostly shown very consistent phenotypes - CPVT or Long QT - and a consistent expressivity; is that the right term?

WITNESS TOFT OVERGAARD: Somewhat - so, that is basically what's summarised in this figure below here, that there's a nice correlation or a good correlation with the severity of the calcium binding and the phenotypic expression, but can I make one more comment?

ROY: Please.

WITNESS TOFT OVERGAARD: So, I'm not aware of any ion channel - single ion channel mutations or variants that basically have a phenotypic expression that includes both CPVT and Long QT, and that's because these are so discrete phenotypes - one is on the cell membrane problem; the other one is a calcium release problem from the sarcoplasmic reticulum. So, already here we can see calmodulin is special because it regulates both sides.

ROY: That's coming back to pre-2012 and before you first identified a calmodulin variant.

WITNESS TOFT OVERGAARD: Yeah.

ROY: These phenotypes were understood as associated with variants that directly affected the individual receptor, and so they had discrete phenotypes that were consistent and relating just to that variant and that receptor.

WITNESS TOFT OVERGAARD: Yes.

ROY: As more calmodulin cases are emerging, they're presenting varied phenotypes, and the explanation for that appears to include - per the Kato article - that calmodulin is acting at two different receptor sites for calcium and channels for potassium and sodium as well.

WITNESS TOFT OVERGAARD: Yeah, so, as we - so, before calmodulin regulation - a range of these - so, in - and the Kato article says, okay, for some calmodulin mutations they - this particular one, the N138K mutation, has an effect on the potassium channel. That seems to be reflected also at the full - whole body phenotype.

ROY: That's a demonstration of the assay predicting what was then exhibited in the phenotypes of that family?

WITNESS TOFT OVERGAARD: Yeah, I would say the assay would help the cardiologist to understand why you see such a complex, never seen before, phenotype.

JUDICIAL OFFICER: I may be misunderstanding this, but in the Kato article they are working from persons who had defined pathogens; is that correct? They were able to identify whether they had LQTS or CPVT, for example.

WITNESS TOFT OVERGAARD: I don't believe it's--

JUDICIAL OFFICER: Is that incorrect?

WITNESS TOFT OVERGAARD: Without having it fully present, I'm not sure that they saw CPVT-like phenotype but they saw various degrees of Long QT.

JUDICIAL OFFICER: Isn't Professor Skinner's criticism directed to this; that, in this case, in the case of the particular children in question, there was no identified phenotype?

WITNESS TOFT OVERGAARD: That's correct, I guess, except they're that--

JUDICIAL OFFICER: Is that right?

WITNESS TOFT OVERGAARD: But typically, for the cardiologist, I - as I

understand it, to be comfortable with assigning a phenotype, they need a good electrocardiogram description plus other circumstances.

ROY: Your Honour, I think we might come back to some of those issues in due course. Is it, to an extent, a valid criticism that this is not being conducted in a living breathing human? These assays are necessarily being conducted in--

WITNESS TOFT OVERGAARD: So, without any ethical considerations, we would prefer to do it in humans, but of course we cannot do that.

ROY: Are there any other alternative ways to study the effect of the variant?

WITNESS TOFT OVERGAARD: Yeah, so, we start at the very basic level with isolated protein, and then you can move on and study isolated proteins, how they interact. That's assay number two. And then you can take it up into a cellular level, and that's assay three and four, where you have the channels in a cellular surrounding with all the membranes and lots of other proteins, of course. So, that's a more complex system but it's still an individual cell in the laboratory. Then you can take it into an animal model; so, try to have the variants put into mice is a preferred one. In our neighbouring lab, we put them into worms to see - because then you have a whole organism. You can see the effect there. But even in a mouse model, you cannot necessarily fully translate to the human model because, let's say the cardiac arrhythmia, some of them are really hard to induce in a mouse because things are different in a mouse.

ROY: In your addendum, you conclude your response to this criticism. I don't know that you need to turn it up, but it's at p 12 of your addendum. You say that "the scientific community rarely uses assays developed for diagnostic purposes for establishing molecular disease mechanisms." Can you explain that?

WITNESS TOFT OVERGAARD: Yeah, so, that was - maybe we got a little bit provoked by the comments from the Skinner report, but they - it seems like they would only use accredited laboratory tests for doing diagnosis, and that's probably the right way in what they do as their primary job. But the scientific community uses the assays like the ones performed in the Brohus article to discover, delineate and figure out the molecular pathways behind these clinical diagnoses, which, in the end, is used for also finding new treatment options. So it's just to say that even though - and there you would typically not use diagnostic assays, because diagnostic assays reflect on something you know. Our assays are trying to investigate what's the cause of the unknown.

ROY: They just fundamentally have different purposes?

WITNESS TOFT OVERGAARD: Yes.

WITNESS NYEGAARD: Yes.

ROY: Professors Kirk and Skinner also note, and this is--

JUDICIAL OFFICER: But ultimately in clinical experience, which occurs over time, tends to confirm like you see in a laboratory you can have far greater certainty with it; is that right?

WITNESS TOFT OVERGAARD: Can you repeat the last part?

JUDICIAL OFFICER: As soon as you know, just take an example, that a reasonable cohort of people who have died from unknown causes have the variant, you become increasingly confident that what you found in the laboratory conforms with the real world?

WITNESS TOFT OVERGAARD: Yes, that's correct.

ROY: On page 9 of Professor Kirk and Skinner's report, which is what we were already looking at, they also provide a couple of examples of false positive results in relation to disorders of cardiac rhythm and conduction. That's at the third paragraph under the heading 2.2 “Functional Data”.

WITNESS TOFT OVERGAARD: That's correct.

ROY: At the bottom of that paragraph they say, "For a functional assay to be applied as a diagnostic test we need to have--

JUDICIAL OFFICER: I'm sorry, Ms Roy, what's that report again?

ROY: I'm sorry, your Honour, it's behind 7-03 and I'm reading from page 54 of the tender bundle.

JUDICIAL OFFICER: Yes, I've got it, thank you.

ROY: Page 8 of the report. I was reading from the third paragraph under 2.2 “Functional Data”, the first sentence. And now the last sentence of that paragraph they say, "For a functional assay to be applied as a diagnostic test we need to have defined thresholds for calling the result abnormal and evidence regarding the probability of false positive results which are a potential for an assay, including validated diagnostic assays." They then say, "Taking these issues into account the ClinGen group provided criteria for assessing the strength of evidence of functional data that have not been validated to a diagnostic standard. The group's recommendations include consideration of the inclusion of benign as well as pathogenic variants. While the calmodulin genes were generally intolerant of variation and there are comparatively few benign variants in the gene,” they say “we could not find evidence that the same types of studies have been performed on benign variants." Meaning the same types of assays have been performed on benign variants. They say, "This means there is no way to determine a threshold for distinguishing benign from pathogenic evidence or to determine the likelihood of a false positive result."

JUDICIAL OFFICER: That assumes benign variants, does it not?

ROY: Yes, your Honour.

WITNESS TOFT OVERGAARD: That's right.

ROY: Can I ask you to respond to that?

WITNESS TOFT OVERGAARD: Yes, I think actually it's a really good point. And if we had a lot of benign variants we would have loved to have include these. So what we did in response to this comment, because we have actually performed a number of these assays with one of the variants that where we do not see any cardiac arrhythmia.

ROY: So to understand that, going back to what Professor Nyegaard was telling us earlier today, you don't readily accept that there are in fact benign variants, but nevertheless in response to this comment you have selected a variant that appears on the gnomAD database?

WITNESS TOFT OVERGAARD: That's correct.

ROY: Can you tell us about that?

WITNESS TOFT OVERGAARD: And also because very recently the same variant became part of the UK Biobank Registry. So there we could look up clinical phenotypes and saw no evidence of cardiac arrhythmia phenotype.

ROY: So you deliberate chose a variant from the gnomAD database that also appeared in the UK Biobank database and therefore gave you far greater information?

WITNESS TOFT OVERGAARD: That's correct.

WITNESS NYEGAARD: Exactly.

ROY: To attempt to find somebody that appears to not have an obvious cardiac phenotype?

WITNESS TOFT OVERGAARD: Yes, that is correct.

ROY: And you then ran the first assay in relation to that variant?

WITNESS TOFT OVERGAARD: Yes. So just last week we repeated the assay with this particular variant and I think if we go to slide 50.

ROY: Yes, slide 50. And at the minute we're on slide 4. And we're going to turn up slide 50. If we can turn up slide 50.

WITNESS TOFT OVERGAARD: Yes.

ROY: I should clarify this is work that you conducted last week?

WITNESS TOFT OVERGAARD: It includes work from last week, that's correct. So if I can explain. It's actually the same picture we saw before with the same binding curves and the same variants, but now we also include this I10T variant.

ROY: So it's I10T?

WITNESS TOFT OVERGAARD: I10T. It's impossible to say, yep.

ROY: We might in a minute look at where that is located on the protein.

WITNESS TOFT OVERGAARD: That's fine.

ROY: But for now can you tell us what the results were?

WITNESS TOFT OVERGAARD: But for now, just for reference we also included data for the F142L variant. The reason for this I highlighted over here to the right that the F142L variant has been seen for severe Long QT and also with sudden cardiac death while asleep below the age of two.

ROY: Did you also conduct the assay for the F142L variant?

WITNESS TOFT OVERGAARD: It is and they have already been published in 2015.

ROY: So what does that comparison tell you?

WITNESS TOFT OVERGAARD: So it's perhaps easier to see on the middle panel because that's the simple measures of the affinity constant. And if we compare then the non-mutated wild type with the I10T we see there's basically no difference in calcium binding capability. And then the F142L have a similar impact, maybe slightly larger impact than the N98S and the G114R mutations.

ROY: So to the extent it was a valid criticism of Professors Skinner and Kirk, at least insofar as they would use tests for diagnostic purposes, this is a partial answer to that criticism?

WITNESS TOFT OVERGAARD: It's about what we believe may be benign in terms of cardiac arrhythmia, the I10T displays no effect in this assay.

ROY: That would help you to identify that you were not arriving at false positives?

WITNESS TOFT OVERGAARD: That is correct.

ROY: The thrust of the criticism was, if I understand it correctly, that without having conducted tests of this kind, you couldn't be certain that all calmodulin variants, benign or pathogenic, were having this effect and, therefore, show

that they were in fact not pathogenic?

WITNESS TOFT OVERGAARD: That is correct. Unfortunately we don't have enough different benign mutations to make this into statistically valid, but we can say this one in particular is no different.

ROY: Moving now to the second assay, which is again reported on page 8 of your report, which is tab 6-02 on page 15 of the tender bundle in volume 1. I believe your results from the Brohus article appear on slide 51, and possibly also 52, but let's start with slide 51.

WITNESS TOFT OVERGAARD: That's correct.

ROY: Can you explain this assay and these results?

WITNESS TOFT OVERGAARD: Yes. So to the left is just depicted the ion channel in a cell membrane, so what we are looking at here is the ability of calmodulin variants to bind to a fragment of the Cav1.2, so the small fragment, we term this a peptide because it's a small sequence of amino acids. We can order these so they are synthetically made and sent to us, and the reason for making them synthetically is we need to have a fluorophore attached, so that's a molecule that has good fluorescence properties. I don't want to become too technical, but what we measure here is called fluorescence polarisation, so that's actually we hit the fluorophore with light and then we measure - it's a little bit of how long it takes to rotate in solution, and the larger it is the longer it takes, so we can see when something binds to it, like calmodulin binding to the small peptide it becomes larger, so we have a readout of when calmodulin binds. What we are seeing here is the summary of that binding. There's a lot of data into these grabs, so the summary of these bindings, we perform an assay - let me step back. We have the peptide. We can measure how quickly it spins in solution when it's only the peptide. Then we add in calmodulin at an increasing concentration, and we see calmodulin starts binding the peptide. So the peptides become larger and spins more slowly. In that way we can measure the binding strength for the calmodulin to the peptide. Now, because the binding strength is intrinsically dependent on the amount of calcium present we do this at different calcium concentrations. So what you see at the bottom of the graph here, so that's the X-axis, is that we perform this at different calcium concentrations indicated by the dots here, so we have eight different calcium concentrations. We see this is a measure of the binding strength here. It's a little bit awkward but the higher the binding strength the lower we are on the graph. Okay. So we start up here, really low calcium. It binds to the channel. When we increase calcium the binding becomes stronger.

ROY: This is the Cav1.2?

WITNESS TOFT OVERGAARD: This is the Cav1.2, yes.

ROY: Which is associated with which phenotype?

WITNESS TOFT OVERGAARD: That's the Long QT phenotype.

ROY: Okay.

WITNESS TOFT OVERGAARD: So if you advance one, so for the Brohus article we took out a condition at low calcium and compared the binding here, so these four dots is reflected down here and the wild-type has a binding strength of 6 in this, and all three variants you can see they are above here, that means that their affinity or their binding strength is lower, so they all have reduced capability of binding this peptide at low calcium concentration. If you step forward two more - we then extract the same information but you can have even more information, of course, from this graph, but to make it more clear, we extracted at a medium calcium concentration, the binding strength is reduced. At medium concentrations, that would constitute when the channel opens and the calcium is let in to the cell, and then at a high calcium concentration, this is when calmodulin is fully saturated with calcium; we don't see any significant difference here, maybe a tendency for one of them to bind stronger but this is also when the calmodulin is saturated so you overcome the deficiency in calcium binding; so calcium overpowers the deficiency.

ROY: What, if anything, does this indicate for the G114R variant in terms of the phenotype?

WITNESS TOFT OVERGAARD: Just like the N98S, for which we know a lot, it has a reduced capability of binding, so it has a potential to not function correctly on the channel, assisting the channel in closing.

ROY: Is there anything further you would say about this before we move to the other calcium channel?

WITNESS TOFT OVERGAARD: No, that's fine.

JUDICIAL OFFICER: The difference seems very marked to the medium calcium. Is there any significance in that?

WITNESS TOFT OVERGAARD: Can you repeat, sorry?

JUDICIAL OFFICER: Yes. In the slide you've got the low calcium at the medium calcium, and the high calcium.

WITNESS TOFT OVERGAARD: Yes.

JUDICIAL OFFICER: The difference between what I call the stand of the wild-type, and the others, it seems far more marked than it does - is at any of the other levels; is there any significance in that?

WITNESS TOFT OVERGAARD: So, if I understand you correctly, so, the difference between wild type and these three is larger than within three?

JUDICIAL OFFICER: Yes.

WITNESS TOFT OVERGAARD: Yeah. That sort of points to the same, that whole - how can I explain? It's - for us, we interpret this as they have a similar effect on calmodulin. So, the wild type is the - so, that's what it's supposed to do. So, wild type is the natural situation, and all the other three are - at least two of them - well-recognised as pathogenic.

JUDICIAL OFFICER: What I'm asking is there seems to be a greater difference at the medium level rather than the low level. Is there any significance in that?

WITNESS TOFT OVERGAARD: Yeah, so, I would translate this to the ability of sensing calcium is disturbed. Once it's fully saturated, it may well look like the wild type situation, so once it fully closed, it may be the same, yep.

ROY: You then repeated this assay but in respect of the other calcium channel, the ryanodine receptor 2?

WITNESS TOFT OVERGAARD: That's correct.

ROY: I think that should be on slide 52.

WITNESS TOFT OVERGAARD: Next slide. And you can forward three - or one or two, three, yes. So, basically, this is exactly the same assay, just using the peptide from the ryanodine receptor that the calmodulin binds to and gets to. And I have to say that, for many of these channels, calmodulin binds to not only one side but to several sides. Some of that is still debated in the scientific community, exactly which side is the right one, but this is - ones we have investigated, the scientific community agrees these are the anchoring binding sites.

ROY: What does that mean, "anchoring binding site"?

WITNESS TOFT OVERGAARD: For - at least the scientific community agrees that this is where calmodulin binds. I've become quiet because it's rather complex. So, it really depends on the calcium concentration, but this is where calmodulin for sure is binding. Yep.

ROY: Can you explain the results?

WITNESS TOFT OVERGAARD: Yes. So, it's similar to what we saw before, so the - at low calcium. The different variants are significantly different, so they will bind with a lower affinity or lower binding strength. The same for the medium calcium concentration. And again, for the high calcium concentration, there's no significant binding if we do statistical testing - so, significant difference if we do statistical measurements. So, maybe, if you have enough calcium, you overpower this calcium - reduction in calcium binding and it's able to bind anyway.

ROY: Trying to grapple with what is particularly significant, because now on both of these assays, it seems like, at the medium calcium level, we see the

greatest divergence from the wild type. Would it be useful to explain what the calcium level is by reference to the sinus rhythm and the heartbeat or the action potential? What would be the best way to understand what the significance is of the medium calcium level--

WITNESS TOFT OVERGAARD: I think for these two particular channels, it's really well-known that these are calcium channels and they depend on - when they open, they depend on calmodulin to sense that calcium increases to help them close again. So, when - and this is the medium calcium range. This is sort of where they are sensing this calcium. So, we say that particular sensing is affected.

ROY: Is the medium calcium level, of the three, the more likely to indicate phenotype?

WITNESS TOFT OVERGAARD: In a way, I can't really say that. I will say a disturbance in any of these medium, low or high would very likely generate a phenotype.

ROY: Is any more significant than the other?

WITNESS TOFT OVERGAARD: I don't think anyone can say that. But I could say, in terms of when you want to sense the calcium - critical sensing of calcium concentration, that is the medium range. So, this is where calmodulin senses calcium, changes confirmation, closes the channel.

ROY: From that point of view, is the particularly marked difference in all three of those variants and the middle calcium level particularly significant?

WITNESS TOFT OVERGAARD: In my opinion, yes, but it's my scientific opinion, so whether that correlates - yep.

ROY: Can you explain that? You already have, but it would help us if you--

WITNESS TOFT OVERGAARD: I've explained it - yeah, so, it's because when you have calmodulin sitting and sensing calcium, if you - that's - the primary function is to employ calmodulin to sense an increase in calcium, to do something. If that sensing is disturbed, which the middle calcium concentration range shows us, that translates more easily to a function or disturbed function of the target - okay.

ROY: I think that makes sense. Thank you. Was this an assay you were able to repeat in relation to the gnomAD variant?

WITNESS TOFT OVERGAARD: Yes, indeed. Let's see. So, we did not have time to repeat for this particular one but we performed another assay for another channel where we included the gnomAD assay.

ROY: That was a different assay to the four that we're looking at from the--

WITNESS TOFT OVERGAARD: Yes. That's the one we performed last week.

ROY: Is that the one that we've already referred to?

WITNESS TOFT OVERGAARD: No, we haven't looked at that yet.

ROY: Can we look at that? Is that slide 56? Would this be the appropriate point to look at that result?

WITNESS TOFT OVERGAARD: I think we haven't discussed this yet so maybe - it's in particular to another channel.

ROY: I think we're at cross-purposes. You're referring to the sodium channel assay?

WITNESS TOFT OVERGAARD: That's correct.

ROY: There wasn't another assay that was done for the Brohus article?

WITNESS TOFT OVERGAARD: No, but we went back and - because we have data for assay number four, I think.

ROY: We'll reach that when we come to it then.

WITNESS TOFT OVERGAARD: Yep.

ROY: So let's come to the third assay on page 8 of your report. I understand this was conducted in another lab, not your lab?

WITNESS TOFT OVERGAARD: So the third assay, yes, that's performed in the lab of Professor Ivy Dick in the USA, Maryland, University of Maryland.

ROY: Are you able to explain the nature of that test to us?

WITNESS TOFT OVERGAARD: I can - I'm not an expert, but I can explain a little bit.

ROY: Would it be slide 53?

WITNESS TOFT OVERGAARD: That is correct.

ROY: So what was this assay testing? Actually, we'll take it a step back. How is this assay different from the first two that you conducted in your lab?

WITNESS TOFT OVERGAARD: So this assay is a single cell based assay. So here you need to add the gene for the calmodulin variant into a human embryonic kidney cell. So it's a - you have the gene and then you have the cell produce a calmodulin variant. Mind you, there will also be wild type calmodulin in there from the cell's own expression. So we have some calmodulin variant and then you measure the - this is an electrophysiology

assay, so you patch clamp the cells and then you measure the current going through the particular ion channel.

ROY: What were the results?

WITNESS TOFT OVERGAARD: Yeah, so--

ROY: I won't ask you more about the technical details, I understand it's not your test.

WITNESS TOFT OVERGAARD: No, but I can say that the two coloured lines say where you - you mechanic - you can say you forcefully depolarise the cell. And in that way you activate the calcium channel, just like in a cardiac cell but instead of having the sodium ion channels to it you do it applying electricity. So you depolarise the cell. That's what's shown on top from minus 80 to 30 millivolts. That opens the voltage-gated calcium channel, the Cav1.2. And you see a current going through. So that's the downward slope. The difference in black and red is the black is the current when you use barium ions. Barium ions can go through the channel, but they're not bound by calmodulin. If you use calcium you see a rapid inactivation. So basically open the channel and there's significant - indicates that the channel is closing. And so the difference here between barium and calcium, that's the calcium dependent inactivation.

ROY: And that describes the calcium dependent inactivation and describes the calcium dependence of the closing of the channel?

WITNESS TOFT OVERGAARD: That is correct. And it is mediated by calmodulin.

ROY: And that again is critical at a slightly later stage in the heartbeat to close the channel?

WITNESS TOFT OVERGAARD: It's bad if you don't close, then it keeps open and maybe you remember phase two will be elongated and you get Long QT.

ROY: Can you describe the results of this assay? And again, you've tested the wild type N98S, G114R and G114W?

WITNESS TOFT OVERGAARD: The same three variants are tested. You see that the N98S, so these are single examples as I understand, and then lots of these are aggregated into the figure on the right. So you see that the difference between the black and the red line is smaller. So that means they become - ability of N98S to assist in the calcium dependent inactivation is reduced. And the same you can see for - these are a little bit hard to compare because the scale is different, but that's also in effect for the G114R and G114W. It's basically summarised to the right. So the wild type, that's the calmodulin, that's the black bar. And then we see a reduction in the CDI here and the calcium dependent inactivation for the three different variants.

ROY: I take it this particular assay is highly relevant to Long QT Syndrome?

WITNESS TOFT OVERGAARD: I think this is the one they used for - it's highly relevant for Long QT Syndrome, yes.

ROY: And the N98S variant suggests that there's a greater chance - the results in respect of the N98S variant would tend to suggest a greater chance of Long QT Syndrome phenotype with that variant as compared to the G114R or the G114W variant?

WITNESS TOFT OVERGAARD: There seems to be a difference, in effect, how that translates, we don't know. But perhaps if we go to the next slide we can put this into perspective.

ROY: Please.

WOODS: I'm sorry, your Honour, I'm not sure if we've got this particular slide.

JUDICIAL OFFICER: Slide 53.

WOODS: It's said to be number what?

WITNESS TOFT OVERGAARD: 53.

JUDICIAL OFFICER: We're on 53 now.

WITNESS TOFT OVERGAARD: So in the - I think the--

ROY: I might indicate, I think my friend is following from a printout that was made available to the other parties. But because there are animations in the PowerPoint slides the printout doesn't include the final part of the PowerPoint.

WOODS: I see.

ROY: And that's part of my intention behind tendering the actual PowerPoint presentation at the conclusion. And we're also, as your Honour established this morning, we're recording this. So we'll be able to see it. So yes, that is a limitation of the printed slides. So this is coming back to slide 53.

WITNESS TOFT OVERGAARD: Yeah, next slide.

ROY: And you're moving onto slide 54.

WITNESS TOFT OVERGAARD: Yeah, just like suggested by the Skinner report it's hard to interpret these in terms of phenotype if you don't compare to relatively benign or maybe even more severe Long QT phenotypes. So this is actually data published in 2014 in the paper where it was suggested that the calmodulin variants inducing severe Long QT was through the effect of the Cav1.2 channel. So here we also have the N98S and you'll see - maybe if you go to the wild type, it's to the left, so we see the strong calcium dependent inactivation, the red curve going up. If you compare it to N98S, it does not go to the same height, so the effect is less, and if we compared to these three

variants, these were the ones found in 2013 by Kato et al. They almost completely cancel the calcium dependent inactivation.

ROY: So those three variants on the right, D96V, D130G, and F142L, I take it carried a very severe Long QT phenotype.

WITNESS TOFT OVERGAARD: Yes, these were very severe, very *de novo* mutations, very early onset and most of them, the carriers died at a very early age. So in that sense, the N98S is an intermediate effect and then, as you pointed out in this assay, the G114R and G114W has a slightly lower effect than the N98S.

ROY: The G114R and G114W would sit in between the wild type on the left and the N98S?

WITNESS TOFT OVERGAARD: It would be up here somewhere, yes.

ROY: I noticed the N54I is included. That was the first variant you discovered?

WITNESS TOFT OVERGAARD: Yes, and this was to point out to the Skinner report saying we did not have a benign variant to include but the N54I has no Long QT significance whatsoever, so in that sense it's a control for this assay that not every calmodulin mutation has an effect in this assay.

ROY: Before we leave this assay, in addition to the reports of Professors Kirk and Skinner, have you had a chance to read the report of Professor Wilde?

WITNESS TOFT OVERGAARD: Yes.

ROY: For the Inquiry's benefit, that appears at tender bundle 9, tab 2. In fact I might ask you to turn it up, if you have it there. Page 28 of the tender bundle, which is page 5 of Professor Wilde's report.

WITNESS TOFT OVERGAARD: Yes.

ROY: He expresses some criticism of one of the conclusions in the Brohus article in connection with this assay and the comparison to the N98S variant. I'll read from the final sentence that starts on that page, where he sets out what he's disagreeing with. He says, "Hence, I do not agree with the sentence" - and this is the sentence from the Brohus article:

> "The effect of the mutations are experimentally similar to the N98S variant, which has been associated with Long QT Syndrome, CPVT, IVF, or a mixed phenotype and sudden unexplained death at a young age has been observed for a number of other Calmodulinopathy cases, citing Crotti, including a carrier of an N98S mutation, sudden unexplained death while asleep, which displays a similar functional impact as the G114R and G114W mutations."

Professor Wilde says, "I do believe that the authors of the Brohus article underestimate the difference in the CDI," that's the Calcium Dependent Inactivation, "for these mutants." I should say we haven't clarified but "mutant" is another word for "variant"?

WITNESS TOFT OVERGAARD: That is correct.

ROY: If we go back to page 5, before he sets out that statement, he says - this is in the sentence above the last one:

> "An important difference between the functional characteristics of the N98S variant compared to the two variants at position 114 is the less calcium dependent inactivation of the N98S variant, which is expected to lead to more QT prolongation and, in my mind, an increased likelihood of sudden cardiac death at a very young age."

Can you explain and respond to that?

WITNESS TOFT OVERGAARD: I highly respect Professor Wilde and he may in a - you can say simple sense, he's completely correct. It seems like the effect is lower than for the N98S. The reason we use the word "similar" is to not discuss whether or not these are statistically significant different or if they translate into significantly different clinical phenotypes, but he is correct that if you want to expect severe Long QT the gene or the severity or Long QT, this particular assay may suggest that the G114R has a lower impact than the N98S.

ROY: Do you accept Professor Wilde's comments more generally that there is something of a "mismatch", to use his words, between the phenotype that is presumed for the Folbigg family, being the death of two infants at 10 months and 18 months while asleep, and what the functional assays reported in the Brohus article predicted, which I understand is CPVT or possibly mild Long QT Syndrome?

WITNESS TOFT OVERGAARD: I think he can be correct in saying that this has not been observed before and it goes against the general correlation between the impact on calcium binding and this particular assay and the severity of Long QT; that is correct.

ROY: Is it right that that critique contributed to some further work that you completed last week?

WITNESS TOFT OVERGAARD: This particular critique and another critique in the Skinner reports surrounding the fever induction, yes.

ROY: I'll make you wait a little bit longer and we'll go to something else before we come to the results of that.

WITNESS TOFT OVERGAARD: That is correct.

ROY: Can we deal with the fourth assay, which I believe will be slide 55? I understand again this is not one that was conducted in your lab, but was conducted in a lab of Professor Wayne Chen at the University of Calgary?

WITNESS TOFT OVERGAARD: That is correct.

ROY: Can you explain this test to us?

WITNESS TOFT OVERGAARD: Yeah, this is rather complex, so.

ROY: Keep it as simple as you like.

WITNESS TOFT OVERGAARD: So, it's inherently more difficult to study the function of the ryanodine receptor because it's not sitting on a membrane accessible from the outside.

ROY: The ryanodine receptor is the one that's internal--

WITNESS TOFT OVERGAARD: Yeah, that's--

ROY: --inside the cell?

WITNESS TOFT OVERGAARD: So, you cannot make a standard patch clamp electrophysiology to study, you can say, the function of the channel. So, Professor Wayne has established a system where he instead looks at how much calcium is retained in the internal calcium store. So, basically, he has a fluorescent marker in the sarcoplasmic reticulum, but which in these cells are called the endoplasmic reticulum. This is the compartment where the ryanodine receptor - this ion channel sits, and when it opens, the calcium ion flows out into the cell and, basically, the calcium in the store will be decreased. So, by measuring the amount inside, that's a proxy for when the channel opens. So, when the calcium concentration goes down, that means the ryanodine receptor has opened.

ROY: What were the results of this analysis on the variants in this case?

WITNESS TOFT OVERGAARD: So, there are several readouts of this particular one. So, the one we depict here on the right-hand side, that is how much calcium is released every time the receptor opens and before it closes again. So - and we - and that is determined in relatively to how much calcium there can be in the ER. So, the percentage of this total calcium in the endoplasmic reticulum, how much of that is released before the channel closes. So, what you can see, we have control. Control here is a cell without having extra calmodulin in it. The wild type is where we add an extra gene encoding calmodulin that is not mutated or is not a variant. And then the G114R and G114W, that's where we add genes encoding these variants. And, to sum-up, you can see that the two variants - the G114R and G114W - the ryanodine receptor releases more calcium per opening than for the wild type and the control.

ROY: Why is too much calcium bad?

WITNESS TOFT OVERGAARD: Too much calcium is bad because then - that's also complicated. This is a - I can say it another way. This is a key signature of what a CPVT mutation in the ryanodine receptor would look like.

ROY: Which is bad?

WITNESS TOFT OVERGAARD: Which is bad.

ROY: That answers my next question. If you were to predict a phenotype from this test in relation to G114R and G114W, would you be predicting CPVT?

WITNESS TOFT OVERGAARD: This would say that they will be prone to CPVT-like phenotypes, yes.

ROY: The G114W phenotype was sudden unexplained death; is that right?

WITNESS TOFT OVERGAARD: That was the IVF, I think, and sudden cardiac death, yeah.

ROY: We haven't covered this specifically, but I take it from everything you've presented, there is nothing in particular that would predict IVF that you're aware of at this stage?

WITNESS TOFT OVERGAARD: As I understand, IVF is a bucket for diagnoses they use when they don't have evidence for any of the other phenotypes. So, imagine a child dying. You didn't have time to record electrocardiography or anything else. I think they would label it IVF, but you would have to ask the cardiologist.

ROY: As far as you're aware, there would not be an assay that you would run to try and predict IVF--

WITNESS TOFT OVERGAARD: No.

ROY: --as distinct from CPVT or Long QT?

WITNESS TOFT OVERGAARD: I think it's because they don't know the mechanisms so they don't know what to look for.

ROY: We've gone through the four assays. Can I understand - and you've already given us some of this background - how they work in relation to each other? I think you did explain that the first assay just tests binding to calcium. If that showed that the variant didn't seem to affect calcium binding, would you stop testing or would you still conduct assays two, three and four?

WITNESS TOFT OVERGAARD: I can say, if you have an effect on calcium

binding, you would most likely see effects in the subsequent assays because that's sort of the next level. I can give you one example of the opposite. So, for the very first mutation, the N54I, there's a really extremely moderate effect on calcium binding, so we don't really see an effect. We don't really see an effect in binding to the ryanodine receptor peptides; that's assay two - three.

ROY: Which would indicate Long QT Syndrome?

WITNESS TOFT OVERGAARD: CPVT.

ROY: CPVT.

WITNESS TOFT OVERGAARD: Yeah. There's no effect in the Cav1.2 functional assay, and it doesn't seem to induce Long QT, but there's dramatic effects in this assay that we're looking at here in the ryanodine receptor functional assays.

ROY: It doesn't show up much at all in assays one, two and three, but in four it's dramatic?

WITNESS TOFT OVERGAARD: So - yes, that is correct. So, that means that it is, yes, more complicated when we go to full channels or even in the full body.

ROY: Do you have a hypothesis for why that is the case?

WITNESS TOFT OVERGAARD: We're still working on that.

ROY: Is it right that there is no accepted theory for why certain calmodulin variants are more likely to affect one receptor over another? I keep saying receptor. Channel.

WITNESS TOFT OVERGAARD: I think for some we can try and predict and expect - depends on how much information we have. So, recently, just in an example, the full ryanodine receptor with calmodulin on it, the structure was determined and published. So, of course, we can go in and see where is the N54 position? Is it close to a binding site in the ryanodine receptor? And it's really borderline close in one particular condition. So, it doesn't - it helps us maybe try and come up with a molecular mechanism if we have available information. And but for many of these it takes years to generate enough information to make a clear picture of the mechanism.

ROY: So the results of the assays were published, as we know, in the Brohus article. I'd like to turn that article up, which is at volume 2 of the tender bundle. So a different folder to the one you've been looking at, behind tab 15-02. I'm going to the conclusions in that article on page 19 of the tender bundle, which is page 449 of the article. From what you've said, and I'd like to step through this paragraph, it's the first complete paragraph that appears in the column on the left-hand side of the page, do you have that there?

WITNESS TOFT OVERGAARD: Sorry, I don't think we have the paper here.

ROY: It's volume 2. I might get some assistance. It's page 449 of the Brohus article, which is page 19 of the tender bundle.

JUDICIAL OFFICER: It's in volume 3 in mine.

ROY: It's appearing in volume 2.

WITNESS TOFT OVERGAARD: But it's fine, you can just read it out.

ROY: It's tab 15-02.

JUDICIAL OFFICER: Yes.

ROY: So page 19 of that bundle, which is page 449 of the article. This is in the discussion section of the article. And the paragraph, that is the first complete paragraph to appear on the page on the left-hand column that begins, "Based on the previous studies", do you have that?

WITNESS TOFT OVERGAARD: Yes.

ROY: I'll read out the first passage. "Based on the previous studies and the functional data presented here, we predict that the G114R and G114W variants are pathogenic and that carriers are therefore prone to cardiac arrhythmias of IVF or CPVT-like phenotypes with a potential component of mild Long QT Syndrome which could cause cardiac arrest during sleep." Do you agree with that conclusion?

WITNESS TOFT OVERGAARD: Yes, that's correct.

ROY: Then it says, "This prediction is based on the following observations", and they're listed. And what I wanted to do is link those, I'll step through them one-by-one, link them to your assays and well, do that and confirm that those are your conclusions. So the first says, "This prediction is based on the following observations." The first is "(i) Both mutations impair the ryanodine 2 receptor, RyR2" - I'm going to stop saying receptor - "binding and regulation to a similar extent as other know CPVT-associated CaM" that's CALM, "mutations", or calmodulin mutations.

WITNESS TOFT OVERGAARD: That's correct.

ROY: Does that relate to your first and second assays?

WITNESS TOFT OVERGAARD: This is the second assay.

ROY: The second assay. And then the second--

WITNESS TOFT OVERGAARD: I'm sorry, it's second and fourth.

ROY: Second and fourth. Thank you.

WITNESS TOFT OVERGAARD: Yep.

ROY: Then, "(ii) Both mutations impair Cav1.2 CDI to an intermediary extent, consistent with a possible mild Long QT Syndrome, but not likely severe Long QT Syndrome." That's the third assay?

WITNESS TOFT OVERGAARD: That is correct.

ROY: "(iii) The mutations are not among the most severe in terms of reduced calcium sensing, likely due to the location in a linker region between calcium binding sites." Does that relate to an assay?

WITNESS TOFT OVERGAARD: Yes, so that's a calcium binding assay. So assay 1.

ROY: And the explanation, "likely due to the location in a linker region between the calcium binding sites"?

WITNESS TOFT OVERGAARD: Yeah, so this is when we look up the structure and where its - the position or the mutation is positioned.

ROY: And we looked at the calcium binding sites, there were four of them, and this is the suggestion that this appears in between. Perhaps actually if we look at it just quickly. If we could turn up slide 4. The G114 is identified as you've put on your - you've marked it there, between the top two calcium binding locations, it appears in between the two.

WITNESS TOFT OVERGAARD: It's between EF-hand 3 and 4, yes.

ROY: EF-hand 3 and 4. So that explanation that we see in the third subpoint in the article that it's likely - "The mutations are not among the most severe in terms of reduced calcium sensing." That first part relates to the assay, the first assay. And the explanation given, "Likely due to the location in a linker region between the calcium binding sites." The hypothesis is because it occurs there, that's the explanation.

WITNESS TOFT OVERGAARD: Yes, so it's not binding directly to calcium. But again it's the N98 - I mean, as it is binding directly to calcium. But because the particular changed into a serine is moderate. Serine can also coordinate calcium because it has free electrons on the oxygen on the site chain. You also get a you can say medium effect from that particular mutation compared to some of the other more devastating.

ROY: So if for example, if we can look at slide 3.

WITNESS TOFT OVERGAARD: Yes.

ROY: So what you're saying is in addition to the mere location, also relevant is

the shape change?

WITNESS TOFT OVERGAARD: Yes.

ROY: Between the amino acids that are being replaced?

WITNESS TOFT OVERGAARD: Shape and chemical.

ROY: Shape and chemical.

WITNESS TOFT OVERGAARD: Yep.

ROY: So it's possible that, as you described with N98S, that is a less dramatic change at the relevant binding site than, for example, the G114R is, albeit the G114R is not at a binding site?

WITNESS TOFT OVERGAARD: That's correct, maybe more - you can see in calm 2, there's also been identified in N98I, I is isoleucine; it doesn't have any extra electrons so that's more severe than the N98S, and I believe - we have to look it up, of course, but I will predict a more severe phenotype from this one than N98S.

ROY: Then back to the article, "(iv) The effect of the mutations are experimentally similar to the N98S variant," that's the use of the word "similar" that you referred to before?

WITNESS TOFT OVERGAARD: Yes.

ROY: "Which has been associated with Long QT Syndrome, CPVT, IVF or a mixed phenotype." Apart from the assays did you otherwise contribute to that conclusion?

WITNESS TOFT OVERGAARD: I think we - those conclusions we discussed extensively between the three main labs of the paper.

ROY: Between the three main labs?

WITNESS TOFT OVERGAARD: Yes, so that's the Vinuesa lab and the Schwartz lab and my lab.

ROY: We will, your Honour, also hear from Professor Vinuesa and Professor Schwartz, who ran the other two labs.

JUDICIAL OFFICER: I understand that.

ROY: Finally:

> "(v) Sudden unexplained death at a young age has been observed for a number of other Calmodulinopathy cases including a carrier of the N98S mutation, sudden unexplained death while asleep, which

> displays similar functional impact as the G114R and G114W mutations. Of note, the girl with the G114W mutation who suffered a cardiac arrhythmia and was diagnosed with IVF."

I assume that in general refers to all of the assays insofar as they're comparing. I take it you agree with that conclusion?

WITNESS TOFT OVERGAARD: Yes.

ROY: The article goes on to compare and to discuss the comparison of the phenotypes that are presumed in relation to Ms Folbigg and her daughters, or that Ms Folbigg is described and her daughters to the extent, sudden unexplained death, is taken as a phenotype and that is compared to what is known about a number of other individuals and families with CALM variants. I'm not proposing to take you through those descriptions of other individuals and families with the CALM variants in that article, and I expect probably Professor Schwartz or Professor Vinuesa will be better placed to do that; would you agree with that?

WITNESS TOFT OVERGAARD: I think Professor Schwartz is the one who has the overview of the entirety of the calmodulin mutations and phenotypes.

ROY: Coming back to the Brohus article, and there's another conclusion expressed in the summary on the first page, which is page 441 of the article and 11 of the tender bundle, and the conclusion passage there says:

> "A novel functional calmodulin variant (G114R) predicted to cause idiopathic fibrillation, catecholaminergic polymorphic ventricular tachycardia or mild Long QT Syndrome was present in the two children. A fatal arrhythmic event may have been triggered by their intercurrent infections, thus calmodulinopathy emerges as a reasonable explanation for a nature cause of their deaths."

Particularly the last passage, "A reasonable explanation for a natural cause of their deaths," again I take it, as co-authors, you agree with that conclusion?

WITNESS TOFT OVERGAARD: That's correct.

ROY: Before we leave the article, to be clear I haven't taken you through - there are other parts, and all of it. There is a reference in that article to the presence of a BSN variant in the Folbigg boys, Caleb and Patrick. Did you have any involvement in the inclusion of that detail in the report?

WITNESS TOFT OVERGAARD: No, that was not part of my work.

ROY: Do you know who was responsible for that?

WITNESS TOFT OVERGAARD: I believe Professor Vinuesa was the main--

ROY: We'll ask her about it. There is also a passage at the beginning of the

discussion on page 447, which is page 17 of the bundle.

JUDICIAL OFFICER: Ms Roy, how long are you going to be on this report?

ROY: On the report?

JUDICIAL OFFICER: Sorry, on the Brohus article?

ROY: That's it on Brohus article, and then I'll come to their Report.

JUDICIAL OFFICER: I was proposing, if anyone wants me to, to take a ten-minute adjournment at this stage. I'm going to sit till 4:15pm, so people know where we are. If Ms Roy is very close to finishing we might sit on, but that's to put no pressure on her or for that matter Professor Toft Overgaard and Nyegaard, so we'll adjourn for ten minutes.

SHORT ADJOURNMENT

Yes, Ms Roy.

ROY: I'd just like to clarify something that happened, the evidence that we've heard since lunch. All of that evidence has come from you, Professor Toft Overgaard and I just wanted to check with you, Professor Nyegaard, whether the evidence that we've heard in the second half of the day, does that fall within your area of expertise?

WITNESS NYEGAARD: So the laboratory work? No.

ROY: To the extent we discussed the matters not to do with the laboratory work, do you take a different view to any of the evidence that was given by Professor Toft Overgaard?

WITNESS NYEGAARD: No.

ROY: Coming to the Brohus article's conclusion, this was the last thing we were doing before the adjournment, and you said that you agree with the conclusion, that "Calmodulinopathy emerges as a reasonable explanation for a natural cause of the girl's deaths." I take it also, Professor Nyegaard, that is your view as well. Can you--

JUDICIAL OFFICER: Did Professor Nyegaard answer that question?

WITNESS NYEGAARD: Yes.

ROY: --say that out loud, please?

WITNESS NYEGAARD: Yes, it is.

ROY: Thank you.

JUDICIAL OFFICER: Happens all the time.

ROY: Then we were coming to a passage at the beginning of the discussion on page 447, 17 of the bundle, which says, "Our main findings are that the mother and her two female children (Child 3 and 4)", that's Sarah and Laura Folbigg, "were carriers of a novel functional variant in the CALM2 gene associated with sudden death in infancy and childhood, and that the variant is predicted to be arrhythmogenic." Now I understand your contribution to the first part of that, that the variant being predicted to be arrhythmogenic, excuse me the second half. In terms of the statement that "the novel and functional variant is associated with sudden death in infancy and childhood"--

JUDICIAL OFFICER: Whereabouts is that, Ms Roy?

ROY: It's under “Discussion” on page--

JUDICIAL OFFICER: Sorry, I see right at the bottom, it's here.

ROY: Page 17, 447 of the article. In terms of the statement, "associated with sudden death in infancy and childhood", are the CALM2 genes associated particularly with sudden death in infancy and childhood, or just with death in general?

WITNESS TOFT OVERGAARD: Depending on the definition of the different words you use. I mean, I think what this is intended to mean is that the mutations in the CALM2 gene has been seen in sudden death in infancy, which is highlighted in the paper, the examples of that.

ROY: Is it also associated with deaths in other age categories?

WITNESS TOFT OVERGAARD: Yes.

ROY: Is it particularly associated with sudden death in infancy and childhood, as against other age categories?

WITNESS TOFT OVERGAARD: Not that I'm aware of.

JUDICIAL OFFICER: I'm sorry, Professor, I didn't catch that answer?

WITNESS TOFT OVERGAARD: So not - not that I'm aware of. I believe there are a range of ages for onset of disease, yep.

ROY: We're finished with the Brohus article. If we can turn up your report. I'm informed, your Honour, that we have different volume numbers depending on whether we are at single or double-sided. So I won't give you volume numbers, I will only give you tab numbers. It's tab 6-02 and at page 19 in the bundle, which is page 12 of the report.

WITNESS TOFT OVERGAARD: I have it, yes.

ROY: Your final conclusion on that page, "In our opinion, based on the available research data and understanding of human genetics the CALM2 G114R mutation is enough to have caused the death of the two children."

WOODS: Sorry, what page is it on?

ROY: It's page 19. Page 12 of their article. Page 19 of the bundle and page 12 of their report. It's paragraph 21. You maintain that conclusion?

WITNESS NYEGAARD: Yes.

WITNESS TOFT OVERGAARD: Yes.

ROY: To be clear, you do not say that these girls in fact died by reason of Calmodulinopathy?

WITNESS TOFT OVERGAARD: No.

ROY: Would you say if it was likely that the girls died by reason of the variant?

WITNESS TOFT OVERGAARD: From what we know, yes.

ROY: You would say it's likely?

WITNESS TOFT OVERGAARD: It's likely. It's - it definitely is with the impact we see, yes.

ROY: Can you repeat that?

WITNESS TOFT OVERGAARD: So yes, we - as we write we think that it is likely that this mutation can have caused the death.

ROY: And that is based on the functional assays?

WITNESS TOFT OVERGAARD: So I think it's based on the functional assays we see, plus the knowledge we have from the calmodulin variants and severity in general. And the - maybe it wasn't written so clear in the Brohus article, but that there are modifying effects. So you can - so you could easily expect an expansion of phenotypes compared to what we see here.

ROY: But you were not commenting on matters outside of the genetics evidence.

WITNESS TOFT OVERGAARD: No.

ROY: And other facts in the case relevant to the cause of death of the two girls.

WITNESS TOFT OVERGAARD: No.

WITNESS NYEGAARD: No, this is from where we stand and what we can see, select pressure combined with a functional assay, it looks like a bad variant.

ROY: It looks like a bad variant.

WITNESS NYEGAARD: Yes.

ROY: Do you necessarily then take into account the fact of the girls' deaths in assessing that it was likely to have caused their deaths? If I can put it another way. If you did not know that Sarah and Laura Folbigg had died, would you predict that this variant would cause their deaths in infancy?

WITNESS TOFT OVERGAARD: So, also having read, of course, the expert reports and the concerns in the other expert reports, you would perhaps not predict a very early death for this particular impact in the functional assays at this point.

ROY: Is it inherent then in your conclusion that it is likely to have caused their deaths, the fact that they died?

WITNESS TOFT OVERGAARD: Yes.

ROY: To be clear, have you placed any weight at all on the fact of the death of their two brothers?

WITNESS TOFT OVERGAARD: No.

JUDICIAL OFFICER: Just to add to that, your opinion has been based on your research and the material which you've been supplied and nothing else.

WITNESS TOFT OVERGAARD: That's correct.

WITNESS NYEGAARD: Yes.

ROY: I want to come now to the final criticisms that were made by some of the other experts, by primarily Professors Skinner and Kirk. We'll start with what's led to the new discovery finally.

WITNESS NYEGAARD: Mm-hmm.

ROY: If we turn to your Addendum, which would now appear behind tab 6-03 in everyone's bundles, and page 5 of that Addendum report at the bottom of the page, you note that both Professors Wilde, Skinner and Kirk raised the concern that given the result of the functional assays and the predictions for CPVT or mild Long QT Syndrome, a clinical manifestation of death before the age of two while asleep is remarkably unlikely. This is also what Professor Wilde described as the mismatch between the functional assays and the hypothesised phenotype.

Professors Kirk and Skinner go further, and you report that over the page on the top of page 6, and critique an observation made in the Brohus article that fever could have trigger arrhythmic events in the girls, given there was some evidence that both had been ill in the days before their deaths. Please explain to the Inquiry where those comments led you.

WITNESS TOFT OVERGAARD: So, in the - as also seen on the top of page 6 in the Skinner report, they say that it's not possible for Calmodulinopathies to be triggered by fever because fever has only been seen as an arrhythmic trigger in sodium channel disease or that is mutations in the SCN5A gene. So, when reading this, of course, it came to mind that calmodulin exactly regulates the sodium channel encoded by the SCN5A gene. So, maybe it's of assistance to have a look at some of our slides.

ROY: Yes, please. I think possibly we're starting with slide 42, but you can correct me.

WITNESS TOFT OVERGAARD: Yes, that's fine. I think you can move - go one forward, please. One forward. Yes, so just to - the concerns are listed here. The phenotypic expression in the Folbigg variant doesn't seem to match what was known at this time about Calmodulinopathies, and the correlation between our functional assays and the phenotypes observed, and then the fact that fever as a trigger of arrhythmia has only been seen for Brugada Syndrome, which is a sodium channel disease in children. So, Brugada Syndrome is caused by mutations in SCN5A, also known as Nav1.5 at the protein level.

So, if you go one further, we have something popping up. Yes. So, to put that into perspective, we went through all the ion channels and the sodium channel, or the Nav1.5 protein, same as the SCN5A gene. That is a gene known to cause Brugada Syndrome and Long QTS 3. So, that's actually two ends of the same spectrum. So, Brugada Syndrome is a loss of function phenotype for this gene. That means that less sodium ions are coming through the channel, where mutations that generate Long QT typical is a gain-of-function phenotype, so then more sodium ions is coming through the channel.

But the same mutation can also result in a mixed phenotype in a person, so a combination of the two, and that may be hard to understand, but if you recall earlier, the sodium channel had actually two different functions in the action potential. It's the very initiator of the action potential, but it still keeps partly open during the later phases so it can affect both the Brugada or lead to Brugada Syndrome and the Long QT Syndrome.

And what came to our mind is that calmodulin is a critical regulator of this sodium channel activity. So, next we ask ourselves what about the Folbigg mutation in particular, where does it sit in this structure? And there are a number of structures of calmodulin bound to a piece of this channel. So, if we go to the next slide.

ROY: This is slide 43.

WITNESS TOFT OVERGAARD: So, slide 43. It's just to illustrate that the G114R mutation is located in a different site than most other known arrhythmogenic calmodulin mutations. And so, if we go to the next slide, next.

ROY: Slide 44; is that what you're looking--

WITNESS TOFT OVERGAARD: Yeah, so, one more forward. Yeah. So, this is an illustration we just looked up in the databases of protein structures. So, this is one where calmodulin - in cyan, this greenish colour - binds to the fragment of the sodium channel Nav1.5, and this is in a condition where there's no calcium present.

JUDICIAL OFFICER: Whereabouts is the binding, I'm sorry?

WITNESS TOFT OVERGAARD: Sorry?

JUDICIAL OFFICER: No, I see, it's okay.

WITNESS TOFT OVERGAARD: Yeah, so, the magenta part here, that's the sodium channel and the cyan part, that's the calmodulin. And then we highlighted where are the G114 residues and the N98S; that's on the back side. And then we also included this presumably benign I10T variant which is located in the other end of the protein. So, what you can see is that the G114R is uniquely positioned close to the sodium channel. It may be hard to see in this representation, so if you move one slide forward.

ROY: Slide 44?

WITNESS TOFT OVERGAARD: Yeah. Here we add it--

ROY: Slide 45.

WITNESS TOFT OVERGAARD: This is a - it's called a space-fill representation, so these were the actual size of the individual atoms. You can see that the G114, the glycine, is basically tucked into the interface between calmodulin and the sodium channel. So, imagine putting - taking the smallest amino acid in the world and putting one of the largest in place of that. We would predict that could interfere with the binding.

ROY: This is binding to the sodium channel?

WITNESS TOFT OVERGAARD: To the sodium channel, yes.

ROY: How did you identify this?

WITNESS TOFT OVERGAARD: So, we just looked - these structures are available in the PDB databases - so, the structural databases - so, if that's what you mean, we can easily bring it up and visualise--

ROY: Yes, so, at this stage, this is a theoretical analysis where you're

modelling how the calmodulin protein with the variant is binding with the sodium channel?

WITNESS TOFT OVERGAARD: Almost. So, this is real data for how this structure was produced. So, this is a wild type calmodulin, so the non-mutated binds to the channel. We're just indicating the position where you would put an arginine instead of the glycine. Yeah.

ROY: Having formed the view that this would predict potentially a significant effect, what did you do next?

WITNESS TOFT OVERGAARD: So, next I asked my laboratory assistants - post-docs and assistants - to go into the lab and test whether the binding would be affected. The reason we could do this is we have been planning and now had funding for studying calmodulin interaction with several different targets to try and come closer to possible effects and phenotypes for the individual mutations. So, we had all the materials ready, so we could, within a couple of days, go into the laboratory and produce similar binding data as assay two of the Brohus article. So, if you go to next slide.

ROY: Slide 46.

WITNESS TOFT OVERGAARD: So, this is the, I would say, preliminary result of the test we did last week. These represent duplicate measurements, and we - again, we have taken some of the data out, so showing you the binding to the sodium channel for four different calmodulin variants at low, medium and high calcium. And the G114R is shown in red. And you may see - say something is missing in the first one; that's actually because the binding is so severely affected that it's - we can almost not measure binding for this variant. So, at low calcium, it's really severely affected, the G114R mutation or variant. And we compared it to this seemingly benign variant but also to the N98S variant, which is located on the other side of this domain. So, it doesn't seem to be affected. We can predict this because it's not really sitting in the interface with this particular channel.

ROY: Put another way, this is consistent with what you would predict by looking at where the G114 sits and where it would bind to sodium?

WITNESS TOFT OVERGAARD: Yeah, so, it doesn't bind well at low calcium. It's also severely affected in the medium calcium range. So, this is a sensing range. And it's affected but less so in the high calcium.

ROY: I think you said this is equivalent to the second assay that you did in the Brohus article?

WITNESS TOFT OVERGAARD: Yes, that's correct.

ROY: Is there any equivalent to the first assay that you could run?

WITNESS TOFT OVERGAARD: But that doesn't change because the first

assay was calcium binding to the Folbigg variant.

ROY: You wouldn't do a sodium binding?

WITNESS TOFT OVERGAARD: No, the calmodulin doesn't bind to sodium, so.

ROY: That answers that.

WITNESS TOFT OVERGAARD: Yep.

ROY: Again, if you had the fullness of time and resources, what would happen next in terms of the further assays that were conducted in the Brohus article? Would they be repeated? Equivalent assays--

WITNESS TOFT OVERGAARD: In terms of scientific competition, I don't want to reveal too much, but - of course, this is a new finding because other people have tried to look at the severe Long QT effects on the sodium channel, and they have determined that there were no effects. So, obviously, it's a new finding that a particular mutation will give a particularly strong effect on the binding of the sodium channel. So, next we would move forward, likely with collaborators because we don't do the next assays ourselves, and characterise the effect of this at the single cell level.

ROY: Do I take it that, quite apart from this case that we're concerned with in the Inquiry, this is a significant discovery?

WITNESS TOFT OVERGAARD: Yes, indeed.

ROY: Would you ordinarily be sharing it with the world this quickly?

WITNESS TOFT OVERGAARD: No. We hope not too many people are listening because of course we - in science we are in a competition with other labs. So the first one to try and publish gets all the glory. So by doing this of course we have invited the competitors to run for the race. But for us I think this is more important.

ROY: I understand the history of the discovery of the calmodulin variants that we discussed is a focused investigation on calcium. But given what's known about calmodulin's interaction and role in so many other processes and specifically potassium and sodium channels, why haven't those assays been done before now?

WITNESS TOFT OVERGAARD: I think it's a historical perspective and I don't know, I have sort of had a walk through that might explain this.

ROY: Yes, please.

WITNESS TOFT OVERGAARD: It's actually the next slide.

JUDICIAL OFFICER: Before you do can I just ask you something, I don't know if I quite understand it. When you dealt with the calcium channel, the first assay you did, was to do with calmodulin's ability to bind calcium. By contrast, calmodulin doesn't bind sodium; is that right?

WITNESS TOFT OVERGAARD: That is correct.

JUDICIAL OFFICER: Thank you, yes, I understand.

ROY: So this is slide 46. No, 47 you wanted.

JUDICIAL OFFICER: 47, I think.

ROY: 47.

WITNESS TOFT OVERGAARD: Yes, so there's a lot of things coming in so we do one at a time. So this is trying to explain when the first calmodulin mutations were identified, that's the N54I and the N98S, these were found in carriers with clear, or we could say clinical phenotype arrhythmic phenotype, that's CPVT. So there to try and explain the mechanism obviously we studied the interaction with known ion channels that have produced and where mutations are producing CPVT. So that became a part of our studies to visualise that these mutations affected the function of the ryanodine receptor or RyR2, also known as the CPVT1 gene. So if you do next slide. Yep. So then the year after in 2013, three other mutations were found producing - found in carriers of severe Long QT Syndrome. So obviously we extend the functional studies to try and explain what's the mechanism behind this. And those functional studies showed us that this - actually collaborators and competitors, but the scientific community showed and agree that this is most likely through the severe effect on the voltage-gated calcium channel. So that would make the phenotype comparable with what we saw in the lab in the functional assays. Then could you do next slide. As more carriers were identified of the N98S variant it became clear that this variant also could be in individuals with Long QT or a mixed phenotype. So of course we compared the effects also in the functional assays for the Cav1.2 and you can see a more moderate effect, which goes along with a more moderate Long QT phenotype. And you see it in individuals with a mixed effect where you can expect that this is the effect on both channels in the same individuals who get sort of a mix of phenotypes.

So next, if you took the next one. We analysed the G114R and it has an effect on both channels. This is what comes out as the conclusion in the Brohus article that we expect this could be a mix of these two phenotypes. And then we can get back to this, but then if you go to the next slide. So as an example of this is a continued expansion of the phenotypic spectrum of the Calmodulinopathies. As we learn more and more we find more and more mutations, more and more carriers. One example of that is the Kato article or paper that we talked about where suddenly a Long QT mutation calmodulin - calmodulin mutation shows you something that you cannot reconcile within known phenotypic spectrum. And then they performed

functional assays for the potassium channel Kv7.1. And they see a contribution of a disturbance of that channel. So that can explain a more complicated phenotype that is not explained by the simplistic view that this is a calcium channel effect. And then if you do the next slide. So now we - it's by the reports of Skinner and we say okay maybe that for this particular variant, the Folbigg variant could be a problem with regulating the sodium channel. And so if we then highlight this particular mutation, it is unable to bind correctly to the sodium channel, so this would then possibly include the phenotypes of this channel mutations into the spectrum of Calmodulinopathies. So meaning that you can expect a combination of Long QT, maybe even Brugada and CPVT in the expression of carriers of this.

ROY: Brugada Syndrome, now I'm not going to ask you for a cardiology description of Brugada Syndrome--

WITNESS TOFT OVERGAARD: Thank you.

ROY: --but is it your understanding that that can be associated with cardiac arrhythmia as triggered by fever?

WITNESS TOFT OVERGAARD: That is correct. That's at least what was written by cardiologist in the Skinner report. And also if we quickly look it up what is Brugada Syndrome is I think it's known as a nocturnal death syndrome gene. And I also believe Professor Schwartz--

ROY: Associated particularly with sleep, nocturnal death syndrome?

WITNESS TOFT OVERGAARD: With sleep and you could see arrhythmic events while at rest, and this is also present in the Schwartz Addendum. I believe he shows that mutations, Long QTS3 mutations in particular, most events are actually while at rest but please refer to Professor Schwartz.

ROY: So that suggests to you this is the beginning a discovery that may explain the death of an infant while asleep or at rest?

WITNESS TOFT OVERGAARD: I think it makes it easier to understand the apparent discrepancy between the functional assays in the Brohus article and the particular phenotypic expression in the family, so understanding that the sodium channel might be a part of this particular mutation, molecular mechanism, would include, let's say, per extension would include the phenotypic spectrum of this particular channel in to the calmodulinopathies.

ROY: In the Kato article, which was considering a different variant, where they reported the discovery of a potassium effect, that had a moderating effect?

WITNESS TOFT OVERGAARD: I believe that can have a moderating effect, yes, because it's on an ion channel that has ion current in the opposite direction, but mind you, we cannot predict whether or not it will be having a mitigating or an enhancing effect for this particular one.

ROY: That was my next question. In this case, is it possible it could also have a moderating effect?

WITNESS TOFT OVERGAARD: Yes, indeed.

ROY: Will you then progress or, with collaborators, as you said, to conducting the other types of assays?

WITNESS TOFT OVERGAARD: We will, and the sodium channel is, in particular, complicated to understand, as I understand, but, of course, we will move on and try to see collaborations for the functional assays for this.

ROY: You mentioned that you were able to turn the assays around very quickly that you did last week because you already had the materials ready to go and you had funding.

WITNESS TOFT OVERGAARD: Yes.

ROY: Ordinarily how long would it take to prepare for one of these assays?

WITNESS TOFT OVERGAARD: If you have the funding--

ROY: Assuming you have funding.

WITNESS TOFT OVERGAARD: A couple of months because you need to design or you can say to determine what materials you need, so what part of the channel to order and then order to have it synthesised and delivered, yes.

ROY: Would funding also be a reason why these assays hadn't been conducted previously for the CALM variants?

WITNESS TOFT OVERGAARD: That is correct, at least in my lab, yes.

ROY: In a perfect world, where you had unlimited resources, what would you--

JUDICIAL OFFICER: There's no such thing, Ms Roy.

ROY: No. I'm not suggesting the Inquiry will supply it. But would you be conducting further assays in relation to other interactions between calmodulin and other genes?

WITNESS TOFT OVERGAARD: Definitely. So that's one of the reasons we're interested in calmodulin; it's so, you can say, unpredictable what the phenotypes will be because, depending on where the mutation is, which one of the, or which set of the hundreds of binding partners is affected by this mutation and that's really hard to predict.

ROY: With the time that remains, I was proposing to go back to the other - there are a handful of other criticisms made in the Professor Skinner and Kirk - but is there anything else you'd like to tell us about this discovery

before we move on? Did you have other slides?

WITNESS TOFT OVERGAARD: I can't remember. You can go forward one just to explain. Yes, just one, we added this just to be clear that this one particular mutation would be out here in the spectrum so it's non-arrhythmogenic, so basically if you go forward one, that's what we're proposing to do. There could be other combinations of phenotypes where other interactions would modulate the phenotype in that particular carrier.

ROY: Professor Nyegaard, you're nodding furiously. Is this within your area of expertise?

WITNESS NYEGAARD: I'm not surprised at all because we know that there is this selective pressure and it's almost like every mutation has its own phenotypic spectrum, so it's really just to find them.

ROY: You consider this entirely consistent with what you would expect from what you took us through this morning in terms of how calmodulin is conserved?

WITNESS NYEGAARD: Yes.

ROY: Is there anything else you would add specifically in relation to this new development?

WITNESS NYEGAARD: No.

ROY: I think this will relate to you, Professor Nyegaard. There's another matter. There are two other matters that you address in your addendum report, that I don't propose to specifically go to, but in your report you address the use of the ACMG guidelines. Excuse me, in the addendum to your report you address the use of the ACMG guidelines which are the American College of Medical Genetics and Genomics and the Association for Molecular Pathology guidelines. That's a diagnostic tool, I take it?

WITNESS NYEGAARD: Yes. So it's a system, so when you start sequencing the individuals it's a really nice guideline to recognise something that we know in advance, kind of the known boxes, so when we do these gene mapping studies, I would never use the ACMG for that. So it's really built on data from science, looking for known connections on known variants, yes.

ROY: You've put it in your addendum report, it's at page 12 of your addendum report, which is at 6-03 in the tender bundle. Are you aware that the classification of the G114R variant, according to the ACMG guidelines, was a significant part of the 2019 Inquiry prior to your--

WITNESS NYEGAARD: Yes, I could understand that on the material that we got recently, yeah.

ROY: You note on p 12 of your addendum report, which is p 44, about halfway

down, that you "respectfully disagree with using this system to make deterministic judgments regarding the arrhythmogenic potential for this novel variant."

WITNESS NYEGAARD: Yes.

ROY: You started to explain, but can you explain why that's so?

WITNESS NYEGAARD: Yeah, I think I did it also a little bit before. So, it is a really useful tool for clinical diagnostics; so, running a lot of patients through, looking for variants in genes that are already known to be linked to particular condition, and then searching for those variants that are already known to be pathogenic. So, it's a really nice tool to recognise something that is already known. But since this variant that we're talking about here, the G114, is really not known, it's really hard for such a variant to be classified. Yeah.

ROY: I might take a step back. The guidelines themselves use five categories: benign, likely benign, variant of uncertain significance, likely pathogenic, and pathogenic. You've expressed the view that this variant is likely or is pathogenic?

WITNESS NYEGAARD: It's detrimental. So, pathogenic is something that is - has a particular meaning in the ACMG guidelines. So, in the kind of research where we link new variants, we call it detrimental, and you could say that it is has a really high effect on phenotype. To actually classify it, it has to kind of earn certain points in that system. So, I'm not - I don't know how it's classified after these new discoveries. I think it's not a tool that should be used so deterministically.

JUDICIAL OFFICER: Leave aside for the moment the points system, if I can call it that, in the ACMG guidelines. Wouldn't your conclusions in your initial report, concerning the possibility of it causing the death of the two children, at least lead to a classification of likely pathogenic?

WITNESS TOFT OVERGAARD: I can--

WITNESS NYEGAARD: Yes.

WITNESS TOFT OVERGAARD: --maybe also answer. So, yes, we would say it's likely pathogenic. Perhaps that's the conclusion that we mean, but we would really rather not have that defined with the restrictions of the ACMG guidelines.

JUDICIAL OFFICER: That's because of that particular points system that they adopt.

WITNESS NYEGAARD: Yep.

WITNESS TOFT OVERGAARD: Yeah. And I can - my understanding from the other reports to reach likely pathogenic, you need a 90% certainty. I'm

unaware of how they actually put these degrees of certainty on the system.

JUDICIAL OFFICER: I was going to ask, how do you measure those degrees?

WITNESS TOFT OVERGAARD: How do you measure that? And if it's, let's say, 89% likely, it's still 89% likely. It's not unlikely, so it's--

WITNESS NYEGAARD: Yes. So, I understand that it has - it's on the - using the ACMG classification, it's on the borderline between variant of unknown significance, so there's absolutely no evidence against it being pathogenic. It's just because it hasn't earned enough money to go into the likely pathogenic. And as I understand it, it's really on the very borderline, so it's almost like taking, like, a quantitative measurement and putting it into boxes.

ROY: If I asked you - and you can decline to answer because I'll be putting you on the spot - to give your own percentage assessment of the likelihood, where would you put it; the likelihood being that it was pathogenic?

JUDICIAL OFFICER: Is that generally?

ROY: Generally; not by reference to the guidelines.

JUDICIAL OFFICER: Generally; not by reference to these particular children.

ROY: I'm not asking you for a probability of causing the death, but a probability it is pathogenic, using your own metric and not the guidelines.

WITNESS TOFT OVERGAARD: So, perhaps--

ROY: We're asking for a degree of confidence.

WITNESS TOFT OVERGAARD: --we can use it in a - normally, in a scientific sense, we say something needs to be 95% sure to be significant, so--

JUDICIAL OFFICER: Ms Roy, I don't know if that question's going to be really helpful.

ROY: It may not be, your Honour, as it was coming out.

WITNESS NYEGAARD: I can say that this variant--

ROY: I might stop you from answering.

WITNESS NYEGAARD: Okay.

JUDICIAL OFFICER: Law has to be cited at some stage.

ROY: Yes. There's two remaining matters that arise from Professors Kirk and Skinner, both of which, even directly, are already addressed, but just for completeness. I won't ask you to turn it up, but Professors Kirk and

Skinner - for the record, at paragraph 2.3 on p 9 of the report, which is tab 7-03, p 55 of the tender bundle - in applying the guidelines, they apply one of the criteria concerning whether the novel variant appears in a data set.

WITNESS NYEGAARD: Yes.

ROY: They advise that they have located one person with a CALM3-G114R variant in the Regeneron data set. They indicate they do not consider that alone is necessarily evidence against pathogenicity, but you comment on it in your addendum. Can you explain the significance in your view of that variant in the Regeneron data set?

WITNESS NYEGAARD: Yes. So, there seem to be a lot of discussion about if Kathleen had any symptoms or not. So, I think it's going along the same line, that there are people who can survive or seem to - yeah, can survive these variants. And it's known in other genetic diseases that there is reduced penetrance of almost all diseases that has a genetic component - also, monogenic diseases. In particular, when you look into these big cohorts of individuals, there is this survival bias that you - if you - it's only people who live and are adults and have been able to sign up to these things. So, the variant there was in the Regeneron database. So, Regeneron was a company that sequenced a lot of the participants from the UK Biobank, so we have located that particular carrier came from the UK Biobank, and so we looked at it, and there is not that much data on that person, but it doesn't seem to have any cardiac phenotype that we can at least see from there.

So, my point is just that this doesn't go against this variant being very detrimental because it's so ultra-rare, so that's why I comment on it. Yeah.

ROY: The final matter, which it's not raised by Professors Kirk and Skinner in this Inquiry and not addressed in your addendum, but it comes out of their 2019 report, which is found in Exhibit 2-Z, which is volume 15 of the tender bundle that is Exhibit 2. We don't need to turn it up. I'll get you to turn it up in case you need it, but I suspect you'll be able to answer it without it. They say, "CALM2 is relatively tolerant to missense variation with a gnomAD Z-score for constraint being 2.79." We were referring to some of these--

WOODS: Sorry, relatively tolerant to what?

ROY: "Relatively tolerant to missense variation."

WITNESS NYEGAARD: Yes.

ROY: "With a gnomAD Z-score for constraint being 2.79." Can you explain that?

WITNESS NYEGAARD: Yes, so this was exactly what we addressed this morning, so I don't know if you want to pull up slide number 25.

ROY: Please.

WITNESS NYEGAARD: Yes. So, this score, I'm not 100% sure how it's calculated, but what we tested is that it's very sensitive to gene length. We did some plots. So, for a smaller gene, it's harder to reach a high Z-score, and so the ACMG uses these, you know, kind of cut offs, and this is generally - you could say that in the gnomAD, they now recommend to use this observed divided by expected because it's a quantitative score and it has confidence intervals. And that cut off, if you have to use a cut off, they say - they really advise against, but if you have to use a cut off, it's 0.35, at least I've understood that, and the gene is much lower than that. So, the upper bound of the confidence interval is way below that.

ROY: First of all, is this the Z-score that's on screen here?

WITNESS NYEGAARD: Yes.

ROY: Okay. You were telling us about it this morning, but--

WITNESS NYEGAARD: Can I say something?

ROY: Please.

WITNESS NYEGAARD: So, he also mentioned that there is another place to look for conservation where, instead of looking at just this one gene, they look for, you could say, similar domains in other proteins so you've got much more statistical power to say if this is conserved or not, and there, he actually say that it's really highly constrained. So, I mean, it's just - you know, I don't want to put - I don't think--

ROY: Is the simplest way to put it that in your view, the Z-score is not a useful measure in relation to calmodulin.

WITNESS NYEGAARD: Exactly, yes.

ROY: Okay, and that is because?

WITNESS NYEGAARD: The gene is small, and there are so few counts, so the statistics is - yeah.

ROY: Excuse me one moment. Would you agree or disagree with the statement they make that CALM2 is relatively tolerant to missense variation?

WITNESS NYEGAARD: I disagree on that.

WITNESS TOFT OVERGAARD: So do I.

ROY: You also disagree.

WITNESS TOFT OVERGAARD: Yes.

ROY: We heard this morning, quite strongly.

WITNESS NYEGAARD: Quite strongly, yeah.

ROY: If you could excuse me one moment. Your Honour, that concludes the examination.

JUDICIAL OFFICER: The cross-examination's not going to take place at this stage.

ROY: No.

JUDICIAL OFFICER: Professors, that's as far as we can take it with you both in this Inquiry at this stage. People haven't had an opportunity to consider your most recent report, which is obviously highly significant. We may need to ask you to give evidence by AVL obviously, unless you want to come to the humidity of Australia in February. We may need you to give evidence by AVL, but those assisting me will get in touch with you as far as that's concerned and we'll work out a convenient time. Can I simply thank you for coming out here and for your presentation today. It's been fascinating, thank you.

<THE WITNESSES WITHDREW

ROY: Your Honour, I will tender a USB of the PowerPoint presentation that we saw just now that we were using. That can be conveniently slotted in at 6-04 of the tender bundle, and I'll call 6-05 of the tender bundle, I would tender a print out that contains the numbering of the slides that we used during that segment.

JUDICIAL OFFICER: A copy added to the tender bundle.

ROY: We'll provide your Honour with a working copy.

JUDICIAL OFFICER: Is there anything else that needs to be done at this stage, Ms Callan?

CALLAN: Your Honour, on Friday of last week, approximately 6:00pm, the Inquiry received from the legal representatives for Ms Folbigg a report dated 9 November 2022 from Dr Pascale Guicheney, Research Director at the Sorbonne Université.

JUDICIAL OFFICER: Dr Guicheney was instrumental in the Kato report, I think.

CALLAN: Certainly an author of the Kato report, and the report, amongst other things, elaborates on and addresses the findings in that Kato article. Your Honour, the Inquiry is, of course, concerned to receive all useful contributions that can be made. There may have been a real difficulty in terms of timing of receipt of this report on the eve of this first hearing segment. As it's transpired, for other reasons, this topic is now going to be more fully dealt with in February of next year, which eases the impact of the late service of the report. In my submission, and I would propose that it be circulated to the parties, perhaps

tendered now but with the parties given an opportunity to consider their position.

More generally, I note your Honour made orders yesterday in respect of the service of any evidence from the parties ahead of the hearing block next year, and all I can say is that the failure to adhere to that timetable may have real implications for whether the Inquiry can receive late served evidence, or what can be achieved in the hearing block in February.

JUDICIAL OFFICER: Just bear with me one moment. What I propose to do is I propose to admit those reports into evidence.

CALLAN: Yes.

JUDICIAL OFFICER: That doesn't mean that Professor Guicheney will be called.

CALLAN: Yes.

JUDICIAL OFFICER: But it does highlight the difficulty of getting material late. Professors Toft Overgaard and Nyegaard's material falls into a quite different category because it was new and quite exceptional. But it's not going to help me very much if, as happened in relation to Professor Guicheney, the report was apparently sought as at 13 October, as I understand the position from what you told me. Whilst I wish to give every party an opportunity to put anything they wish to put, I also want to complete the Inquiry, and it's in Ms Folbigg's interest that the Inquiry be completed as soon as possible.

In those circumstances, I do expect these directions to be complied with, and whilst I'm not going to say categorically that any evidence served in non-compliance with those directions will be rejected, there would have to be fairly good reasons as to (a) why it's late, and (b) why it's so significant that it be introduced. I don't want to say any more than that at this stage, but I do want this Inquiry's hearing to complete in February so we can comply with the timetable.

WOODS: Very well. I understand, your Honour, that Dr Guicheney's report will be admitted. Is it conditionally?

JUDICIAL OFFICER: No, it's not conditionally, but it doesn't necessarily mean that she'll be called to give evidence.

WOODS: We've heard what you say about the timetable.

JUDICIAL OFFICER: I do mean it.

WOODS: Yes, thank you.

JUDICIAL OFFICER: Anything else?

CALLAN: Can I provide a copy for the Court record of that report? No, your Honour, nothing else to raise.

JUDICIAL OFFICER: Very well.

ADJOURNED PART HEARD TO MONDAY 13 FEBRUARY 2023