Supplementary information

A Calculations of recurrence risks based on oocyte sampling: Vincent Macaulay

Case 2 Pearson's syndrome

Assumptions
Given the sensitivity of the measurement of the proportion of deleted molecules and the typical bottleneck sizes (~10 in single selection, ~200 in the repeated selection model), we can assume that no deleted molecules survived the bottleneck in the 10 samples oocytes, since the smallest possible non-zero proportion is 1/(bottleneck size) and this could have been detected.

We assume that each oocyte has passed independently through the bottleneck, i.e. that they are not derived from cells after the bottleneck.

Models
Single selection (binomial): bottleneck size n_s
Multiple selection (repeated binomial sampling in a Markov chain): bottleneck size n_m, repeated for g cycles

(Remember, that as far the variance of oocytes at least is concerned, the parameters are related roughly as $n_s = n_m / g$.)

Analysis
There are two sources of uncertainty that we have to describe in probability terms. One is of the actual level of deleted molecules in the germline, given the observation that in 10 oocytes we don't see anything. The other is the probability that any particular oocyte would cross the threshold of disease (here taken to be 66% deletion) if we knew the level in the germline. We can evaluate each of these and combine them using the laws of probability to determine the probability of disease given the oocyte data. (This is done by multiplying the two probabilities for each possible value of the unknown germline level and adding them up.) We can do this exactly for the single selection case, since the maths is easier, but we have to simulate the results on a computer for the multiple selection model.

Parameters
Single selection: we take n_s = 1, 2, ..., 20, 50, 100.
Multiple selection: we take n_m = 100, g = 20 (which should mimic n_s = 5), n_m = 200, g = 20 (which should mimic n_s = 10) and n_m = 20, g = 20 (which should mimic n_s = 1).

Results
Single selection:

n_s	Prob. of disease

1	8.33%
2	0.40%
3	0.53%
4	0.028%
5	0.0014%
6	0.0020%
7	0.0001%
> 7	< 0.0001%

Multiple selection:

n_m	g	Prob. of disease
20	20	2.60%
100	20	0.003%
200	20	< 0.001%

Supplementary tables 1 and 2

Probability of disease using single and multiple selection models in patient 2

Conclusion

Using typical bottleneck sizes (~10 in single selection, ~200 in the repeated selection model) the recurrence risk is <<1%. The risk given to the patient was conservative at <1%.

Case 1: 9176

Because the oocyte data is much less easy to model in this case (because mutant is present and because two oocytes are confounded), I've taken a slightly different approach.

Assumptions:

- 5% average mutant load in oocytes reflects the value before the sampling process
- Asymptomatic if <80% mutant load [1]

Method:

Calculate probability that either in a single selection or multiple selection an oocyte would have ≥80% mutant, if at the start the level was 5%.

Parameters:
Single selection: we take n_s = 1, 2, ..., 20, 50, 100.

Multiple selection: we take n_m = 100, g = 20 (which should mimic n_s = 5), n_m = 200, g = 20 (which should mimic n_s = 10) and n_m = 20, g = 20 (which should mimic n_s = 1).

Single selection (binomial sampling distribution)

n_s	Prob. of disease
1	5%
2	0.25%
>3	<0.01%

Multiple selection (repeated binomial samplings)

n_m	g	Prob. of disease
20	20	1.8%
100	20	<0.01%
200	20	<0.01%

Supplementary tables 3 and 4

Probability of disease using single and multiple selection models in patient 1

Alternative approach using the probability distribution model: David Samuels

An alternative to the simple binomial model is the Kimura probability distribution model[2]. For this approach we need the average mutant load in the sampled oocytes, which we take to be 5%, and the heteroplasmy variance in the sampled oocytes. The two non-separated oocytes complicate the measurement of the heteroplasmy variance. These two oocytes had an average heteroplasmy of 40%, and the most conservative assumption (meaning the assumption leading to the highest recurrence estimate) is that this pair of oocytes consisted of one oocyte with 80% heteroplasmy and the other with 0% heteroplasmy. This assumption gives a heteroplasmy variance of 0.035. Based on the heteroplasmy mean and variance, the probability distribution for the oocyte heteroplasmy values from 0 to 1 can be calculated as described in Wonnapinij and Samuels [2]. A KS test comparing the observed heteroplasmies in the 18 oocytes to the Kimura distribution yielded a p-value of 0.82, indicating that the measured values are consistent with the Kimura distribution model.

The recurrence probability was estimated by integrating the Kimura probability distribution from a threshold of 80% to 100%. The calculated recurrence probability was 3%, consistent with the largest recurrence estimates from the binomial model, and consistent with the observation of 1 in 18 sampled oocytes with a heteroplasmy of 80%.

Conclusion

In order to counsel the patient, we quoted the result as "risk ≤ 5%", the range because we were computing "model-based" estimates of risk (i.e. they depend on how we model the bottleneck). Confidence intervals in this context are not appropriate.

B Detailed Methods: analysis of oocytes

Oocytes were collected following a single course of gonadotrophin stimulation (20 for case 1 and 17 for case 2). The oocytes were washed 3 times in sterile PBS (phosphate buffered saline) and placed in individual 500 µl tubes in 1-3 µl PBS. 20 µl of Gene Releaser (Cambio Ltd) was added and the tubes were microwaved for 4 min 45 sec in a 900W microwave to release DNA. Prior to PCR amplification, the tubes were heated at 80°C for 10 min and 1 µl aliquots removed for PCR amplification using ABgene PCR Mastermix with 1.5 mM $MgCl_2$. Five pmoles of primer were used in a 50 µl reaction volume. Amplifications were performed for 40 cycles (1 min each at 94°C, 55°C, 72°C, with an initial denaturing step of 4 min at 94°C and a final extension of 10 min at 72°C).

The 9176T>C mutation can be detected by restriction digestion because it introduces a novel *Scr*F I restriction site [3]. After PCR amplification, 5 µl of fresh mastermix and 27 nmoles of ^{32}P radiolabelled dCTP were added and a further cycle of PCR amplification was performed with an extension step of 10 min. Ten microlitre aliquots were incubated overnight with 5 units of *Scr*F I restriction enzyme in 5 µl 1x digest buffer and 6 µl aliquots were electrophoresed on 6% acrylamide TBE gels. Radioactive bands were quantitated by Phosphorimager analysis. As little as 0.1% 9176T>C mtDNA could be detected on a wild type background (results not shown). This detection limit was determined by serial dilutions of DNA from the affected child into wild type DNA prior to PCR.

For the rearrangement, the PCR products were end-labelled and electrophoresed as for the point mutation samples. The sensitivity of the method was again high (1% could be detected).

C Preliminary study of human blastomeres

To study mtDNA segregation in preimplantation embryos and placentas we used naturally occurring polymorphic variants, because deriving sufficient clinical material from families with mtDNA disease would be a formidable task. Following our studies of mtDNA segregation in human oocytes [4], this approach has now been used to investigate early embryos[5] and placenta[6]. Inferences from all these studies are consistent with data on pathogenic variants [7-9].

Blastomere donors

Approval was obtained from the Oxford Research Ethics Committee and the UK Human Fertility and Embryology Authority (HFEA) to obtain, with informed consent, spare

embryos from women undergoing IVF, 26 embryos plus 1 unfertilised egg and 1 parthenogenetically activated egg from 5 patients were available for analysis (6, 13, 1, 2 and 6 eggs/embryos respectively from controls 1Bla-5Bla respectively).

Results

We aimed to determine whether mtDNA segregation could be detected in blastomeres from normal, healthy women attending an infertility clinic. It was necessary to identify heteroplasmic embryos in order to investigate mtDNA segregation. We therefore adapted a previously described [6] assay that detects heteroplasmy at two positions within mtDNA for single blastomeres (at bp 16,224 and 16,311). We assayed 26 embryos plus 1 unfertilised egg and 1 parthenogenetically activated egg from 5 donors (who provided 6, 13, 1, 2 and 6 eggs/embryos respectively). We then analysed embryos from three donors in detail, comparing the variation in level of heteroplasmic variant between runs, between embryos and between blastomeres within embryos.

Prevalence of heteroplasmy in the embryos

All of the embryos were homoplasmic at bp 16,311. Embryos from three mothers were assayed for heteroplasmy at bp 16,224 and all were found to have at least one embryo that was heteroplasmic (table 1).

Heteroplasmy between embryos/oocytes: Control 1Bla (n=6)

Six embryos were analysed from one of the patients with heteroplasmy at bp 16,224 who had the lowest overall level of variant mtDNA (range 0-2.8%, average across embryos 0.54%). In 4/6 embryos only normal type mtDNA was detectable. In one embryo, variant mtDNA was only detectable in 1/3 blastomeres, the other 2 had 100% normal mtDNA. Only one blastomere was available for analysis from the sixth embryo and this contained a low level of variant mtDNA.

Heteroplasmy between embryos/oocytes: Control 2Bla (n=13)

Twelve embryos and one parthenogenetically activated egg were analysed from a second patient with heteroplasmy at bp 16,224 (see figure 2). Only one blastomere was available for analysis from 3 of these embryos; 2-6 blastomeres per embryo were available from the remaining 10 embryos.

Range of mutant levels within single embryos

In all cases the level of heteroplasmic variant was low, with average loads of 1.9% per blastocyst (range 0-3.8%) and 2.0% per embryo range (1.7 -3.0%). The variance between embryos was slightly greater than that between blastomeres within an embryo, but neither reached statistical significance. The parthenogenetically activated egg was the only 'embryo' from which 6 blastomeres were available (asterisked in figure 2), and this had

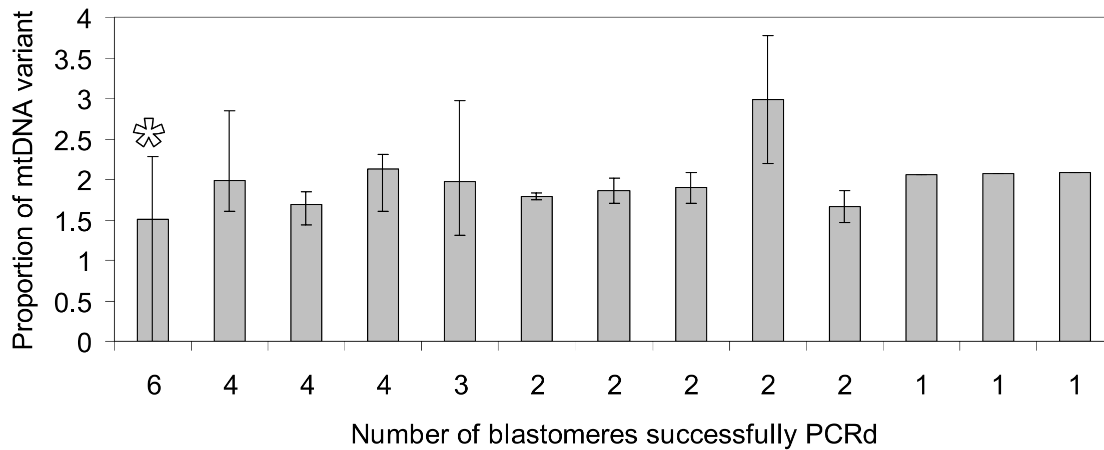
the widest range of load of polymorphic variant between blastomeres (that is 0 - 2.3%.) In most embryos the range between blastomeres was less than 1% (mean range 0.9%).

Heteroplasmy between embryos/oocytes: Control 3Bla (n=1)

Only one embryo was available from the 3rd patient and the levels of variant were similar in each of the 5 blastomeres (average 3.0%, range 2.4-3.6%).

Mother	Number of Blastomeres	Average %variant	Range (%)
Control Bla1	3	0.4	0-1.3
n=6	2	0	0
	1	2.8	
	1	0	
	1	0	
	1	0	
Control Bla3	5	3	2.4-3.6
n=1			

Supplementary Table 5 Load of T16,224C variant mtDNA in 20 embryos or unfertilized ooctyes from 3 control women.



Supplementary figure 1: Bar chart showing the level and range of heteroplasmy in 13 embryos from control 2Bla.

Similar levels of heteroplasmy at bp 16,224 in 13 embryos from one woman, arranged by number of blastomeres successfully amplified by PCR (error bars indicate the range of variant level in individual blastomeres, asterisk indicates one parthenogenetic embryo in which there was a single blastomere with undetectable mutant).

Conclusion of blastomere study

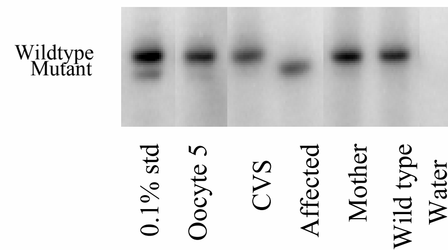
The use of polymorphic variants to investigate mtDNA segregation in the germline enabled us to use the small quantities of human material that are available for study, data on pathogenic variants being very scanty. Levels of heteroplasmy were similar within an embryo.

D Supplementary Figure 2:

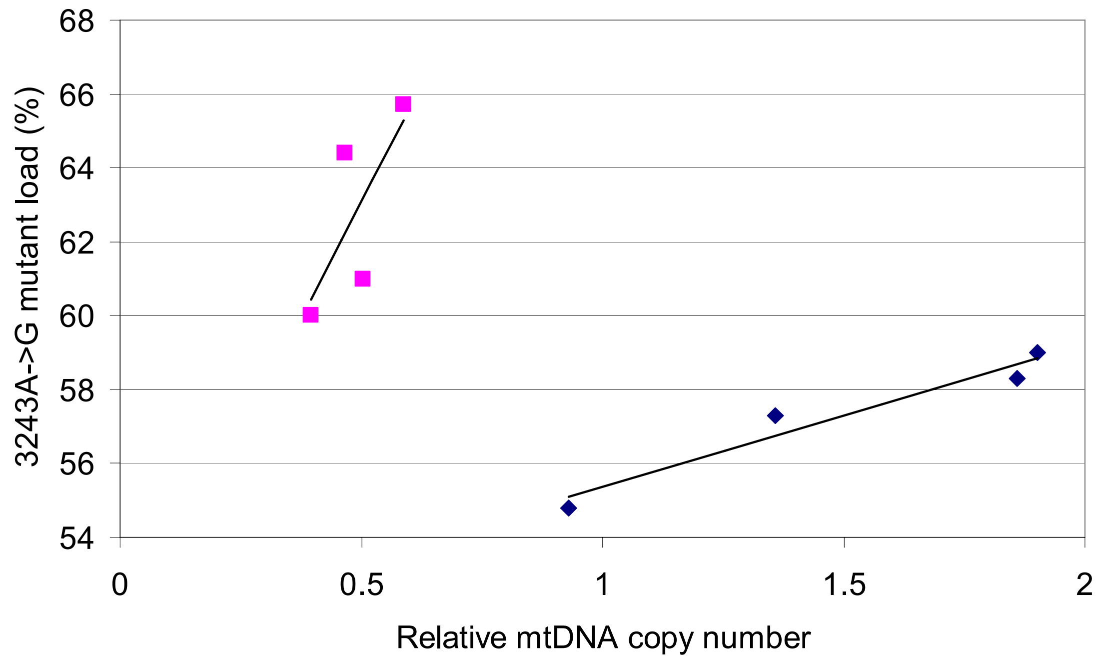
No 9176T>C mutant mtDNA was detectable in PCR product from CVS

The 9176T>C mutant mtDNA generates a new restriction site, so that, in the affected child the upper wild type (normal) band is replaced by a lower (mutant) fragment. The child carried ~99% 9176T>C mutant mtDNA in all tissues examined (marked "affected"), but none was detectable in the mother's blood, oocyte 5 or CVS. Lane 1 shows a 0.1% standard, demonstrating a sensitivity of >0.01%. While on this image, there could be a trace of mutant mtDNA in oocyte 5 by eye (but not on quantitative analysis), this would have been below 0.1%. Traces at this level would not affect the results of the recurrence risks calculation.

Supplementary Figure 2: No 9176T>C mutant mtDNA was detectable in CVS



E Supplementary Figure 3



MtDNA mutant load plotted against mtDNA copy number in each of 8 placental samples from a woman carring the 3243A>G mutations

The placental samples appear to fall into two groups, in each case the mutant load being proportional to mtDNA copy number. We were unable to identify the basis for the apparent separation into the two groups, micro satellite analysis indicating that all samples were fetal and not maternal in origin.

References

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