Bachelor Thesis of

Raphaël Morsomme

Acknowledgment

I am grateful to my supervisor Patrick Lindsey, who has devoted countless hours to this project. His investment far exceeded my expectations and without the time and energy he put into it, this thesis would not have been half as enriching and educative. I also want to thank Rick Kamps, who shared his data with us. I can promise him that we took good care of them and I hope that the results of this thesis will be of some help in his work on IVF. Finally, this project would not have been possible without the initial help of Ronald Westra. I thank him for putting me in contact with Patrick Lindsey and wish him a prompt recovery from his recent stay at the hospital.

Introduction

In-vitro fertilization (IVF) treatments such as intracytoplasmic sperm injection (ICSI) and pre-implementation genetic diagnosis (PGD) procedures are types of assisted reproductive technologies used for infertility treatment. Conducted with success for the first time in 1978 by Dr. Steptoe and Dr. Edwards in the U.K., IVF has since helped more than 5 million couples to conceive children (Adamson et al., 2013). IVF treatments are used to overcome numerous causes of infertility including, but not limited to, a defect in sperm quality, serious genetic conditions of one of the partners, a weaknesses in the mitochondria of the female partner or menopause.

IVF treatments are procedures that are emotionally and financially demanding for the couples who resort to them. Firstly, IVF procedures are an important source of stress for the couple (Beutel et al., 1999). Women are, for instance, advised to prepare their body for at least three months before starting an IVF procedure in order to maximize the chance of having a successful pregnancy. Besides paying attention to their nutrition and level of stress, they must, with their male partner, also complete a series of checks at the hospital to establish the optimal IVF treatment. Secondly, IVF treatments are costly. In the Netherlands, the total cost of a cycle can amount to more than 10,000€. While the Netherlands had subsidized public-sector programs, these are too scant to accommodate the large number of couples resorting to IVF each year and although some Dutch insurance companies cover the procedure, for instance *Hollandzorg* reimburses the first three attempts in case of medical indication, the majority of couples resorting to IVF cover most of the costs themselves.

Yet, despite advances in assisted reproductive technology, the success rate of IVF procedures was only 42% for women younger than 35 in the U.S.A. in 2015 and decreases with age (SART Clinic Summary Report, 2015). This means that the majority of couples resorting to an IVF treatment to conceive a child can expect to have to go through this emotionally and financially demanding process multiple times before having a successful pregnancy.

Clinicians are therefore constantly trying to increase the success rate of IVF treatments. In this line, researchers have recently started to appreciate the role played by mitochondrial activity in early embryonic development. Mitochondria are cellular structures which have long been solely conceptualized as the energy powerhouse of the cell. Yet, recent researches have shown that mitochondria not only convert the energy

from food into a form that cells can use, but are also an integrated component of the cell with a central function in the execution of diverse cellular events (McBride, 2006). Besides regulating metabolism via the production of ATP (the source of energy that the cell uses), they also act as a signaling platform for cell-cycle progression, and are involved in cellular differentiation, cell death (Youle, 2005; Bossy-Wetzel et al., 2003), antiviral response (Seth et al., 2005), as well as cell development (Chen et al., 2003). Several studies highlight the importance of mitochondrial dynamics during embryonic development (Chen et al., 2002; Chen et al, 2003; Fragouli et al., 2015; May-Panloup, 2016). The oocyte is the richest cell of the body in mitochondria and depends largely on these cellular structures for fertilization and early embryonic development. These studies show that suboptimal levels of mitochondria-generated ATP contribute to a variety of embryo developmental defects, and therefore adversely affect the success rates of IVF treatments. Furthermore, Ogino et al. (2015) show that the mitochondrial content of oocyte cells is a good predictor of good-quality embryos. The role that mitochondrial activity plays in early cell development is thus of particular importance to the clinicians operating at the department of reproductive medicine of Maastricht University Medical Center+.

The overarching goal of this thesis is to assist clinicians in their quest to increase the overall success rates of IVF procedures by gaining a deeper understanding of how mitochondrial activity is related to early embryonic development. To accomplish this, clinicians are mostly attentive to two elements: the quality of the embryo that is transferred back to the woman and the state of the receiving woman. On the one hand, they investigate new potential criteria for the selection of embryos in order to re-implant embryos that are, on average, of better quality; on the other hand, they can ensure that the conditions of the uterine tissues that will receive the embryo in question are optimal for pregnancy. The first project of this thesis deals with the former of these two elements and the second project with the latter. Project 1 studies how to model the mitochondrial content of embryos to predict their quality. Ogino et al. (2015) show that the copy number of the mitochondrial DNA (mtDNA) in oocyte cells is a good predictor for the quality of the embryo. We investigate whether the copy number of the mtDNA in cumulus cells would be a suitable surrogate for this purpose. Project 2 aims at gaining a better understanding of the mitochondria of uterine cells. In particular, we model the evolution of the mtDNA copy number in the uterine tissues that will receive the embryo during a

menstrual cycle and over the lifetime of women. The third and final project of this thesis aims to establish the circumstances under which a mitochondrial replacement therapy (MRT) procedure would be more suitable than a classic IVF treatment. Next to the cellular DNA, which is packaged in chromosomes within the nucleus of the cell, mitochondria also have their own DNA, known as mitochondrial DNA or mtDNA. Since children receive the entirety of the mitochondria found in their cells from their mother, there is strong correlation between the quality of the mother's mitochondria and that of the child's. If the woman's mtDNA is characterized by a high level of mutation load, then it may prove impossible to obtain an embryo suitable for re-implantation using classic IVF treatments. In such cases, the mitochondrial replacement therapy (MRT) may offer an alternative as it substitutes the unhealthy mitochondria of the mother with healthy mitochondria from a third-party woman. Yet, the procedure raises serious ethical concerns (for instance, since mtDNA may have an impact on people's personality, children conceived via MRT effectively have inherited traits from three individuals (Liao, 2017)) and the Netherlands has yet to approve the practice. Project 3 attempts to develop a model that determines the probability of obtaining an embryo with mitochondria that are healthy enough to be suitable for re-implantation using classic IVF treatments when the woman's mitochondria suffer from high loads of genetic mutation. This model can then be used to identify cases in which the probability that classic IVF treatments succeed is so small that an MRT procedure should be preferred.

This thesis is a bundle of these three projects. For each, I analyzed the data using the software RStudio[1] under the supervision assistant professor P. Lindsey and produced a report presenting the main findings. These reports form the chapters of my thesis and follow the following structure: I first briefly introduce the context in which the research is realized and its relevance in the context of IVF, I then present the data that was used and the methods we employed to analyze them, I finally discuss the main findings as well as suggestions for potential follow-up research projects. Since my knowledge of cellular biology is limited and the goal of my thesis is to assist clinicians analyze their data, the discussion of the results mainly focuses on recommendations for future studies.

I would like to conclude this introduction with a brief reflection on the nature of the data analysis projects that I conducted in this thesis – especially those in project 1 and project 2. The study of mitochondrial activity in the context of IVF treatment is a new area

[1] Ask the coordinator of the Capstone project for the code.

of research in biology which still needs to be further developed. Clinicians have not yet established clear relations between variables such as the mitochondrial activity of embryos, that of uterine cells, the quality of the embryos or the age of the mothers. The data analyses that I conducted are the first steps of larger-scale research projects conducted at the department of reproductive medicine of Maastricht University Medical Center+ and their main objective is to offer guidance for follow-up research. As such, they are pilot studies and my objective is to *explore* the data, rather than testing a given set of hypotheses. I seek to identify potential relations between the variables that are worth investigating further, rather than drawing inferential conclusions. Since no clear relations between the different variables have been established yet, I approached the analysis of the data with an open mind and modelled the data in a fairly free manner. I used the Akaike Information Criterion (AIC) in order to identify which of the created models offers the best fit of the data. The AIC is an estimator of the complete quality of a statistical model (Akaike, 1998). It takes into account not only the likelihood of the model, i.e. how well it fits the data points, but also its simplicity, i.e. how many parameters the model has. By penalizing models with large numbers of parameters, the AIC takes into consideration the trade-off between goodness-of-fit and parsimony. The main advantage of using models' AIC as a selection criterion rather than conducting a Chi-square test to compare them is that, unlike the latter approach, it is not limited to models that are nested. As long as the data on which they are fitted are the same, models – no matter how drastically different they are – can be compared using the AIC. Given the exploratory nature of the projects of this thesis, this characteristic of the AIC makes this measure of the quality of a model the ideal selection criterion.

Project 1: Surrogacy of mitochondrial content in cumulus cells for that in oocyte cells in the prediction of embryo quality.

Abstract

In the context of *in-vitro* fertilization (IVF), clinicians use a series of criteria to select which of the embryos that they have fertilized in vitro is to be transferred back to the woman. This selection procedure is chosen to have a positive impact on the success rate of the consequent pregnancies. Yet, despite advances in assisted reproductive technology, the success rate of IVF procedures was only 42% for women younger than 35 in the U.S.A. in 2015 and decreases with age (SART Clinic Summary Report, 2015). Improving the success rate of these emotionally as well as financially demanding procedures would not only benefit the couples that resort to them, but also decrease public expenditure for this type of programs. Recent researches have established the existence of a relationship between the mitochondrial content of embryos and their quality (Ogino et al., 2015). Drawing on these results, we investigate whether the copy number of mitochondrial DNA (mtDNA) in oocyte cells is a good predictor of the quality of the embryos, and whether the copy number of mtDNA in cumulus cells can be used as a surrogate for that in oocyte cells when predicting embryo quality. We observe that the mitochondrial content of the oocyte cells of the embryo can be modelled to predict its quality with an accuracy level superior to 80%. Yet, the mitochondrial content of cumulus cells does not appear to be a suitable surrogate for that of oocyte cells for this purpose.

In-vitro fertilization (IVF) procedures are types of assisted reproductive technology used for infertility treatment. In these programs, clinicians collect 12 to 20 oocytes from the mother's ovaries and fertilize them *in-vitro*. The in-vitro fertilized eggs are kept in culture between 3 and 5 days where they undergo cleavage. At the end of the culture period, clinicians transfer back to the woman's womb the embryo which they deem most likely to yield to a successful pregnancy. In the Netherlands, only one embryo is re-implanted in

order to avoid multiple pregnancies. The selection procedure is based upon several criteria including, but not limited to, the embryo's morphology, the mutation load of its mitochondria DNA (mtDNA) and, in the case of parents with serious genetic inherited conditions, whether or not the embryo carries the condition. To assess the embryo's morphology, clinicians conduct a visual assessment (Ziebe et al., 1997). This non-invasive procedure enables them to determine the number and distribution of blastomeres. Embryos with a satisfying level of fragmentation and composed of a large number of (multinucleated) blastomeres are more likely to yield to a successful pregnancy and are thus preferred for re-implantation. Clinicians also evaluate the mutation load of the embryo's mtDNA in the case of couples who resort to pre-implementation genetic diagnosis (PGD) because the woman has a high level of mtDNA mutation load (see project 3). This is an excluding criterion: embryos with a mutation load superior to 15% are too unlikely to yield to a successful pregnancy and are automatically discarded. Furthermore, in the case of parents who resort to PGD in order to prevent passing on a particular genetic condition to their child, clinicians analyze the embryo's nuclear DNA. Clinicians analyze the gene(s) concerned by the condition in order to determine whether or not the embryo carries it. This is an excluding criterion too. Embryos carrying the condition are deemed unsuitable for re-implantation and are discarded. Using these criteria, clinicians transfer back to the woman's womb the embryo that they deem most likely to yield to a successful pregnancy. This selection procedure is chosen to have a positive impact on the success rate of the consequent pregnancies in the context of IVF; implantation rates after elective embryo transfer are significantly higher as compared to non-elective embryo transfer (Staessens et al., 1993).

Clinicians recently started to appreciate the role played by mitochondrial activity in embryonic development (Ogino et al., 2015; Klinge, 2017). Recent studies have for instance established the existence of a relationship between the mitochondrial content of embryos and their quality (Ogino et al., 2015). Their results indicate that the mtDNA copy number in oocyte cells ($\mathrm{mtDNA_{oocyte}}$) of embryos is a good predictor for their quality. Drawing on these results, the goal of this research project is twofold: (i) investigating if the $\mathrm{mtDNA_{oocyte}}$ copy number can be modelled to predict the quality of the embryo, and (ii) if part (i) of the project is conclusive, determining whether the copy number of mtDNA in cumulus cells ($\mathrm{mtDNA_{cumulus}}$) can be used as a surrogate for the $\mathrm{mtDNA_{oocyte}}$ copy number when predicting the quality of the embryo.

If the mitochondrial content of embryos is a good predictor for their quality, then clinicians can use it as an additional criterion when selecting which of the embryos kept in culture is to be transferred back to the woman's womb. If correctly integrated to the existing procedure, making use of this additional criterion should ensure that the embryos that are re-implanted are, on average, of better quality. This will result in an increase in the overall birth rate, thereby decreasing the stress for couples resorting to IVF procedures and reducing the total amount of public expenditure for this type of programs.

Yet, obtaining the $\mathrm{mtDNA}_{\mathrm{oocyte}}$ copy number is, in practice, not possible. Collecting an oocyte cell to analyze its mitochondrial content would cause too much damage to the embryo and significantly decrease its likelihood to yield to a successful pregnancy. The embryo is composed of two types of cells: oocyte cells and cumulus cells. The core of the embryo is composed of oocytes cells, which will eventually become the fetus. This core is surrounded by a layer of cumulus cells that act as a protective belt during the maturation of the embryo. This protective layer of cumulus cells will later become the placenta. Since oocyte cells divide into two daughter cells on average once per day, the embryos contain at most 32 oocyte cells during the five days in which they are kept into culture before being transferred back to the mother's womb. Harvesting one of them in order to determine its mtDNA copy number would thus significantly decrease the likelihood that the embryo has to yield to a successful pregnancy. Hence, in practice, clinicians cannot use the mitochondrial content of oocyte cells as a selection criterion when choosing which embryo to re-implant. However, harvesting cumulus cells does not cause any harm to the embryo. In fact, the IVF procedure requires clinicians to wash away the cumulus cells. Since the mitochondria of the oocytes cells and those of the cumulus cells exclusively come from the same individual i.e. the mother, one can expect the mitochondrial content of both types of cells to be closely related. Hence, clinicians would like to know whether they can use the $\mathrm{mtDNA}_{\mathrm{cumulus}}$ copy number of embryos, which they have access to, as a surrogate for that of the $\mathrm{mtDNA}_{\mathrm{oocyte}}$ to predict the quality of the embryo.

Data

The dataset is composed of 16 oocytes collected on 8 women. It contains the following variables: the age of the women, the $\mathrm{mtDNA}_{\mathrm{cumulus}}$ copy number per single cell, the $\mathrm{mtDNA}_{\mathrm{oocyte}}$ copy number per single cell and the quality grade of the embryos as

determine by the clinicians operating at the department of reproductive medicine of Maastricht University Medical Center+ (see Table 1.1). In the context of assisted procreation, clinicians grade the quality of the embryos using the following categories:

- *germinal vesicle* (GV): the embryo has not begun meiosis (a type of cellular division) yet and is thus considered as immature,
- *metaphase 1* (M1): the embryo is in the first phase of meiosis but is not completely mature yet since it has not entered the second phase of meiosis
- *no pronucleus* (0PN): the embryo has not been fertilized and therefore shows no pronucleus,
- *two distinct pronuclei* and two polar bodies (2PN): the embryo has been fertilized but has not divided correctly,
- *healthy*: the embryo has been fertilized, has divided correctly and the clinician deems it fit for being transferred back to the woman's womb.

It is worth noting that since clinicians are, for ethical reasons, not allowed to analyze *healthy* embryos that were not re-implanted, the sample only contains *unhealthy* embryos i.e. embryos with quality grades GV, M1, 0 PN or 2 PN. Table 1.2 offers presents the minima, maxima and average value of each variable across these four quality grades.

Table 1.1 – Dataset

Date	Quality Grade	$\mathbf{MtDNA_{oocyte}}$ Copy Number	$\mathbf{MtDNA_{cumulus}}$ Copy Number	Age of Mother
3/24/2017	2 PN	1,360	256	29
3/24/2017	0 PN	8,653	68	29
4/4/2017	GV	16,249	52	29
4/4/2017	0 PN	67,206	160	29
4/4/2017	0 PN	63,950	589	29
5/24/2017	0 PN	186,023	186	29
5/24/2017	0 PN	239,067	246	29
5/24/2017	M1	1,188	373	29
6/27/2017	GV	163,091	293	30
6/27/2017	GV	39,286	290	30
10/2/2017	M1	234,918	303	32
10/2/2017	0 PN	17,489	334	32
10/2/2017	0 PN	43,340	251	32
10/2/2017	missing	29,721	290	32
9/19/2017	M1	115,738	328	35
2/7/2017	2 PN	217,177	390	39
2/21/2017	0 PN	46,181	123	40

Table 1.2 – Descriptive Statistics per Quality Grade

Quality Grade	Count	$\mathbf{MtDNA_{oocyte}}$ Copy Number per Single Cell			$\mathbf{MtDNA_{cumulus}}$ Copy Number per Single Cell			Age of Women		
		Min	Mean	Max	Min	Mean	Max	Min	Mean	Max
GV	3	16,249	72,875	163,091	52	212	293	29	30	30
M1	3	1,188	117,281	234,918	303	335	373	29	32	35
0PN	8	8,653	83,989	239,067	68	245	589	29	31	40
2PN	2	1,360	109,268	217,177	256	323	390	29	34	39
Total	16	1,188	91,307	239,067	52	265	589	29	31	40

Method

We develop several regression models to determine how to model the mitochondrial content of embryos in order to predict their quality. More specifically, we develop three multinomial regression models that (i) model the $\mathrm{mtDNA}_{\mathrm{oocyte}}$ copy number of embryos to predict their quality grade (model 1, see table 1.3) and (ii) assess whether the $\mathrm{mtDNA}_{\mathrm{cumulus}}$ copy number is an appropriate surrogate for that of oocyte cells to predict the quality grade of embryos (model 2 and model 3). Although the categories of the response variable, i.e. the embryo's quality grade, are ordinal – with, in increasing order of quality: GV, M1, 0PN and 2PN – we consider them as nominal because it is not obvious how to define the step size between the categories e.g. constant, exponential or another mathematical relationship. Furthermore, since this is a pilot study, we limit ourselves to determining whether there exists a relation between the mitochondrial content of embryos and their quality, not what the exact nature of this relation is. This would be the aim of a follow-up study. Thus, for our purpose, considering the quality grades as nominal instead or ordinal categories is sufficient. Since some embryos come from the same parents, the models include a random effect in order to take inter-individual variability into account. We use the Akaike Information Criterion (AIC) to evaluate how well the models fit the data and to compare the models with one another (Akaike, 1998). Since the data on which we fit the models are the same, the one with the lowest AIC is preferred. We further evaluate the level of accuracy of the obtained models with the proportion of embryos that they classify correctly. We consider that a model classifies an embryo correctly if it assigns the largest probability to the appropriate quality grade. To have a point of comparison, since the largest category of the response variable, i.e. 0PN, possesses 8 out of the 16 observations, a naïve model that classifies all embryos as 0PN would have an accuracy level of 50%. We finally conduct a sensitivity-specificity analysis and compute the mean area under the curve (AUC) across the four categories of the response variable to evaluate the performance of the models.

Table 1.3 offers an overview of the three multinomial regression models that we developed. Model 1 predicts the quality grade of embryos using their $\mathrm{mtDNA}_{\mathrm{oocyte}}$ copy number and the age of the woman from whom they were collected as predictors (research question 1). It includes an interaction between the two predictors. We develop two additional multinomial regression models (model 2 and model 3) in order to determine whether the $\mathrm{mtDNA}_{\mathrm{cumulus}}$ copy number can be used as a surrogate for the $\mathrm{mtDNA}_{\mathrm{oocyte}}$

copy number to predict the quality grade of the embryos (research question 2). Model 2 is an extension of model 1 that includes the $\text{mtDNA}_{\text{cumulus}}$ copy number as a third predictor. It includes an interaction between the age of the woman and the $\text{mtDNA}_{\text{cumulus}}$ copy number, and an interaction between the age of the woman and the $\text{mtDNA}_{\text{oocyte}}$ copy number. In model 3, we use the same settings as in model 1 and substitute the $\text{mtDNA}_{\text{cumulus}}$ copy number for the $\text{mtDNA}_{\text{oocyte}}$ copy number. If the $\text{mtDNA}_{\text{cumulus}}$ copy number is a suitable surrogate for the $\text{mtDNA}_{\text{oocyte}}$ copy number, we will observe the following: first, the latter variable will not be statistically significant in model 2 and second, model 3 will be as performant as model 1.

Table 1.3 – Overview of Multinomial Models for Quality Grade

Model	Predictors	Interaction Term	AIC	Accuracy Level	AUC
Model 1	$\text{mtDNA}_{\text{oocyte}}$ copy number, women's age	$\text{mtDNA}_{\text{oocyte}}$ copy number with women's age	34	0.81	0.97
Model 1 bis	$\text{mtDNA}_{\text{oocyte}}$ copy number, women's age	/	55.1	0.5	0.51
Model 2	$\text{mtDNA}_{\text{oocyte}}$ copy number, $\text{mtDNA}_{\text{cumulus}}$ copy number, women's age	$\text{mtDNA}_{\text{cumulus}}$ copy number with women's age and $\text{mtDNA}_{\text{oocyte}}$ copy number with women's age	42	0.875	0.98[2]
Model 3	$\text{mtDNA}_{\text{cumulus}}$ copy number, women's age	$\text{mtDNA}_{\text{cumulus}}$ copy number with women's age	52	0.56	0.79
Naïve Model	/	/	45.5	0.5	0.5

[2] This value has to be taken with caution since the AUC is 1 for two categories i.e. M1 and 2PN. Obtaining an AUC of 1 is an indication that the model overfits the data.

Results

Model 1's AIC is smaller (34) than that of the naïve model (45.5). This indicates that the $\mathrm{mtDNA}_{\mathrm{oocyte}}$ copy number of an embryo is, in combination with the age of the woman from whom it is collected, a good predictor for the quality of the embryo (question 1). The interaction between the two predictors of model 1 is required in the model since removing the interaction term gives a model (model 1 bis) that offers a less good fit of the data (AIC of 55.1). Figure 1.1 presents the quality grade predicted by model 1 for each embryo. Model 1 correctly classifies 81% of the data points. The AUC for the quality grades GV, M1, 0PN and 2PN are respectively 0.97, 0.97, 0.96 and 0.96 (see figure 1.2), giving a mean AUC of 0.97. Figure 1.3 presents the probability computed by model 1 for the four quality grades. More specifically, the plots show the probability for an embryo to have a particular quality grade across various combinations of woman's age and $\mathrm{mtDNA}_{\mathrm{oocyte}}$ copy number as estimated by model 1. The darker the color, the larger the probability is. For instance, we can see that there are two distinct types of embryos that are very likely to have a quality grade 2PN: embryos with a low $\mathrm{mtDNA}_{\mathrm{oocyte}}$ copy number (less than 50,000) and coming from a woman older than 30, *and* embryos with an $\mathrm{mtDNA}_{\mathrm{oocyte}}$ copy number larger than 75,000 and coming from a young mother (less than 30 years old).

Figure 1.3 indicates that model 1 produces a clear cut prediction for the quality grade of embryos. Model 1 assigns either a very high (close to 1) of very low (close to 0) probability of being of a certain quality grade for most combinations of the woman's age and embryo's $\mathrm{mtDNA}_{\mathrm{oocyte}}$ copy number. In other words, given an embryo with a particular combination of $\mathrm{mtDNA}_{\mathrm{oocyte}}$ copy number and woman's age, the model is very likely to give an *unambiguous* classification i.e. close to 0 or close to 1 for each of the four quality grade. Together with the high accuracy level of model 1 (81% of accurate classification) and the large mean AUC (superior to 97%), the clear cut predictions of the model indicates that the mitochondrial content of oocyte cells, in combination with the age of the mother, is a *reliable* predictor for the quality of the embryo.

The $\mathrm{mtDNA}_{\mathrm{cumulus}}$ copy number of an embryo does, however, not appear to be a suitable surrogate for the $\mathrm{mtDNA}_{\mathrm{oocyte}}$ copy number to predict the quality grade of the embryo. The AIC of model 2 (42) and model 3 (52) are superior to that of model 1 (34) and the accuracy level of model 3 is lower (0.56) than that of model 1 (0.81) (see figure 1.4). The AUC of model 3 for the quality grades GV, M1, 0PN and 2PN are respectively

0.74, 0.85, 0.59 and 0.96, giving a mean AUC of 0.79 which is smaller than that of model 1 (0.97).

Figure 1.1 – Classification of Model 1

		Predicted Quality Grade			
		GV	M1	0PN	2PN
Actual Quality Grade	GV	2	0	1	0
	M1	0	2	0	1
	0PN	1	0	7	0
	2PN	0	0	0	2

Figure 1.2 – ROC Curves of Model 1

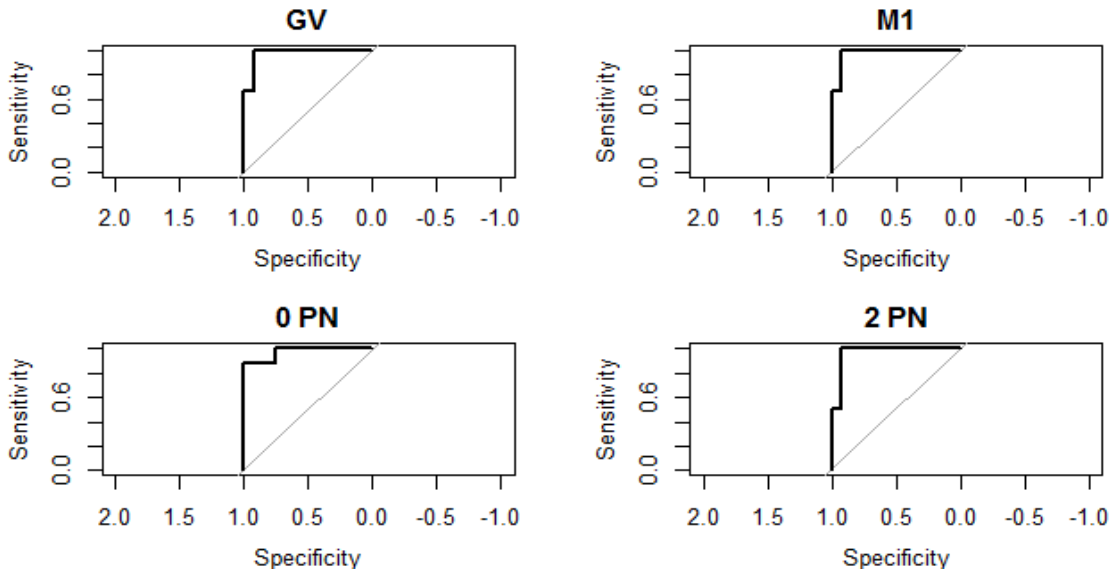


Figure 1.3 – Probabilities Estimated by Model 1

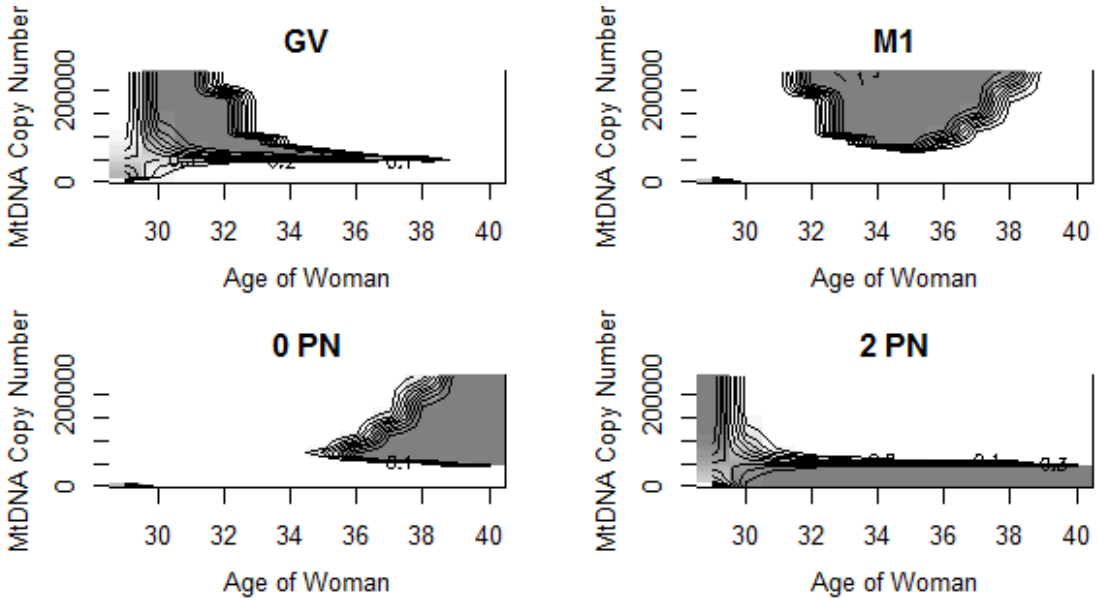


Figure 1.4 – Classification of Model 3

		Predicted Quality Grade			
		GV	M1	0PN	2PN
Actual Quality Grade	GV	0	0	3	0
	M1	0	1	2	0
	0PN	0	1	7	0
	2PN	0	0	1	1

Discussion

These findings confirm the claim that clinicians could use the mitochondrial content of embryos as an additional criterion for the selection of embryos in the context of IVF. How to integrate this criterion to the existing framework in the most efficient way – whether the mitochondrial content of embryos should replace existing criteria, or complement them – should be investigated in a follow-up research. More specifically, the $\mathrm{mtDNA}_{\mathrm{oocyte}}$ copy number of an embryo appears to be an accurate predictor for its quality grade. Together with the age of the woman, it produces a clear-cut prediction that is accurate in 81% of the cases, meaning that it could potentially be an effective selection criterion.

For instance, model 1 tells us that if a woman below age 30 has an embryo which is deemed unsuitable for re-implantation, then the embryo in question is extremely likely (probability close to 1) to have a quality grade of 2PN, regardless of its $\mathrm{mtDNA}_{\mathrm{oocyte}}$ copy number (see figure 1.1 – 2PN). This means that when women below age 30 have an embryo that is deemed unsuitable for re-implantation, it will still have been fertilized, have two distinctive pronuclei two polar bodies (2PN) but will have failed to divide correctly. Since 2PN is the quality grade that is closest to *healthy*, model 1 suggests that women below the age of 30 either produce healthy embryos or embryos that are almost healthy, i.e. embryo with a quality grade 2PN.

Since the $\mathrm{mtDNA}_{\mathrm{oocyte}}$ copy number is, in practice, not accessible to clinicians, we also investigated whether the $\mathrm{mtDNA}_{\mathrm{cumulus}}$ copy number is a suitable surrogate to predict the quality of embryos. The data do not corroborate this hypothesis. The $\mathrm{mtDNA}_{\mathrm{cumulus}}$ copy number of an embryo does not appear to be a suitable surrogate for the $\mathrm{mtDNA}_{\mathrm{oocyte}}$ copy number to predict the quality grade of the embryo.

It is important to note that the sample has a relatively small size (16 embryos). Although we failed to model the $\mathrm{mtDNA}_{\mathrm{cumulus}}$ copy number of an embryo to predict its quality, this does not mean that there is no relation between the two variables and that the $\mathrm{mtDNA}_{\mathrm{cumulus}}$ copy number of an embryo is *not* a suitable surrogate for its $\mathrm{mtDNA}_{\mathrm{oocyte}}$ copy number. Conducting a similar analysis on a larger dataset would make the regression models more powerful and could potentially allow us to identify trends that we failed to detect in the small dataset used in this project. Since the mitochondria of the oocytes cells and those of the cumulus cells exclusively come from the same individual i.e. the mother, we can expect the mitochondrial content of both types of cells to be closely related. Model 1 shows that the $\mathrm{mtDNA}_{\mathrm{oocyte}}$ copy number of embryo is a good predictor for their quality, it is thus reasonable to expect the mitochondrial content of cumulus cells to have a certain predictive power too. Collecting more data may thus allow us to identify the relation between the $\mathrm{mtDNA}_{\mathrm{cumulus}}$ copy number of embryo and their quality.

Furthermore, taking the mutation load of mtDNA into account should improve the accuracy of the model. The copy number only indicates the *quantity* of mitochondria present in a single cell, but this number does not directly say how much energy they produce. The mutation load of the mtDNA also has an impact on the amount of energy produce by the mitochondria. Certain mutations on the mtDNA cause mitochondria to

produce less – or even no – energy. Computing the proportion of mitochondria that do not suffer from such mutation and multiplying this number by the copy number in order to have an estimate of the number of *healthy* mitochondria per single cell would produce a more reliable estimate of how much energy the cell is able to produce. Modelling the number of healthy mitochondria per single cell instead of their copy number could thereby help us identify the relation between the mitochondrial content of cumulus cells and the quality of embryos.

The data analysis also suffered from the fact the dataset only contains embryos that were deemed unfit for re-implantation i.e. embryos with quality grades GV, M1, 0 PN or 2 PN. This bias in the data that were available for this study results from the fact that clinicians are, for ethical reasons, not allowed to analyze the *healthy* embryos that were not re-implanted. This is problematic since in order to determining how the mitochondrial content of embryos can be used by clinicians to distinguish between embryos that will yield to a successful pregnancies and those that will not, it is necessary to have data on both types of embryos. Since our dataset was limited to unhealthy embryos, the regression models we developed are only able to distinguish between the following four quality grades: GV, M1, 0 PN and 2 PN; but not between *healthy* and *unhealthy* oocytes. Would the hospital be allowed to analyze the mitochondrial content of embryos deemed suitable for re-implantation, we would be able to develop a model that would help clinicians achieve their stated goal i.e. distinguishing between *healthy* and *unhealthy* oocytes. We therefore suggest the hospital to lobby the Dutch national committee for ethics to allow clinicians to analyze the mitochondrial content of embryos that were deemed suitable for re-implantation before discarding them. In most cases, embryos that were deemed suitable for re-implantation will be frozen in case the IVF procedure fails or in case the parents would like to have more children in the future. However, there will be cases where the pregnancy is successful and the parents have no desire to have more children. If clinicians were allowed, in those specific cases, to analyze the mitochondrial content of healthy embryos before discarding them, we would possess data on both healthy and unhealthy embryos and could develop a regression model that determines how the mitochondrial content of embryos can be used to distinguish between embryos that are likely to yield to a successful pregnancy and those that are not.

Finally, the models we have developed run the risk of *overfitting* the data; that is, they model the specific data points of the sample instead of modelling the underlying

mechanism connecting the predictors and the response variable. For instance, our most parsimonious models (model 1 and model 3) contain a total of 12 parameters to classify the 16 observations of the sample. This is problematic because, since they fail to detect the underlying trends, overfitting models may not be generalizable to the broader population (although they fit the data points of the sample relatively well). This could, for instance, be the case for model 1, which, although it has a high level of accuracy with more than 80% of correctly classified data points, might not be generalized to the broader population of embryos. Besides making the models even more parsimonious – which would, in this case, produce models with very little predictive power and is thus not a viable option (e.g. model 1 bis and naïve model, see table 1.3) – the only solution would be to collect a larger amount of data. As Long (1997) pointed out, "it is risky to use logistic regression with samples smaller than 100, while sample over 500 seem adequate" (p. 54) and added that "a rule of at least 10 observations per parameter seem reasonable" (p. 54). Since multinomial regression models are more complex that logistic regression models, one should therefore dispose of dataset of at least 100 observations.

Conclusion

In the Netherlands, clinicians who conduct IVF treatments transfer back to the woman's womb only one embryo. Clinicians use several criteria the select which of the eggs they have fertilized *in-vitro* is to be re-implanted including the embryo's morphology, its mtDNA mutation load of its mitochondria DNA and, in some cases, if it carries genetic conditions of the parents. This selection procedure is chosen to have a positive impact on the success rate of the consequent pregnancies.

Clinicians recently started to appreciate the role played by mitochondrial activity in embryonic development. In this project, we investigate whether the mitochondrial content of and embryo can be modelled to predict its quality and could therefore be a potential selection criterion. We find that the $\mathrm{mtDNA}_{\mathrm{oocyte}}$ copy number of an embryo appears to be an accurate predictor for its quality grade. Together with the age of the woman, it produces a clear-cut prediction that is accurate in 81% of the cases, meaning that it could potentially be an effective selection criterion. Yet, the mitochondrial content of cumulus cells, which are the only embryonic cells that can, in practice, be analyzed by clinicians, does not appear to be a suitable surrogate for that of oocyte cells.

We recommend clinicians to collect more data. The dataset used in this project is of relatively small size (16 embryos). Conducting a similar analysis on a larger dataset would make the regression models more powerful and could allow us to identify the potential relation between the $\mathrm{mtDNA}_{\mathrm{cumulus}}$ copy number of embryo and their quality which we failed to detect in the small dataset used in this project. In addition, fitting the models to a larger dataset will make them less likely to overfit the data. We also suggest that the proportion of healthy mitochondria in cumulus cells i.e. mitochondria that do not suffer from genetic mutations impairing their activity be recorded. Taken together with the copy number, this variable will provide a more accurate estimate of the mitochondrial activity in the cumulus cell. Finally, we recommend the hospital to lobby the Dutch national committee for ethics to allow clinicians to analyze the mitochondrial content of embryos that were deemed fit for re-implantation before discarding them. This way, we would possess data on both healthy and unhealthy embryos and could develop a regression model that determines how the mitochondrial content of embryos can be used to distinguish between embryos that are likely to yield to a successful pregnancy and those that are not.

Project 2: Evolution of the mitochondrial content in ovarian cells during the menstrual cycle and over the lifetime of women.

Abstract

One of the main goals of clinicians involved in *in-vitro* fertilization (IVF) is to increase the success rate of the treatments. Besides the quality of the embryo that is re-implanted, the quality of the uterine tissues that will receive the embryo in question plays an important role in the success of the consequent pregnancies. Recent researches have established the existence of a relationship between the mitochondrial content of the embryos and the success rate of IVF treatments (Ogino et al., 2015). Drawing on these results, we investigate whether the mitochondrial content of the uterine tissues that will receive the embryo is also related to the success rate of the procedure. In this pilot study, we investigate how the copy number of mitochondrial DNA in uterine cells ($\mathrm{mtDNA_{uterine}}$) evolve during the menstrual cycle and over the lifetime of women. We observe that the $\mathrm{mtDNA_{uterine}}$ copy number peaks around day 9 of the menstrual cycle and that it is increases during the menopause (around 55 years). A follow-up study could investigate whether a minimum copy number must present in the uterine tissues that will receive the embryo for pregnancies to be possible, and, if this is the case, how hormones can be used to regulate the $\mathrm{mtDNA_{uterine}}$ copy number and thereby make the uterine conditions more favorable for the embryo.

Despite advances in assisted reproductive technology, the success rate of IVF procedures was only 42% for women younger than 35 in the U.S.A. in 2015 and decreases with age (SART Clinic Summary Report, 2015). This means that the majority of couples resorting to an IVF treatment to conceive a child can expect to have to go through this emotionally and financially demanding process multiple times before having a successful pregnancy. For this reason, clinicians involved in IVF procedures are constantly looking for ways to improve the success rate of these procedures. To accomplish this, they are attentive to two

elements: the quality of the embryo that is re-implanted in the woman's womb (see project 1) and the conditions of the uterine tissues that will receive the embryo. On the one hand, they can investigate new criteria for the selection of embryos in order to improve its effectiveness and thereby re-implanting embryos of better quality; on the other hand, they can ensure that the receiving woman is in the optimal conditions for a successful pregnancy. In particular, they can make sure that the uterine tissues that will receive the embryo are in optimal conditions for embryonic development. By better understanding how the uterine cells function, clinicians hope to be able to more effectively identify cases where the uterine conditions are not optimal for embryonic development early enough so that they can improve the conditions before the re-implantation occurs or decide to postpone the procedures if these are too unfavorable.

Among the many elements that determine how favorable the uterine conditions are for embryonic development, clinicians are particularly interested in the mitochondrial content of uterine cells for two reasons. Firstly, studies have shown that mitochondrial activity is related to embryonic development (Ogino et al., 2015; Klinge, 2017). By being the main source of energy of the cell, mitochondrial activity is related to cellular functions that are essential to the early growth of the embryo such as cell division. Secondly, clinicians can fairly easily regulate the activity of mitochondria. Klinge (2017) shows that the level of estrogen has an impact of mitochondrial activity. Would studies show that the level of mitochondrial activity in uterine cells has an impact on the success rate of the consequent pregnancy, clinicians could easily integrate this element to their current practice. For instance, if it appears that a minimum level of mitochondrial activity is necessary for a pregnancy to be possible, they could, for instance, use injections of estrogen to boost the level of mitochondrial activity in the uterine cells in cases it is too low to sustain the early development of the embryo.

The goal of this project is twofold: (i) modelling the evolution of the $\text{mtDNA}_{\text{uterine}}$ copy number over a menstrual cycle and (ii) modelling the evolution of the $\text{mtDNA}_{\text{uterine}}$ copy number over the lifetime of women. Since this is a pilot study, we are not looking for a particular pattern but explore the data with an open mind.

Data

We dispose of five datasets, each containing information on a specific type of women: women trying to become pregnant (Preg), women taking a contraceptive pill (Pill), women

suffering from endometrial cancer (Canc), women suffering from endometriosis (Endo) and women in their post-menopausal phase (Meno) (see Table 1). These datasets are composed of respectively 25, 4, 5, 7 and 4 individuals and contain the following variables: the age of the women and three measurements of their $\mathrm{mtDNA}_{\mathrm{uterine}}$ copy number realized from the same sample. This enables researchers to detect any measurement error due to the instruments used. In addition the dataset Preg contains an additional variable indicating the day of the cycle on which the 3 measurements of the $\mathrm{mtDNA}_{\mathrm{uterine}}$ copy number were realized. After filtering out individuals with missing age or missing cycle day, we have a total of 41 individuals. Finally, since the datasets Pill, Canc, Endo and Meno have only a few data points each and since merging them together would make little biological sense as they contain individuals that are significantly different, we do not conduct any analysis on them.

Table 2.1 – Descriptive Statistics per Dataset

Dataset	Pre-filtering Count	Post-filtering Count	Age of Women			$\mathrm{MtDNA}_{\mathrm{uterine}}$ Copy Number		
			Min	Mean	Max	Min	Mean	Max
Preg	25	21	30	43.1	58	129	611	2,220
Pill	4	4	38	46	56	240	1,475	4,666
Canc	5	5	70	84	91	5	877	1,998
Endo	7	7	41	46	56	0	940	2,744
Meno	4	4	70	77	87	593	2,276	7,847
All	45	41	30	52	91	0	947	7,847

Method:

We develop a non-linear regression model that estimates the evolution of the $\mathrm{mtDNA}_{\mathrm{uterine}}$ copy number during the menstrual cycle and over the lifetime of women using the day of the menstrual cycle and the age of the woman as predictors (model 1, see table 2.2). In order to obtain initial estimates for the parameters of the model, we start by building two simpler models (model 2 and model 3). Model 2 models the evolution of the $\mathrm{mtDNA}_{\mathrm{uterine}}$ copy number during the menstrual cycle and model 3 models its evolution over the lifetime of women. Since we ignore what the relation between the variables is, we start by fitting a smooth curve to the data to identify the main trend that each model should capture. We use different levels of smoothness in order to identify the relevant trend: a curve that is too smooth will tend to be linear and is thereby likely to miss important patterns while a curve that is too wiggly is likely to overfit the data. Using the right level of smoothness, we identify a kink around day 9 of the menstrual cycle and an increase in the $\mathrm{mtDNA}_{\mathrm{uterine}}$ copy number around age 45 preceded and followed by two plateaus (figure 2.1).

We then use well-known functions to model these patterns: we use three different bell-shaped distributions to model the kink around day 9 and a logistic curve to capture the increase around age 45. The three bell-shaped distributions that we fitted are the probability distributions of the normal distribution, that of the Cauchy distribution and of the gamma distribution. We consider the Cauchy distribution because its kink is sharper than that of the normal distribution and the gamma distribution because it allows for an asymmetrical kink. These three bell-shaped curves have the further advantage of ensuring that the estimated $\mathrm{mtDNA}_{\mathrm{uterine}}$ copy numbers at the end and at the beginning of the cycle are the same, which is a biological constraint that must be satisfied by the model since menstrual cycles continuously follow one another. The logistic curve is also a suitable way to model the evolution of the $\mathrm{mtDNA}_{\mathrm{uterine}}$ copy number over the lifetime of women since the curve is constrained between its lower plateau (before the increase) and its upper plateau (after the increase). This satisfies the biological constraint that the $\mathrm{mtDNA}_{\mathrm{uterine}}$ copy number is positive (at least 0) and probably bounded above by some maximum value (due to the limited size of cells). The $\mathrm{mtDNA}_{\mathrm{uterine}}$ copy number can thus not increase or decrease infinitely. Unlike a linear curve, a logistic curve satisfies these biological constraints.

The logistic and the bell-shape curves that we fit follow the formula

$$\text{mtDNA}_{\text{uterine}} \text{ copy number} \sim \beta_0 + \beta_1 * f(x, \beta_2, \beta_3)$$

where the function f denotes the probability distribution function of the chosen distribution i.e. bell-shaped curve or logistic curve, β_0 represents the intercept of the model – in this case, the minimum $\text{mtDNA}_{\text{uterine}}$ copy number possible – β_1 the factor by which to expand the chosen distribution, the variable x denotes the predictor i.e. cycle day or woman's age, and β_2 and β_3 are parameters determining the shape of the distribution. β_2 determines the location of the kink (bell-shaped curve) or of the increase (logistic curve) and β_3 determines the steepness of the increase. Smaller values of β_3 result in steeper increases in the $\text{mtDNA}_{\text{uterine}}$ copy number. Since the $\text{mtDNA}_{\text{uterine}}$ copy number cannot be negative (biological constraint), we force the coefficient β_0 to remain positive.

Furthermore, for each model (logistic and bell-shaped) we model two distributions of the data points around the trend: normally distributed and following a Cauchy distribution. We consider the Cauchy distribution because its fatter tails may provide a better fit for the several outliers we observe in the data. Finally, since the data contains three measurements for each individual, the models include a random effect in order to take inter-individual variability into account. The models are fitted on the Preg dataset since it is the only one containing women who have a menstrual cycle. We optimize the value of the sequence of coefficients $\beta_0, \dots, \beta_3$ for each of these models using a nonlinear optimizer.

We then select the specification of model 2 and of model 3 with the lowest AIC and use their coefficients as initial estimators for the coefficients of model 1. Model 1 assumes that the logistic trend is constant across the different cycle days and that the kink around day 9 is constant throughout the lifetime of the women, and therefore includes no interaction term between the two trends. The regression of model 1 follows the following formula

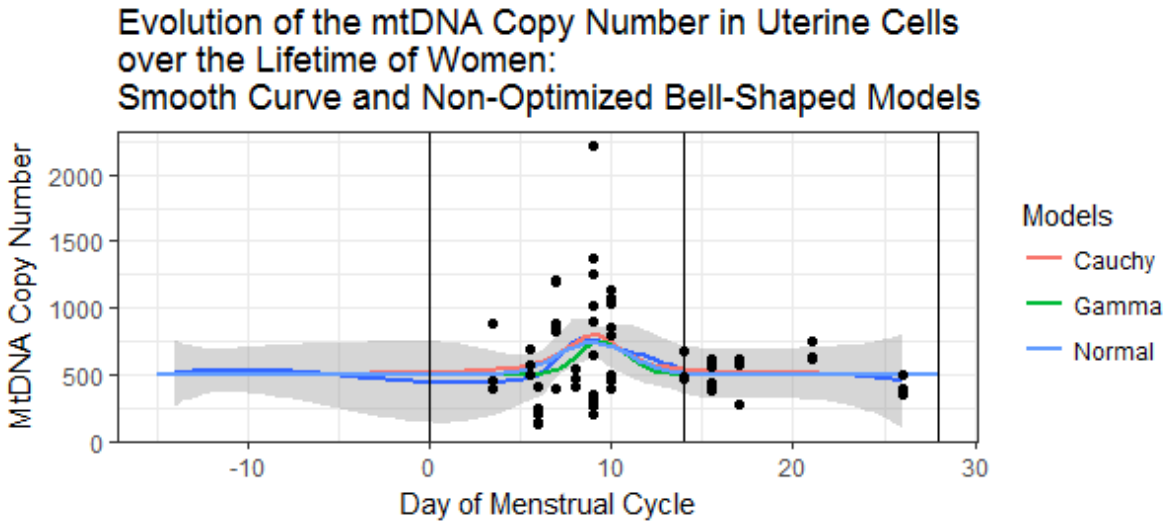
$$\text{mtDNA}_{\text{uterine}} \text{ copy number} \sim \beta_0 + \beta_1 * f(x_1, \beta_2, \beta_3) + \beta_4 * g(x_2, \beta_5, \beta_6)$$

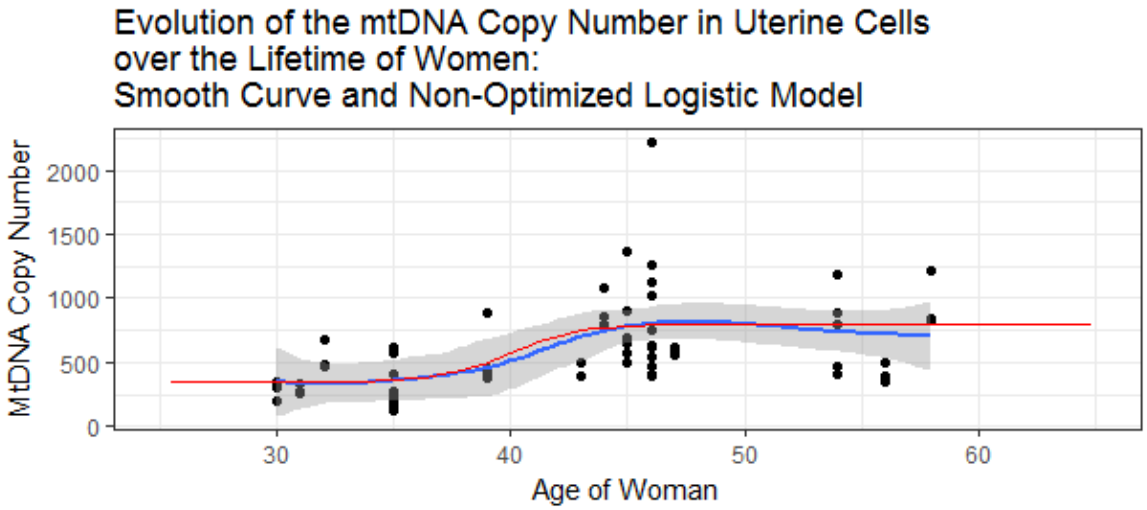
where the function f represents the bell-shaped curve of the best model of the kink around day 9 and the function g represents the logistic curve. The variable x_1 denotes the day of the menstrual cycle and x_2 the age of the women. β_0 represents the intercept of the model, and β_1 and β_4 fulfill the same function as β_1 in the previous model. The parameters β_2, β_3, β_5, β_6 determine the shape of the functions f and g.

Table 2.2 – Overview of Models for $mtDNA_{uterine}$ copy number (best models are put in bold)

Model	**Predictors**	**Function modelling the kink / the increase**	**Distribution**	**AIC**
Model 1a	Cycle Day	Normal	Normal	257.8
Model 1b	**Cycle Day**	**Normal**	**Cauchy**	**244.1**
Model 1c	Cycle Day	Cauchy	Normal	257.9
Model 1d	Cycle Day	Cauchy	Cauchy	244.7
Model 1e	Cycle Day	Gamma	Normal	254.8
Model 1f	Cycle Day	Gamma	Cauchy	247
Model 2a	Age of Woman	Logistic	Normal	254.2
Model 2b	**Age of Woman**	**Logistic**	**Cauchy**	**241.8**
Model 3	Cycle Day and Age of Woman	Normal and Logistic	Cauchy	244.6

Figures 2.1 – Smooth Curves and Initial Models





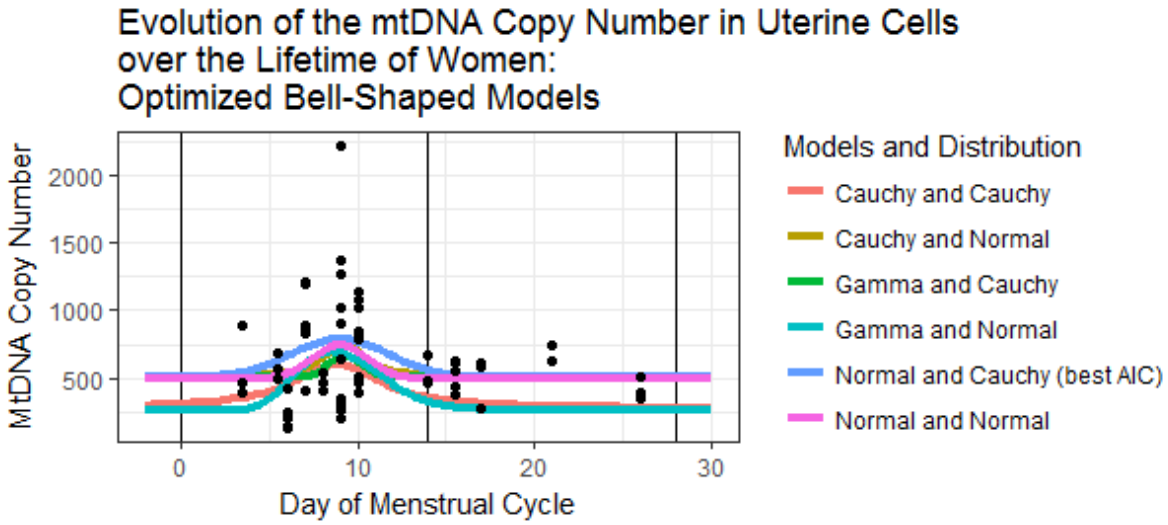
Results

All 6 specifications of model 1 model a kink around day 9 (see figure 2.2). This strongly suggests that the regression curves produced by these models capture the true trend of the data and are not the results of how the models are specified. This is furthermore confirmed by model 3 which shows a similar kink around day 9 after taking into account the effect of age on the $\mathrm{mtDNA}_{\mathrm{uterine}}$ copy number. A comparison of the AIC of the various configurations of model 1 indicates that model 1b offers the best fit of the data, that is, the kink around day 9 is best modelled with a normal curve and the distribution of the data points around this trend follows a Cauchy distribution (fatter tails) rather than a normal distribution (thinner tails). Since the gamma distribution, which unlike the normal distribution can be asymmetrical, does not offers a better approximation of the kink, the results suggests that the kink is symmetrical.

Model 2a and model 2b both indicate that the $\mathrm{mtDNA}_{\mathrm{uterine}}$ copy number is stable at 350 until age 40 before increasing to 700 and stabilize again at that level at age 50. Yet, model 3 shows that, once the effects of the kink around day 9 of the menstrual cycle are taken into account, the increases appears to start at age 55.

Finally, by comparing the AIC of models 1a vs 1b, 1c vs 1d, 1e vs 1f and 2a vs 2b, we observe that in all cases, the Cauchy distribution better models the distribution of the data points around the trend than a normal distribution. This indicates that the dataset contains a significant amount of data points that do not follow the pattern. Indeed, because it has fatter tails than the normal distribution, the Cauchy distribution gives a smaller penalty to outliers than the normal distribution; hence it is a better model for data that are more dispersed around the trend.

Figures 2.2 – Fitted Models



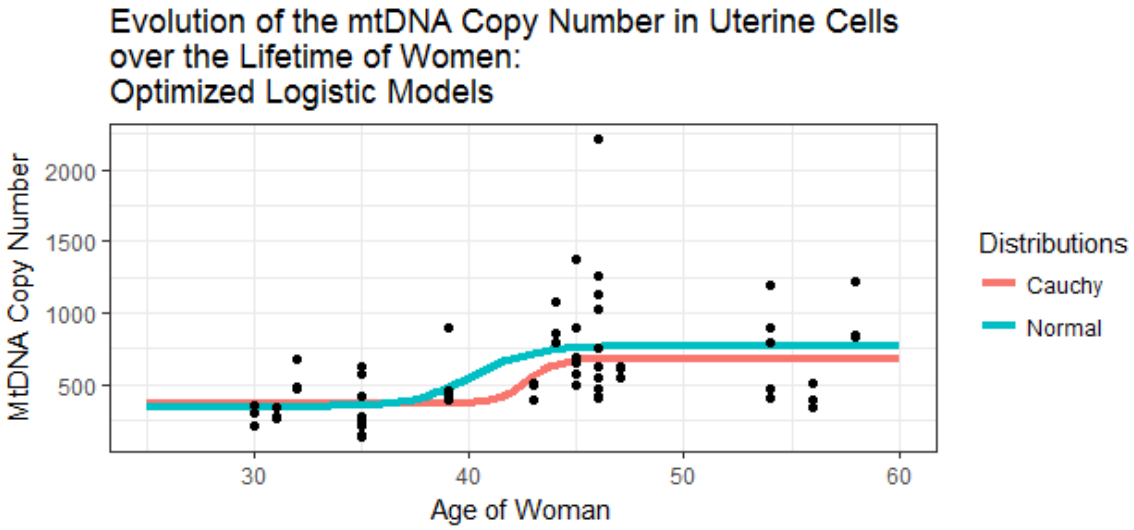
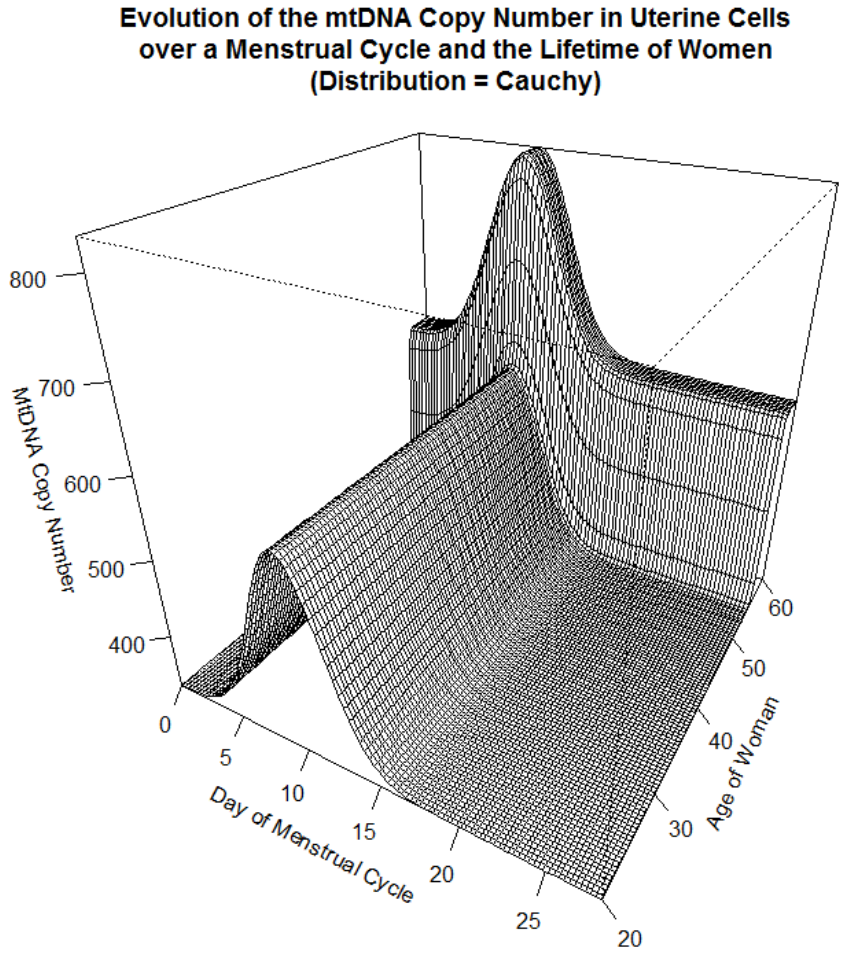


Figure 2.3 – Fitted Model



Discussion

The increase in the $\mathrm{mtDNA}_{\mathrm{uterine}}$ copy number that we observe around day 9 of the menstrual cycle precedes the ovulation, which usually occurs around day 14, by a few days. This increase in mitochondrial activity seems to occur between day 5 and day 15 and may thus be a preparation for the potential fertilization of the oocyte that would follow the ovulation. Furthermore, the evolution of the level of mitochondrial activity in uterine tissues seems to match that of estrogen during the follicular phase (the pre-ovulation period of the menstrual cycle), but not the dip in level of estrogen that occurs at the beginning of the luteal phase (post-ovulation period of the menstrual cycle). This is due to the fact that, since most of our data were recorder on individuals in their follicular phase, we only attempted to model the kink around day 9 and assume the trend to be linear in the luteal phase. Yet, since researches have shown that the production estrogen is associated with an increase in mitochondrial activity, a follow-up study could gather more data for the luteal phase and investigate the extent to which the mitochondrial activity in uterine cells and the level of estrogen are correlated throughout the whole menstrual cycle, not just the follicular phase. Furthermore, another follow-up study could investigate whether the intensity of the kink at day 9 varies with age. We assume that it did not, yet one could expect that it fades as women become older and their level of fertility decreases. Researchers could do so by let the coefficient β_1 in the formula of model 1 depend on the age of the women, say, in a linear fashion, instead of keeping it constant as we did in this study.

The increase in the $\mathrm{mtDNA}_{\mathrm{uterine}}$ copy number that we observe at age 55 seems to occur after the moment that menopause is expected to occur i.e. between 49 and 52 years of age. These results indicate that women who still have a menstrual cycle at age 55, i.e. past the usual age for menopause, experience an increase in the level of mitochondrial activity in their uterine tissues. Future research could investigate the biological reason for this increase in mitochondrial activity during the last menstrual cycles of those women who enters menopause at a later age.

The absence of data from women younger than 30 after day 20 of the menstrual cycle had an impact of the models we developed. Under certain specifications, the model predicted a negative $\mathrm{mtDNA}_{\mathrm{uterine}}$ copy number after day 20 of the cycle for women younger than 30, which does not make sense biologically. A solution to this problem is to force the intercept to be positive (or to be greater than some minimum amount of

mitochondria known in the literature to be biologically necessary for the cell to survive). Yet, a less artificial approach would be to collect data covering all combinations of age and cycle day so that the models we develop do not predict negative $\mathrm{mtDNA}_{\mathrm{uterine}}$ copy numbers. This would ensure that the obtained models make sense from a biological point view.

Drawing on the results of this study, we suggest clinicians to conduct two follow-up studies. First, they could investigate whether a minimum level of mitochondrial activity is necessary in uterine cells for a pregnancy to be possible. For this purpose, they should record the outcome of the IVF procedures, i.e. whether or not it led to a successful pregnancy, and use a logistic regression to predict the outcome of the cycle based on the $\mathrm{mtDNA}_{\mathrm{uterine}}$ copy number. As discussed in project 1 – clinicians should also record the proportion of healthy mitochondria in uterine cells, since this variable, together with the mtDNA copy number, would give a more accurate estimation of the true level of mitochondrial activity in uterine tissues. Second, clinicians could study the long term impact that contraception methods such as birth control pills have on the mitochondrial activity in uterine cells. To do so, researchers should collect data on pill users and non-pill users. In this study, we possessed date on both groups, but the size of the sample was too limited (4 individuals for the pill users) for us to investigate any difference in mitochondrial activity between the two groups.

Conclusion

One of the main goals of clinicians involved in *in-vitro* fertilization (IVF) is to increase the success rate of the treatments. Besides the quality of the embryo that is re-implanted, the quality of the uterine tissues that will receive the embryo in question plays an important role in the success of the consequent pregnancies. Among the many elements that determine how favorable the uterine conditions are for embryonic development, clinicians are particularly interested in the mitochondrial content of uterine cells. In this project, we attempted to gain a better understanding of the mitochondrial activity of uterine cells. In particular, we model the evolution of the mtDNA copy number in the uterine tissues that will receive the embryo during a menstrual cycle and over the lifetime of women.

We observe that the level of mitochondrial activity in uterine cells peaks around day 9 of the menstrual cycle and is characterize by an increase around age 55. Follow-up

studies could investigate whether a minimum level of mitochondrial activity is necessary in uterine cells for a pregnancy to be possible. If this turns out to be the case, clinicians could use injections of estrogen to boost the level of mitochondrial activity in the uterine cells in cases it is too low to sustain the early development of the embryo. Clinicians could also investigate the long term impact that contraception methods such as birth control pills have on the level of mitochondrial activity in uterine cells.

Project 3: Probability of obtaining a suitable embryo from women with large mutation loads of their mitochondrial DNA.

Abstract

Traditional *in-vitro* fertilization (IVF) procedures such as pre-implementation genetic diagnosis (PGD) methods or intracytoplasmic sperm injection (ICSI) have helped couples suffering from a wide range of infertility conditions to conceive children. Yet, in the case of couples where the woman's mitochondrial DNA (mtDNA) is characterized by a high mutation load, these methods can be of little help – or even no help at all in the most severe cases. If the mtDNA mutation load of the woman is too large, it may prove too difficult to obtain an embryo with an mtDNA mutation load below 15% – one of the criteria an embryo must satisfy to be deemed suitable for re-implantation. In such cases, the mitochondrial replacement therapy (MRT), by ensuring that the mitochondria of the obtained embryo are healthy, would be a more suitable approach. The purpose of this study is to estimate the probability of obtaining an embryo that fulfills the criteria for re-implantation in the case of women with high mtDNA mutation load. More specifically, we model the mtDNA mutation load in urine to estimate the mtDNA mutation load in blastomeres and, using this estimate, we attempt to predict the probability of obtaining an embryo that is suitable for re-implantation.

About 1 in 4,000 people are concerned by mitochondrial diseases. It the Netherlands, the number of people suffering from defective mitochondria amounts to more than 4,000. The most common of these conditions is the mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS), a condition that results from mutation in the DNA of mitochondria (mtDNA) and which affects the nervous and muscular systems. The list of symptoms includes – but is not limited to – muscle weakness, seizures, vision abnormalities and stroke-like episodes. Repeated episodes of the latter symptom can result

in severe damage to the brain which could lead to dementia and other long term declines in cognitive abilities.

Unlike their nuclear DNA, which they receive from both their biological mother and biological father, the children's mitochondria are not a mixture from those of their parents. Mitochondria are transmitted to the children via the blastomere of the mother; the sperm of the father does not transmit any mitochondria to the embryo. As a result, children receive the entirety of the mitochondria found in their cells from their mother. The level of mtDNA mutation load of embryos is thus directly related to that of the blastomeres of the mother. Embryos of women with blastomeres characterized by a high level of mtDNA mutation load are also likely to have high levels of mtDNA mutation loads. Since the production of energy by the mitochondria is related to cellular functions that are essential to the early stages of embryonic development such as cell differentiation and cell division, embryos with high levels of mtDNA mutation loads are less likely to yield to a successful pregnancy than embryos whose mitochondria do not suffer from genetic mutation. For this reason, clinicians automatically discard embryos with an mtDNA mutation load superior to 15%.

For each IVF cycle, clinicians collect at most 20 of female gametes. Given this limited number, it may thus prove very difficult – or even impossible in some cases – to obtain an embryo with an mtDNA mutation load inferior to 15%[3], from women that have an mtDNA mutation load relatively large. In such cases, resorting to the mitochondrial replacement therapy (MRT) instead of classical PGD techniques seems to be a natural move. The MRT procedure is a special form of *in-vitro* fertilization in which the embryo is composed from the reproductive cells of three different individuals. The sperm of the father and the blastomere of the mother compose the nucleus of the obtained oocyte and the rest of the cell, including its mitochondria, comes from the blastomere of a third-party woman that does not suffer from mitochondrial diseases. In practice, after fertilizing *in-vitro* the blastomere of the mother with the sperm of the father, clinicians extract the nucleus of the obtained egg and transfer it into a blastomere that has healthy mitochondria. The resulting cell is then placed in culture for 5 days, at the end of which it is transferred to the womb of the mother provided that the resulting embryo is deemed suitable for re-implantation.

[3] which in addition must satisfy other criteria to be deemed suitable for re-implantation (see project 1).

Although the MRT procedure appears to be a good substitute to classical IVF techniques for women that have a high level of mtDNA mutation load, it raises serious ethical concerns which have limited its use worldwide. Researchers have recently discovered that, next to the DNA present in the nucleus of the cell, the DNA of the mitochondria also has an influence on the personality of a person (Liao, 2017). This means that children conceived with the MRT procedure have in effect inherited traits from three individuals. For this reason, the Netherlands has, for instance, yet to approve the practice. As explained above, traditional IVF treatments may be of no help for women that have a high level of mtDNA mutation load – or require a large number of cycles to obtain a suitable embryo. Since these procedures are psychologically and financially demanding for the couples resorting to them, we argue that couples where the woman has a high level of mtDNA mutation load should be allowed to use the MRT procedure instead of classical IVF techniques.

The goal of this study is to determine the level of mtDNA mutation load above which the probability of obtaining a suitable embryo is too low for classical IVF treatments to be suitable and couples should instead resort to an MRT procedure. For this purpose we model the mtDNA mutation load of the urine ($\mathrm{mtDNA}_{\mathrm{urine}}$) of the woman in order to predict the mtDNA mutation load present in her blastomeres ($\mathrm{mtDNA}_{\mathrm{blastomere}}$). We in turn use this estimate to approximate the probability of obtaining an embryo that is suitable for re-implantation given the $\mathrm{mtDNA}_{\mathrm{blastomere}}$ mutation load of the woman. The final model provides the probability of obtaining an embryo that is suitable for re-implantation given the level of $\mathrm{mtDNA}_{\mathrm{urine}}$ mutation loads in the woman. The advantage of using urine as a predictor is that it can be obtained in a non-intrusive way and its level of mtDNA mutation load is known to be more stable than that of other tissues.

Data

The sample is composed of 11 women on which the following variables are recorded: birth year, age at the beginning of the IVF procedure, type of mtDNA mutation, between 10 and 16 measurements of their $\mathrm{mtDNA}_{\mathrm{blastomere}}$ mutation load and a measurement of the mtDNA mutation load in their blood cells ($\mathrm{mtDNA}_{\mathrm{blood}}$), hair cells ($\mathrm{mtDNA}_{\mathrm{hair}}$) and urine ($\mathrm{mtDNA}_{\mathrm{urine}}$) (see table 3.1). Table 3.2 present the main descriptive statistics of the dataset.

Table 3.1 - Dataset

Birth Year	Age at Beginning of Procedure	$MtDNA_{blood}$ Copy Number	$MtDNA_{hair}$ Copy Number	$MtDNA_{urine}$ Copy Number	Average $MtDNA_{blast.}$ Copy Number
1972	36	0.13	0.26	0.55	0.42
1979	30	0.25	0.1	missing	0.34
1982	28	0.27	0.29	0.59	0.39
1981	32	0.19	0.26	0.38	0.33
1983	31	0.16	0.29	0.28	0.16
1992	24	0.26	0.31	0.54	0.34
1990	25	0.15	0.31	0.39	0.37
1990	25	0.23	0.3	0.37	0.47
1987	29	0.1	missing	0.27	0.12
1979	37	0.15	0.45	0.48	0.25
1982	31	0	missing	missing	0

Table 3.2 – Descriptive Statistics

	Age at Beginning of Procedure	$MtDNA_{blood}$ Copy Number	$MtDNA_{hair}$ Copy Number	$MtDNA_{urine}$ Copy Number	Average $MtDNA_{blast.}$ Copy Number
Min	24	0.	0.1	0.27	0
Mean	29.6	0.17	0.29	0.43	0.29
Max	47	0.27	0.45	0.59	0.47

Method

The estimation of the probability of obtaining an embryo that fulfills the criteria for re-implantation given the $\mathrm{mtDNA}_{\mathrm{urine}}$ mutation load of the mother follows two steps. In the first step, we model the $\mathrm{mtDNA}_{\mathrm{urine}}$ mutation load in order to estimate the $\mathrm{mtDNA}_{\mathrm{blastomere}}$ mutation load. In the second step, we use the $\mathrm{mtDNA}_{\mathrm{blastomere}}$ mutation load estimated in step 1 to predict the probability of obtaining an embryo that fulfills the criteria for re-implantation.

Step 1

Similarly to what we did in project 2, we fit a nonlinear regression curve that models the $\mathrm{mtDNA}_{\mathrm{urine}}$ mutation load in order to estimate the $\mathrm{mtDNA}_{\mathrm{blastomere}}$ mutation load. We focus solely on the mutation load in urine since clinicians can obtain it using a non-invasive procedure and its level of mtDNA mutation load is known to be more stable than that of other tissues. We fit two types of curves: linear (model 1) and logistic curves (model 2 and model 3) (see table 3.3). While the intercept of model 2 is free, the intercept of model 3 is fixed to 0. This ensures that the curve of model 3 passes through the point $(0,0)$. In this regard, model 3 is closer than model 2 to the biological reality that individuals with no mutation load in the mtDNA of their urine also have no mutation in the mtDNA of their blastomeres. We consider the logistic curve because it satisfies the biological requirement that the $\mathrm{mtDNA}_{\mathrm{blastomere}}$ mutation load must be comprised between 0 and at most 1.

We fit the model only on the individuals suffering from the mutation A3243G, i.e. patient suffering from MELAS, and discard the individuals with other types of mutations in their mtDNA. We do so because our the model that we build specifically targets the MELAS condition: the model for transmission used in step 2 is specific to the A3243G mutation. Moreover, although we know the age of the person at the beginning of the procedure, since we ignore when the different measurements were effectuated and since it seems that the measurement span over several years, we are reluctant to assume that the person had the same age at each measurement and therefore decide not to include an age variable in the model. Since some individuals went through multiple cycles and several measurements of their $\mathrm{mtDNA}_{\mathrm{blastomere}}$ mutation load were realized each occasion, the models include a random effect in order to take inter-individual variability into account. Furthermore, as the response variables is comprised between 0 and 1, we model the

distribution of the data points around the trend with a beta distribution since it is comprised in this interval too. We optimize the value of the parameters of the three models using a nonlinear optimizer. We finally conduct a visual assessment of the obtained models to ensure that they are biologically plausible.

Step 2

We use the $\text{mtDNA}_{\text{blastomere}}$ mutation load estimated by the model developed in step 1 to predict the probability of obtaining an embryo that fulfills the criteria for re-implantation.
To accomplish this, we use a truncated Kimura distribution found to describe best the transmission of the A3243G mutation (Otten et al., 2018). This model estimates that the $\text{mtDNA}_{\text{blastomere}}$ mutation load is comprised between 0 and 0.765. Blastomeres never have a mutation load superior to the latter value since this would be lethal for the embryo. Finally, since the beta distribution has a probability of zero of having a value of 0, we add a very small increment (10^{-4}) to the $\text{mtDNA}_{\text{blastomere}}$ mutation load for the individuals where this variable is equal to 0 so that. Because the instruments used to measure $\text{mtDNA}_{\text{blastomere}}$ mutation load are not able to detect very small levels of mutation loads, add a very small increment to the $\text{mtDNA}_{\text{blastomere}}$ mutation load that are equal to 0 does not alter the nature of the measurements.

Table 3.3 – Overview of Models for $\text{mtDNA}_{\text{blastomere}}$ mutation load

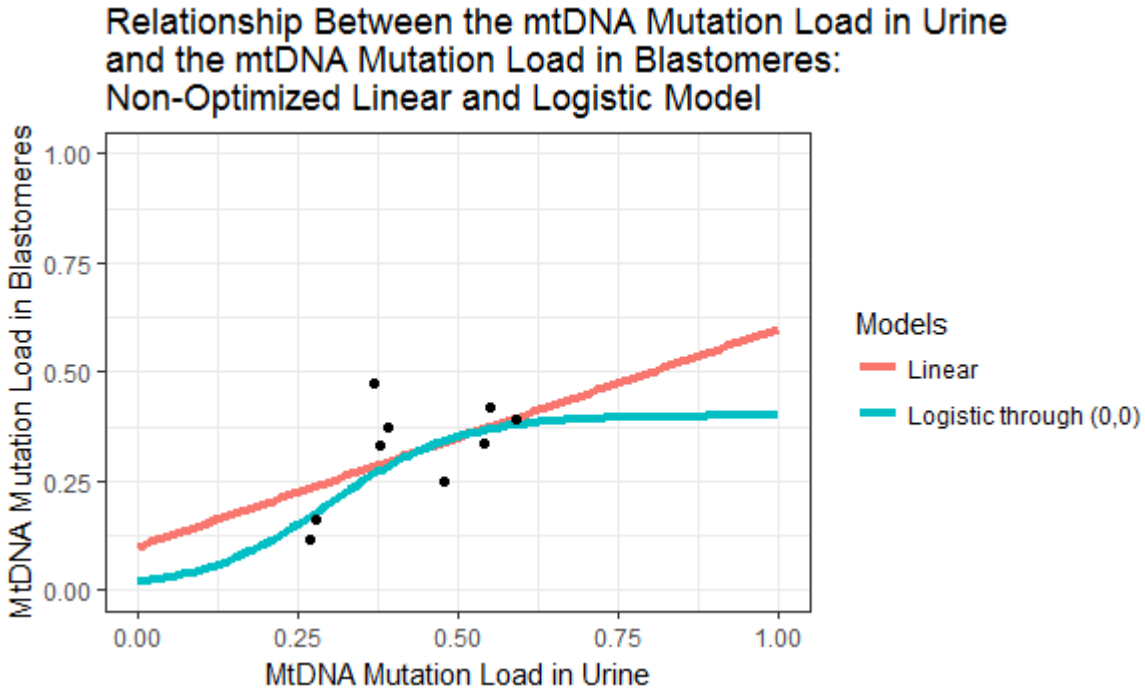
Model	**Predictor**	**Function modelling the trend**	**Distribution**	**AIC**
Model 1	$\text{mtDNA}_{\text{urine}}$ mutation load	Linear	Beta	-973.4
Model 2	$\text{mtDNA}_{\text{urine}}$ mutation load	Logistic	Beta	-652.8
Model 3	$\text{mtDNA}_{\text{urine}}$ mutation load	Logistic without an intercept	Beta	-1,073.6

Results

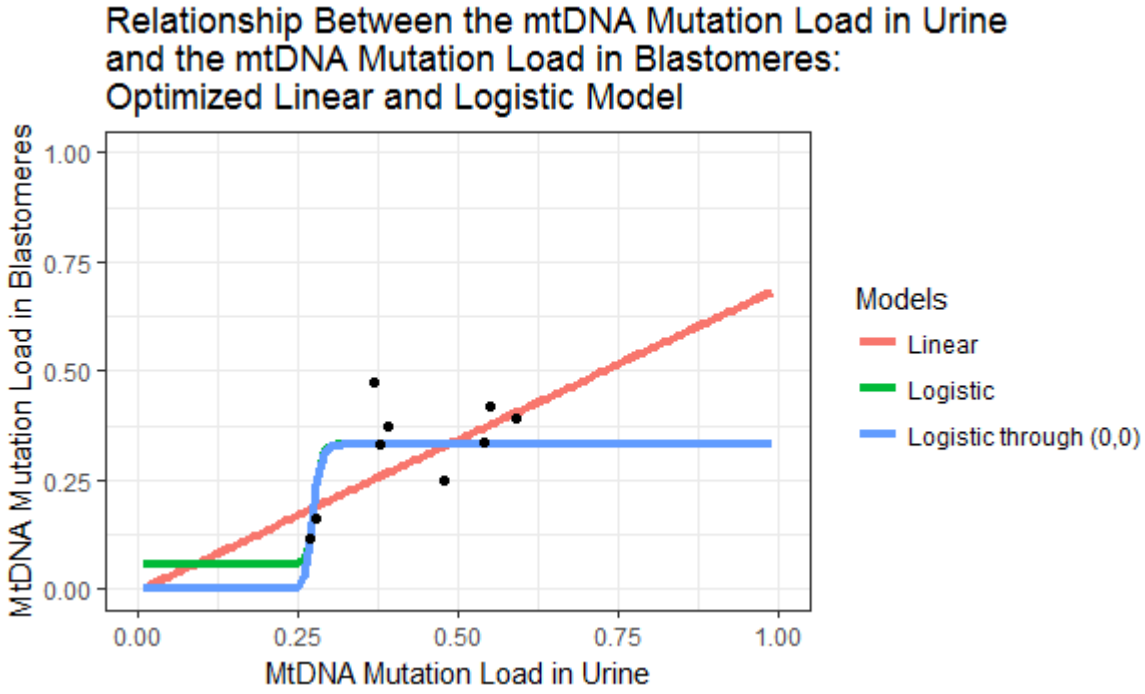
Figure 3.2 shows the regressions obtained by the three models. The two logistic regressions are characterized by an increase that is similar in terms of steepness (the increase is relatively sharp in both cases) and location (around 26%), and by an upper plateau at a similar level (30%) after the increase. Since it has no intercept, model 3 goes through the point $(0,0)$; unlike model 2 whose intercept is 0.05. The curve of model 1 also passes extremely close to the point $(0,0)$. The AIC of model 3 is the smallest (-1,073.6), meaning that this model offers the best fit of the data; yet, a visual assessment of the logistic regression reveals that the curve may fail to capture the underlying mechanism linking the two variables. Instead, to maximize the goodness-of-fit of the model, the optimizer makes the curve pass through the two data points with an $\mathrm{mtDNA}_{\mathrm{urine}}$ mutation load close to 0.27. This results in a very sharp increase starting at 0.25 which, given our current knowledge of mtDNA, is arguably not biologically plausible. For this reason, although the logistic curve has a better AIC than the linear one, we use both model 1 and model 3 in the second step of the project.

Figure 3.3 shows the estimated probability of obtaining an embryo that fulfills the criteria for re-implantation given the $\mathrm{mtDNA}_{\mathrm{urine}}$ mutation load of the woman. We observe that the sharp increase of the logistic curve from step 1 is preserved in the model of step 2. That is, if we use a logistic regression to estimate the $\mathrm{mtDNA}_{\mathrm{blastomere}}$ mutation load from the $\mathrm{mtDNA}_{\mathrm{urine}}$ mutation load, then there is a clear cut-off point around 26%: women with a level of $\mathrm{mtDNA}_{\mathrm{urine}}$ mutation load inferior to that value have a very high probability (close to 1) of having a suitable oocyte and those with a level of $\mathrm{mtDNA}_{\mathrm{urine}}$ mutation load superior to it have a probability of 26%. On the other hand, if we use a linear model to estimate $\mathrm{mtDNA}_{\mathrm{blastomere}}$ mutation load from the $\mathrm{mtDNA}_{\mathrm{urine}}$ mutation load, then we obtain a curve that smoothly decreases from 1 to 0 as the $\mathrm{mtDNA}_{\mathrm{urine}}$ mutation load increases. With the linear model for the $\mathrm{mtDNA}_{\mathrm{blastomer}}$ mutation load, a woman with a $\mathrm{mtDNA}_{\mathrm{urine}}$ mutation load of 25% is estimated to have a 55% chance of having an embryo that fulfills the criteria for re-implantation.

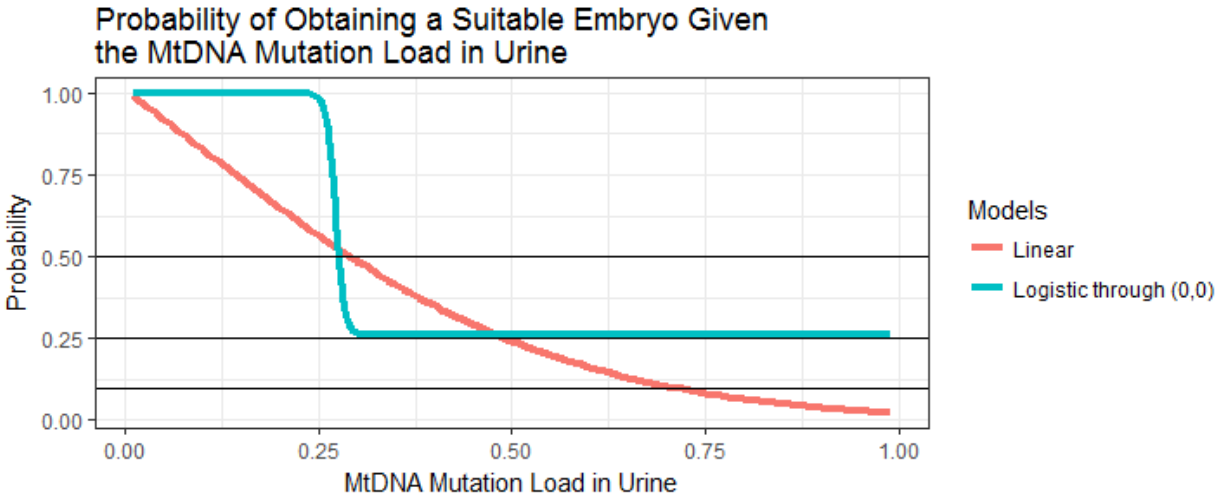
Figures 3.1 – Initial Models (Step 1)



Figures 3.2 – Fitted Models (Step 1)



Figures 3.3 – Fitted Models (Step 2)



Discussion

I am reluctant to interpret the trends obtained in step 1 at face value. The linear and logistic models yield fairly different results (figure 3.2). For instance, the linear model predicts that an individual with an $\text{mtDNA}_{\text{urine}}$ mutation load of 0.75 has an $\text{mtDNA}_{\text{blastomere}}$ mutation load of 0.5, the logistic model predicts, for the same individual, a value of 0.32. Furthermore, the trend captured by the logistic model is arguably not plausible form a biological point of view. While the linear model predicts that the level of the response variable continually increases with the predictor, the logistic model predicts a sharp increase at 0.26, which is preceded and followed by plateaus. Given our current knowledge of mtDNA, it is difficult to justify such increase in $\text{mtDNA}_{\text{blastomere}}$ mutation load when the $\text{mtDNA}_{\text{urine}}$ mutation load reaches 0.26. For this reason, and because the two models give results that are so different, I am reluctant to interpret the obtained trends at face value.

Furthermore, since the dataset contains no observation with low levels of $\text{mtDNA}_{\text{urine}}$ mutation load, we ignore how the two variables are related when the $\text{mtDNA}_{\text{urine}}$ mutation load is inferior to 0.25. For instance, the plateau predicted by the logistic model for mutation load in urine below 0.25, is solely due to the nature of the model and the absence of data points with a $\text{mtDNA}_{\text{urine}}$ mutation load below this value. We also had to force the logistic model through the point $(0,0)$ to ensure that it predicts a $\text{mtDNA}_{\text{blastomere}}$ mutation load of 0 for individuals without any mutation in their mtDNA. A less artificial approach to satisfy this biological constraint would be to record

more data from individuals with low levels of $\mathrm{mtDNA_{urine}}$ mutation load. Collecting such data would have a second advantage: make the logistic model for the $\mathrm{mtDNA_{blastomere}}$ mutation load more realistic from a biological point of view. The sharp increase we observe in the fitted logistic model at an $\mathrm{mtDNA_{urine}}$ mutation load of 0.26 (figure 3.2) results from the fact that the optimizer makes the curve pass through the two data points with an $\mathrm{mtDNA_{urine}}$ mutation load close to 0.27. The logistic model is therefore likely to offer a relatively poor representation of the true nature of the underlying mechanism linking the mtDNA mutation load of in urine and that in blastomeres.

Collecting more data – especially from individuals with low levels of $\mathrm{mtDNA_{urine}}$ mutation load – would thus help us refine the relation between the two variables and improve the quality of the model we fit to the data in step 1. Figure 3.3 illustrates the impact that the model used to predict $\mathrm{mtDNA_{blastomere}}$ mutation load from $\mathrm{mtDNA_{urine}}$ mutation load in step 1 has on the estimated probability of obtaining of suitable embryo. While the linear model suggests that the probability decreases in a fairly linear fashion with $\mathrm{mtDNA_{urine}}$ mutation load, the logistic model suggests that past a certain threshold i.e. a $\mathrm{mtDNA_{urine}}$ mutation load of 0.26, the probability sharply decreases from almost 1 to 0.26. Again, because the two models give results that are so different and because the logistic model is not realistic from a biological point of view, I am reluctant to interpret these results at face value and strongly encourage clinicians to collect more data, especially from individuals with low levels of $\mathrm{mtDNA_{urine}}$ mutation load.

Conclusion

Traditional *in-vitro* fertilization (IVF) procedures such as pre-implementation genetic diagnosis (PGD) methods or intracytoplasmic sperm injection (ICSI) have helped couples suffering from a wide range of infertility conditions to conceive children. Yet, in the case of couples where the woman's mitochondrial DNA (mtDNA) is characterized by a high mutation load, these methods can be of little help – or even no help at all in the most severe cases. In such cases, the mitochondrial replacement therapy (MRT) would be a more suitable approach. Yet, due to ethical concerns, the Netherlands has yet to approve the practice.

The purpose of this study is to establish the circumstances under which a mitochondrial replacement therapy (MRT) procedure would be more suitable than a classic IVF treatment. For this purpose, we estimate the probability of obtaining an

embryo that fulfills the criteria for re-implantation in the case of women with high mtDNA mutation load. This model can then be used to identify cases in which the probability that classic IVF treatments succeed is so small that an MRT procedure should be preferred. Yet, because the two models give results that are so different and because the logistic model is not plausible from a biological point of view, I am reluctant to interpret these results at face value and strongly encourage clinicians to collect more data, especially from individuals with low levels of $\mathrm{mtDNA}_{\mathrm{urine}}$ mutation load in order to refine the relation between the two variables.

General Conclusion

In-vitro fertilization (IVF) treatments are types of assisted reproductive technologies used to treat infertility. These treatments are emotionally and financially demanding for the couples who resort to them. Despite advances in assisted reproductive technology, the success rate of IVF procedures was only 42% for women younger than 35 in the U.S.A. in 2015 and decreases with age, meaning that the majority of couples resorting to an IVF treatment to conceive a child have to go through this process multiple times before having a successful pregnancy. Clinicians are therefore trying to increase the success rate of IVF treatments. In this line, researchers have recently started to appreciate the role played by mitochondrial activity in early embryonic development.

The overarching goal of this thesis is to assist clinicians in their quest to increase the overall success rates of IVF procedures by gaining a deeper understanding of how mitochondrial activity is related to early embryonic development. Project 1 investigates whether the mitochondrial content of an embryo can be modelled to predict its quality and could therefore be a potential criterion for selecting which embryo is to be re-implanted. We find that the $\mathrm{mtDNA}_{\mathrm{oocyte}}$ copy number of an embryo appears to be an accurate predictor for its quality grade; but the mitochondrial content of cumulus cells, which are the only embryonic cells that can, in practice, be analyzed by clinicians, does not appear to be a suitable surrogate for that of oocyte cells. Project 2 models the evolution of the mitochondrial activity in uterine cells during the menstrual cycle and over the lifetime of women. We find that the $\mathrm{mtDNA}_{\mathrm{uterine}}$ copy numbers peaks around day 9 of the menstrual cycle and increases around age 55. Project 3 attempts to estimate the probability of obtaining an embryo that fulfills the criteria for re-implantation in the case of women with high levels of mtDNA mutation load so that clinicians can identify cases where the probability that classic IVF treatments succeed is so small that an MRT procedure should be preferred. Yet, because the two models give results that are so different and because the logistic model is not plausible from a biological point of view, I am reluctant to interpret these results at face value.

The data analyses that I conducted are pilot studies whose results should guide the future work of clinicians working at the department of reproductive medicine of Maastricht University Medical Center+. Drawing on these results, the principal suggestions I have are the following. Firstly, I recommend the hospital to lobby the Dutch national committee for ethics to allow clinicians to analyze the mitochondrial content of

embryos that were deemed fit for re-implantation before discarding them. This way, we would possess data on both healthy and unhealthy embryos and could develop a model that determines how the mitochondrial content of cumulus cells can be used to distinguish between embryos that are likely to yield to a successful pregnancy and those that are not (project 1). Secondly, in the context of project 2, clinicians should record the outcome of the IVF procedures so that they could establish whether a minimum level of mitochondrial activity is necessary in uterine cells for a pregnancy to be possible. Finally, in order to refine the relation between the mutation load of $\mathrm{mtDNA}_{\mathrm{urine}}$ and that of $\mathrm{mtDNA}_{\mathrm{blastomere}}$, I encourage clinicians to collect more data, especially from individuals with low levels of $\mathrm{mtDNA}_{\mathrm{urine}}$ mutation load.

References

Adamson, G. D., Tabangin, M., Macaluso, M., & de Mouzon, J. (2013). The number of babies born globally after treatment with the assisted reproductive technologies (ART). Fertility and Sterility, 100(3), S42. Bossy-Wetzel, E., Barsoum, M. J., Godzik, A., Schwarzenbacher, R., & Lipton, S. A. (2003). Mitochondrial fission in apoptosis, neurodegeneration and aging. *Current opinion in cell biology*, *15*(6), 706-716.

Akaike, H. (1998). Information theory and an extension of the maximum likelihood principle. In Selected Papers of Hirotugu Akaike (pp. 199-213). Springer, New York, NY.

Beutel, M., Kupfer, J., Kirchmeyer, P., Kehde, S., Köhn, F. M., Schroeder-Printzen, I., ... & Weidner, W. (1999). Treatment-related stresses and depression in couples undergoing assisted reproductive treatment by IVF or ICSI. Andrologia, 31(1), 27-35.

Cassina, P., Cassina, A., Pehar, M., Castellanos, R., Gandelman, M., de León, A., ... & Radi, R. (2008). Mitochondrial dysfunction in SOD1G93A-bearing astrocytes promotes motor neuron degeneration: prevention by mitochondrial-targeted antioxidants. *Journal of Neuroscience*, *28*(16), 4115-4122.

Chen, D. Y., Wen, D. C., Zhang, Y. P., Sun, Q. Y., Han, Z. M., Liu, Z. H., ... & Kou, Z. H. (2002). Interspecies implantation and mitochondria fate of panda-rabbit cloned embryos. *Biology of Reproduction*, *67*(2), 637-642.

Chen, H., Detmer, S. A., Ewald, A. J., Griffin, E. E., Fraser, S. E., & Chan, D. C. (2003). Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. *The Journal of cell biology*, *160*(2), 189-200.

Desquiret-Dumas, V., Clément, A., Seegers, V., Boucret, L., Ferré-L'Hotellier, V., Bouet, P. E., ... & May-Panloup, P. (2017). The mitochondrial DNA content of cumulus granulosa cells is linked to embryo quality. Human Reproduction, 32(3), 607-614.

Fragouli, E., & Wells, D. (2015, November). Mitochondrial DNA assessment to determine oocyte and embryo viability. In *Seminars in reproductive medicine* (Vol. 33, No. 06, pp. 401-409). Thieme Medical Publishers.

Huang, D. W., Sherman, B. T., & Lempicki, R. A. (2008a). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature protocols, 4(1), 44.

Huang, D. W., Sherman, B. T., & Lempicki, R. A. (2008b). Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic acids research, 37(1), 1-13.

Liao, S. M. (2017). Do mitochondrial replacement techniques affect qualitative or numerical identity?. Bioethics, 31(1), 20-26.

Long, J. (1997). Regression models for categorical and limited dependent variables. Advanced quantitative techniques in the social sciences, 7.

Lowell, B. B., & Shulman, G. I. (2005). Mitochondrial dysfunction and type 2 diabetes. *Science*, *307*(5708), 384-387. McBride, H. M., Neuspiel, M., & Wasiak, S. (2006). Mitochondria: more than just a powerhouse. *Current biology*, *16*(14), R551-R560.

May-Panloup, P., Boucret, L., Chao de la Barca, J. M., Desquiret-Dumas, V., Ferré-L'Hotellier, V., Morinière, C., ... & Reynier, P. (2016). Ovarian ageing: the role of mitochondria in oocytes and follicles. Human reproduction update, 22(6), 725-743.

Mihaylova, M. M., & Shaw, R. J. (2011). The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. *Nature cell biology*, *13*(9), 1016.

Ogino, M., Tsubamoto, H., Sakata, K., Oohama, N., Hayakawa, H., Kojima, T., ... & Shibahara, H. (2016). Mitochondrial DNA copy number in cumulus cells is a strong predictor of obtaining good-quality embryos after IVF. *Journal of assisted reproduction and genetics*, *33*(3), 367-371. Seth, R. B.,

Otten, A. B., Sallevelt, S. C., Carling, P. J., Dreesen, J. C., Drüsedau, M., Spierts, S., ... & Samuels, D. C. (2018). Mutation-specific effects in germline transmission of pathogenic mtDNA variants. Human Reproduction.

Staessen, C., Janssenswillen, C., Van den Abbeel, E., Devroey, P., & Van Steirteghem, A. C. (1993). Infertility: Avoidance of triplet pregnancies by elective transfer of two good quality embryos. *Human Reproduction*, *8*(10), 1650-1653.

Sun, L., Ea, C. K., & Chen, Z. J. (2005). Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-κB and IRF3. *Cell*, *122*(5), 669-682.

Youle, R. J., & Karbowski, M. (2005). Mitochondrial fission in apoptosis. *Nature reviews Molecular cell biology*, *6*(8), 657.

Yu-Wai-Man, P., Votruba, M., Burté, F., La Morgia, C., Barboni, P., & Carelli, V. (2016). A neurodegenerative perspective on mitochondrial optic neuropathies. *Acta neuropathologica*, *132*(6), 789-806.

Ziebe, S., Petersen, K., Lindenberg, S., Andersen, A. G., Gabrielsen, A., & Andersen, A. N. (1997). Embryo morphology or cleavage stage: how to select the best embryos for transfer after in-vitro fertilization. *Human reproduction (Oxford, England)*, *12*(7), 1545-1549. Gardner, D. K., Lane, M., Stevens, J., Schlenker, T., & Schoolcraft, W. B. (2000). Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. Fertility and sterility, 73(6), 1155-1158.

Appendix

Additional Project: Gene expression analysis in adult zebrafish exposed to swimming-induced exercise.

Introduction

The main purpose of this project is to familiarize myself with genomics, the branch of biology that studies the structure, function and mapping of genetic material and learn about the different steps involved in the analysis of genetic material. While I conducted the analysis of the data in project 1, project 2 and project 3, in this project, I mostly shadowed my supervisor who conducted the vast majority of the data preparation and analysis. For this reason, I have included this project as an appendix rather than as a fourth project. In this study, we analyze the effect of physical activity (swimming) on the expression of genes in zebrafish and map the obtained results on the genetic pathways related to mitochondrial activity.

Data

The dataset is composed of 16 zebrafish exposed to either of two physical activities of different intensities: 8 fish were randomly assigned to the first treatment group where they had to produce a light physical activity (swimming at low speed), and the remaining 8 formed the second treatment group where they had to produce an intense physical activity (swimming at their optimal swimming speed). We investigate the genetic data of zebrafish because this species has a comprehensive genomic database. The dataset contains the level of expression of 45,220 probes (including dark corners) for each of the 16 fish of the experiment as well as the background level for each probe of the chip. The measurements were realized on a chip per subject. We therefore possess no information on the potential effect of the dye on the measurements.

Since mapping pathways related to mitochondrial activity is the ultimate goal of this project, we used the following steps to filter the probes and only keep those that have a known Ensembl ID (see table 1). We first filtered out the 2,417 dark corners and the 1,707 probes whose identification number was missing (and could therefore not be identified). From the 42,096 remaining probes, we then used the Agilent chip description available on the NCBI online database to identify the 33,295 unique probes of the dataset. We further used the DAVID platform to identify the 10,579 probes that are identified in

and described by the Ensembl database (Huang et al., 2008a and 2008b). Keeping only the unique probes, we end up with 8,615 probes with known function for the analysis.

Table 4.1: Filtering Steps

Filtering Step	Probe Count	Tool
All probes, including dark corners	45,220	-
All probes, excluding dark corner	43,803	-
All probes with existing ID	42,096	-
Uniquely identified probes	33,295	Agilent description (NCBI)
Probes with analogous elements in the human DNA	10,576	DAVID platform (Ensembl database)
Unique Probes with analogous elements in the human DNA	8,615	-

Method

After filtering the 45,220 probes of the original dataset to keep only the 8,615 probes with known function, we build three regression models for each probe. Model 1 only uses an intercept to predict the level of expression of each probe. Its formula is

$$y_i \sim \beta_{0i}, \qquad \text{for } i = 1, \dots, 8615$$

where the variable y_i represents the level of expression of probe i and the coefficient β_{0i} is the intercept corresponding to probe i. Model 2 is an extension of model 1 that includes the level of the background as a predictor. The formula of model 2 is

$$y_i \sim \beta_{0i} + \beta_{1i} * BG_i, \qquad \text{for } i = 1, \dots, 8615,$$

where the variable BG_i represents the level of the background of probe i. The other elements of the equation are defined as above. Model 3 extends model 2 and includes the treatment group to which each fish belongs as a predictor. The formula of model 3 is

$$y_i \sim \beta_{0i} + \beta_{1i} * BG_i + \beta_{2i} * t, \qquad \text{for } i = 1, \dots, 8615,$$

where the binary variable t indicates the treatment group to which fish f_j belongs:

$$t = \begin{cases} 0, & \text{if the fish belongs to the first treatment groups} \\ 1, & \text{if the fish belongs to the second treatment groups} \end{cases}.$$

The other elements of the equation are defined as above. We then compare, for each probe, the AIC of the three models to determine whether or not the exercise had an effect on its level of expression. We conclude that the treatments have a causal effect on the level of expression of a specific probe if the AIC of its model 3 is smaller than those of model 1 and model 2. The value of the coefficient β_2 of model 3 furthermore indicates the size of the effect. Since we are using the log intensities of the response variable, the group difference is to be interpreted as a fold change. For instance, a β_{2i} of 1.5 indicates that the level of expression of probe i is 1.5 larger in the second treatment group than it is in the first one; a β_2 inferior to 1 indicates that the probe is up regulated in the first treatment group.

We finally use the program PathVisio to identify pathways whose probes are down and up regulated under intense physical activity and visualize their distribution in the genetic pathways related to the activity of mitochondria. To accomplish this, we identify as *differentially expressed* the genes for which the difference in level of expression in the two treatment groups is (i) statistically significant and (ii) large enough to be biologically significant. For (i), we select genes whose best model in terms of AIC is model 3 and for (ii), we select genes for which the level of expression in of one the two treatment groups is more than 1.2 that in the other group. I chose this value fairly arbitrarily; clinicians may wish to use another one. Finally, we identify pathways characterized by a large number of genes identified as positive. To accomplish this, we consider pathways with an associated p-value inferior to 0.05. This p-value takes in account the proportion of positive probes in the pathways as well as the proportion of positive probes among those that were measured in the pathways. For instance, 50% of the measured probes of the pathway Osteoclast are positive; yet, since only 2 probes of this pathway were measured, its z-score is only 0.91 and the associated p-value is 0.36 (not statistically significant). On the contrary, 39% of the measured probes of the pathway Glycolysis and Gluconeogenesis are positive; yet, since 33 of the 57 probes composing this pathway are measured, the associated z-score is equal to 2.28, with a p-value of 0.021 (statistically significant).

Results

Table 2 presents the 14 pathways whose proportion of positive probes is statistically significant at the α-level 0.05. The pathway Mitochondrial LC-Fatty Acid Beta-Oxidation Pathway in Zebrafish, which is related to mitochondrial activity, has a z-score equal to

2.67 and a p-value equal to 0.005 (statistically significant). It is composed of 16 probes and 5 of the 8 probes whose level of expression is measured in this study are positive. Figure 1 presents the pathway and the importance of the different level of expression of the probes among the two treatment groups. A red color indicates that the probe is upregulated in the second treatment group i.e. the group of zebrafish that was subject to an intense physical activity and a green color indicates that the probes is downregulated in the second treatment group. The darker the color is, the larger the difference between the two treatment groups is. A grey color indicates that the probe is not included in the dataset. Given my limited knowledge of cellular biology and genetics, I leave the interpretation of the results to the clinicians operating at the department of reproductive medicine of Maastricht University Medical Center+. They can use the results of the analysis to identify the pathways characterized by large numbers of upregulated probes and use the platform PathVisio to visualize the difference of level of expression of probes across the two treatment groups in the pathways they are interested in. For the DNA of the zebrafish, the platform contains a total of 84 pathways.

Table 4.2: Significant Pathways

Pathway	Positive (r)	Measured (n)	total	%	Z-Score	p-value (Permuted)
Electron Transport Chain	24	40	109	60.00%	5.65	0
Oxidative phosphorylation	17	24	61	70.83%	5.62	0
TCA Cycle	12	24	45	50.00%	3.18	0.003
Nuclear receptors in lipid metabolism and toxicity	6	10	26	60.00%	2.8	0.005
Mitochondrial LC-Fatty Acid Beta-Oxidation	**5**	**8**	**16**	**62.50%**	**2.67**	**0.005**
DNA Replication	0	30	41	0.00%	-3.01	0.005
Cytoplasmic Ribosomal Proteins	5	62	81	8.06%	-2.83	0.006
Polyol pathway	2	2	12	100.00%	2.6	0.008
Glycolysis and Gluconeogenesis	13	33	57	39.39%	2.28	0.021
Cell cycle	6	58	91	10.34%	-2.31	0.023
Estrogen Signaling	4	44	68	9.09%	-2.21	0.027
G13 Signaling Pathway	0	17	35	0.00%	-2.26	0.028
Noncanonical Wnt Pathway	18	117	155	15.38%	-2	0.044
Nodal Signaling Pathway	10	75	100	13.33%	-2.01	0.046

Figure 4.1: Mitochondrial LC-Fatty Acid Beta-Oxidation Pathway in Zebrafish