ΕΘΝΙΚΟ ΚΑΙ ΚΑΠΟΔΙΣΤΡΙΑΚΟ ΠΑΝΕΠΙΣΤΗΜΙΟ ΑΘΗΝΩΝ
ΙΑΤΡΙΚΗ ΣΧΟΛΗ
&
ΠΑΝΕΠΙΣΤΗΜΙΟ ΔΥΤΙΚΗΣ ΑΤΤΙΚΗΣ
ΤΜΗΜΑ ΜΑΙΕΥΤΙΚΗΣ

ΠΡΟΓΡΑΜΜΑ ΜΕΤΑΠΤΥΧΙΑΚΩΝ ΣΠΟΥΔΩΝ
«ΕΡΕΥΝΑ ΣΤΗ ΓΥΝΑΙΚΕΙΑ ΑΝΑΠΑΡΑΓΩΓΗ»

ΤΙΤΛΟΣ ΔΙΠΛΩΜΑΤΙΚΗΣ ΕΡΓΑΣΙΑΣ

«Μελέτη της φυσιολογίας του φαινομένου δεύτερου ωοθυλακικού κύματος: αξιολόγηση επιπέδων ελεύθερου κυτταρικού DNA σε ωοθυλακικό υγρό πτωχών απαντητριών, που προκύπτει από την λήψη ωαρίων κατά την ωχρινική φάση, σε φυσικούς κύκλους εξωσωματικής γονιμοποίησης»

TITLE OF THESIS

"Concurring on the physiology of the second follicular wave phenomenon: evaluation of cell-free DNA levels in follicular fluid of poor responders undergoing Luteal Phase Oocyte Retrieval (LuPOR) during natural IVF cycles"

Όνομα μεταπτυχιακής φοιτήτριας : Τσιούλου Πετρούλα
Ιδιότητα : Βιολόγος
Α.Μ. : 20160714

Τριμελής Εξεταστική Επιτροπή:
1. κα. Σιμοπούλου Μάρα : Επιβλέπουσα
2. κ. Μαστοράκος Γεώργιος
3. κ. Κουτσιλιέρης Μιχαήλ

Αθήνα, 2019

AKNOWLEDGMENTS

It was a great pleasure for me to conduct my thesis for the master degree of "Research in Female Reproduction", that provides the unique opportunity to collaborate with highly-esteemed researchers in the context of reproductive physiology.

I would like to cordially thank my exceptional supervisor Dr. Mara Simopoulou, Assistant Professor of Physiology of Medical School of Athens, for her precious multilateral support throughout my thesis' conduction who further encouraged me to be acquainted with the world of assisted reproduction. I am grateful for her appreciation, as well as for all the scientific guidance and advice on this specific research field, along with promoting my introduction into the world of assisted reproduction technologies, that led to broaden my horizons with respect to the field of clinical embryology. Her eagerness to help and orchestrate every difficulty raised is obviously invaluable. She also gave me the chance to become a published author on important articles to peer-reviewed journals, to participate to notable congresses, thus contributing to my personal and educational qualification.

Furthermore, I would like to kindly thank Dr. George Mastorakos, for contributing with his peerless scientific insights, in line with the grant that he approved for the conduction of the experimental part of the present thesis, and I would also like to express my appreciation towards Professor Michael Koutsilieris of Medical School of Athens, for his honoring participation in my examination committee, providing helpful instructions on the topic of physiology of reproduction.

Moreover, I would like to particularly express my thanks towards the Centre for Human Reproduction "Genesis Athens Clinic", as it provided the participants for this study, in conjunction with entrusting all available data in encoding form stemming from patients' medical history and progress following in vitro fertilization procedure. In the same line, a great thank should undoubtedly be given to the Laboratory of physiology of Medical School of Athens for providing the optimal conditions and place for conducting every experimental procedure, with a special focus on Dr. Andrianos Nezos for his assistance and directions regarding standardization of protocol and several technical parts of the current thesis.

What is more, I would kindly like to thank the "fantastic five team", consisting of my brilliant collaborators, namely Polina Giannelou, Vaggelis Maziotis, Anna Rapani, Sokratis Grigoriadis, for the excellent team work, our daily incredible cooperation accompanied with their priceless help.

Last but not least, a great thank should be attributed to my friends for their personal support, as well as to my family for their belief, standing by me at every new step, as well as for the financial and psychological support all these years that encouraged me to continue the hard work.

TABLE OF CONTENTS

ABSTRACT		p. 4-5
ΠΕΡΙΛΗΨΗ		p. 6-7
I. GENERAL CHAPTER	**A. INTRODUCTION**	p. 8-25
	1) THE PROCESS OF OOGENESIS/ FOLLICULOGENESIS	p. 8-10
	2) OVARIAN RESERVE	p. 10-12
	3) POOR RESPONDERS IN ASSISTED REPRODUCTION	p. 12-15
	4) THE LUTEAL PHASE OOCYTE RETRIEVAL (LuPOR) APPROACH FOR POOR RESPONDERS	p. 15-22
	5) CELL-FREE DNA IN FOLLICULAR FLUID AS A POTENTIAL BIOMARKER OF IVF OUTCOMES	p. 22-25
II. SPECIFIC SHAPTER	**A. AIM OF THE STUDY**	p. 26
	B. MATERIALS AND METHODS	p. 26
	1) STUDY POPULATION: INCLUSION AND EXCLUSION CRITERIA	p. 26-27
	2) OUTCOMES: PRIMARY AND SECONDARY	p. 27-28
	3) NATURAL CYCLE PROTOCOL, OOCYTE RETRIEVAL AND FERTILIZATION	p. 28-29
	4) FOLLICULAR FLUID SAMPLE PREPARATION AND cfDNA EXTRACTION	p. 29-30
	5) QUANTIFICATION OF cfDNA USING RT-PCR METHOD	p. 30-33
	6) ELECTROPHORESIS METHOD	p. 33-34
	C. STATISTICAL ANALYSIS	p. 34
	D. RESULTS	p. 34-38
	E. DISCUSSION	p. 39-41
III. REFERENCES		p. 42-52
APPENDIX*		p. 53-58

ABSTRACT

Poor ovarian response represents an in-depth studied etiology of infertility, accompanied with low quantity and quality of oocytes that leads to poor prognosis of in vitro fertilization (IVF) outcomes. The heterogeneity of this group leads to the absence of consensus regarding diagnosis and definition of poor responders, which challenges their efficient management. Recently, the novel approach of double oocyte retrieval during both the follicular and luteal phases of a single menstrual cycle was introduced to IVF clinical practice. Our team introduced the acronym of LuPOR to describe the practice of luteal phase oocyte retrieval. This is based on previous observations in animal models reporting the existence of two or three follicular waves during a single menstrual cycle, which is averse to the prevailing theory of folliculogenesis. The aforementioned phenomenon is known as "Second Follicular Wave", being also detected in women. Current literature mainly focuses on dual stimulation and dual oocyte retrieval in the same menstrual cycle, indicating similar embryological parameters stemming from data regarding both phases. Moreover, studies that compared the approach of dual stimulation and oocyte retrieval to the conventional protocol, showed encouraging results with respect to the first option, providing a higher cumulative number of available oocytes in a shorter time frame. However, little is known about the practice of double oocyte retrieval during natural cycles, along with the underlying physiology with respect to the mechanism of the second follicular wave phenomenon. Concurrently, numerous biomarkers have been proposed to evaluate oocyte and embryo competency, with the novel option of the non-invasive biomarker of cell-free DNA (cfDNA) in follicular fluid (ff) to be at the spotlight of research. The aim of this prospective study is to uniquely investigate the physiology of the second follicular wave phenomenon, through the evaluation of ff cfDNA levels resulting from follicular and luteal phases of the same menstrual cycle, during natural IVF cycles. The aforementioned intents to reveal a potential association with the number and maturation status of corresponding oocytes, as well as the number of subsequent zygotes following insemination. The study population included 47 poor responders aged from 32 to 48 years, being characterized according to Bologna criteria, and enrolled based on strict inclusion and exclusion criteria. All women were detected with a second follicular wave and thus they underwent double oocyte retrieval in the same menstrual cycle during natural IVF cycles. The preparation of collected ff samples and the subsequent cfDNA extraction were conducted appropriately, resulting in cfDNA quantification through real-time polymerase chain reaction (RT-PCR) method. For RT-PCR, we opted for ALU115 and ALU247 primer sets, with the first to amplify both short and long cfDNA fragments, while the second only the long ones.

Statistical analysis was performed employing R statistical programming language. The results showed that the mean levels of ALU115 were statistically significant lower during FoPOR when compared to LuPOR (0.79 ± 0.72 vs 1.46 ± 1.59 ng/μl, p-value=0.02). Regarding FoPOR's group, a statistically significant positive correlation of serum estradiol levels and ALU115 concentration (p-value=0.04) was revealed, along with a negative correlation of cfDNA integrity and estradiol levels (p-value=0.03). The latter was also observed with respect to LuPOR's group (p-value=0.03). No other statistically significant difference was observed between ALU115, ALU247 and cfDNA integrity for any of the examined parameters. Finally, a statistically significant lower number of oocyte retrieved (1.29±0.58 vs 1.09 ± 0.28, p-value=0.02) and MII oocytes (0.77 ± 0.55 vs 1.08 ± 0.61, p-value=0.02) regarding FoPOR were recorded when comparing FoPOR to LuPOR groups. However, no statistically significant difference was demonstrated regarding the number of 2PN zygotes. The aforementioned results highlight the quality and safety of performing retrieval in both the follicular and luteal phases, as evaluated through the apoptotic marker of ff cfDNA. Furthermore, they buttress the validity of LuPOR approach, rendering it as an alternative and highly-promising option for the time-sensitive poor responders. However, further randomized controlled trials are imperative to be conducted with the intent to strengthen our results regarding LuPOR practice during natural IVF cycles.

ΠΕΡΙΛΗΨΗ

Η πτωχή ωοθηκική απόκριση αποτελεί μια καλά μελετημένη αιτία υπογονιμότητας, συνοδευόμενη από λίγα ωάρια χαμηλής ποιότητας, γεγονός που οδηγεί σε κακή πρόγνωση των αποτελεσμάτων της εξωσωματικής γονιμοποίησης. Η ετερογένεια της ομάδας αυτής επιφέρει την απουσία ομοφωνίας αναφορικά με τη διάγνωση και τον ορισμό των πτωχών απαντητριών, κάτι που δυσκολεύει τη διαχείρισή τους. Πρόσφατα, η καινοτόμος προσέγγιση της διπλής ωοληψίας κατά τη διάρκεια ωοθυλακικής και ωχρινικής φάσης σε ένα έμμηνο κύκλο έχει εισαχθεί στην κλινική πρακτική της εξωσωματικής γονιμοποίησης. Η ομάδα μας πρότεινε το ακρωνύμιο LuPOR για να περιγράψει την πρακτική της ωοληψίας κατά την ωχρινική φάση. Αυτή βασίζεται σε προηγούμενες παρατηρήσεις σε ζωικά μοντέλα που αναφέρουν την ύπαρξη δύο ή τριών ωοθυλακικών κυμάτων κατά τη διάρκεια ενός έμμηνου κύκλου, το οποίο αντιβαίνει στην υπάρχουσα θεωρία της ωοθυλακιογένεσης. Το προαναφερθέν φαινόμενο είναι γνωστό ως «δεύτερο ωοθυλακικό κύμα» κι έχει εντοπιστεί και στις γυναίκες. Η τρέχουσα βιβλιογραφία εστιάζει στην διπλή διέγερση των ωοθηκών και στη διπλή ωοληψία στον ίδιο εμμηνορρυσιακό κύκλο, δείχνοντας παρόμοιες εμβρυολογικές παραμέτρους από δεδομένα των δυο φάσεων. Επιπλέον, μελέτες που συγκρίνουν την προσέγγιση της διπλής διέγερσης και ωοληψίας με το συμβατικό πρωτόκολλο αποκαλύπτουν ενθαρρυντικά αποτελέσματα αναφορικά με την πρώτη επιλογή, καθώς παρέχει ένα συγκεντρωτικό αριθμό διαθέσιμων ωαρίων σε συντομότερο χρονικό διάστημα. Ωστόσο, ελάχιστα είναι γνωστά σχετικά με την πρακτική της διπλής ωοληψίας κατά τη διάρκεια φυσικών κύκλων εξωσωματικής γονιμοποίησης, αλλά και για την υποκείμενη φυσιολογία του μηχανισμού του φαινομένου του δεύτερου ωοθυλακικού κύματος. Ταυτόχρονα, πολλοί βιοδείκτες έχουν προταθεί για την αξιολόγηση των ωαρίων και εμβρύων, με την καινοτόμο επιλογή του μη επεμβατικού βιοδείκτη του ελεύθερου κυτταρικού DNA (cfDNA) στο ωοθυλακικό υγρό να βρίσκεται στο επίκεντρο της έρευνας. Σκοπός της παρούσας προοπτικής μελέτης είναι να διερευνήσει για πρώτη φορά τη φυσιολογία του φαινομένου του δεύτερου ωοθυλακικού κύματος μέσω της αξιολόγησης των cfDNA επιπέδων στο ωοθυλακικό υγρό από την ωοθυλακική και ωχρινική φάση, σε φυσικούς κύκλους εξωσωματικής γονιμοποίησης. Το προαναφερθέν στοχεύει στο να αποκαλύψει μια πιθανή συσχέτιση του αριθμού και της ωριμότητας των ωαρίων, καθώς και του αριθμού των γονιμοποιημένων ζυγωτών έπειτα από την έγχυση. Ο πληθυσμός της μελέτης αποτελείται από 47 πτωχές απαντήτριες ηλικίας από 32 έως 48 ετών, που έχουν χαρακτηριστεί βάσει των κριτηρίων της Bologna και έχουν ενταχθεί βάσει αυστηρών κριτηρίων ένταξης και αποκλεισμού. Όλες οι

γυναίκες ανιχνεύτηκαν με δεύτερο ωοθυλακικό κύμα κι έτσι υποβλήθηκαν σε δεύτερη ωοληψία του ίδιου έμμηνου κύκλου κατά τη διάρκεια φυσικών κύκλων εξωσωματικής γονιμοποίησης. Πραγματοποιήθηκε η προετοιμασία των δειγμάτων ωοθυλακικού υγρού και η επικείμενη απομόνωση του cfDNA, με σκοπό την ποσοτικοποίηση της συγκέντρωσης του cfDNA μέσω της μεθόδου αλυσιδωτής αντίδρασης πολυμεράσης σε πραγματικό χρόνο (RT-PCR). Για την RT-PCR, επιλέξαμε τους εκκινητές ALU115 και ALU247, με τους πρώτους να ενισχύουν τόσο τα μικρά όσο και τα μεγάλα θραύσματα cfDNA, ενώ οι δεύτεροι μόνο τα μεγάλα. Η στατιστική ανάλυση πραγματοποιήθηκε με τη χρήση της R γλώσσας προγραμματισμού. Τα αποτελέσματα έδειξαν ότι η μέση τιμή των επιπέδων των ALU115 ήταν στατιστικά σημαντικά μικρότερη κατά την ωοθυλακική φάση σε σχέση με την ωχρινική (0.79 ± 0.72 vs 1.46 ± 1.59 ng/μl, p-value=0.02). Εστιάζοντας στα αποτελέσματα της ωοθυλακικής φάσης, αποκαλύφθηκε μια στατιστικώς σημαντική συσχέτιση των επιπέδων οιστραδιόλης ορού και συγκέντρωσης ALU115 concentration (p-value=0.04) καθώς και αρνητική συσχέτιση της ακεραιότητας του cfDNA με τα επίπεδα οιστραδιόλης (p-value=0.03). Το τελευταίο παρατηρήθηκε επίσης και στην ομάδα LuPOR (p-value=0.03). Καμία άλλη στατιστικώς σημαντική συσχέτιση δε βρέθηκε μεταξύ των συγκεντρώσεων ALU115, ALU247 και ακεραιότητας cfDNA με οποιαδήποτε παράμετρο που εξετάστηκε. Τέλος, βρέθηκε στατιστικά σημαντικός μικρότερος αριθμός ωαρίων κατά την ωοληψία (1.29±0.58 vs 1.09 ± 0.28, p-value=0.02) και ώριμων MII ωαρίων (0.77 ± 0.55 vs 1.08 ± 0.61, p-value=0.02) αναφορικά με την ωοληψία ωοθυλακικής φάσης, ύστερα από σύγκρισή του με την LuPOR ομάδα. Ωστόσο, καμία στατιστικά σημαντική διαφορά δεν αποδείχθηκε ως προς τον αριθμό των γονιμοποιημένων ζυγωτών. Τα αποτελέσματα αυτά τονίζουν την ποιότητα και της ασφάλεια την ωοληψίας τόσο κατά την ωοθυλακική όσο και κατά την ωχρινική φάση, όπως αξιολογήθηκε μέσω του αποπτωτικού δείκτη του cfDNA. Επιπροσθέτως, ενισχύουν την εγκυρότητα της LuPOR πρακτικής, καθιστώντας την σαν μια εναλλακτική και πολλά υποσχόμενη πρακτική για την χρονοευαίσθητη ομάδα των πτωχών απαντητριών. Ωστόσο, είναι αναγκαία η διεξαγωγή επιπλέον τυχαιοποιημένων μελετών με σκοπό να ενδυναμώσουν τα αποτελέσματά μας σχετικά με την αξιοποίησης της LuPOR πρακτικής σε φυσικούς κύκλους εξωσωματικής γονιμοποίησης.

I. GENERAL CHAPTER

A. INTRODUCTION

1) THE PROCESS OF OOGENESIS/FOLLICULOGENESIS

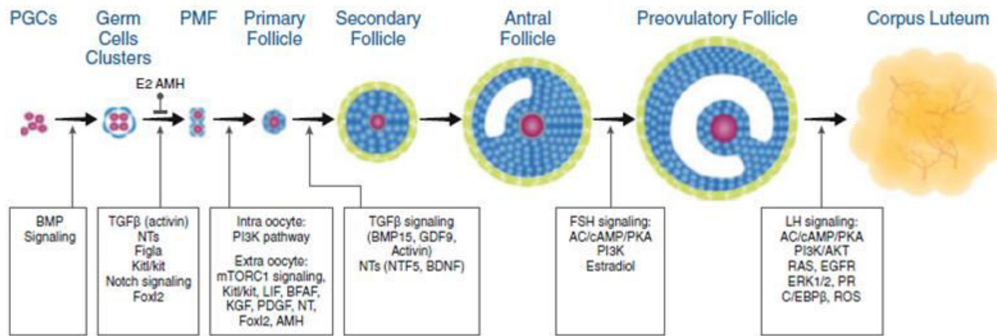
The word “ovary” is derived from the Latin term “ovum”, meaning “egg” (“ovary | Origin and meaning of ovary by Online Etymology Dictionary,” n.d.; Soutis, 2006). Ovaries depict the principal gonads of the female reproductive system, playing the dual pivotal role of gamete production, and hormonal secretion. In fact, they can successfully orchestrate follicular growth as well as ovulation of mature oocyte, in line with their endocrinological function of producing sex hormones, mainly referring to estrogens and progesterone (Rimon-Dahari, Yerushalmi-Heinemann, Alyagor, & Dekel, 2016). In 1827, Karl Ernst von Baer discovered that mammalian oocyte was enclosed in ovarian follicle (Cobb, 2006)

In the first arm, female gamete production-known as “oogenesis” which goes hand in hand with “folliculogenesis”-is initiated at the embryonic stage, when primordial germ cells proliferate, along with their migration from the dorsal axis of the inferior mesendery, to the developing genital niche in a female XX embryo (Rimon-Dahari et al., 2016; Virant-Klun, 2015). This physiological process is mainly controlled by signals originating from extraembryonic ectoderm and visceral endoderm, which in turns expresses several Proteins of Bone Morphogenetic family (Figure 1) (Rimon-Dahari et al., 2016). The clusters of primordial germ cells break down to primordial follicles, each of them containing a single oogonium encompassed by a flattened epithelium of pre-granulosa cells. Oogonia continue to proliferate through mitotic divisions, until they reach prophase I of meiotic divisions, where they become arrested at diploid primary oocyte (Nikolic, Volarevic, Armstrong, Lako, & Stojkovic, 2016). Similarly, the aforementioned process is regulated by various molecules, mostly activin and FOXL2 (Figure 1) (Rimon-Dahari et al., 2016). During this developmental period, chromosomes remain attached inside the oocyte’s nucleus that is called “Germinal Vesicle” (GV) within the primordial follicles (Heiligentag & Eichenlaub-Ritter, 2018). Following birth, they turn into primary oocytes in primordial follicles. At this stage, primordial follicles remain inactive until the commencement of menarche, in which the first selected pool of primary oocytes complete meiotic division, separating into a haploid secondary oocyte accompanied with the subsequent extrusion of the first polar body (Virant-Klun, 2015).

Knowledge concerning follicular growth was obtained through the evaluation of ovarian histology after laparotomy, in combination with endocrinologic results and transvaginal ultrasonography (Chikazawa, Araki, & Tamada, 1986). Indeed, a series of different developmental stages of follicles occurs, including the transition from primordial follicle to primary and secondary follicle. Several factors inside and outside the primordial follicles contribute to their development to primary follicles, whereas the evolution to preantral secondary follicle consists of the proliferation of granulosa cells enclosed within theca cells, under the control of TGF-β pathway (Figure 1) (Rimon-Dahari et al., 2016). The early stages of primordial to preantral follicles are independent from the secretion of pituary gonadotropins, namely Follicle Stimulation Hormone (FSH) and Luteinizing Hormone (LH), are factors such as inhibin, activin, follistatin, transforming growth factor-b are mostly involved. Follicular maturation occurs in conjunction with the differentiation of granulosa cells into mural and cumulus granulosa cells, along with the formation of a single cavity widely known as antrum (Heiligentag & Eichenlaub-Ritter, 2018; Rimon-Dahari et al., 2016). Mural granulosa cells mediate to the formation of the inner lining of follicles, while cumulus granulosa cells develop the cumulus-oocyte complex (Hennet & Combelles, 2012). Antral stage follicles increase in size and are mostly dependent from the pulsatile-fashion hormonal stimuli of FSH and LH that are produced by the activation of Hypothalamus-Pituitary-Ovary axis (HPA) (Plant, 2015). Follicle Stimulation Hormone is essential for survival and proliferation of granulosa cells. The selection of “dominant” or “privileged” or so called “Grafian” follicle is succeeded by LH surge that triggers ovulation, with the subsequent release of oocyte into fallopian tube, while the remaining granulosa cells differentiate into corpus luteum under progesterone production (Heiligentag & Eichenlaub-Ritter, 2018).

To conclude, folliculogenesis represents a crucial physiological process, including recruitment of follicles during menstruation, the selection of a subgroup of primordial ones accompanied by atresia and maturation of the primary follicle that lead to a competent oocyte characterized by both nuclear and cytoplasmic maturation. The latter requires a complex bilateral dialog between oocyte and cumulus cells, exchanging numerous molecules, namely fatty acids, transcripts, nutrients or miRNAs via gap junctions or transporters into oolema (Heiligentag & Eichenlaub-Ritter, 2018).

Figure 1: A schematic presentation of the stages of ovarian folliculogenesis and the major factors and signaling pathways involved in this process (Rimon-Dahari et al., 2016).



PGCS: Primordial Germ Cells; PMF: Primordial Follicle

2) *OVARIAN RESERVE*

During the 20th week of gestation, about 7 million primordial follicles are observed in the developing ovary, from which the vast majority degenerate through the natural atresia process. Following birth, almost a million primordial follicles are presented in the gonad with the intent to be recruited and further grow or contrarily to undergo the physiological atretic process. Thenceforth at puberty, almost 300,000-400,000 follicles are available to be recruited for each menstrual cycle, throughout the reproductive age until menopausal period, which in turns signs the end of fecundity, as the follicular pool is totally diminished (Figure 2) (Persani, Rossetti, & Cacciatore, 2010).

Figure 2: Follicular dynamics in physiology (Persani et al., 2010).

Life stages	Number of total follicles (primordial/primary)
Gestation (20th week)	7×10^6
Birth	1×10^6
Puberty	300 000
Pre-menopause	<1000
Menopause	0

Atresia

Ovulation

400 – 500

The physiological transition from puberty to menopause coincides with ovarian or reproductive aging. Interestingly, the rate of follicles' depletion differs among women and it is affected by a wide pallet of factors, mainly by the age, race, along with genetics and

environmental parameters (Tal & Seifer, 2017). Therefore, several contributing aspects, such as endometrioma, specific pelvic infections, ovarian surgery, chemotherapy or radiotherapy for cancer diseases, reduced aromatase activity, alterations in blood flow, or even obesity and chronic smoking, seem to play a vital role to follicular decline (Jirge, 2016). Concerning genetics, experiments on mouse models documented an association of mutations on the fragile X mental retardation 1 (FMR1) gene with ovarian aging (Hoffman et al., 2012). These results were confirmed by prospective studies in humans, observing that reduced number of FMR1 gene alleles was related with poor ovarian reserve (Gleicher, Weghofer, & Barad, 2010; Gleicher et al., 2015). Other defects of X chromosomes, including depletions, translocations or rearrangements, conduce to the appearance of premature ovarian failure or insufficiency (Laven, 2016), which is defined as the cessation of menstruation prior the age of 40 (Cohen, Chabbert-Buffet, & Darai, 2015). Importantly, polymorphisms or alternatively spliced variants of FSH receptor in cumulus and granulosa cells showed that elicit unexpected poor response in young women (Desai, Roy, & Mahale, 2013). Additionally, over the last decades, women tend to delay the time of childbearing, commonly due to social reasons stemming from their infinite focus on career success or educational progress (Simoni, Mu, & Collins, 2017). However, advanced maternal age may exert an impact on pregnancy outcome, with several studies to demonstrate that pregnancy rates seem to be intensely decreased over the age of 35, with pregnancy loss rates, along with chromosomal abnormalities to be increased (Crawford & Steiner, 2015).

It is notable that the aforementioned subgroups of women reflect different sides of the same coin, with the common denominator being the reduced quantity of primordial follicular pool during the reproductive period. This special population presents huge heterogeneity, including patients presenting with premature ovarian failure, poor responders, or women of advanced maternal age. They can plainly be included in the general category known as "Poor Ovarian Reserve" (POR), a term that was introduced in an attempt to concur on the reproductive potential of women, with respect to the quantity and quality of their oocytes (Jirge, 2016). Unfortunately, their fecundity is irrevocably jeopardized, and thus they tend to fail to achieve a pregnancy through natural conception. According to the World Health Organization, infertility is defined as the failure to achieve a clinical pregnancy following 12 months of regular and unprotected sexual intercourse (Zegers-Hochschild et al., 2009). Therefore, infertile couples often turn to Assisted Reproduction units, with the intent to seek *in vitro* fertilization (IVF) treatment, prior to opting for oocyte donation programs which may be viewed as the last resort. The demanding cases of patients presenting with reduced ovarian follicular pool pose a challenge to the highly-esteemed scientists, who in turn propose various markers to guide IVF management and enable accurate prediction of ovarian reserve in an efficient manner.

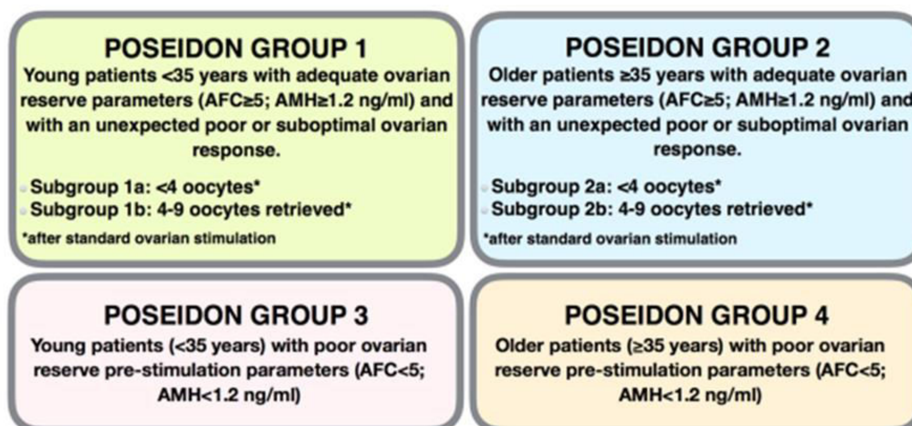
The first ovarian reserve test was voiced by Scott and his colleagues concerning FSH measurement on day-3 of menstrual cycle (Scott, Hofmann, Oehninger, & Muasher, 1990). Thenceforth, numerous tests were emerged, including clomiphene citrate challenge test, inhibin B, estradiol (E_{2}), gonadotropin-releasing hormone (GnRH), antral follicle count (AFC), and anti-müllerian hormone (AMH) (Fleming, Seifer, Frattarelli, & Ruman, 2015; Tal & Seifer, 2017). However, the indirect estimations served as confounders for the results. Nowadays, the most sensitive and accurate biomarkers that are commonly opted include the AFC, and AMH, with the latter to be rendered as the "queen" among the prognostic markers (Jirge, 2016). On the one hand, AFC represents the total number of follicles that are observed via ultrasound in both ovaries during day 2-4 of menstrual cycle and they are measured at 2-10 mm in diameter. On the other hand, AMH is produced ultimately by granulosa cells of small and large preantral and small antral follicles (Fleming et al., 2015; Jirge, 2016). Estimating women's ovarian reserve may enhance specialist's adjustment for a personalized treatment, tying to predict the response for individual woman which may favor the subsequent IVF outcomes (La Marca & Sunkara, 2014).

3) POOR RESPONDERS IN ASSISTED REPRODUCTION

During the IVF procedure, a crucial step is controlled ovarian stimulation, in order to increase the number of oocytes retrieved (Pacchiarotti et al., 2016). In Assisted Reproductive Technologies (ART) world, the cases that under-response to stimulation ovarian protocols is defined as poor ovarian response. Poor ovarian response is considered to be an important etiology of infertility, with a prevalence that ranges from 9% to 24% among women who undergo ovarian stimulation (N. P. Polyzos & Devroey, 2011). Following literature search, it is obvious that there are numerous terms employed, namely "poor", "low", "bad", "slow", "inadequate", "suboptimal" that are joined with "response", "responder" and "ovarian reserve" (A. P. Ferraretti et al., 2011). Garcia and his colleagues were the first to report on poor responders, resulting in reduced number of oocytes retrieved, following ovarian stimulation with human menopausal gonadotropin and human chorionic gonadotropin (hCG), (Garcia, Jones, Acosta, & Wright Jr, 1983). However until now, there is an absence of a consensus regarding POR diagnosis and definition, due to overlapping among the different subgroups, which adds another level of complexity to their management (N. P. Polyzos & Devroey, 2011).

In light of the wide range of definitions employed for the description of poor responders throughout the studies (Surrey & Schoolcraft, 2000), it was imperative to decide on a universally accepted definition. Thus, a workshop was organized by the European Society of Human Reproduction (ESHRE) and Embryology, in which numerous research groups participated, resulting in setting the appropriate criteria-known as the "Bologna criteria"-regarding POR definition. According to the "Bologna criteria", a woman could be characterized as a poor responder, if she fulfills at least two of the following three parameters: i) advanced maternal age (≥40 years) or any other risk factor for POR; ii) a previous POR, which entails ≤3 oocytes with a conventional stimulation protocol; iii) an abnormal ovarian test, meaning AFC: 5-7 follicles or AMH: 0.5-1.1 ng/ml (A. P. Ferraretti et al., 2011). Particularly, Bologna criteria fail to define precisely the risk factors, the cut-off points of AFC and AMH measured, or even to take into account oocyte quality. Particularly, several risk factors that may harbor the peril during the treatment of the distinct category of infertile women are both medical and genetic parameters, with a great emphasis on short menstrual cycle, ovarian cystectomy and chronic smoking (Younis, Ben-Ami, & Ben-Shlomo, 2015). Undoubtedly, the primary challenge was to approach the category of the true poor responders who fail to conform to Bologna criteria. Later, the POSEIDON group (Patient-Oriented Strategies Encompassing IndividualizeD Oocyte Number) suggested a novel classification, enlisting patients with poor response into four separate groups, based on the following parameters: i) age and expected aneuploidy rate; ii) ovarian biomarkers, namely AFC and AMH; iii) ovarian response, which requires a previous stimulation cycle. The four groups are portrayed in Figure 3 (Humaidan, Alviggi, Fischer, & Esteves, 2016).

Figure 3: Four groups of 'low prognosis patients' in ART, according to the POSEIDON's stratification based on oocyte quantity and quality (Humaidan et al., 2016).



Efforts should be focused on the efficient management of poor responders, in light of the poor prognosis regarding IVF outcomes. Studies have demonstrated reduced oocyte yield concerning poor responders, with up to 7% of them to appear one oocyte, coupled with decreased pregnancy rate at almost 14.8%, following the comparison to normal responders (Oudendijk, Yarde, Eijkemans, Broekmans, & Broer, 2012). Furthermore, studies have observed a higher pregnancy loss rate, with a percentage of 47% originated from poor responders in contrast to 25% from normal responders (Haadsma et al., 2010).

Available data from current reports reveal that the management of poor responders still remains a heated debate among IVF experts (Giovanale, Pulcinelli, Ralli, Primiero, & Caserta, 2015). In this context, there are two principal schools of thoughts, with the first to support that stimulation protocols ascertain the optimal results for poor responders, while the second one to support the option of the natural cycle approach (Jirge, 2016; F Ubaldi et al., 2007; Filippo Ubaldi, Vaiarelli, D'Anna, & Rienzi, 2014).

Concerning the stimulation approach, its main purpose is to increase the patients' ovarian response, employing a broad range of protocols (Jirge, 2016; Filippo Ubaldi et al., 2014). Following literature search, the respective stimulation protocols involve gonadotropins (Anna Pia Ferraretti et al., 2004), GnRH agonists or antagonists (Pu, Wu, & Liu, 2011; M Schimberni et al., 2016), clomiphene citrate (Haas & Casper, 2017; Song et al., 2016) or combination of clomiphene citrate and gonadotropins (D'Amato et al., 2004), or alternatively protocols that employ hCG (Berkkanoglu, Isikoglu, Aydin, & Ozgur, 2007), growth hormone (X.-L. Li et al., 2017), letrozole (Haas & Casper, 2017), melatonin (Pacchiarotti et al., 2016; Vitale et al., 2016), low-dose aspirin (Frattarelli, McWilliams, Hill, Miller, & Scott Jr, 2008; Lok, Yip, Cheung, Yin Leung, & Haines, 2004), heparin of low molecular weight, adrogens such as deydroepiandrosterone (DHEA) (Bosdou et al., 2012; Chern et al., 2018), myo-inositol (Unfer, Raffone, Rizzo, & Buffo, 2011; Vitale et al., 2016), or even corifollitropin alfa (Andrisani et al., 2019). It seems that the antagonist stimulation protocol is the most widely employed for poor responders by clinicians, as it was ranked on the top of the list among the other choices (Papathanasiou, Searle, King, & Bhattacharya, 2016). A randomized controlled trial (RCT) compared the IVF outcomes resulting from minimal stimulation protocol and those from conventional GnRH antagonist protocol in 77 poor responders. The results demonstrated that there was no statistically significant difference regarding the number of oocytes retrieved as well as the pregnancy rate, rendering minimal stimulation as a valid option, avoiding IVF treatment's medication and cost (Pilehvari et al., 2016).

An alternative and moderate treatment for poor responders represents the employment of natural IVF cycles, which can be chosen by all women who want to bypass the administration of drugs, which in turns entails less somatic and financial burden (Jirge,

2016). Natural IVF cycles may embrace several subcategories. In the first arm, it may include the retrieval of the dominant oocyte during woman's spontaneous cycle, followed by the impending single embryo transfer (J. Li, Xu, Zhou, Guo, & Xin, 2011). In the second arm, it may be reflected through repeated natural cycles, followed by the subsequent cryopreservation of zygote-stage embryos through vitrification, a method known as "freeze and collect" or "single embryo banking". In this case, clinicians make an attempt to resemble the situation that occurs in normal responders, resulting in remarkable live birth outcomes (Cobo, Garrido, Crespo, José, & Pellicer, 2012). The final arm includes the modified natural cycle with the administration of hCG during women's menstrual cycle. It is characterized as second-line option for the management of poor responders and it is accompanied with notable live birth outcomes (Ho & Paulson, 2017; Lainas et al., 2015). In fact, various studies support the option of natural cycles for poor responders who are presented with one or two follicles (J. Li et al., 2011; Loutradis, Drakakis, Vomvolaki, & Antsaklis, 2007; Morgia et al., 2004; Filippo Ubaldi et al., 2014). Furthermore, a large retrospective study including 500 poor responders revealed promising results following natural IVF cycles, with pregnancy rates to reach the percentage of 9.7% per patient aged over 40 years, while a percentage of 31.7% per patient aged younger than 35 years (Mauro Schimberni et al., 2009). Contrarily, another retrospective cohort study showed lower birth rates regarding poor responders-who were defined according to Bologna criteria-when comparing to normal responders, leading to the conclusion that the therapeutic potential of natural IVF cycle is restricted in cases of poor responders (N. Polyzos et al., 2012). Consequently, natural cycle is apparent more cost-effective and patient-friendly therapeutic option compared to stimulation protocols, and it is further accompanied with remarkable IVF outcomes (Practice Committee of the American Society for Reproductive Medicine, 2018).

Interestingly, the lack of standardization of treatment usually leads clinicians to reclaim their experience and employ an empirical approach, in order to dictate the ideal management of this special group. To conclude, it is apparent that the management of poor responders is still characterized by controversy, as the heterogeneous nature of this category of patients pose difficulties in establishing a common treatment strategy.

4) THE LUTEAL PHASE OOCYTE RETRIEVAL (LuPOR) APPROACH FOR POOR RESPONDERS

A restrictive factor for POR patients of advanced maternal age is time. The latter goes hand in hand with IVF cycle cancellation, due to several reasons, such as failure of oocyte retrieval, or response to stimulation protocol, or even gametes' fertilization (Gonda, Domar, Gleicher, & Marrs, 2018). Under the prism of time and cycle postponing, couples are inevitably discouraged, presented with further psychological strain and physical burden, especially on behalf of women (Gameiro, Boivin, Peronace, & Verhaak, 2012; Garel et al., 2009). On that concept, it becomes apparent that it is of paramount importance to determine both time and oocyte yield for these patients. Recently, IVF specialists introduced to their clinical practice the novel approach of double oocyte retrieval during the same menstrual cycle, in light of the increased E_2 levels and developing follicles recorded via ultrasound monitoring (Kuang et al., 2014). This can be explained by previous observations of the phenomenon of "Second Follicular Wave", mostly in animal models (Baerwald, Adams, & Pierson, 2003a). Our team proposed the acronym LuPOR, in an attempt to describe the clinical practice of **Lu**teal **P**hase **O**ocyte **R**etrieval (Sfakianoudis et al., 2019). The emergence of LuPOR approach represents a highly promising practice that could revolutionize the therapeutic strategy for time-sensitive patients, such as poor responders.

The "Second Follicular Wave" phenomenon

The traditional theory of folliculogenesis process includes the recruitment of a single cohort of 2-5 mm antral follicles (follicular wave), with the subsequent development of one dominant follicle throughout the follicular phase of the menstrual cycle (Gougeon & Lefèvre, 1983).

However, robust evidence resulting from observations of the estrous cycle of various animal models, such as ruminants (G. Adams, 1999), heifer (G. P. Adams, Matteri, Kastelic, Ko, & Ginther, 1992; Knopf, Kastelic, Schallenberger, & Ginther, 1989; Sirois & Fortune, 1988), cattle (Ginther, Kastelic, & Knopf, 1989; Pierson & Ginther, 1988), equine (Ginther, 1993), suggest the existence of more than one "cohorts" or "waves" of follicles, leading to the development of at least two privileged follicles, during the same menstrual cycle (Vaiarelli et al., 2018). Particularly, researchers detected two or three follicular waves during interovulatory interval that were characterized as "major" and "minor", with the final wave to result in ovulation. It was supported that major waves could be either ovulatory or not, whereas the minor waves to be ultimately anovulatory. The changes regarding the follicular morphology and hormonal status during two or three follicular waves are describing in Figure 4 and Figure 5 respectively (Baerwald, Adams, & Pierson, 2003b). Concurrently, specific hormonal changes were noted, namely increased levels of FSH, which ensued by the raise of

E_2 levels, when follicular waves were in progress, in contrast to the decline of FSH concentrations along with the expression of LH receptors in granulosa cells at the end of the phenomenon (Gastal, Gastal, & Ginther, 1999).

In the same line, similar wave patterns and hormonal observations were documented in humans. Interestingly, the evaluation of FSH, LH, and E_2 levels was conducted, in conjunction with the monitoring of the number and diameter of developing follicles via high-resolution transvaginal ultrasound, in an effort to describe the demonstration of the current phenomenon in women (Baerwald et al., 2003b). The results were in accordance to those originated from animal models, a fact that confirms the existence of the phenomenon of multiple follicular waves in humans. The above clinical observations in women challenge the traditional notion of folliculogenesis, updating the field of reproduction. As a consequence, the assumption behind follicular selection could be aptly depicted through the theory of "continuous recruitment", or a "single recruitment episode", or finally "follicular waves" (Baerwald, Adams, & Pierson, 2012).

The underlying mechanisms of this phenomenon still remain the "black box" in physiology (Vaiarelli et al., 2018). It is widely known the contributing role of HPA axis to follicular dynamics, mainly through the secretion of GnRH and gonadotropins. In fact, FSH is crucial for preventing new developing follicles from atretic process (van Santbrink, Hop, van Dessel, de Jong, & Fauser, 1995). Additionally, when corpus luteum is formed during luteal phase, it produces progesterone which is coupled with the development of major and minor follicular waves in women. Contrarily to this, progesterone contributes to pituitary suppression which in turn prevents minor waves from being ovulatory (Kuang et al., 2014; Vaiarelli et al., 2018). Robust evidence support that various markers of inflammation seem to be crucial for the follicular wave patterns (Salmassi et al., 2001; Vinatier et al., 1995), mainly with serum C reactive protein (CRP) to be secreted during ovulation, in the middle of luteal phase, as well as during menstruation. Studies investigated high CRP levels in blood serum of women appeared with two follicular waves when compared to those with two follicular waves (Clancy, Baerwald, & Pierson, 2013).

Figure 4: Morphologic and endocrinologic changes associated with two waves of follicle development during the human menstrual cycle. Dotted vertical lines indicate the days of wave emergence. Follicles are shown in pink and corpus luteum in yellow (Baerwald et al., 2012)

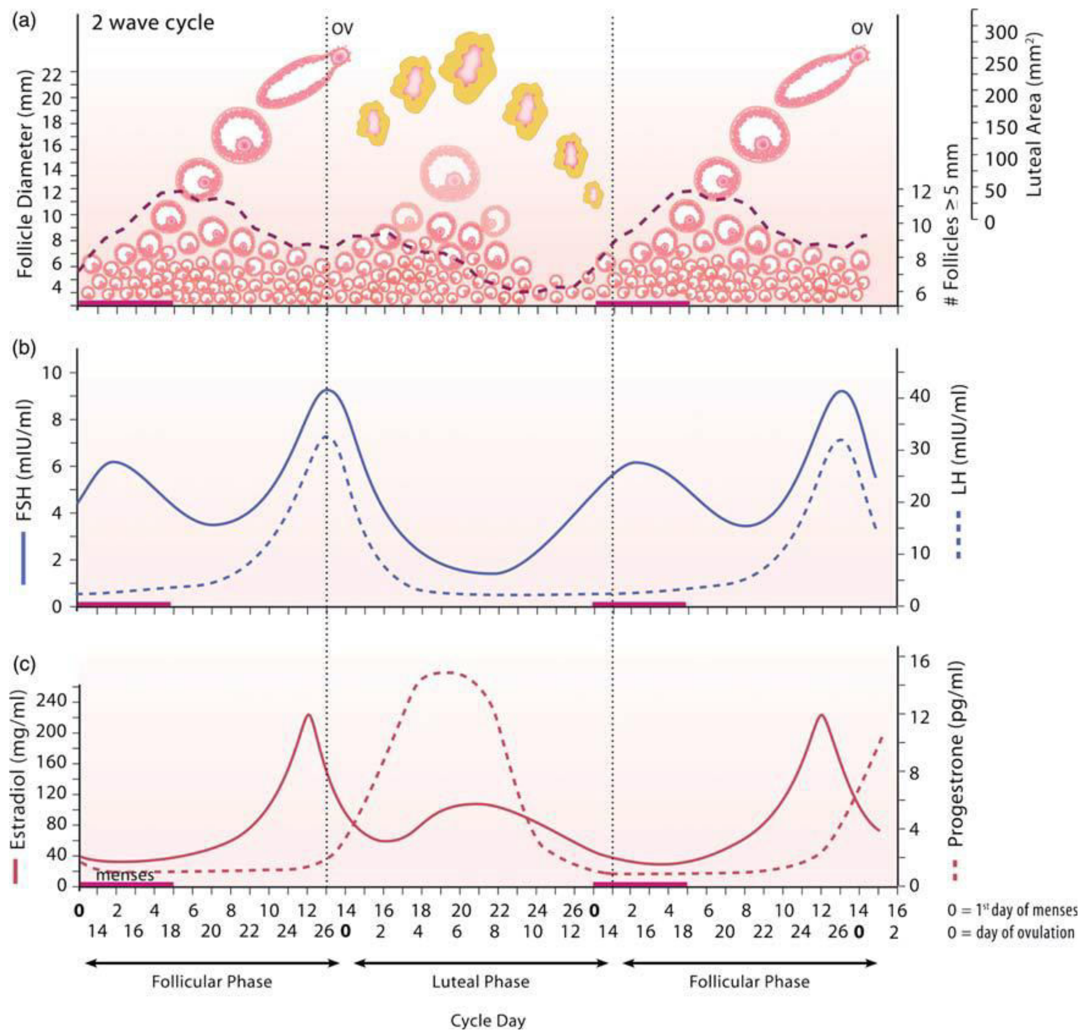
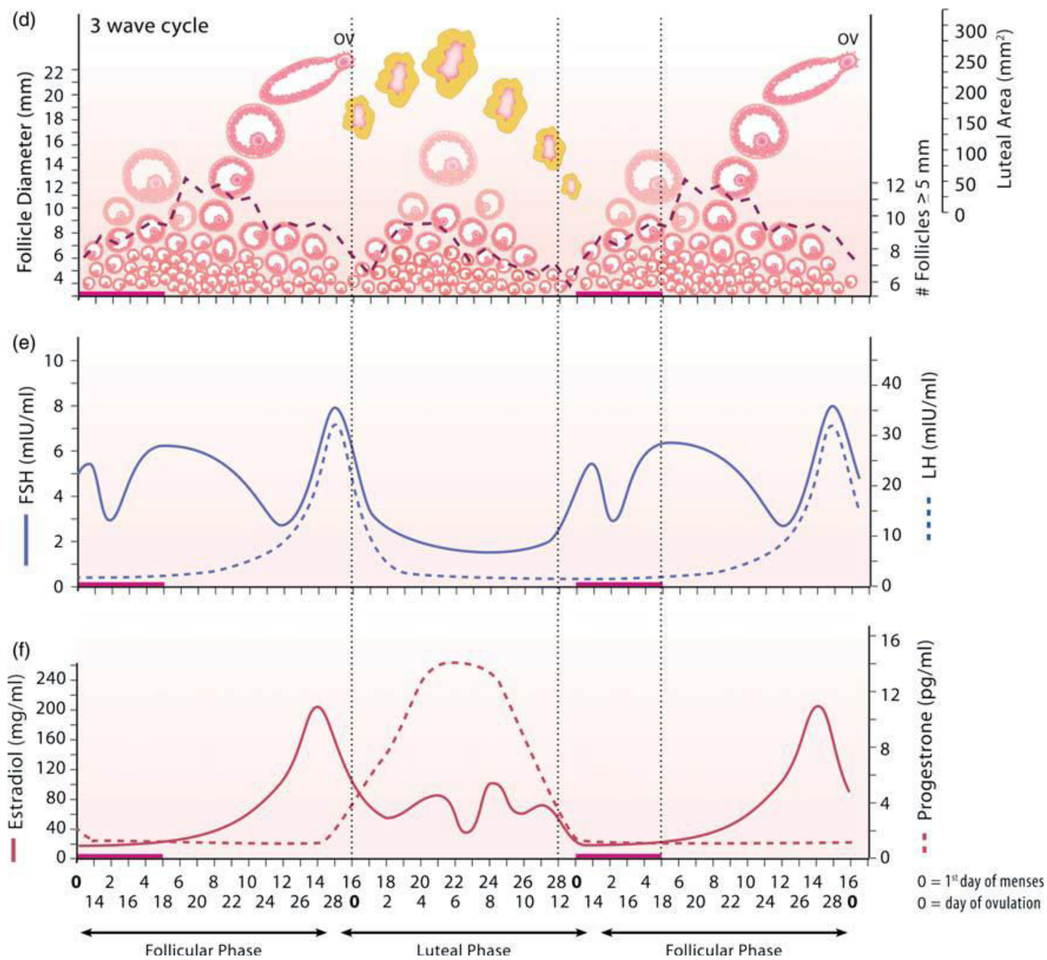


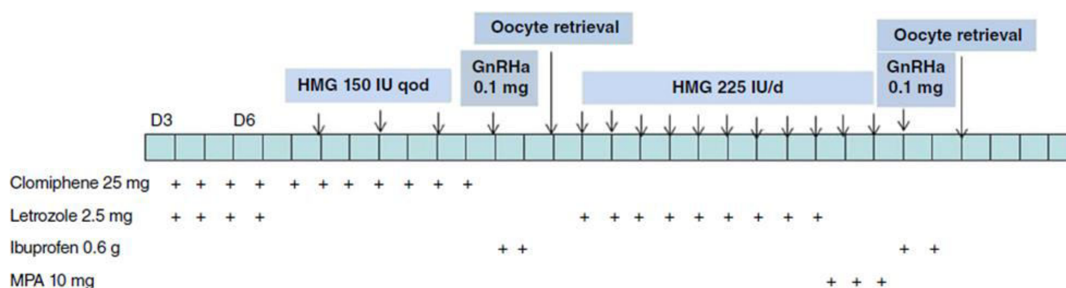
Figure 5: Morphologic and endocrinologic changes associated with three waves of follicle development during the human menstrual cycle. Dotted vertical lines indicate the days of wave emergence. Follicles are shown in pink and corpus luteum in yellow (Baerwald et al., 2012)



"DuoStim" Approach

The new discovery of the "Second Follicular Wave" phenomenon was introduced in the ART world. In fact, IVF clinicians performed ovarian stimulation for both the follicular and luteal phase of a single menstrual cycle, proceeding with the subsequent oocyte retrieval during follicular phase (Follicular Phase Oocyte Retrieval: FoPOR) and during luteal phase (LuPOR) as well. Kuang and his colleagues were the forerunners in proposing the approach of the double controlled ovarian stimulation for the management of poor responders, which is widely known as the "Shanghai protocol", giving the acronym of "DuoStim". This pilot study employed a combination of gonadotropins, clomiphene Figure 6. The results showed that hold double stimulation along with double retrieval in the same menstrual cycle may hold a remarkable promise, as the number of oocytes retrieved, MII oocytes, and top-quality embryos, in conjunction with clinical pregnancy rates, were comparable between the two phases (Kuang et al., 2014).

Figure 6: The protocol of double stimulation during the follicular and luteal phases in patients with poor ovarian response (Kuang et al., 2014)



Thenceforth, numerous retrospective and prospective studies showed results that were in accordance to the aforementioned. Particularly, they focused on comparing the embryological and clinical parameters resulting from follicular phase and those resulting from luteal phase of a single menstrual cycle of patients appeared with low IVF prognosis. The majority of them observed no statistically significant difference regarding the mean number of oocytes retrieved, mature MII oocytes, fertilization rate, embryos' development to cleavage stage, blastocyst formation, and euploidy rate (Jin, Niu, Xu, Chen, & Zhang, 2018; Liu, Jiang, Zhang, & Yin, 2017; Madani, Hemat, Arabipoor, Khodabakhshi, & Zolfaghari, 2018; Rashtian & Zhang, 2018; F. M. Ubaldi et al., 2016; Vaiarelli et al., 2018; W. Zhang et al., 2018). In terms of clinical pregnancy and live birth rates, it was obvious that data from both phases did not demonstrate any statistically significant difference (Jin et al., 2018; Liu et al., 2017; F. M. Ubaldi et al., 2016; W. Zhang et al., 2018). Interestingly, a retrospective study observed higher implantation rates regarding embryos that resulted from luteal phase in contrast to those from follicular phase, possibly due to higher doses of exogenous gonadotropins during luteal phase stimulation (Q. Zhang, Guo, & Li, 2017). In the same line, other studies made an effort to compare "DuoStim" approach with conventional stimulation protocols, indicating higher mean number of oocytes retrieved concerning the employment of "DuoStim" protocol, though providing oocytes and embryos that appear equal developmental competence and quality (Cardoso et al., 2017; Jin et al., 2018; Liu et al., 2017; Madani et al., 2018; W. Zhang et al., 2018). Concerning clinical pregnancy and live birth rates, the reported results were similar between "DuoStim" and conventional protocols (Jin et al., 2018; W. Zhang et al., 2018).

The alternative protocol of "DuoStim" may favor specific time-related categories of patients, namely women suffering from cancer who are in the urge of fertility preservation in the light of preventing delay of chemotherapy or radiotherapy (Creux, Monnier, Son, Tulandi, & Buckett, 2017; Maman et al., 2011), as well as patients with poor ovarian reserve, including those of adcanced maternal age, poor or diminished ovarian reserve (Cardoso et al., 2017; Kuang et al., 2014; Xu & Li, 2013). Moreover, it may be a viable solution for women who are accompanied with previous unsuccessful IVF cycles due to fertilization failure or women who undergo preimplantation genetic diagnosis for aneuploidies or monogenetic diseases and they did not manage to develop blastocyst for biopsy (Cardoso et al., 2017). Finally, it may depict an alternative option for oocyte donors (Martinez et al., 2014).

Natural cycle and LuPOR approach

It is apparent that the "DuoStim" protocol contributes to a higher oocyte yield leading to the achievement of pregnancy in a shorter period of time (Kuang et al., 2014). This may undoubtedly relieve the patients' anxiety and stress which is mainly sourced from previous futile IVF attempts (Chan, Lau, Tam, & Ng, 2016). On the other hand, performing a "DuoStim" protocol entails dual administration of stimulation regime, as well as dual ovarian penetration and anesthesia for oocyte retrieval procedure. Obviously, the duration and dosage of gonadotropins during "DuoStim" are higher than the conventional one (Wu, Zhao, Sun, & Liu, 2017). However, there is a lack of studies to examine if stimulation protocols may jeopardize hormonal profile of women for long-term. Furthermore, a recent meta-analysis revealed a and increased possibilities of adverse perinatal outcomes, concerning preterm birth and low birth weight when controlled ovarian stimulation is employed instead of natural IVF cycles (Kamath, Kirubakaran, Mascarenhas, & Sunkara, 2018). On top of that, a second administration of gonadotropins augments the cost of IVF treatment.

On the antipode, little is known about the developmental competence of oocytes and embryos resulting from LuPOR during natural IVF cycle. A retrospective study that was conducted by Sfakianoudis and his colleagues concluded that LuPOR approach during natural IVF cycles may represent a highly-promising and patient-friendly approach during the clinical management of extreme poor responders, accompanied with remarkable embryological results (Sfakianoudis et al., 2019). Another benefit stemming from the employment of the aforementioned moderate approach is the improved prognosis for the successive IVF cycle, as oocyte retrieval during luteal phase prevents the formation of a cystic follicle that is caused by

the existence of both increased progesterone and E_2 concentrations (Herman et al., 1990). The current study makes an effort to uniquely bring new insight regarding the "Second follicular wave" phenomenon through the employment of natural IVF cycles, as well as buttress the alternative LuPOR approach as an option for the efficient management of poor responders.

5) CELL-FREE DNA IN FOLLICULAR FLUID AS A POTENTIAL BIOMARKER OF IVF OUTCOMES

The Holy Grail in the ART world is to ascertain the IVF outcome and hence increase success rates (Wade, MacLachlan, & Kovacs, 2015). Following literature search, several prognostic and diagnostic tools have been proposed, such as time-lapse imaging (Chen, Wei, Hu, Yuan, & Liu, 2017) or other computational applications for embryo selection (Simopoulou et al., 2018), in an effort to accurately predict IVF prognosis and thus navigate infertility management. Among these, great emphasis is given on defining potential non-invasive biomarkers contained in follicular fluid (ff), as the latter depicts the ambient microenvironment that "accommodates" the oocyte and easy access to research material is ensured. During folliculogenesis, antral follicles consist of an "antrum", which is characterized as a cavity filled with ff. Follicular fluid is rich in hormones, enzymes, anticoagulants and electrolytes and contributes to communication between granulosa cells and the enclosed oocyte (Hennet & Combelles, 2012). Consequently, ff plays a vital role for follicular maturation, oocyte growth as well as oocyte developmental competence (Basuino & Silveira, 2016).

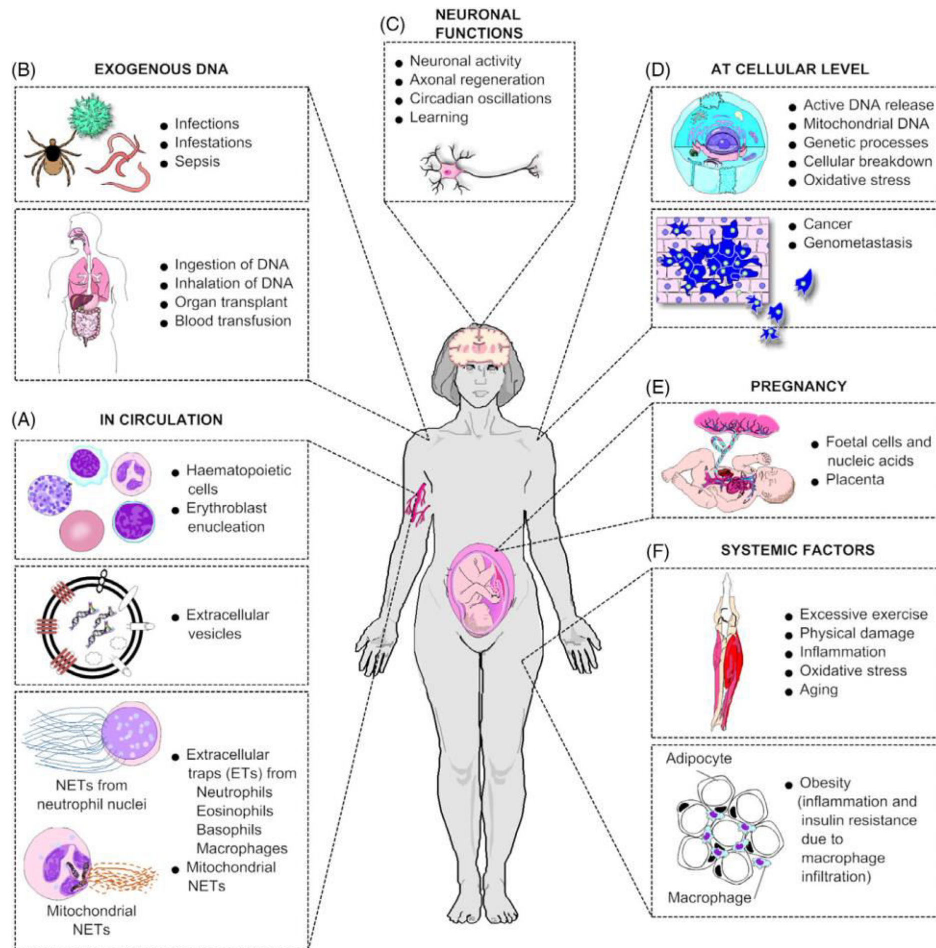
It is notable that ff seems to denote a great source of biomarkers that can be employed as additional tool for both oocyte and embryo outcome (Ledee et al., 2011). Molecular and metabolomics analyses of ff have proposed a wide pallet of potential ff components that are originated from blood plasma or even secreted from granulosa and theca cells (Castiglione Morelli et al., 2019; Revelli et al., 2009; Scalici et al., 2016). These components involve hormones-such as gonadotropins, estogens, androgens, progesterone, prolactin, corticoids (Revelli et al., 2009)- growth hormones and factors of transforming growth factor-beta (TGF-β)-such as inhibin, activin, AMH (Revelli et al., 2009)- other growth factors-such as, insulin-like growth factor (IGF), epidermal growth factor (EGF), basic fibroblast growth factor (BFGF) (Vural, Vural, Doğer, Çakıroğlu, & Çekmen, 2016; Zhou et al., 2003)- interleukins and chemokines- such as interleukin 15 (Spanou et al., 2018), granulocyte colony-stimulating factor (GCSF) (Ledee et al., 2013)- reactive oxygen species (ROS), anti-apoptotic factors-

such as tumor necrosis factor (TNF), Fas-ligand- protein, peptides and amino-acids-such as leptin, alanine- sugars, such as hyaluronan, lactate, glucose- prostanoids, such as prostaglandin F2alpha (Baka & Malamitsi-Puchner, 2006; Field, Dasgupta, Cummings, & Orsi, 2014; Revelli et al., 2009), and finally extracellular microRNA (miRNA), as it was recently examined (Scalici et al., 2016).

The last decade, the novel biomarker of cell-free DNA (cfDNA) has introduced in various fields of medicine, especially in cardiology (Dinakaran et al., 2014), in oncology with the intent to examine cancer progression (Jahr et al., 2001; Pinzani, Salvianti, Pazzagli, & Orlando, 2010; S. Traver et al., 2014; Volckmar et al., 2018), as well as in obstetrics and gynecology, for early screening for genetic abnormalities, diagnosis of preeclapsia or determination of fetal sex through fetal cfDNA in the circulation (Barrett et al., 2017; Silver et al., 2017; S. Traver et al., 2014). In fact, the levels of cfDNA in blood plasma- known as "liquid biopsy" (Gravina, Sedivy, & Vijg, 2016; Ivanov, Baranova, Butler, Spellman, & Mileyko, 2015)- of patients diagnosed with a type of cancer, such as ovarian, were higher compared to healthy controls. Similarly, detected fetal cfDNA concentration in maternal plasma was raised in cases of abnormal placentation, or common aneuploidies, mainly in trisomy 13, 18, and 21 (S. Traver et al., 2014).

Mandel and Metais were the first to discover the existence of cfDNA in blood plasma, reporting that it is released by all cell types (Mandel, 1948). Cell-free DNA represents double-stranded fragments of DNA that can be detected in either blood circulation of other biological fluids, such as ff, of patients or healthy individuals (Dimopoulou et al., 2014a; Guan et al., 2017; Scalici et al., 2014). In fact, cfDNA may vary in terms of the triptych of length, integrity and concentration (Ivanov et al., 2015). It can be derived from apoptotic or necrotic processes, resulting in short or long fragments, namely of about 160-180 base pairs (bp) that are cleaved by endogenous caspase-activated DNAase during apoptotic cell death (Aucamp, Bronkhorst, Badenhorst, & Pretorius, 2018a; Nagata, 2000), whereas of almost 10000 bp that are cleaved during necrosis, which is characterized by the combination of cell disintegration, organelle swelling and random chromatin digestion. Furthermore, cfDNA is reported to be released either passively from damaged, dead or dying cells or actively, only from living cells. In Figure 7, a detailed schematic description of the various sources of cfDNA in human body is presented (Aucamp et al., 2018a). It is assumed that cfDNA may harbor the peril of mutagenesis, as it appears to be randomly integrated into healthy cells (Gravina et al., 2016). The aforementioned arise the need for mapping cfDNA fragments, emerging the era of "fragmentomics" (Ivanov et al., 2015).

Figure 7: A schematic description of putative origins of cell-free DNA in the human body; NET: neutrophil extracellular trap (Aucamp et al., 2018a).



Focusing in the field of ART, the employment of cfDNA levels in ff as a potential non-invasive prognostic biomarker is not fully examined. Dimopoulou and her colleagues were the forerunners in introducing the evaluation of cfDNA in ff from women who undergo ovarian stimulation during their IVF/ICSI treatment (Dimopoulou et al., 2014b). The results revealed a positive correlation between ff cfDNA levels and FSH value, along with an association between increased ff cfDNA levels and small number of oocytes, which may be attributed to ovarian stimulation protocol that activates the apoptotic pathway. Thereafter, the study conducted by Scalici and her colleagues, examined 100 ff samples that were individually aspired from women with normal response or ovarian insufficiency. They concluded that high ff cfDNA concentrations are related to small follicular size, reduced E_2 levels and finally poor embryo quality, coupled with slow division rate and increased fragmentation rate (Scalici et al., 2014). Finally, Traver and her colleagues performed a

prospective study including 117 pooled ff samples from women presented with ovarian reserve disorders and those with normal response. They observed an association between raised ff cfDNA levels and women with poor reserve status, with long duration of stimulation protocols in line with the high doses of gonadotropins used, with small number of retrieved oocytes, and finally with poor cleavage embryo outcomes regarding their number and quality. Following ROC curve analysis, they clarified a significantly and independently association between ff cfDNA levels and clinical pregnancy outcomes, accompanied with 88% specificity and 60% sensitivity (Sabine Traver et al., 2015a). All the aforementioned studies support that ff cfDNA levels may depict a highly promising non-invasive biomarker for IVF prognosis, especially in conjunction with embryonic morphological criteria.

So far, available data demonstrated that cfDNA in human ff was mainly originated from apoptotic events instead of necrotic ones (Scalici et al., 2014; Sabine Traver et al., 2015a). The evaluation of cfDNA integrity was performed via real-time Polymerase Chain Reaction (RT-PCR) with the aid of Arthrobacter luteus (ALU) sequences that are short interspersed elements (SINEs) of 300 nucleotides characterized by their abundance in the human genome countering almost 1.4 million copy numbers (Hussein, Mohamed, & Ahmed, 2019). In the present study, we employed ALU-specific primers, namely ALU115 and ALU247, with the first to amplify both short fragments stemming from apoptosis, along with long fragments stemming from necrosis, whereas the second set of primers to amplify only long DNA fragments. Thenceforth, the ratio of Q_{247}/Q_{115} was estimated, with the value Q_{247} to corresponds to cfDNA concentration resulting from ALU247 primer, while the value Q_{115} to that from ALU115 primer (Umetani, 2006). However, the existing studies appear with diversity with respect to their design, namely the wide population selected, or the collected ff samples to be either pooled or individual (Dimopoulou et al., 2014b; Scalici et al., 2014; Sabine Traver et al., 2015b). Finally, throughout current literature, no study examines cfDNA levels in ff during natural IVF cycles, in order to delineate the exact origin of cfDNA without the impact of ovarian stimulation protocols, rendering this project highly novel.

II. SPECIFIC CHAPTER

A. AIM OF THE STUDY

The aim of the present study is to investigate the physiology of the second follicular wave and concur on its clinical importance regarding poor responders in IVF. This was achieved through the comparison of ff cfDNA levels resulting from FoPOR and LuPOR in a single menstrual cycle, and their respective association with the number and maturation status of corresponding oocytes, as well as the number of subsequent 2PN zygotes following insemination. The study uniquely brings to literature new insight concerning the second follicular wave phenomenon, employing natural cycles and cfDNA as an apoptotic biomarker for the first time. Data from this research project will contribute towards buttressing the validity of the practice of LuPOR, so as to enable the optimal practice of employing oocytes and embryos originating from luteal phase follicular wave, in the context natural cycles. It also focuses on delineating the optimal and efficient management regarding the time-sensitive category of patients presenting with poor ovarian response, highlighting the highly promising option of the LuPOR approach. The practice of LuPOR appears as a promising route towards a higher oocyte yield in a shorter time period than available conventional approaches. The current study sets out to test this theory employing observational morphological along with molecular approaches.

B. MATERIALS AND METHODS

The current prospective study was conducted during the period from September 2018 to May 2019 and it represents a collaboration between the Department of Physiology of Medical School in Athens and the Centre of Human Reproduction "Genesis Athens Clinic". Patients enrolled signed written consent forms prior to their recruitment to this study. The study protocol (133-26/03/2018) was approved by the Hospital Ethics Board in accordance to the Helsinki declaration.

1) STUDY POPULATION: INCLUSION AND EXCLUSION CRITERIA

The study population included 47 infertile women seeking IVF treatment. The patients were characterized as poor responders according to the Bologna criteria (A. P. Ferraretti et al., 2011). Moreover, a second follicular wave was observed during the luteal phase of their menstrual cycle and thus they underwent double oocyte retrieval during the same menstrual cycle. The patients' baseline hormonal levels of FSH, LH, and prolactin were evaluated on day 3 of the menstrual cycle of each patient, while the concentrations of progesterone on day 21 of the menstrual cycle, and E_2 levels prior to oocyte pick-up procedure. Further data included concerns ALU115 and ALU247 concentrations regarding FoPOR and LuPOR, oocyte maturation referring to GV, MI, MII, and abnormal, as well as to fertilization status referring to 1PN, 2PN, 3PN, lysed, and degenerated.

A typical male factor investigation included history evaluation, physical examination, and semen analysis. Semen analysis was performed according to World Health Organization (WHO) criteria, regarding volume, concentration, motility, and morphology. The reported male infertility was mainly on the grounds of oligozoospermia that is characterized, accompanied with the semen concentration $<15x10^6$/ml (World Health Organization, 2010). For the evaluation of cfDNA levels, 47 ff samples from LuPOR were collected and served as the study group, whereas 47 collected ff samples from FoPOR of the same menstrual cycle of each patient served as the control group. Only natural cycles were included, in an effort to exclude any detrimental effects on the oocytes' microenvironment that could be stemming from stimulation ovarian protocols. Furthermore, only Intracytoplasmic Sperm Injection (ICSI) cycles on the grounds of mild male infertility were included, in order to ensure access to data regarding the oocyte maturation status. On the other hand, women diagnosed with polycystic ovary syndrome, inflammatory diseases such as endometriosis or even chronic endometritis, infectious diseases involving sexually transmitted diseases, and cancer diseases were excluded from the current study. Another exclusion criterion was lack of oocyte detection following the oocyte retrieval procedure during either FoPOR or LuPOR.

2) OUTCOMES: PRIMARY AND SECONDARY

The primary outcomes of the present study are the number and the maturation status of oocytes resulting from LuPOR in comparison to those from FoPOR of the same menstrual cycle for each patient. The secondary outcomes are the number of fertilized zygotes and DNA integrity. The latter can be evaluated employing the ratio of Q_{247}/Q_{115}, in order to conclude on the origin of cfDNA. The value Q_{247} corresponds to cfDNA concentration resulting from employing the ALU 247 primer, while the value Q_{115} corresponds to cfDNA concentration resulting from employing ALU 115 primer. If the value of the aforementioned ratio is equal to 1, it corresponds to the understanding that cfDNA is mainly originated from necrosis process, whereas if the value of respective ratio is equal to 0, then it is deduced that cfDNA is mainly originated from the apoptotic process.

3) NATURAL CYCLE PROTOCOL, OOCYTE RETRIEVAL AND FERTILIZATION

At the first appointment, baseline levels of FSH, LH and E_2 were assessed by chemiluminescent microparticle immunoassay on a Roche Cobas E-411 Immunoassay analyzer (Roche Diagnostics GmbH, Mannheim, Germany). Clinicians monitored follicular growth employing transvaginal ultrasonography on the 8th day of menstrual cycle, in combination to daily evaluations of serum LH and E_2 levels. When the dominant follicle was detected with a diameter of >18mm and serum E_2 levels over 100 pg/ml, an intramuscular injection of 5000 IU of hCG or recombinant hCG was administered, in order to trigger ovulation. Following 36 hours from hCG injection, follicular aspiration was performed using ultrasonically guided vaginal probe, under mild anesthesia.

Using an emCell work chamber that provides long term stable environment for handling gametes, the collected follicular fluid following retrieval was placed into a collection petri dish, in order to detect oocyte(s). The collected oocyte(s) was/were conserved in Fert Media (ORIGIO Sequential Media) for 2 hours incubation time. Thereafter, 38 hours post hCG the oocyte(s) was/were stripped from cumulus-oocyte complexes, using 80 IU/ml hyaluronidase (FertiPro) and mechanically denudated. Oocyte maturation status was recorded. As mature (Metaphase II: MII) oocyte is considered that with the appearance the first polar body, those presenting at GV or Metaphase I (MI) stage are considered immature and finally those with irregular morphology-as regards to oocyte shape, size, ooplasm characteristic, structure of perivitelline space, zona pellucida or polar body morphology-were defined as abnormal (Lazzaroni-Tealdi et al., 2015). Mature oocytes were placed into dishes with Step-1

culture media (ORIGIO Continuous Culture Media) for 1-hour post denudation. ICSI dishes have been prepared with two drops of Quinn's Advantage Medium with HEPES (SAGE) with 5% Human Serum Albumin (HSA) under mineral oil. The mature oocytes is/are being inseminated employing ICSI procedure 39 hours post hCG insemination. Following ICSI procedure, oocyte(s) was/were cultured in culture dishes containing Step-1 culture media (ORIGIO Continuous Culture Media) with 5% HSA under mineral oil into incubator at 37°C, with 5% O_2, 6% CO_2, 89% N_2, and 95% humid atmosphere.

Fertilization check was performed 16 to 18 hours post insemination. Hence, normally fertilized zygotes were identified by two pronuclei and the extrusion of the second polar body. The aforementioned zygotes were cryopreserved according to standard laboratory protocols until the collection of an ideal number of embryos. Employing slow freezing technique, in this developmental stage where the two pronuclei are visible, day-1 embryos are been cryopreserved using the FreezeKit Cleave (Vitrolife).

Several previous clinical observations-such as elevated serum E_2 during luteal phase-regarding this category of patients showed that they presented new follicle recruitment. These patients were recommended to be monitored for follicular growth via transvaginal ultrasonography, 7 days post FoPOR. Similarly, when the dominant follicle was detected with a diameter of >18mm, an intramuscular injection of 5000 IU of hCG or subcutaneous injection of 250 μg of hCG was administered, in order to trigger ovulation. Following 36 hours from hCG injection, LuPOR performed using ultrasonically guided vaginal probe, with or without the need for sedation or anesthesia. Identical laboratory protocols were performed as described above, resulting to another round of cryopreserved embryos during the same menstrual cycle.

4) FOLLICULAR FLUID SAMPLE PREPARATION AND cfDNA EXTRACTION

Following oocyte pick-up, the remaining ff was placed into round-bottom falcon tubes and centrifuged at 1000g for 15 minutes, in order to remove any histologic remnant from the oocyte retrieval procedure. Supernatants of ff samples were immediately transferred to the laboratory of Physiology, using a cool box containing crushed ice. Bloodstained and cloudy ff samples were excluded. The samples were filtered using a 0.45 μm filter to eliminate cell debris, and supernatants were immediately stored at -80°C. The preparation of filtered ff samples was based on the protocol of Umetani and colleagues (Umetani, 2006).

According to this protocol, 50 μl of each ff sample was mixed with 50 μl of a solution buffer, containing 25 ml/l Tween 20, 50 mmol/l Tris and 1 mmol/l EDTA and then digested with 8 μl of proteinase K (PK) (Macherey-Nagel, Germany) at 70°C for 20 min followed by heat-inactivation and insolubilisation at 95°C for 5 min. Digestion was performed in Veriti 96-well Thermal Cycler (Applied Biosystems). A final centrifugation was conducted at 10.000g for 5 min and collected supernatants were then stored at -20°C until cfDNA quantification.

5) QUANTIFICATION OF cfDNA USING RT-PCR METHOD

The concentration of cfDNA was quantified by RT-PCR for human ALU repeats (Custom DNA Oligos, Eurofins, Genomics, Austria), using two primer sets that generate a 115-bp amplicon (ALU115 primers) and a 247-bp amplicon (ALU247 primers), respectively (Umetani, 2006). The primer set for ALU115 amplifies both shorter (stemming from apoptosis) and longer DNA fragments (stemming from necrosis), whereas the primer set for ALU247 amplifies only longer DNA fragments. The sequences of each primer set are the following (Umetani, 2006):

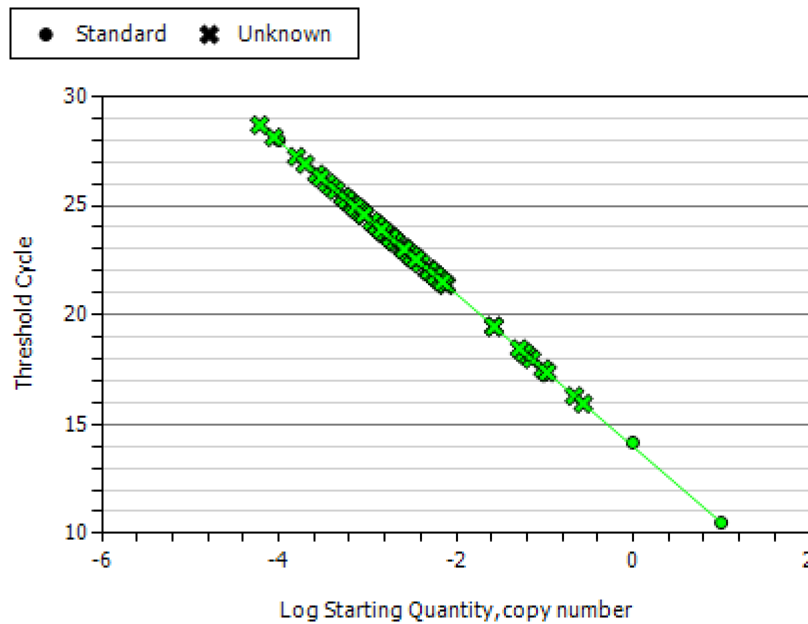
ALU115 primer:
Forward: 5'-CCTGAGGTCAGGAGTTCGAG-3',
Reverse: 5'-CCCGAGTAGCTGGGATTACA-3'.

ALU247 primer:
Forward: 5'-GTGGCTCACGCCTGTAATC-3'
Reverse: 5'-CAGGCTGGAGTGCAGTGG-3'.

For each 96-well PCR plate, a 100x reaction mixture was prepared by adding 800 μl nano-filtration (NF) H_2O, 1000 μl SYBR Green I Master Mix (Kapa Biosystems), 50 μl of 0.25μM forward and 50 μl of 0.25μM reverse primers (either ALU115 or ALU247). In each well, 1 μl of each PK-digested ff sample was added to 9 μl of reaction mixture (final volume: 10μl). Thereafter, a film carefully covered the whole loaded PCR plate. The PCR plate was placed onto the PCR instrument (Bio-Rad iCycler Thermal Cycler IQ5 Multicolor Real-Time PCR Detection System). Real-time PCR amplification was performed with precycling heat activation of DNA polymerase at 95°C for 3 min, followed 35 cycles of denaturation at 95°C for 3 seconds, annealing at 63°C for 30 seconds, and extension at 55°C-95°C for 15 seconds

and 20°C for hold. A negative control (without template) and 2 intercontrol samples were added in each qPCR plate. All measures were performed in duplicate. Follicular fluid cfDNA concentrations were calculated based on a standard curve prepared with successive dilutions (10 ng to 0.01 pg) of gently prepared genomic DNA, obtained from peripheral blood of healthy volunteer (Figure 8). The detection limit of the method was 0.01pg.

Figure 8: Standard curve chart

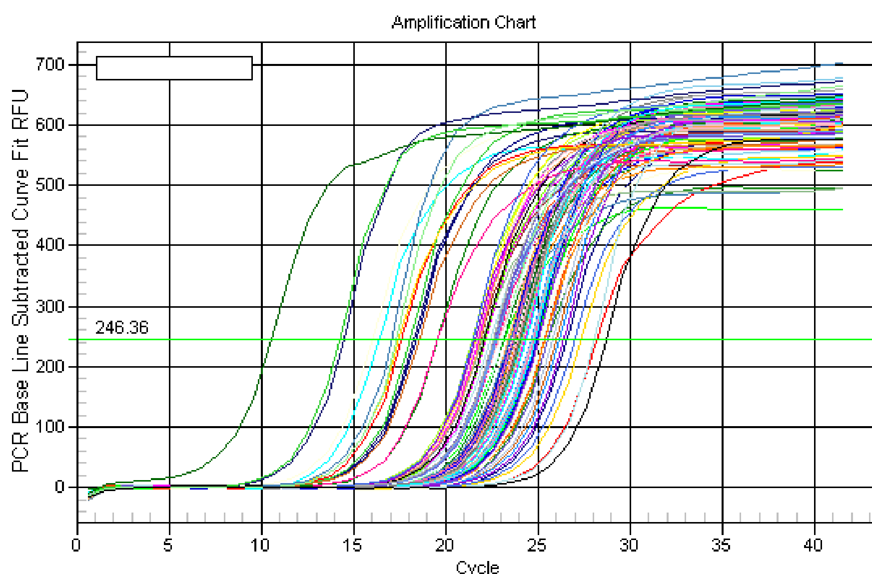


Principal of RT-PCR method

The method of PCR depicts a cutting-edge option that is widely applied in biological sciences, especially in the field of molecular biology. This technique provides the opportunity of amplification a small amount of DNA template or target sequence, resulting in amplicons of a specific segment of DNA. With the successful isolation of thermal stable DNA polymerase from the bacterium *Thermus aquaticus*, the technique was remarkably improved and automated. Regarding RT-PCR technique, it seems that enables the monitoring of reaction as it occurs in real time, as well as it allows the precise measurement of the amount of amplicons at each cycle, resulting in accurate quantification of the initial sample. Furthermore, it eliminates post-PCR manipulations, as both amplification and detection occur

in a single tube. Several parameters in RT-PCR of paramount importance are DNA polymerase, oligonucleotides (dNTPs) selection, design of the appropriate primers for the target sequence and undoubtedly a decent experimental technique. Following thermal cycling procedure, including about 35-40 cycles, PCR product is detected and quantified. Regarding RT-PCR, the product can easily be measures at the end of each cycle using fluorescent dyes that yield increasing fluorescent signal in direct proportion to the number of PCR product molecules (amplicons) generated. The latter dye often binds to the minor groove of any double-stranded DNA. Excitation of DNA-bound SYBR Green dye produces a robust fluorescent signal, in contrast to the unbound dye. In the early PCR cycles, a horizontal baseline is observed. If the target was present in the sample, sufficient accumulated PCR product will be produced at some point so that amplification signal becomes visible. The change in fluorescence over the course of the reaction is measured by an instrument that combines thermal cycling with fluorescent dye scanning capability, resulting in an amplification chart that represents the accumulation of product over the duration of the entire PCR reaction. There are three major steps that constitute PCR technique, namely "denudation", "annealing", and "extension". During "denudation", incubation in high temperatures, reaching up to 95°C in case of Taq DNA polymerase, leads to "melt" double-stranded DNA into two single strands and loosen secondary structure in single-stranded DNA. Denudation time can be increased if GC bases content of DNA template is high. Concerning "annealing", complementary sequences are hybridized, employing appropriate temperature that is based on the calculated melting temperature (Tm) of the primers. The option of 5°C below the Tm of the primer is mostly preferred. During the final step called "extension", DNA polymerase accomplishes the extension of primer sequences, appearing with the rate of 100 bases per second under the optimal temperature of 70°C-72°C. If an amplicon is small, this step is often combined with an additional "annealing" step using the temperature of 60°C (Lorenz, 2012; Waters & Shapter, 2014).

Figure 9: Real-time PCR amplification chart



6) ELECTROPHORESIS METHOD

Following RT-PCR for quantification of ff cfDNA, electrophoresis method was performed, with the aim to observe the final PCR product resulting from almost 35 thermal cycles. The first step was the preparation of a 2% agarose gel electrophoresis. For this purpose, we dissolved 0.6 gr of agarose powder in 30 ml TAE buffer, with the latter to be a solution containing Tris base, acetic acid and EDTA (Ethylenediaminetetraacetic acid). When the agarose powder was well-dispersed in the buffer, it was heated in a microwave, with caution to avoid boiling. Thereafter, almost 1 μl of ethidium bromide was added into the melted agarose prior to cooling stage, which binds to major grooves of double-stranded DNA. The aforementioned mixture was poured into a suitable cast, and a comb was placed with the intent to form wells for cfDNA samples' loading. When the agarose gel was solidified, the comb was removed, leaving 8 available wells. The first well was loaded with 2 μl DNA ladder (Fast Gene DNA ladder 50bp) for the evaluation of molecular weight, while the rest wells were loaded with 9 μl ff cfDNA samples diluted with 1 μl loading dye, in order to monitor the electrophoresis' progress. Thenceforth, the set-up of electrophoresis device was performed. The prepared gel was placed onto the box of the respective electrophoresis apparatus, being ultimately submerged in the buffer throughout the procedure. The electrodes of the device were correctly connected with a power source of 100 Volt, running for 20 minutes. At the end of the running, the gel was carefully placed onto the glass surface of illuminator of ultraviolent light. Hence, the visualization of the produced bands of separated cfDNA fragments post-electrophoresis was enabled, being connected to camera and giving the opportunity to capture images.

Principal of electrophoresis method

The method of electrophoresis is widely used in the field of molecular biology. It efficiently enables the separation of macromolecules, namely proteins, or nucleic acids, employing a gel that usually contains agarose. Concerning the separation of DNA employing agarose gel electrophoresis, it is based on electric events. Particularly, DNA is known to be negatively charged due to phosphate chain. Thus, during the procedure, DNA fragments migrate towards the positive pole, being separated according to their size, with the shorter ones to move further through agarose's pores (Koontz, 2013). Agarose is known to be produced by the seaweed of *Gelidium* and *Gracilaria species*, containing L- and D-galactose subunits that form pores during polymerization. Notably, the migration is affected by several parameters, giving emphasis on the size of respective DNA, the concentration of agarose of the prepared gel, and the voltage supplied (Lee, Costumbrado, Hsu, & Kim, 2012).

.

C. STATISTICAL ANALYSIS

Statistical analysis was performed employing R statistical programming language through the RStudio interpreter (Boston, MA, USA). Spearman's correlation coefficient was employed to evaluate possible associations. Normality of the distribution was examined via Shapiro-Wilk's test. The distribution of most parameters was not normal and thus Wilcoxon rank-sum test (Mann-Whitney U) test was preferred to examine potential differences between groups. In cases of distributions of both groups were normal, Student's t-test was preferred.

D. RESULTS

Real-time PCR method

A total of 47 women classified as poor responders according to the Bologna criteria participated in the present study. All patients underwent 2 natural cycle oocyte retrievals – one in the follicular phase and one in the luteal phase – during the same menstrual cycle. The mean age of our patients was 42.91 ± 3.77 years old. The serum hormonal levels of FSH, LH, prolactin, progesterone and AMH, along with their range are depicted in Table 1. Antral follicle count via ultrasound, number of oocytes retrieved, maturity status and number of pronuclei are presented in Table 2.

The mean levels of ALU115 present as statistically significantly lower during FoPOR when compared to LuPOR (0.79 ± 0.72 ng/μl vs 1.46 ± 1.59 ng/μl, p-value=0.02) (Figure 10). No statistically significant difference was observed between the two groups neither regarding the concentration of ALU247 (0.07 ± 0.14 ng/μl vs 0.22 ± 0.47 ng/μl) nor the Q_{247}/Q_{115} ratio (0.15±0.14 vs 0.55 ± 0.87 ng/μl). In both procedures the cfDNA integrity on average was measured to be <0.5 ng/μl, which corresponds to mainly apoptotic events.

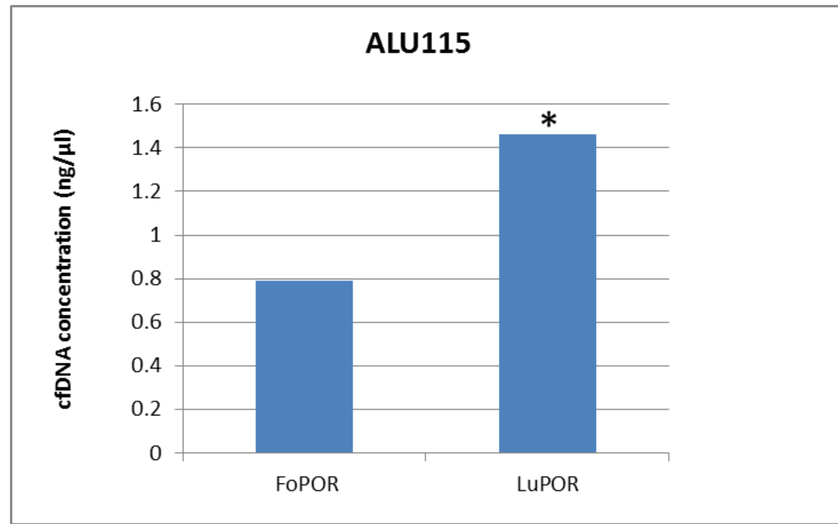
In the FoPOR group, the results revealed a statistically significant positive correlation of serum E_2 levels and ALU115 concentration (p-value=0.04). ALU247 was not associated with any of the examined parameters. The cfDNA integrity was negatively correlated with serum E_2 levels (p-value=0.03). This negative correlation was observed in the LuPOR group as well (p-value=0.03). No other statistically significant difference was observed between ALU115, ALU247 and cfDNA integrity for any of the examined parameters.

Table 1: Patients' age and hormonal characteristics

	Mean ± SD	**Range**
Age (years)	42.91 ± 3.77	32 – 48
FSH (mIU/ml)	11.22 ± 2.81	4.56 - 15.6
LH (mIU/ml)	8.58 ± 4.54	2.5 - 22.1
Prolactin (ng/ml)	16.67 ± 7.90	4.7 – 40
Progesterone (ng/ml)	15.83 ± 5.01	11.1 - 30.2
E_2 (pg/ml)	197.51 ± 87.94	38 – 520
AMH (ng/ml)	0.55 ± 0.41	0.01 - 1.9

FSH: follicle-stimulating hormone; LH luteinizing hormone; E_2: estradiol; AMH: anti-müllerian hormone; FoPOR: follicular phase oocyte retrieval; LuPOR: luteal phase oocyte retrieval

Figure 10: Comparison of the mean levels of ALU115 during FoPOR and LuPOR approaches



*FoPOR: follicular phase oocyte retrieval; LuPOR: luteal phase oocyte retrieval; *: statistically significant*

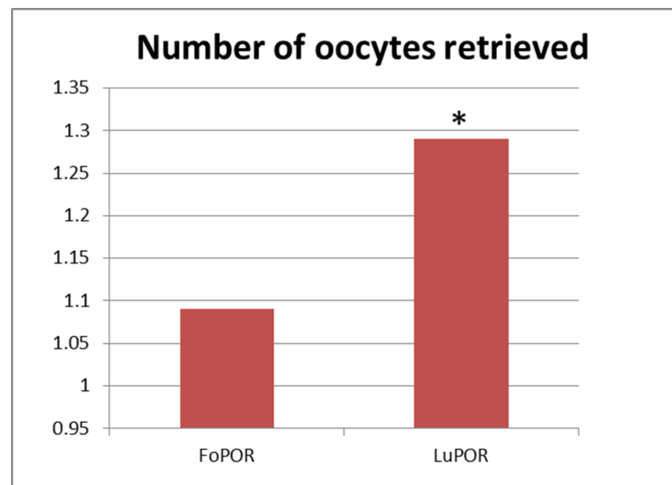
Table 2: Oocytes' characteristics and molecular results during FoPOR and LuPOR approaches during natural IVF cycles

		FoPOR		LuPOR	
		Mean ± SD	**Range**	**Mean ± SD**	**Range**
U/S follicle count		1.77 ± 0.90	1 - 4	1.77 ± 0.90	1 - 5
Number of oocytes retrieved		1.09 ± 0.28	1 - 2	1.29 ± 0.58	1 - 4
Maturation status of oocytes retrieved	***MII***	0.77 ± 0.55	0 - 2	1.08 ± 0.61	0 - 3
	MI	0.02 ± 0.14	0 - 1	0.08 ± 0.28	0 - 1
	GV	0.15 ± 0.36	0 - 1	0.06 ± 0.24	0 - 1
	Abnormal	0.11 ± 0.31	0 - 1	0.04 ± 0.20	0 - 1
Fertilization status following insemination	***2PN***	0.49 ± 0.58	0 - 2	0.68 ± 0.62	0 - 2
	1PN	0.13 ± 0.33	0 - 1	0.11 ± 0.37	0 - 2
	3PN	0.06 ± 0.24	0 - 1	0.09 ± 0.28	0 - 1
	Lysed	0.04 ± 0.20	0 - 1	0.13 ± 0.33	0 - 1
Molecular results	***ALU 115 (ng/µl)***	0.79 ± 0.72	0.078 - 2.475	1.46 ± 1.59	0.11 - 6.5
	ALU 247(ng/µl)	0.15 ± 0.14	0.0353 - 0.53	0.55 ± 0.88	0.007 - 2.83
	cfDNA integrity	0.24 ± 0.12	0.04 - 9.51	0.23 ± 0.19	0.03 - 0.71

FoPOR: follicular phase oocyte retrieval; LuPOR: luteal phase oocyte retrieval; U/S: ultrasoun; PN: pronuleus/i; MI: metaphase I; MII: metaphase II

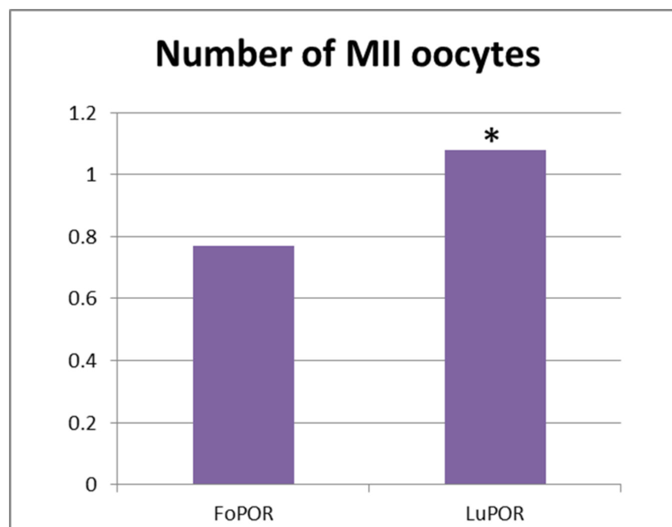
A lower number of oocytes was retrieved during FoPOR when compared to LuPOR (1.29 ± 0.58 vs 1.09 ± 0.28, p-value=0.02) (Figure 11). Number of MII oocytes collected in FoPOR also decreased when compared to LuPOR (0.77 ± 0.55 vs 1.08 ± 0.61, p-value=0.02) (Figure 12). No statistically significant difference was observed regarding the number of 2PN zygotes, or any other of the parameters examined.

Figure 11: Comparison of the mean number of oocytes retrieved during FoPOR and LuPOR approaches



*FoPOR: follicular phase oocyte retrieval; LuPOR: luteal phase oocyte retrieval; *: statistically significant*

Figure 12: Comparison of the mean number of mature MII oocytes during FoPOR and LuPOR approaches

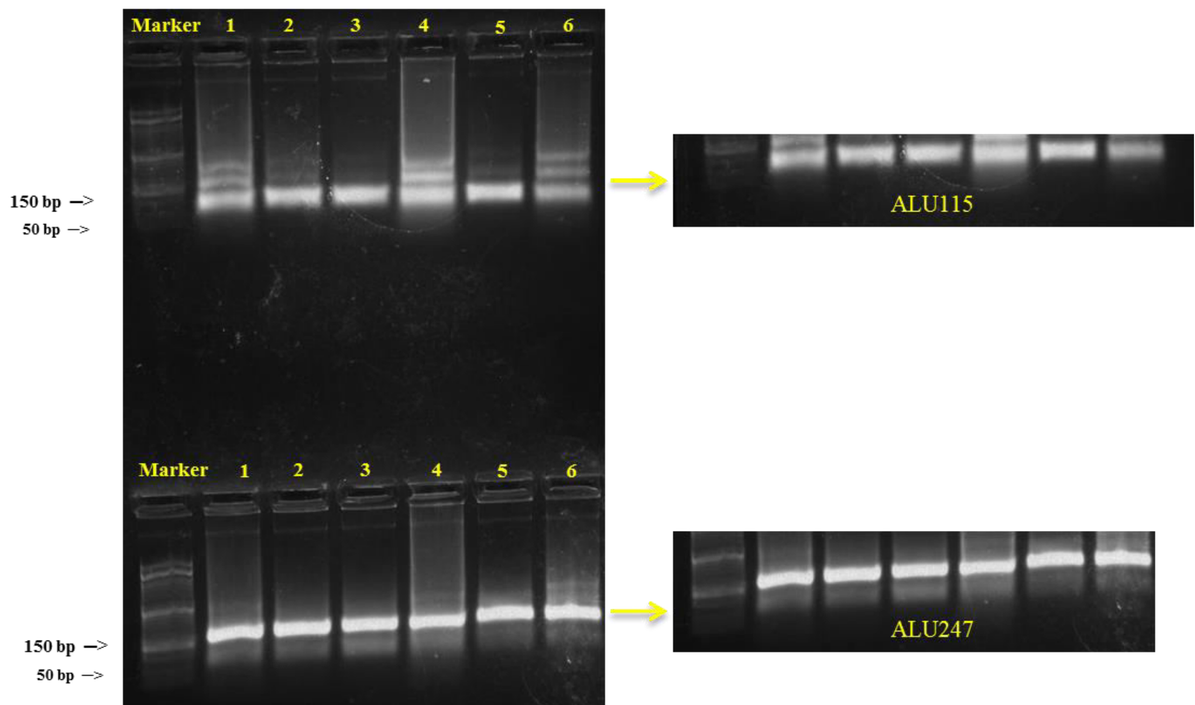


*FoPOR: follicular phase oocyte retrieval; LuPOR: luteal phase oocyte retrieval; *: statistically significant*

Electrophoresis method

Following the electrophoresis method, all the bands that corresponded to the examined samples were in the same point with the bands that corresponded to standard samples. At the left side of Figure 13, the overall results of electrophoresis are presented, focusing at each ALU115 and ALU247 primers at the right side. Indeed, the wells with numbers 2, and 3 refer to intra-control samples, while the wells with numbers 3, 4, 5, 6, and 7 refer to examined samples, being randomly selected from each group. The aforementioned ascertain the validity of the results of RT-PCR method, ending up with RT-PCR products presented with a length above the 150 bps and below the 500 bps, as it was expected after 35 thermal cycles.

Figure 13: Bands of cfDNA RT-PCR product following electrophoresis



Z. DISCUSSION

The present prospective study focuses on examining the second follicular wave phenomenon employing a combination of morphological observations of oocytes and zygotes, and molecular approaches, during natural IVF cycles for poor responders. The underlying reason was the need to concur on the clinical validity of LuPOR practice, being thereafter adopted as an alternative option for poor responders' management.

Except for the evaluation of specific morphological parameters regarding oocyte maturation and subsequent fertilization, the molecular analysis of cfDNA in ff was achieved through measuring ALU specific repeats, with ALU115 to amplify both short cfDNA fragments stemming from apoptosis, and long fragments stemming from necrosis, whereas ALU247 to amplify only long cfDNA fragments.

Following the comparison of FoPOR and LuPOR approaches, our results demonstrated no statistically significant difference regarding the mean levels of ALU247 and the Q_{247}/Q_{115} ratio, which indicates the absence of necrotic events during either the follicular or luteal phases. This may reassure the safety of both practices on the respective phases of the menstrual cycle with regards to the peril of sudden cell death ultimately triggered by necrosis (Aucamp, Bronkhorst, Badenhorst, & Pretorius, 2018b). However interestingly, the mean levels of ALU115 during FoPOR were statistically significant lower, in contrast to the LuPOR approach (0.79 ± 0.72 vs 1.46 ±1.59 ng/μl, p-value=0.02). This may represent an apoptotic origin of cfDNA during the luteal phase, which buttresses the school of thought that several physiological apoptotic events may occur. Indeed, the luteal phase is known to be characterized by the formation of corpus luteum in the absence of pregnancy, with its subsequent degradation to be achieved through apoptosis (Vaskivuo & Tapanainen, 2003). Concurrently, emphasizing on the patients demonstrating a second follicular recruitment during luteal phase, the atresia of the cohort of antral follicles following the selection of the dominant one may contribute to the reported increased levels of ALU115 on behalf of LuPOR.

Focusing on FoPOR's data, the results revealed a statistically significant positive correlation of serum E_2 levels and ALU115 levels (p-value=0.04), along with a statistically significant negative correlation of serum E_2 levels and the Q_{247}/Q_{115} ratio (p-value=0.03). However, no correlation was observed regarding the ALU247 concentrations and other examined parameters. This may be attributed to the atresia process occurring during the follicular phase. Particularly, the cohort of small antral and preantral follicles that are presented with inferior developmental potential compared to the dominant follicular apoptosis initiates in the internal layers of granulosa cells, being hormonally triggered by FSH

(Matsuda, Inoue, Manabe, & Ohkura, 2012). Concerning the privileged follicle, its development involves the proliferation of granulosa cells, which goes hand in hand with an extent of degeneration via apoptosis. In turn, granulosa cells of the dominant follicle secrete estrogens that inhibit FSH production through a negative feedback, along with expressing estrogen receptors (Matsuda et al., 2012; Plant, 2015). When referring to LuPOR's data, only a statistically significant negative correlation of serum E_2 levels with the Q_{247}/Q_{115} ratio was observed (p-value=0.03). This is in accordance with the explanations mentioned above concerning FoPOR's data. Remarkably, a potential mechanism has been proposed by studies performed in animal models that revealed the impact of tumor necrosis factor alpha (TNFα)- which is known for its contributing role in the necrosis process- on ovarian stereoidogenesis via the inhibition of E_2 production (Sakumoto, Shibaya, & Okuda, 2003).

In clinical practice, when assessing the oocytes' parameters of FoPOR and LuPOR approaches, it was observed that a statistically significant lower number of oocytes was retrieved (1.29 ± 0.58 vs 1.09 ± 0.28, p-value=0.02), in conjunction with less MII oocytes (0.77 ± 0.55 vs 1.08 ± 0.61, p-value=0.02) originating from the FoPOR approach. This may render a biological paradox. Following literature search, various prospective and retrospective studies recorded a higher number of oocytes retrieved, along with MII oocytes following LuPOR (Kuang et al., 2014; Liu et al., 2017; Vaiarelli et al., 2018; Q. Zhang et al., 2017). However, it should be noted that the above studies performed a double ovarian stimulation during the follicular and luteal phase prior to FoPOR and LuPOR respectively and thus these differences may be attributed mainly to stimulation protocols. On the other hand, our results further reported no statistically significant difference regarding the number of 2PN zygotes post-insemination, which ascertain both phases as equally efficient.

Limitations of our study involve the small size of the included population, as well as the lack of several data regarding the all-inclusive patients' history, namely body max index, exercise activity or smoking, which may be considered as distant relative being identified as possible sources of cfDNA (Aucamp et al., 2018b). Furthermore, studies should be focused on examining the half-life of cfDNA in the biological fluid of ff, which may further provide crucial information imperative for the improvement of technical aspects of the protocol employed for the manipulation of the corresponding samples.

This study brings for first time in the literature ffcfDNA originating data with respect to the employment of LuPOR approach in the service of natural IVF cycles. Our protocol examined natural IVF cycles in poor responders employing a combination of biomarkers of ALU115 and ALU247 representing markers related to apoptotic or necrotic processes. To conclude, the results of the present study ascertain that both follicular and luteal phases of the menstrual cycle present as of equal dynamic on the grounds of physiology. Particularly, through ff cfDNA evaluation, no difference was revealed concerning necrotic events

including both FoPOR and LuPOR practices, which renders the aforementioned as equally promising approaches to be adopted for the management of poor responders. Our results buttress the validity, safety and efficiency of the LuPOR approach, shedding light to certain questions posed. Conduction of additional, well designed and executed, Randomized Controlled Trials (RCTs) to strengthen this evidence, are essential. The phenomenon of the double follicular wave explored through the practice of LuPOR has taken the IVF world by storm. Nonetheless, little is still known regarding optimal practice while practitioners acknowledge the absence of a widely accepted consensus. This very fact adds another level of complexity to the triplex of definition, diagnosis, and management of this distinct group of time-sensitive and management-demanding patients of poor ovarian reserve. One of the focal points of this work concentrated on reporting back to the IVF practitioner regarding clinical practice and decision-making when considering the employment of LuPOR. The time-sensitive nature of poor responders is usually accompanied with advanced age and compromised quality and quantity of oocytes (Gonda et al., 2018). Could the second follicular recruitment be characterized as the ovarian “swansong”, especially in cases of extreme poor responders, towards the path to gradually be faced with anticipated oocyte depletion? This question still stands. Undoubtedly, ascertaining a positive IVF outcome for this challenging cohort of patients renders a current challenge. Further studies should be performed employing a wider pallet of biomarkers, namely cytokines, in an attempt to investigate more in depth and concur on an efficient treatment for poor responders.

B. REFERENCES

Adams, G. (1999). Comparative patterns of follicle development and selection in ruminants. *Journal of Reproduction and Fertility. Supplement*, *54*, 17–32.

Adams, G. P., Matteri, R., Kastelic, J., Ko, J., & Ginther, Oj. (1992). Association between surges of follicle-stimulating hormone and the emergence of follicular waves in heifers. *Reproduction*, *94*(1), 177–188.

Andrisani, A., Marin, L., Ragazzi, E., Donà, G., Bordin, L., Dessole, F., … Ambrosini, G. (2019). Is corifollitropin alfa effective in controlled ovarian stimulation among all poor ovarian responders? A retrospective comparative study. *Gynecological Endocrinology: The Official Journal of the International Society of Gynecological Endocrinology*, 1–5. https://doi.org/10.1080/09513590.2019.1613360

Aucamp, J., Bronkhorst, A. J., Badenhorst, C. P. S., & Pretorius, P. J. (2018a). The diverse origins of circulating cell-free DNA in the human body: a critical re-evaluation of the literature. *Biological Reviews*. https://doi.org/10.1111/brv.12413

Aucamp, J., Bronkhorst, A. J., Badenhorst, C. P. S., & Pretorius, P. J. (2018b). The diverse origins of circulating cell-free DNA in the human body: a critical re-evaluation of the literature: The diverse origins of circulating cell-free DNA. *Biological Reviews*, *93*(3), 1649–1683. https://doi.org/10.1111/brv.12413

Baerwald, A. R., Adams, G. P., & Pierson, R. A. (2003a). A new model for ovarian follicular development during the human menstrual cycle. *Fertility and Sterility*, *80*(1), 116–122. https://doi.org/10.1016/S0015-0282(03)00544-2

Baerwald, A. R., Adams, G. P., & Pierson, R. A. (2003b). Characterization of Ovarian Follicular Wave Dynamics in Women1. *Biology of Reproduction*, *69*(3), 1023–1031. https://doi.org/10.1095/biolreprod.103.017772

Baerwald, A. R., Adams, G. P., & Pierson, R. A. (2012). Ovarian antral folliculogenesis during the human menstrual cycle: a review. *Human Reproduction Update*, *18*(1), 73–91. https://doi.org/10.1093/humupd/dmr039

Baka, S., & Malamitsi-Puchner, A. (2006). Novel follicular fluid factors influencing oocyte developmental potential in IVF: a review. *Reproductive BioMedicine Online*, *12*(4), 500–506. https://doi.org/10.1016/S1472-6483(10)62005-6

Barrett, A. N., Xiong, L., Tan, T. Z., Advani, H. V., Hua, R., Laureano-Asibal, C., … Choolani, M. (2017). Measurement of fetal fraction in cell-free DNA from maternal plasma using a panel of insertion/deletion polymorphisms. *PloS One*, *12*(10), e0186771.

Basuino, L., & Silveira, C. F. (2016). Human follicular fluid and effects on reproduction. *JBRA Assisted Reproduction*, *20*(1). https://doi.org/10.5935/1518-0557.20160009

Berkkanoglu, M., Isikoglu, M., Aydin, D., & Ozgur, K. (2007). Clinical effects of ovulation induction with recombinant follicle-stimulating hormone supplemented with recombinant luteinizing hormone or low-dose recombinant human chorionic gonadotropin in the midfollicular phase in microdose cycles in poor responders. *Fertility and Sterility*, *88*(3), 665–669.

Bosdou, J., Venetis, C., Kolibianakis, E., Toulis, K., Goulis, D., Zepiridis, L., & Tarlatzis, B. (2012). The use of androgens or androgen-modulating agents in poor responders undergoing in vitro fertilization: a systematic review and meta-analysis. *Human Reproduction Update*, *18*(2), 127–145.

Cardoso, M. C. de A., Evangelista, A., Sartorio, C., Vaz, G., Werneck, C. L. V., Guimaraes, F. M., … Erthal, M. C. (2017). Can ovarian double-stimulation in the same menstrual cycle improve IVF outcomes? *JBRA Assisted Reproduction*, *21*(3), 217–221. https://doi.org/10.5935/1518-0557.20170042

Castiglione Morelli, M. A., Iuliano, A., Schettini, S. C. A., Petruzzi, D., Ferri, A., Colucci, P., … Ostuni, A. (2019). NMR metabolic profiling of follicular fluid for investigating the different causes of female infertility: a pilot study. *Metabolomics: Official Journal of the Metabolomic Society*, *15*(2), 19. https://doi.org/10.1007/s11306-019-1481-x

Chan, C. H. Y., Lau, H. P. B., Tam, M. Y. J., & Ng, E. H. Y. (2016). A longitudinal study investigating the role of decisional conflicts and regret and short-term psychological adjustment after IVF treatment failure. *Human Reproduction*, *31*(12), 2772–2780. https://doi.org/10.1093/humrep/dew233

Chen, M., Wei, S., Hu, J., Yuan, J., & Liu, F. (2017). Does time-lapse imaging have favorable results for embryo incubation and selection compared with conventional methods in clinical in vitro fertilization? A meta-analysis and systematic review of randomized controlled trials. *PLOS ONE*, *12*(6), e0178720. https://doi.org/10.1371/journal.pone.0178720

Chern, C.-U., Tsui, K.-H., Vitale, S. G., Chen, S.-N., Wang, P.-H., Cianci, A., … Lin, L.-T. (2018). Dehydroepiandrosterone (DHEA) supplementation improves in vitro fertilization outcomes of poor ovarian responders, especially in women with low serum concentration of DHEA-S: a retrospective cohort study. *Reproductive Biology and Endocrinology*, *16*(1), 90.

Chikazawa, K., Araki, S., & Tamada, T. (1986). Morphological and endocrinological studies on follicular development during the human menstrual cycle. *The Journal of Clinical Endocrinology and Metabolism*, *62*(2), 305–313. https://doi.org/10.1210/jcem-62-2-305

Clancy, K. B. H., Baerwald, A. R., & Pierson, R. A. (2013). Systemic Inflammation Is Associated with Ovarian Follicular Dynamics during the Human Menstrual Cycle. *PLoS ONE*, *8*(5). https://doi.org/10.1371/journal.pone.0064807

Cobb, M. (2006). *Generation: The Seventeenth-Century Scientists Who Unraveled the Secrets of Sex, Life, and Growth* (1 edition). New York: Bloomsbury USA.

Cobo, A., Garrido, N., Crespo, J., José, R., & Pellicer, A. (2012). Accumulation of oocytes: a new strategy for managing low-responder patients. *Reproductive Biomedicine Online*, *24*(4), 424–432.

Cohen, J., Chabbert-Buffet, N., & Darai, E. (2015). Diminished ovarian reserve, premature ovarian failure, poor ovarian responder—a plea for universal definitions. *Journal of Assisted Reproduction and Genetics*, *32*(12), 1709–1712. https://doi.org/10.1007/s10815-015-0595-y

Crawford, N. M., & Steiner, A. Z. (2015). Age-related infertility. *Obstetrics and Gynecology Clinics of North America*, *42*(1), 15–25. https://doi.org/10.1016/j.ogc.2014.09.005

Creux, H., Monnier, P., Son, W.-Y., Tulandi, T., & Buckett, W. (2017). Immature oocyte retrieval and in vitro oocyte maturation at different phases of the menstrual cycle in women with cancer who require urgent gonadotoxic treatment. *Fertility and Sterility*, *107*(1), 198–204. https://doi.org/10.1016/j.fertnstert.2016.09.041

D'Amato, G., Caroppo, E., Pasquadibisceglie, A., Carone, D., Vitti, A., & Vizziello, G. M. (2004). A novel protocol of ovulation induction with delayed gonadotropin-releasing hormone antagonist administration combined with high-dose recombinant follicle-

stimulating hormone and clomiphene citrate for poor responders and women over 35 years. *Fertility and Sterility*, *81*(6), 1572–1577.

Desai, S. S., Roy, B. S., & Mahale, S. D. (2013). Mutations and polymorphisms in FSH receptor: functional implications in human reproduction. *Reproduction*, *146*(6), R235–R248.

Dimopoulou, M., Anifandis, G., Messini, C. I., Dafopoulos, K., Kouris, S., Sotiriou, S., … Messinis, I. E. (2014a). Follicular fluid oocyte/cumulus-free DNA concentrations as a potential biomolecular marker of embryo quality and IVF outcome. *BioMed Research International*, *2014*. https://doi.org/10.1155/2014/289306

Dimopoulou, M., Anifandis, G., Messini, C. I., Dafopoulos, K., Kouris, S., Sotiriou, S., … Messinis, I. E. (2014b). Follicular Fluid Oocyte/Cumulus-Free DNA Concentrations as a Potential Biomolecular Marker of Embryo Quality and IVF Outcome. *BioMed Research International*, *2014*, 1–5. https://doi.org/10.1155/2014/289306

Dinakaran, V., Rathinavel, A., Pushpanathan, M., Sivakumar, R., Gunasekaran, P., & Rajendhran, J. (2014). Elevated Levels of Circulating DNA in Cardiovascular Disease Patients: Metagenomic Profiling of Microbiome in the Circulation. *PLOS ONE*, *9*(8), e105221. https://doi.org/10.1371/journal.pone.0105221

Ferraretti, A. P., La Marca, A., Fauser, B. C. J. M., Tarlatzis, B., Nargund, G., Gianaroli, L., & on behalf of the ESHRE working group on Poor Ovarian Response Definition. (2011). ESHRE consensus on the definition of “poor response” to ovarian stimulation for in vitro fertilization: the Bologna criteria. *Human Reproduction*, *26*(7), 1616–1624. https://doi.org/10.1093/humrep/der092

Ferraretti, Anna Pia, Gianaroli, L., Magli, M. C., D’angelo, A., Farfalli, V., & Montanaro, N. (2004). Exogenous luteinizing hormone in controlled ovarian hyperstimulation for assisted reproduction techniques. *Fertility and Sterility*, *82*(6), 1521–1526.

Field, S. L., Dasgupta, T., Cummings, M., & Orsi, N. M. (2014). Cytokines in ovarian folliculogenesis, oocyte maturation and luteinisation: CYTOKINES IN FOLLICULOGENESIS. *Molecular Reproduction and Development*, *81*(4), 284–314. https://doi.org/10.1002/mrd.22285

Fleming, R., Seifer, D. B., Frattarelli, J. L., & Ruman, J. (2015). Assessing ovarian response: antral follicle count versus anti-Müllerian hormone. *Reproductive BioMedicine Online*, *31*(4), 486–496. https://doi.org/10.1016/j.rbmo.2015.06.015

Frattarelli, J. L., McWilliams, G. D., Hill, M. J., Miller, K. A., & Scott Jr, R. T. (2008). Low-dose aspirin use does not improve in vitro fertilization outcomes in poor responders. *Fertility and Sterility*, *89*(5), 1113–1117.

Gameiro, S., Boivin, J., Peronace, L., & Verhaak, C. M. (2012). Why do patients discontinue fertility treatment? A systematic review of reasons and predictors of discontinuation in fertility treatment. *Human Reproduction Update*, *18*(6), 652–669. https://doi.org/10.1093/humupd/dms031

Garcia, J. E., Jones, G. S., Acosta, A. A., & Wright Jr, G. (1983). Human menopausal gonadotropin/human chorionic gonadotropin follicular maturation for oocyte aspiration: phase II, 1981. *Fertility and Sterility*, *39*(2), 174–179.

Garel, M., Blondel, B., Karpel, L., Blanchet, V., Breart, G., Frydman, R., & Olivennes, F. (2009). Women’s views on Friendly IVF: A qualitative preliminary study. *Journal of Psychosomatic Obstetrics & Gynecology*, *30*(2), 101–104. https://doi.org/10.1080/01674820802604896

Gastal, E. L., Gastal, M. O., & Ginther, O. J. (1999). Experimental assumption of dominance by a smaller follicle and associated hormonal changes in mares. *Biology of Reproduction*, *61*(3), 724–730. https://doi.org/10.1095/biolreprod61.3.724

Ginther, O. J. (1993). Major and Minor Follicular waves During the Equine Estrous Cycle. *Journal of Equine Veterinary Science*, *13*(1), 18–25. https://doi.org/10.1016/S0737-0806(07)80012-8

Ginther, O. J., Kastelic, J. P., & Knopf, L. (1989). Composition and characteristics of follicular waves during the bovine estrous cycle. *Animal Reproduction Science*, *20*(3), 187–200. https://doi.org/10.1016/0378-4320(89)90084-5

Giovanale, V., Pulcinelli, F. M., Ralli, E., Primiero, F. M., & Caserta, D. (2015). Poor responders in IVF: an update in therapy. *Gynecological Endocrinology*, *31*(4), 253–257. https://doi.org/10.3109/09513590.2014.987228

Gleicher, N., Weghofer, A., & Barad, D. H. (2010). Ovarian reserve determinations suggest new function of FMR1 (fragile X gene) in regulating ovarian ageing. *Reproductive BioMedicine Online*, *20*(6), 768–775. https://doi.org/10.1016/j.rbmo.2010.02.020

Gleicher, N., Yu, Y., Himaya, E., Barad, D. H., Weghofer, A., Wu, Y.-G., … Kushnir, V. A. (2015). Early decline in functional ovarian reserve in young women with low (CGGn < 26) FMR1 gene alleles. *Translational Research: The Journal of Laboratory and Clinical Medicine*, *166*(5), 502-507.e1-2. https://doi.org/10.1016/j.trsl.2015.06.014

Gonda, K. J., Domar, A. D., Gleicher, N., & Marrs, R. P. (2018). Insights from clinical experience in treating IVF poor responders. *Reproductive BioMedicine Online*, *36*(1), 12–19. https://doi.org/10.1016/j.rbmo.2017.09.016

Gougeon, A., & Lefèvre, B. (1983). Evolution of the diameters of the largest healthy and atretic follicles during the human menstrual cycle. *Journal of Reproduction and Fertility*, *69*(2), 497–502.

Gravina, S., Sedivy, J. M., & Vijg, J. (2016). The dark side of circulating nucleic acids. *Aging Cell*, *15*(3), 398–399. https://doi.org/10.1111/acel.12454

Guan, Y., Zhang, W., Wang, X., Cai, P., Jia, Q., & Zhao, W. (2017). Cell-free DNA induced apoptosis of granulosa cells by oxidative stress. *Clinica Chimica Acta; International Journal of Clinical Chemistry*, *473*, 213–217. https://doi.org/10.1016/j.cca.2016.11.023

Haadsma, M. L., Groen, H., Mooij, T. M., Burger, C. W., Broekmans, F. J., Lambalk, C. B., … Hoek, A. (2010). Miscarriage risk for IVF pregnancies in poor responders to ovarian hyperstimulation. *Reproductive Biomedicine Online*, *20*(2), 191–200.

Haas, J., & Casper, R. F. (2017). In vitro fertilization treatments with the use of clomiphene citrate or letrozole. *Fertility and Sterility*, *108*(4), 568–571. https://doi.org/10.1016/j.fertnstert.2017.08.017

Heiligentag, M., & Eichenlaub-Ritter, U. (2018). Preantral follicle culture and oocyte quality. *Reproduction, Fertility and Development*, *30*(1), 18. https://doi.org/10.1071/RD17411

Hennet, M. l., & Combelles, C. M. H. (2012). The antral follicle: a microenvironment for oocyte differentiation. *The International Journal of Developmental Biology*, *56*(10-11–12), 819–831. https://doi.org/10.1387/ijdb.120133cc

Herman, A., Ron-El, R., Golan, A., Nahum, H., Soffer, Y., & Caspi, E. (1990). Follicle cysts after menstrual versus midluteal administration of gonadotropin-releasing hormone analog in in vitro fertilization. *Fertility and Sterility*, *53*(5), 854–858.

Ho, J. R., & Paulson, R. J. (2017). Modified natural cycle in in vitro fertilization. *Fertility and Sterility*, *108*(4), 572–576. https://doi.org/10.1016/j.fertnstert.2017.08.021

Hoffman, G. E., Le, W. W., Entezam, A., Otsuka, N., Tong, Z.-B., Nelson, L., … Usdin, K. (2012). Ovarian Abnormalities in a Mouse Model of Fragile X Primary Ovarian Insufficiency. *Journal of Histochemistry & Cytochemistry*, *60*(6), 439–456. https://doi.org/10.1369/0022155412441002

Humaidan, P., Alviggi, C., Fischer, R., & Esteves, S. C. (2016). The novel POSEIDON stratification of 'Low prognosis patients in Assisted Reproductive Technology' and its proposed marker of successful outcome. *F1000Research*, *5*, 2911. https://doi.org/10.12688/f1000research.10382.1

Hussein, N. A., Mohamed, S. N., & Ahmed, M. A. (2019). Plasma ALU-247, ALU-115, and cfDNA Integrity as Diagnostic and Prognostic Biomarkers for Breast Cancer. *Applied Biochemistry and Biotechnology*, *187*(3), 1028–1045. https://doi.org/10.1007/s12010-018-2858-4

Ivanov, M., Baranova, A., Butler, T., Spellman, P., & Mileyko, V. (2015). Non-random fragmentation patterns in circulating cell-free DNA reflect epigenetic regulation. *BMC Genomics*, *16*(S13), S1. https://doi.org/10.1186/1471-2164-16-S13-S1

Jahr, S., Hentze, H., Englisch, S., Hardt, D., Fackelmayer, F. O., Hesch, R. D., & Knippers, R. (2001). DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Research*, *61*(4), 1659–1665.

Jin, B., Niu, Z., Xu, B., Chen, Q., & Zhang, A. (2018). Comparison of clinical outcomes among dual ovarian stimulation, mild stimulation and luteal phase stimulation protocols in women with poor ovarian response. *Gynecological Endocrinology : The Official Journal of the International Society of Gynecological Endocrinology*, *34*(8), 694–697. https://doi.org/10.1080/09513590.2018.1435636

Jirge, P. R. (2016). Poor ovarian reserve. *Journal of Human Reproductive Sciences*, *9*(2), 63–69. https://doi.org/10.4103/0974-1208.183514

Kamath, M. S., Kirubakaran, R., Mascarenhas, M., & Sunkara, S. K. (2018). Perinatal outcomes after stimulated versus natural cycle IVF: a systematic review and meta-analysis. *Reproductive Biomedicine Online*, *36*(1), 94–101. https://doi.org/10.1016/j.rbmo.2017.09.009

Knopf, L., Kastelic, J., Schallenberger, E., & Ginther, O. (1989). Ovarian follicular dynamics in heifers: test of two-wave hypothesis by ultrasonically monitoring individual follicles. *Domestic Animal Endocrinology*, *6*(2), 111–119.

Koontz, L. (2013). Agarose Gel Electrophoresis. In *Methods in Enzymology* (Vol. 529, pp. 35–45). https://doi.org/10.1016/B978-0-12-418687-3.00004-5

Kuang, Y., Chen, Q., Hong, Q., Lyu, Q., Ai, A., Fu, Y., & Shoham, Z. (2014). Double stimulations during the follicular and luteal phases of poor responders in IVF/ICSI programmes (Shanghai protocol). *Reproductive Biomedicine Online*, *29*(6), 684–691. https://doi.org/10.1016/j.rbmo.2014.08.009

La Marca, A., & Sunkara, S. K. (2014). Individualization of controlled ovarian stimulation in IVF using ovarian reserve markers: from theory to practice. *Human Reproduction Update*, *20*(1), 124–140. https://doi.org/10.1093/humupd/dmt037

Lainas, T. G., Sfontouris, I. A., Venetis, C. A., Lainas, G. T., Zorzovilis, I. Z., Tarlatzis, B. C., & Kolibianakis, E. M. (2015). Live birth rates after modified natural cycle compared with high-dose FSH stimulation using GnRH antagonists in poor responders. *Human Reproduction (Oxford, England)*, *30*(10), 2321–2330. https://doi.org/10.1093/humrep/dev198

Laven, J. (2016). Primary Ovarian Insufficiency. *Seminars in Reproductive Medicine*, *34*(04), 230–234. https://doi.org/10.1055/s-0036-1585402

Lazzaroni-Tealdi, E., Barad, D. H., Albertini, D. F., Yu, Y., Kushnir, V. A., Russell, H., … Gleicher, N. (2015). Oocyte Scoring Enhances Embryo-Scoring in Predicting Pregnancy Chances with IVF Where It Counts Most. *PLOS ONE*, *10*(12), e0143632. https://doi.org/10.1371/journal.pone.0143632

Ledee, N., Gridelet, V., Ravet, S., Jouan, C., Gaspard, O., Wenders, F., … d'Hauterive, S. P. (2013). Impact of follicular G-CSF quantification on subsequent embryo transfer decisions: a proof of concept study. *Human Reproduction*, *28*(2), 406–413. https://doi.org/10.1093/humrep/des354

Ledee, N., Petitbarat, M., Rahmati, M., Dubanchet, S., Chaouat, G., Sandra, O., … Foidart, J. M. (2011). New pre-conception immune biomarkers for clinical practice: interleukin-18, interleukin-15 and TWEAK on the endometrial side, G-CSF on the follicular side. *Journal of Reproductive Immunology*, *88*(2), 118–123. https://doi.org/10.1016/j.jri.2011.01.007

Lee, P. Y., Costumbrado, J., Hsu, C.-Y., & Kim, Y. H. (2012). Agarose Gel Electrophoresis for the Separation of DNA Fragments. *Journal of Visualized Experiments : JoVE*, (62). https://doi.org/10.3791/3923

Li, J., Xu, Y., Zhou, G., Guo, J., & Xin, N. (2011). Natural cycle IVF/IVM may be more desirable for poor responder patients after failure of stimulated cycles. *Journal of Assisted Reproduction and Genetics*, *28*(9), 791–795. https://doi.org/10.1007/s10815-011-9597-6

Li, X.-L., Wang, L., Lv, F., Huang, X.-M., Wang, L.-P., Pan, Y., & Zhang, X.-M. (2017). The influence of different growth hormone addition protocols to poor ovarian responders on clinical outcomes in controlled ovary stimulation cycles: A systematic review and meta-analysis. *Medicine*, *96*(12), e6443. https://doi.org/10.1097/MD.0000000000006443

Liu, C., Jiang, H., Zhang, W., & Yin, H. (2017). Double ovarian stimulation during the follicular and luteal phase in women >/=38 years: a retrospective case-control study. *Reproductive Biomedicine Online*, *35*(6), 678–684. https://doi.org/10.1016/j.rbmo.2017.08.019

Lok, I. H., Yip, S.-K., Cheung, L. P., Yin Leung, P. H., & Haines, C. J. (2004). Adjuvant low-dose aspirin therapy in poor responders undergoing in vitro fertilization: a prospective, randomized, double-blind, placebo-controlled trial. *Fertility and Sterility*, *81*(3), 556–561. https://doi.org/10.1016/j.fertnstert.2003.07.033

Lorenz, T. C. (2012). Polymerase Chain Reaction: Basic Protocol Plus Troubleshooting and Optimization Strategies. *Journal of Visualized Experiments : JoVE*, (63). https://doi.org/10.3791/3998

Loutradis, D., Drakakis, P., Vomvolaki, E., & Antsaklis, A. (2007). Different ovarian stimulation protocols for women with diminished ovarian reserve. *Journal of Assisted Reproduction and Genetics*, *24*(12), 597–611. https://doi.org/10.1007/s10815-007-9181-2

Madani, T., Hemat, M., Arabipoor, A., Khodabakhshi, S. H., & Zolfaghari, Z. (2018). Double mild stimulation and egg collection in the same cycle for management of poor ovarian responders. *Journal of Gynecology Obstetrics and Human Reproduction*. https://doi.org/10.1016/j.jogoh.2018.12.004

Maman, E., Meirow, D., Brengauz, M., Raanani, H., Dor, J., & Hourvitz, A. (2011). Luteal phase oocyte retrieval and in vitro maturation is an optional procedure for urgent

fertility preservation. *Fertility and Sterility*, *95*(1), 64–67. https://doi.org/10.1016/j.fertnstert.2010.06.064

Mandel, P. (1948). Les acides nucleiques du plasma sanguin chez 1 homme. *CR Seances Soc Biol Fil*, *142*, 241–243.

Martinez, F., Clua, E., Devesa, M., Rodriguez, I., Arroyo, G., Gonzalez, C., … Barri, P. N. (2014). Comparison of starting ovarian stimulation on day 2 versus day 15 of the menstrual cycle in the same oocyte donor and pregnancy rates among the corresponding recipients of vitrified oocytes. *Fertility and Sterility*, *102*(5), 1307–1311. https://doi.org/10.1016/j.fertnstert.2014.07.741

Matsuda, F., Inoue, N., Manabe, N., & Ohkura, S. (2012). Follicular Growth and Atresia in Mammalian Ovaries: Regulation by Survival and Death of Granulosa Cells. *Journal of Reproduction and Development*, *58*(1), 44–50. https://doi.org/10.1262/jrd.2011-012

Morgia, F., Sbracia, M., Schimberni, M., Giallonardo, A., Piscitelli, C., Giannini, P., & Aragona, C. (2004). A controlled trial of natural cycle versus microdose gonadotropin-releasing hormone analog flare cycles in poor responders undergoing in vitro fertilization. *Fertility and Sterility*, *81*(6), 1542–1547. https://doi.org/10.1016/j.fertnstert.2003.11.031

Nagata, S. (2000). Apoptotic DNA fragmentation. *Experimental Cell Research*, *256*(1), 12–18. https://doi.org/10.1006/excr.2000.4834

Nikolic, A., Volarevic, V., Armstrong, L., Lako, M., & Stojkovic, M. (2016). Primordial Germ Cells: Current Knowledge and Perspectives. *Stem Cells International*, *2016*. https://doi.org/10.1155/2016/1741072

Oudendijk, J. F., Yarde, F., Eijkemans, M. J. C., Broekmans, F. J. M., & Broer, S. L. (2012). The poor responder in IVF: is the prognosis always poor? A systematic review. *Human Reproduction Update*, *18*(1), 1–11. https://doi.org/10.1093/humupd/dmr037

ovary | Origin and meaning of ovary by Online Etymology Dictionary. (n.d.). Retrieved April 30, 2019, from https://www.etymonline.com/word/ovary

Pacchiarotti, A., Selman, H., Valeri, C., Napoletano, S., Sbracia, M., Antonini, G., … Pacchiarotti, A. (2016). Ovarian Stimulation Protocol in IVF: An Up-to-Date Review of the Literature. *Current Pharmaceutical Biotechnology*, *17*(4), 303–315.

Papathanasiou, A., Searle, B. J., King, N. M., & Bhattacharya, S. (2016). Trends in 'poor responder'research: lessons learned from RCTs in assisted conception. *Human Reproduction Update*, *22*(3), 306–319.

Persani, L., Rossetti, R., & Cacciatore, C. (2010). Genes involved in human premature ovarian failure. *Journal of Molecular Endocrinology*, *45*(5), 257–279. https://doi.org/10.1677/JME-10-0070

Pierson, R. A., & Ginther, O. J. (1988). Ultrasonic imaging of the ovaries and uterus in cattle. *Theriogenology*, *29*(1), 21–37. https://doi.org/10.1016/0093-691X(88)90029-5

Pilehvari, S., ShahrokhTehraninejad, E., Hosseinrashidi, B., Keikhah, F., Haghollahi, F., & Aziminekoo, E. (2016). Comparison Pregnancy Outcomes Between Minimal Stimulation Protocol and Conventional GnRH Antagonist Protocols in Poor Ovarian Responders. *Journal of Family & Reproductive Health*, *10*(1), 35–42.

Pinzani, P., Salvianti, F., Pazzagli, M., & Orlando, C. (2010). Circulating nucleic acids in cancer and pregnancy. *Methods (San Diego, Calif.)*, *50*(4), 302–307. https://doi.org/10.1016/j.ymeth.2010.02.004

Plant, T. M. (2015). 60 YEARS OF NEUROENDOCRINOLOGY: The hypothalamo-pituitary–gonadal axis. *Journal of Endocrinology*, *226*(2), T41–T54. https://doi.org/10.1530/JOE-15-0113

Polyzos, N., Blockeel, C., Verpoest, W., De Vos, M., Stoop, D., Vloeberghs, V., … Tournaye, H. (2012). Live birth rates following natural cycle IVF in women with poor ovarian response according to the Bologna criteria. *Human Reproduction*, *27*(12), 3481–3486.

Polyzos, N. P., & Devroey, P. (2011). A systematic review of randomized trials for the treatment of poor ovarian responders: is there any light at the end of the tunnel? *Fertility and Sterility*, *96*(5), 1058-1061.e7. https://doi.org/10.1016/j.fertnstert.2011.09.048

Practice Committee of the American Society for Reproductive Medicine. (2018). Comparison of pregnancy rates for poor responders using IVF with mild ovarian stimulation versus conventional IVF: a guideline. *Fertility and Sterility*, *109*(6), 993–999.

Pu, D., Wu, J., & Liu, J. (2011). Comparisons of GnRH antagonist versus GnRH agonist protocol in poor ovarian responders undergoing IVF. *Human Reproduction*, *26*(10), 2742–2749.

Rashtian, J., & Zhang, J. (2018). Luteal-phase ovarian stimulation increases the number of mature oocytes in older women with severe diminished ovarian reserve. *Systems Biology in Reproductive Medicine*, *64*(3), 216–219. https://doi.org/10.1080/19396368.2018.1448902

Revelli, A., Piane, L. D., Casano, S., Molinari, E., Massobrio, M., & Rinaudo, P. (2009). Follicular fluid content and oocyte quality: from single biochemical markers to metabolomics. *Reproductive Biology and Endocrinology*, *7*(1), 40. https://doi.org/10.1186/1477-7827-7-40

Rimon-Dahari, N., Yerushalmi-Heinemann, L., Alyagor, L., & Dekel, N. (2016). Ovarian Folliculogenesis. In R. P. Piprek (Ed.), *Molecular Mechanisms of Cell Differentiation in Gonad Development* (Vol. 58, pp. 167–190). https://doi.org/10.1007/978-3-319-31973-5_7

Sakumoto, R., Shibaya, M., & Okuda, K. (2003). Tumor necrosis factor-alpha (TNF alpha) inhibits progesterone and estradiol-17beta production from cultured granulosa cells: presence of TNFalpha receptors in bovine granulosa and theca cells. *The Journal of Reproduction and Development*, *49*(6), 441–449. https://doi.org/10.1262/jrd.49.441

Salmassi, A., Lu, S., Hedderich, J., Oettinghaus, C., Jonat, W., & Mettler, L. (2001). Interaction of interleukin-6 on human granulosa cell steroid secretion. *Journal of Endocrinology*, *170*(2), 471–478.

Scalici, E., Traver, S., Molinari, N., Mullet, T., Monforte, M., Vintejoux, E., & Hamamah, S. (2014). Cell-free DNA in human follicular fluid as a biomarker of embryo quality. *Human Reproduction*, *29*(12), 2661–2669. https://doi.org/10.1093/humrep/deu238

Scalici, E., Traver, S., Mullet, T., Molinari, N., Ferrières, A., Brunet, C., … Hamamah, S. (2016). Circulating microRNAs in follicular fluid, powerful tools to explore in vitro fertilization process. *Scientific Reports*, *6*, 24976. https://doi.org/10.1038/srep24976

Schimberni, M, Ciardo, F., Schimberni, M., Giallonardo, A., De Pratti, V., & Sbracia, M. (2016). Short gonadotropin-releasing hormone agonist versus flexible antagonist versus clomiphene citrate regimens in poor responders undergoing in vitro fertilization: a randomized controlled trial. *Eur Rev Med Pharmacol Sci*, *20*(20), 4354–4361.

Schimberni, Mauro, Morgia, F., Colabianchi, J., Giallonardo, A., Piscitelli, C., Giannini, P., … Sbracia, M. (2009). Natural-cycle in vitro fertilization in poor responder patients: a survey of 500 consecutive cycles. *Fertility and Sterility*, *92*(4), 1297–1301.

Scott, R. T., Hofmann, G. E., Oehninger, S., & Muasher, S. J. (1990). Intercycle variability of day 3 follicle-stimulating hormone levels and its effect on stimulation quality in in vitro fertilization. *Fertility and Sterility*, *54*(2), 297–302.

Sfakianoudis, K., Simopoulou, M., Maziotis, E., Giannelou, P., Tsioulou, P., Rapani, A., … Pantos, K. (2019). Evaluation of the Second Follicular Wave Phenomenon in Natural Cycle Assisted Reproduction: A Key Option for Poor Responders through Luteal Phase Oocyte Retrieval. *Medicina (Kaunas, Lithuania)*, *55*(3). https://doi.org/10.3390/medicina55030068

Silver, R. M., Myatt, L., Hauth, J. C., Leveno, K. J., Peaceman, A. M., Ramin, S. M., … Reddy, U. M. (2017). Cell-Free Total and Fetal DNA in First Trimester Maternal Serum and Subsequent Development of Preeclampsia. *American Journal of Perinatology*, *34*(2), 191–198. https://doi.org/10.1055/s-0035-1570383

Simoni, M. K., Mu, L., & Collins, S. C. (2017). Women's career priority is associated with attitudes towards family planning and ethical acceptance of reproductive technologies. *Human Reproduction*, *32*(10), 2069–2075. https://doi.org/10.1093/humrep/dex275

Simopoulou, M., Sfakianoudis, K., Maziotis, E., Antoniou, N., Rapani, A., Anifandis, G., … Koutsilieris, M. (2018). Are computational applications the "crystal ball" in the IVF laboratory? The evolution from mathematics to artificial intelligence. *Journal of Assisted Reproduction and Genetics*, *35*(9), 1545–1557. https://doi.org/10.1007/s10815-018-1266-6

Sirois, J., & Fortune, J. (1988). Ovarian Follicular Dynamics during the Estrous Cycle in Heifers Monitored by Real-Time UItrasonograph. *Biology of Reproduction*, *39*(2), 308–317.

Song, D., Shi, Y., Zhong, Y., Meng, Q., Hou, S., & Li, H. (2016). Efficiency of mild ovarian stimulation with clomiphene on poor ovarian responders during IVF\ICSI procedures: a meta-analysis. *European Journal of Obstetrics, Gynecology, and Reproductive Biology*, *204*, 36–43. https://doi.org/10.1016/j.ejogrb.2016.07.498

Soutis, M. (2006). Ancient Greek terminology in pediatric surgery: about the word meaning. *Journal of Pediatric Surgery*, *41*(7), 1302–1308.

Spanou, S., Kalogiannis, D., Zapanti, E., Gazouli, M., Sfontouris, I. A., Siristatidis, C., & Mastorakos, G. (2018). Interleukin 15 concentrations in follicular fluid and their effect on oocyte maturation in subfertile women undergoing intracytoplasmic sperm injection. *Journal of Assisted Reproduction and Genetics*, *35*(6), 1019–1025. https://doi.org/10.1007/s10815-018-1170-0

Surrey, E. S., & Schoolcraft, W. B. (2000). Evaluating strategies for improving ovarian response of the poor responder undergoing assisted reproductive techniques. *Fertility and Sterility*, *73*(4), 667–676.

Tal, R., & Seifer, D. B. (2017). Ovarian reserve testing: a user's guide. *American Journal of Obstetrics and Gynecology*, *217*(2), 129–140. https://doi.org/10.1016/j.ajog.2017.02.027

Traver, S., Assou, S., Scalici, E., Haouzi, D., Al-Edani, T., Belloc, S., & Hamamah, S. (2014). Cell-free nucleic acids as non-invasive biomarkers of gynecological cancers, ovarian, endometrial and obstetric disorders and fetal aneuploidy. *Human Reproduction Update*, *20*(6), 905–923. https://doi.org/10.1093/humupd/dmu031

Traver, Sabine, Scalici, E., Mullet, T., Molinari, N., Vincens, C., Anahory, T., & Hamamah, S. (2015a). Cell-free DNA in human follicular microenvironment: New prognostic biomarker to predict in vitro fertilization outcomes. *PLoS ONE*, *10*(8), 1–18. https://doi.org/10.1371/journal.pone.0136172

Traver, Sabine, Scalici, E., Mullet, T., Molinari, N., Vincens, C., Anahory, T., & Hamamah, S. (2015b). Cell-free DNA in Human Follicular Microenvironment: New Prognostic Biomarker to Predict in vitro Fertilization Outcomes. *PLOS ONE*, *10*(8), e0136172. https://doi.org/10.1371/journal.pone.0136172

Ubaldi, F. M., Capalbo, A., Vaiarelli, A., Cimadomo, D., Colamaria, S., Alviggi, C., … Rienzi, L. (2016). Follicular versus luteal phase ovarian stimulation during the same menstrual cycle (DuoStim) in a reduced ovarian reserve population results in a similar euploid blastocyst formation rate: new insight in ovarian reserve exploitation. *Fertility and Sterility*, *105*(6), 1488-1495.e1. https://doi.org/10.1016/j.fertnstert.2016.03.002

Ubaldi, F, Rienzi, L., Baroni, E., Ferrero, S., Iacobelli, M., Minasi, M., … Litwicka, K. (2007). Hopes and facts about mild ovarian stimulation. *Reproductive Biomedicine Online*, *14*(6), 675–681.

Ubaldi, Filippo, Vaiarelli, A., D'Anna, R., & Rienzi, L. (2014). Management of poor responders in IVF: is there anything new? *BioMed Research International*, *2014*.

Umetani, N. (2006). Increased Integrity of Free Circulating DNA in Sera of Patients with Colorectal or Periampullary Cancer: Direct Quantitative PCR for ALU Repeats. *Clinical Chemistry*, *52*(6), 1062–1069. https://doi.org/10.1373/clinchem.2006.068577

Unfer, V., Raffone, E., Rizzo, P., & Buffo, S. (2011). Effect of a supplementation with myo-inositol plus melatonin on oocyte quality in women who failed to conceive in previous in vitro fertilization cycles for poor oocyte quality: a prospective, longitudinal, cohort study. *Gynecological Endocrinology*, *27*(11), 857–861.

Vaiarelli, A., Cimadomo, D., Trabucco, E., Vallefuoco, R., Buffo, L., Dusi, L., … Ubaldi, F. M. (2018). Double Stimulation in the Same Ovarian Cycle (DuoStim) to Maximize the Number of Oocytes Retrieved From Poor Prognosis Patients: A Multicenter Experience and SWOT Analysis. *Frontiers in Endocrinology*, *9*, 317. https://doi.org/10.3389/fendo.2018.00317

van Santbrink, E. J., Hop, W. C., van Dessel, T. J., de Jong, F. H., & Fauser, B. C. (1995). Decremental follicle-stimulating hormone and dominant follicle development during the normal menstrual cycle. *Fertility and Sterility*, *64*(1), 37–43.

Vaskivuo, T. E., & Tapanainen, J. S. (2003). Apoptosis in the human ovary. *Reproductive Biomedicine Online*, *6*(1), 24–35.

Vinatier, D., Dufour, P., Tordjeman-Rizzi, N., Prolongeau, J., Depret-Moser, S., & Monnier, J. (1995). Immunological aspects of ovarian function: role of the cytokines. *European Journal of Obstetrics & Gynecology and Reproductive Biology*, *63*(2), 155–168.

Virant-Klun, I. (2015). Postnatal oogenesis in humans: a review of recent findings. *Stem Cells and Cloning : Advances and Applications*, *8*, 49–60. https://doi.org/10.2147/SCCAA.S32650

Vitale, S. G., Rossetti, P., Corrado, F., Rapisarda, A. M. C., La Vignera, S., Condorelli, R. A., … Buscema, M. (2016). How to achieve high-quality oocytes? The key role of myo-inositol and melatonin. *International Journal of Endocrinology*, *2016*.

Volckmar, A.-L., Sültmann, H., Riediger, A., Fioretos, T., Schirmacher, P., Endris, V., … Dietz, S. (2018). A field guide for cancer diagnostics using cell-free DNA: From

principles to practice and clinical applications. *Genes, Chromosomes & Cancer*, *57*(3), 123–139. https://doi.org/10.1002/gcc.22517

Vural, F., Vural, B., Doğer, E., Çakıroğlu, Y., & Çekmen, M. (2016). Perifollicular blood flow and its relationship with endometrial vascularity, follicular fluid EG-VEGF, IGF-1, and inhibin-a levels and IVF outcomes. *Journal of Assisted Reproduction and Genetics*, *33*(10), 1355–1362. https://doi.org/10.1007/s10815-016-0780-7

Wade, J. J., MacLachlan, V., & Kovacs, G. (2015). The success rate of IVF has significantly improved over the last decade. *Australian and New Zealand Journal of Obstetrics and Gynaecology*, *55*(5), 473–476. https://doi.org/10.1111/ajo.12356

Waters, D. L. E., & Shapter, F. M. (2014). The Polymerase Chain Reaction (PCR): General Methods. In R. J. Henry & A. Furtado (Eds.), *Cereal Genomics* (Vol. 1099, pp. 65–75). https://doi.org/10.1007/978-1-62703-715-0_7

World Health Organization (Ed.). (2010). *WHO laboratory manual for the examination and processing of human semen* (5th ed). Geneva: World Health Organization.

Wu, Y., Zhao, F.-C., Sun, Y., & Liu, P.-S. (2017). Luteal-phase protocol in poor ovarian response: a comparative study with an antagonist protocol. *Journal of International Medical Research*, *45*(6), 1731–1738.

Xu, B., & Li, Y. (2013). Flexible ovarian stimulation in a poor responder: a case report and literature review. *Reproductive Biomedicine Online*, *26*(4), 378–383. https://doi.org/10.1016/j.rbmo.2012.11.020

Younis, J. S., Ben-Ami, M., & Ben-Shlomo, I. (2015). The Bologna criteria for poor ovarian response: a contemporary critical appraisal. *Journal of Ovarian Research*, *8*(1), 76. https://doi.org/10.1186/s13048-015-0204-9

Zegers-Hochschild, F., Adamson, G. D., de Mouzon, J., Ishihara, O., Mansour, R., Nygren, K., … World Health Organization. (2009). International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) revised glossary of ART terminology, 2009. *Fertility and Sterility*, *92*(5), 1520–1524. https://doi.org/10.1016/j.fertnstert.2009.09.009

Zhang, Q., Guo, X. M., & Li, Y. (2017). Implantation rates subsequent to the transfer of embryos produced at different phases during double stimulation of poor ovarian responders. *Reproduction, Fertility, and Development*, *29*(6), 1178–1183. https://doi.org/10.1071/RD16020

Zhang, W., Wang, M., Wang, S., Bao, H., Qu, Q., Zhang, N., & Hao, C. (2018). Luteal phase ovarian stimulation for poor ovarian responders. *JBRA Assisted Reproduction*, *22*(3), 193–198. https://doi.org/10.5935/1518-0557.20180045

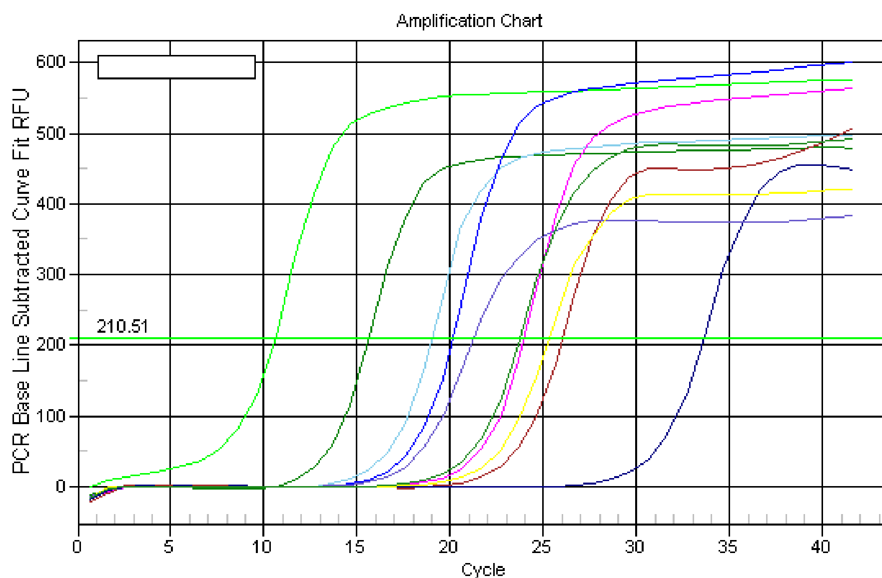
Zhou, J., Wang, J., Penny, D., Monget, P., Arraztoa, J. A., Fogelson, L. J., & Bondy, C. A. (2003). Insulin-Like Growth Factor Binding Protein 4 Expression Parallels Luteinizing Hormone Receptor Expression and Follicular Luteinization in the Primate Ovary. *Biology of Reproduction*, *69*(1), 22–29. https://doi.org/10.1095/biolreprod.102.009191

APPENDIX*

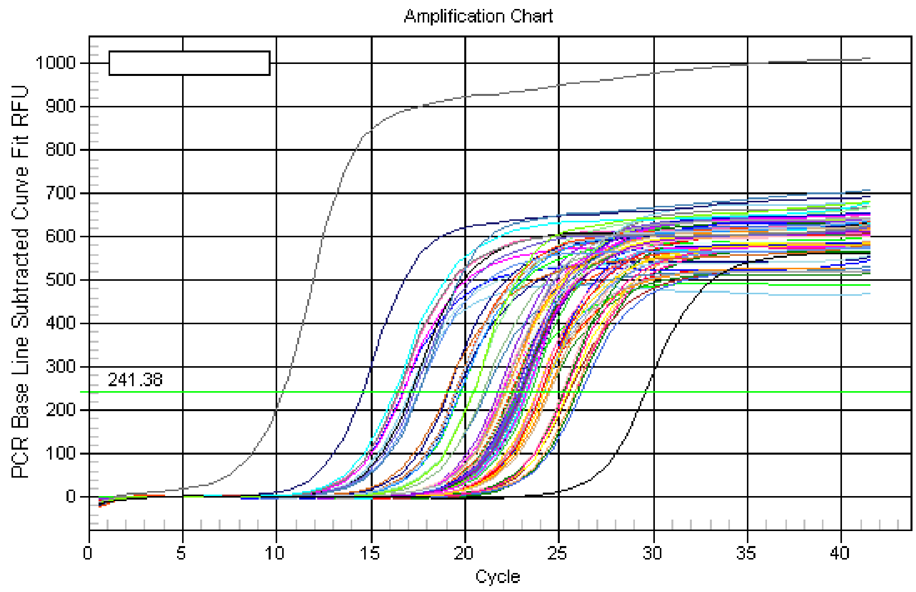
Hereby, we provide supplementary figures with PCR amplification charts for both ALU115 and ALU 247 primers, as depicted in PCR quantification detailed reports, which were obtained at the end of RT-PCR procedure. It should be noted, that among the curves that correspond to cfDNA levels from FoPOR and LuPOR ff samples obtained from poor responders on behalf of the present study, there are additional curves that correspond to cfDNA levels from ff samples obtained by normal responders on behalf of another study.

ALU 115 PRIMERS

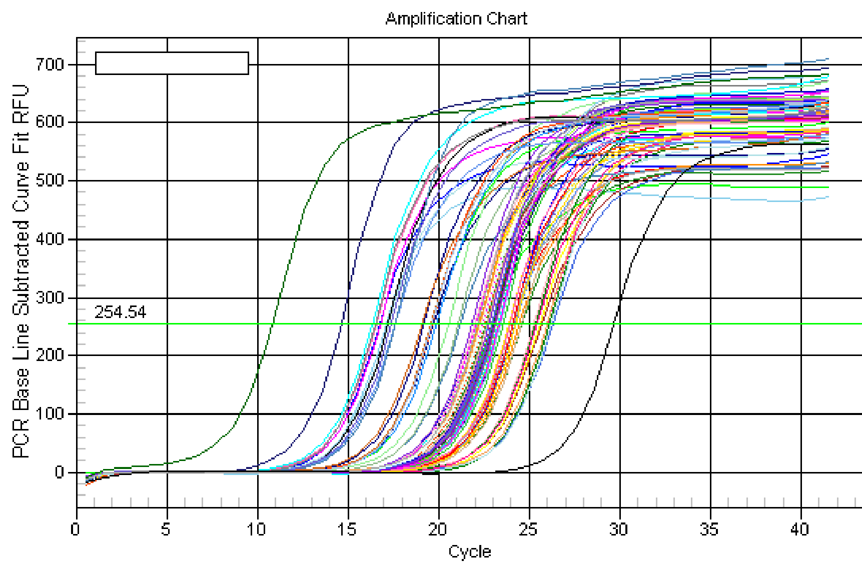
Supplementary Figure 1: Test PCR plate for ALU115 primers



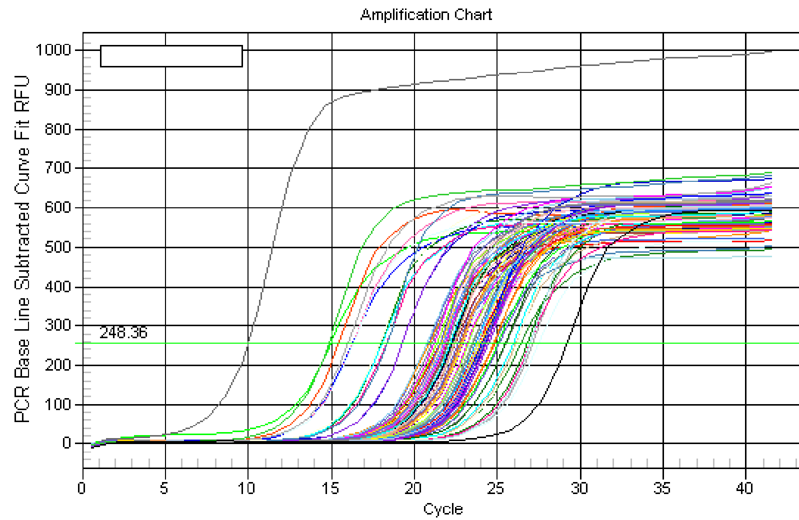
Supplementary Figure 2: First PCR plate for ALU115 primers



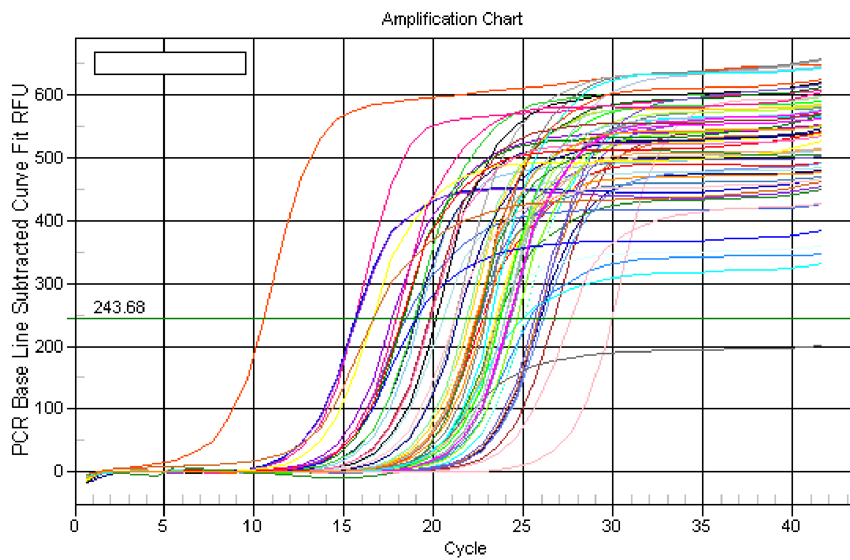
Supplementary Figure 3: Second PCR plate for ALU115 primers



Supplementary Figure 4: Third PCR plate for ALU115 primers

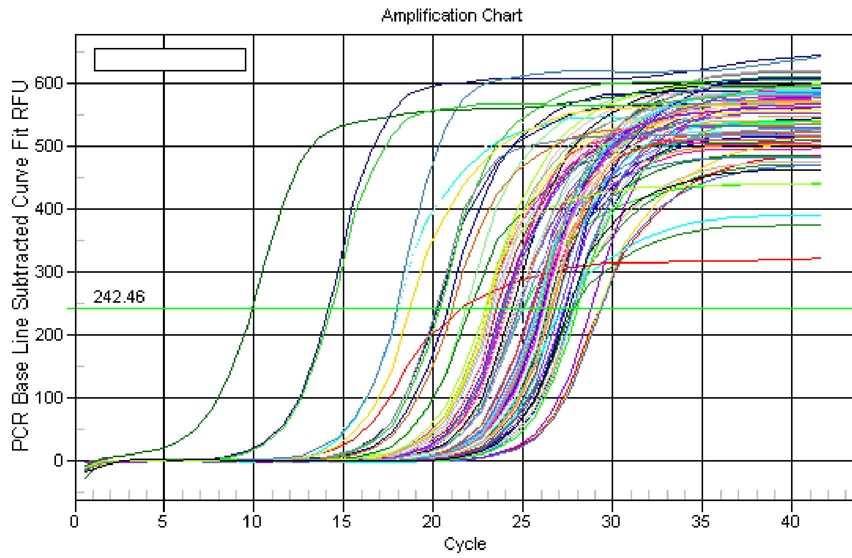


Supplementary Figure 5: Fourth PCR plate for ALU115 primers

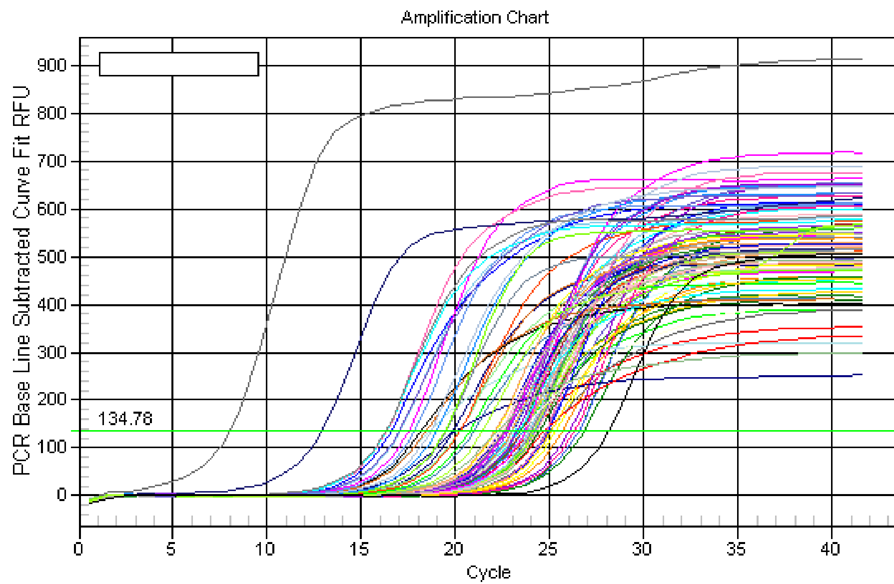


ALU247 PRIMERS

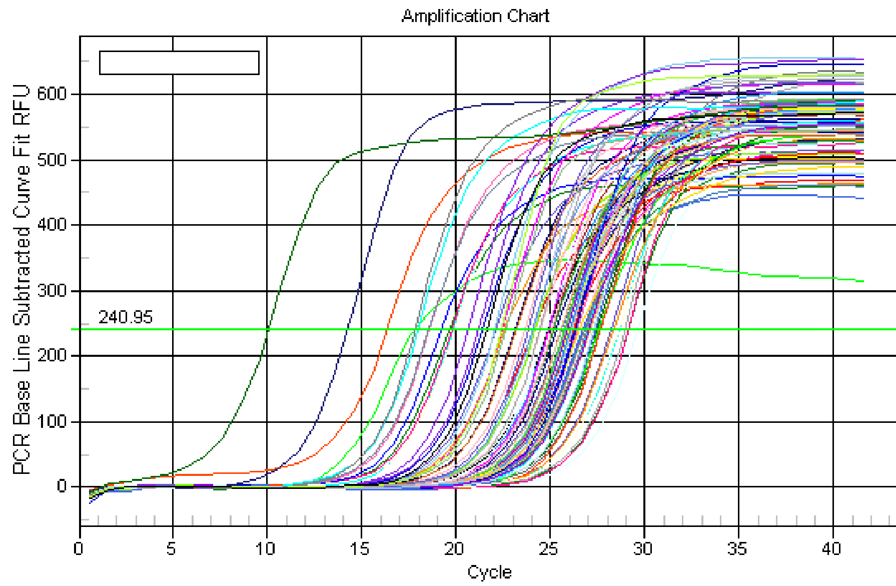
Supplementary Figure 6: First PCR plate for ALU247 primers



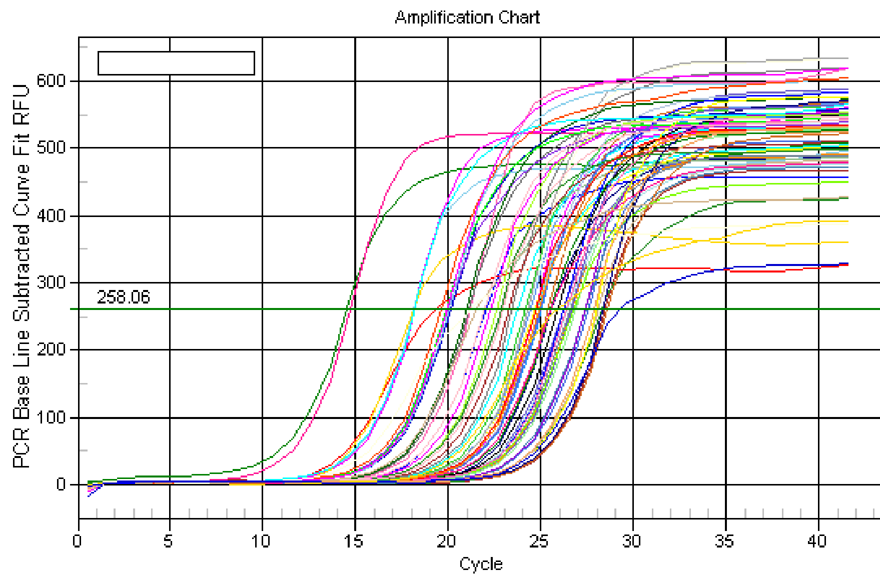
Supplementary Figure 7: Second PCR plate for ALU247 primers



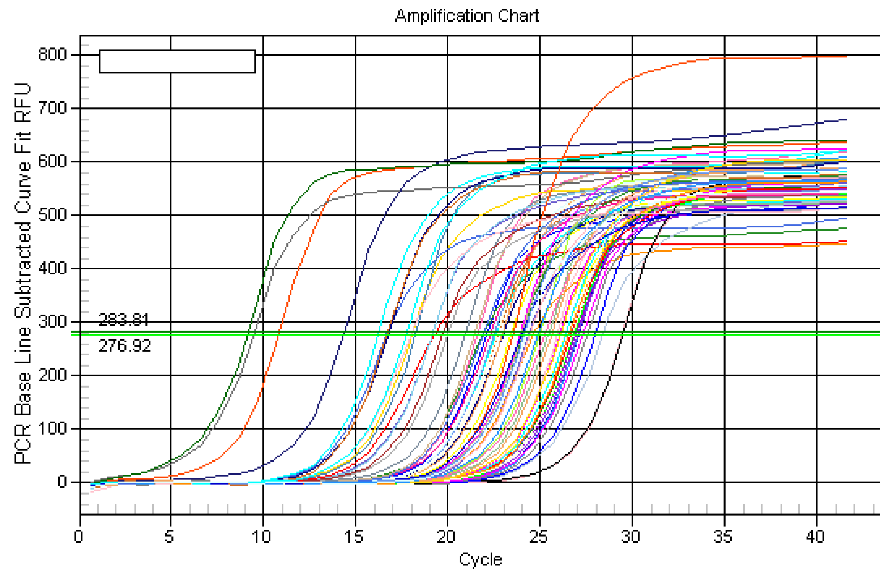
Supplementary Figure 8: Third PCR plate for ALU247 primers



Supplementary Figure 9: Fourth PCR plate for ALU247 primers



Supplementary Figure 10: Repeat PCR plate for ALU115 and ALU247 primers


Amplification Chart
PCR Base Line Subtracted Curve Fit RFU
800
700
600
500
400
300
200
100
0
283.81
276.92
0
5
10
15
20
25
30
35
40
Cycle