T.R.N.C

NEAR EAST UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

THE EFFECT OF REPEATED CONTROLLED OVARIAN HYPERSTIMULATION CYCLES ON OOCYTE AND EMBRYO DEVELOPMENT

AND

THE EFFECT OF THROMBOPHILIA ASSOCIATED POLYMORPHISMS ON RECURRENT MISCARRIAGES

by

LIZZY TELEBOSHE PAUL

Master of Science in Medical Biology and Genetics

Supervisor:

Assoc. Prof. Pinar Tulay

January 2019

Nicosia, North Cyprus 2019

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APPROVAL

Thesis submitted to the Institute of Health Sciences of Near East University in partial fulfillment of the requirement for the degree of Master of Science in Medical Biology and Genetics.

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DEDICATION

I am dedicating this research work to my parents Mr and Mrs Paul K. Taleboshe and my beloved siblings for their love, support and encouragement throughout my education.

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First, I give thanks to God almighty for his substantial grace, blessings, strength, wisdom and good health upon my life, i bless you Lord Jesus for your guidance throughout this Thesis project. I especially want to extend my appreciation to my lovely family; my parents Mr. and Mrs. Paul Taleboshe, my Brother Randy Paul and my two beloved sisters Dekar and Vina Paul and also to my best friend Idris Adewale Lateef for their encouragement and moral support towards my education.

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ABSTRACT

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AND

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Background: Gametogenesis is a process of differentiation and proliferation of gametes into mature sperm cells and oocytes, a process called spermatogenesis and oogenesis, respectively. The fusion of sperm and oocyte produces zygote through a process called fertilization. Infertility is the inability to reproduce after a year of frequent unprotected sexual intercourse without using any form of contraceptives. The etiology of most miscarriages remains idiopathic while some cases have been characterized by habitual abortion in correlation with aneuploidies, or genetic polymorphisms associated with inherited or acquired thrombophilia genetic predispositions. The aim of the first part of this thesis was to investigate the effect of multiple ovarian hyperstimulation cycles on the maturity of oocytes (MI and MII), fertilization rate, embryo developmental qualities and clinical pregnancy rate in donation cycles. The study group involved donors who underwent conventional controlled ovarian hyperstimulation cycles with an interval of 120 days. The other part of the thesis aimed to examine the effect of five prophylactic

treatments on the clinical pregnancy rates of women with thrombophilia gene variations. The study group of the second part of the thesis involved women undergoing infertility assessment due to habitual abortion at 8 to 13 weeks of gestation. **Material and methods**: In the first part of the thesis, a total of 65 patients undergoing oocyte donation cycles multiple times were included in this study. Patients were grouped into four groups; A-D. Group A, consisted of donors with less than (<=2) stimulation cycles while B consisted of donors with (=>3) stimulation cycles. Donors were further grouped into group C and D according to number of oocyte retrieved per donor. Group C included donors who had <=15 oocytes, while group D had donors with =>16 oocytes. All embryos obtained were morphologically graded and scored according to European Society of Human Reproduction and Embryology (ESHRE) criteria. Variables such as number of oocytes obtained, immature (MI) and mature (MII) oocyte retrieved, fertilization rate, embryo quality and clinical pregnancy outcomes of donors grouped A-D were compared. The Mann Whitney non parametric test was used to investigate the statistical significance of total number of oocytes obtained, maturity of oocytes (MI and MII), fertilization rate, embryo grading and clinical pregnancy outcomes of all donors grouped; A to D.

In the second part of the thesis, the association of recurrent fetal loss and the use of prophylactic medication for polymorphism associated with thrombophilia were evaluated. The study group involved a total of 62 women. The polymorphisms associated with thrombophilia, which includes for *methyltetrahydrofolate reductase* (*MTHFR*) 1298 and 677, *Factor V Leiden* (*FVL*) 1691, *plasminogen activator inhibitor-1* (*PA1-1*) G/G and *Factor II prothrombin 20210*, were genotyped using the real time polymerase chain reaction. The Pearson Chi Square and Mann

Whitney test were used for investigating the association of recurrent pregnancy loss and the use of anticoagulant for polymorphisms associated with thrombophilia.

Results: Significant statistical differences were observed in the total number of oocytes obtained, maturity of oocytes (MI and MII), fertilization rate, day 3 embryos qualities and clinical pregnancy outcomes of donors in group A-D. Donors with <=2 ovarian hyperstimulation cycles had lower number of immature oocytes than donors with 3 or more stimulation cycles. While on the other hand, donors with 3 or more stimulation cycles had higher number of mature oocytes than donors with <=2 stimulation cycles. Furthermore, donors with 3 or more hyperstimulation cycles had more number of fertilized oocytes, with better day 3 embryo qualities with minor to moderate fragmentation (grade 1 to grade 3) and higher clinical pregnancy rates compared to donors stimulated <=2 times, $P < 0.05$. However, no difference was observed in the number of embryos of unequal size with heavy fragmentation (grade 4) and embryos arrested at cleavage stage. Similarly to group A and B, donors with <=15 oocytes retrieved had fewer number of immature oocytes than donors in group D with =>16 oocytes. Conversely, donors with =>16 oocytes retrieved (group D) had higher number of mature oocytes retrieved than group C. In addition donors with =>16 oocytes had more fertilized oocytes with better quality of day 3 embryos with mild to moderate fragmentation (G1-G3) and increased clinical pregnancy rates compared to donors with <=15 oocytes retrieved. No differences were observed in the number of bad quality embryo grade with heavy fragmentation (G4) and arrested embryos between donors in group C and D.

For the second part of the thesis, the clinical pregnancy outcomes of women with the genotypes of genes associated with thrombophilia showed that treatment with zinco c significantly led to higher positive pregnancy outcomes in *MTHFR* 677CC-wild type cases. In contrast, no benefits were found for treatments with enoxaparin, 75 mg of aspirin, 81mg aspirin or folic acid in patient with *MTHFR* 1298 (AA, AC and CC), *MTHFR* 677 (CT and TT), *FVL* 1619 (GG, GA and AA), *PAI-1* (4G/4G, 4G/5G and 5G/5G) and *FII 20210* (GG, and GA) genotypes. Also treatment with 75mg of aspirin resulted in more negative pregnancy outcomes than positive outcomes in *MTHFR* A1298C genotypes.

Conclusions: The findings of the first part of the thesis indicates that multiple stimulation cycles do not have any negative effect on ovarian response in terms of quality and quantity of oocytes retrieved, ratio of fertilized oocytes, day 3 embryo qualities and clinical pregnancy outcomes.

The findings of the second part of this thesis suggest that there is a benefit in using of zinco c medication as a potential treatment for preventing possible recurrent pregnancy loss in women with *MTHFR* 677 CC genotype. Conversely, 75mg dose of aspirin led to more negative pregnancy outcome than positives in women with *MTHFR* A1298C genotypes. No further benefits were found for treatment with enoxaparin, 75mg dose of aspirin, 81mg dose of aspirin and folic acid against other thrombophilia genotype cases.

Keywords: Gametogenesis, controlled ovarian hyperstimulation, oocytes, thromphobilia polymorphisms, miscarriages.

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1. ± (Addition or subtraction)
2. (+) positive pregnancy group
3. (-)Negative pregnancy group

LIST OF ABBREVIATION

hPGC	: Human Primordial Germ Cells
PGC	: Primordial Germ Cells
BMP	: Bone Morphogenic Protein
MI and MII	: Meiosis I and II
GV	: Germinal Vesicle
FSH	: Follicle Stimulating Hormone
LH	: Luteinizing Hormone
BTB	: Blood Testes Barrier
IVF	: In Vitro Fertilization
COS	: Controlled Ovarian Stimulation
HCG	: Human Chronic Gonadotropins
RPL	: Recurrent Pregnancy Loss
MTHFR	: Methyl tetra hydro folate Reductase
PAI-1	: Plasminogen Activator Inhibitor 1
FVL	: Factor V Leiden
LMWH	: Low Molecular Weight Heparin

CHAPTER ONE

PART 1: The effect of repeated controlled ovarian hyperstimulation cycles on the quantity and maturity of oocytes obtained, fertilization rate, day 3 embryo qualities and clinical pregnancy outcomes

2.1 Introduction

Gametogenesis is a process whereby diploid cells (2n) undergo meiotic division to produce haploid number of cells (n) called germ cells or gametes. The initiation of gametogenesis occurs through the migration of primordial germ cells (PGCs) at the embryonic stage of life. In gastrulating mouse embryo, the progenitors of PGCs reside mainly at the epiblast, a region above the hypoblast which separates to form the three primary distinctive germ layers:(i) ectoderm (ii) extraembryonic mesoderm posterior to the primitive steak and (iii) endoderm (Manku & Culty, 2015).

The PGCs in mouse embryo migrate from the extraembryonic mesoderm, towards the yolk sac where they divide into two populations of cells which either migrates to the left or right genital ridge (Gilbert, 2000). The PGCs migrates to the caudal region through the newly developed hindgut by moving towards the dorsal mesentery region of the genital ridge, the post-migratory PGCs reach the genital ridge on the eleven week of gestation (Ibtisham et al., 2017).

In humans, scarce amount of information is known about the origin of PGCs at fetal life, though it is proven to be similar to that of mice. However, according to Gilbert and colleagues (2000), human primordial germ cell (hPGC) originates from differentiated pluripotent stem cells of the posterior epiblast under the influence of bone morphogenic proteins (Gilbert, 2000). The hPGCs

migrate from the yolk sac (endoderm) bypassing through the hindgut, down to the dorsal mesentery and to the genital ridge region to become germ line cells (Hogarth & Griswold, 2010).

2.2: Spermatogenesis

The hPGCs undergo mitotic and meiotic cell division alongside proliferation, differentiation and apoptosis to become mature gamete, though the processes occur separately in male and female, named spermatogenesis and oogenesis respectively. Spermatogenesis in males results in the production of spermatozoa (He et al., 2010) , whereas in females oogenesis produces oocytes (Arkoun & Livera, 2018). Although spermatogenetic cycles differ from species to species, the process is similar between humans and rodent, hence today, rodent species are used as animal models due to difficulties in accessing human testis for experimental and research purposes.

In humans, the entire process of gametogenesis is divided into four different phases: (i) germ line cell migration, differentiation and proliferation by mitotic cell division, (ii) the development of gonads from primordial germ cells, (iii) gonad maturation, (iv) structural and functional features of the mature gamete which produces either sperm cells in males or oocytes in females (Xiao et al., 2013).

In males, the process of sperm production starts at adolescent stage of life, in the seminiferous tubules of the testis, a stratified complex epithelium lined by a basal membrane layer and connective tissues containing myeloid and fibroblast cells. Spermatogenesis takes approximately 74 days in humans, while in mouse it takes approximately 6 weeks (Ibtisham et al., 2017). As mentioned above, the process involves four phases, the epithelial germ line cell of the seminiferous tubules undergo mitotic cell division to produce two identical daughter cells called spermatogonia (A dark and A pale), alongside spermatogonia B (Manku & Culty, 2015).

The newly produced spermatogonia type “A dark” forms a reserved pool of stem cells while type A pale play an important role in maintaining balance for further differentiation of spermatogonia type B. At puberty, spermatogonia type B undergo cell growth, proliferation and division to produce two more identical daughter cells called primary spermatocyte linked together by cytoplasmic bridges (Xiao et al., 2013). The produced daughter cells undergo meiosis I and meiosis II where they divide to produce two identical secondary spermatocytes which later divide to produce four haploid cells called round spermatids at the end of meiosis II.

The produced round spermatids undergo spermiation, a process which facilitate the release of mature spermatids from sertoli cells of the testis into the seminiferous tubule lumen before been transported to the rete testis a structure located at the hilum, which carries them to the epididymis where they are stored (Hermo et al., 2009).

Adjacent to the seminiferous tubules are the Leydig cells which secretes androgen in the presence of luteinizing hormone (LH). During the entire process of spermatogenesis, the tight junction of the sertoli cells form the blood testes barrier (BTB) which separates the seminiferous tubule structure from blood circulation. Sertoli cells are somatic cells of the human testis which function in the following process (i) production of anti-Müllerian hormone, (ii) secretion of inhibin and activin required for the production of follicle stimulating hormone (FSH) from the anterior pituitary glands and (iii) secretion of androgen binding protein to maintain adequate testosterone level throughout spermatogenesis (Wen et al., 2017).

The blood testis barrier (BTB) formed by sertoli cells during spermatogenesis also function in the removal of unwanted hormones and other systematic circulatory constituents that can interfere with the process of spermatogenesis. BTB also helps in averting immune response by

preventing the male immune system from recognizing sperm cells as foreign molecules, as a result of their different genetic origin which gives them the potential of expressing different surface antigens recognized as foreign by the human body (Xiong et al., 2006). After puberty, sperm production occurs continuously throughout life time until death, though it is believed to decrease in quantity through aging (Xiao et al., 2013).

2.3: Oogenesis

The process of gametogenesis in females varies from that of the males. In humans, the process is termed oogenesis and it is highly specialized and a long process. Oogenesis begins prior to birth (Stefanski et al., 2018). In females, the hPGCs migrate from the yolk sac of the embryo to the cortex of the primordial gonads where they are replicated at a peak of 700 million, at exactly 20 weeks mid-gestation. A number of these produced cells undergo atresia, "cell death" or apoptosis, decreasing the peak to 2 million cells called immature oocytes.

The immature oocytes each with germinal vesicle (GV) nucleus proceed to meiosis I where they are surrounded by a stratified layer of cuboidal cells of the epithelium called granulosa cells or follicular cell. The produced immature oocytes remain entrapped (arrested) at the prophase stage of meiosis I until birth. At puberty, the suspended immature oocytes continue meiosis (I) where they further undergo frequent cell atresia reducing the number of oocytes to 40,000. The GV of the oocytes breakdown and the granulosa cells surrounding the oocytes secretes glycoprotein forming the zona pellucida composed of several connective tissue which differentiates to form the follicule luteinizing hormone (LH) responsive layer responsible for androgen secretion (Johnson et al., 2005). A space filled with fluid forms between the granulosa cells called antrum or secondary follicle, which later becomes mature oocytes under the influence of estrogen,

follicle stimulating hormone and luteinizing hormone secreted by the anterior pituitary gland of the hypothalamus, also the presence of these hormones stimulates ovulation.

At the end of meiosis I, two identical diploid cells of unequal size are produced. The two daughter cells proceed to meiosis II; one among the daughter cell becomes the first polar body, while the other oocytes undergo arrest 3 hours before ovulation until fertilization. During ovulation the granulosa follicle becomes the graffian follicle and upon the LH surge, the oocyte is released from the ovaries. The released ovum gets picked up by a finger link structure called the fimbriae into the fallopian tube where fertilization occurs. In the absence of sperm cells, the secondary oocyte degenerates, while in the presence of sperm cells, meiosis II is completed by the fusion of mature oocytes and sperm nucleus to form zygote (Coticchio et al, 2012).

2.4: Fertilization

In vivo fertilization in humans occurs as a result of fusion between the nucleus of mature egg and sperm to form a zygote. The process begins with the ejaculation of spermatozoon into the female's reproductive tract through the vagina. The released sperm cells get transported along the cervix, down the uterus by peristalsis, and upon reaching the uterus they undergo capacitation, a process which allows them to bind to the follicle layer of the mature oocyte called the corona radiate.

The process of capacitation also facilitates the degradation of sperm membrane and increases sperm motility in order for acrosome reaction to take place. As the sperm approaches the zona pellucida of the mature oocyte, the acrosome membrane of the sperm exposes surface antigens necessary for binding to the oocyte membrane. The acrosome membrane also secretes enzymes required for breaking through the oocyte's tough coating.

The sperm and oocyte membrane binding process initiates the release of cortical granules which prevents further sperm cells from entering the fertilized oocyte, enabling enzymatic penetration of sperm cells through the zona pellucida of the mature oocyte, this result to the fusion of the plasma membrane of the released spermatozoon and that of the mature oocyte (Jones, 2008). Thus facilitating exocytosis and crosslinking between the glycoprotein of the zona pellucida and that of the sperm. This entire process completes meiosis II in the female reproductive tract, allowing crossing over of maternal and paternal genetic material (Jodar et al., 2013).

2.5: Pre-Implantation Embryo Development

Once fertilization occurs, pre-implantation development of the fertilized embryo initiates 5-6 days after conception. Several studies involving mouse embryogenesis have provided key insight into various developmental pathways. However, certain differences in developmental timing and factors, such as (i) gene expression patterns, (ii) genome activation and (iii) epigenetic modifications are said to be species specific differences which limits the use of certain research findings of mice embryogenesis in human embryonic development (Niakan et al.,2012).

Currently, research studies conducted on human pre-implantation embryo development process involves embryos obtained through *in vitro* fertilization (IVF) where they are exposed to culture media (Legault et al., 2018). Cleavage stage divisions occur from time of fertilization until the time of implantation, at day 3 to 6 post fertilization. On approximately day 4 post fertilization, the embryo consist of 16 to 32 spherical cells, called morula (Niakan et al., 2012). The outer cavity surrounding the morula cells become tightly bound together by gap junctions forming thicker structures around the cavity (Audibert et al.,2009)

After asymmetrical division, two distinct cells emerge five days post fertilization. The cells

differentiate into inner cell (ICM) and trophectoderm, the inner cell mass is a collection of immature stem cells that are pluripotent in all mammals except monotremes. They differentiate to form the definitive structures of the fetus, while the other distinct cell formed after asymmetrical division takes the outer position to become the trophoblast, which later differentiates to form the placenta and other extraembryonic tissues required for normal embryonic growth (Chang, Qiao, & Leung, 2016).

2.6 : Objectives of the research

The first aim of this thesis was to investigate the effect of multiple ovarian hyperstimulation cycles on the quantity and maturity of oocytes (MI and MII), day 3 pre-implantion embryo qualities, fertilization rates and clinical pregnancy outcomes of donors undergoing multiple stimulation cycles. Furthermore, the research aimed to examine whether obtaining higher number of oocytes from donors will result to any adverse effect on the quality of oocytes retrieved, fertilization rates, embryo development and clinical pregnancy outcomes.

2.7 : Significance of the study

The result of the first part of this thesis will help ascertain the effect of multiple controlled stimulation cycles on oocyte quality, pre-implantation embryo quality on day 3 and clinical pregnancy outcomes. In summary, the finding of the first part of this research will aid health organization and other health allied research bodies gain more insight and deeper understanding of the effect of multiple ovarian hyperstimulation cycles on ovarian response.

2.8 : Thesis Structure

The first chapter provides background information about the study. At this stage, basic information about gametogenesis, as well as the process of fertilization and pre-implantation

embryo development was discussed.

Chapter two provides information about spontaneous abortion, recurrent pregnancy loss and thrombophilia polymorphisms.

The third chapter discusses the methodology used for data collection and the statistical methods used for analyzing data for the research. In addition, basic information about the results obtained for this research will be explained in the fourth chapter.

Chapter five explains the results in detailed and compare the data with literature review and other necessary recommendation for future research purposes. Also, the conclusions and summary of this research is included in this chapter.

CHAPTER TWO

PART 2: The effect of thrombophilia associated polymorphisms on recurrent miscarriages and the effect of anti-coagulant drugs

2.1 : Introduction

Spontaneous abortion also known as miscarriage is defined as the loss of fetus or pregnancy before the 20th week of gestation. About 70-80% of most miscarriage occur at the first trimester of pregnancy while 20% occur at the second trimester of pregnancy and are referred to as early and late miscarriages respectively. Miscarriage is the most common type of pregnancy complications and the rate differ, for women under the age of 35 the risk is about 10-20% while for women above the age of 40 the risk is about 40-45% (Stouffs & Lissens, 2012). However, 1-5% of most women experience two or more consecutive miscarriages before the twentieth week of gestation, often referred to as recurrent pregnancy loss (RPL) (Practice Committee of American Society for Reproductive Medicine, 2013). RPL in women is currently the most challenging area of reproductive medicine due to it emotional and psychological burden on the couple affected (Barut et al., 2018).

The pathogenesis of most miscarriages remains idiopathic, as such related diagnosis and treatments are very limited. Although due to other genetic related causes, thrombophilia has also been suggested as a risk factor for RPL (Stefanski et al., 2018).

2.2 : Thrombophilia

Thrombophilia is an inherited or acquired condition that can lead to thromboembolism, the increased risk of unhealthy blood coagulation or clotting in blood vessels (Stefanski et al., 2018). Pregnancy complications such as intrauterine growth retardation, intrauterine fetal death, pre-eclampsia and placenta abruption have been associated with thrombophilia (Pabinger &

Vormittag, 2005).

In normal pregnancy, the trophoblasts invade the spiral arteries and lose their muscular wall to become soft, allowing maximum blood flow through the placenta of the mother to the unborn child. Abnormal pregnancies result in an abnormal trophoblastic invasion of the spiral arteries, narrowed blood vessels and lower level thrombosis from hyper-coagulation which leads to inadequate perfusion of the intervillious space (Kupferminc, 2003). The etiology of some placental abnormalities and hemostatic imbalances which results to inappropriate placental perfusion and feto-maternal circulation during pregnancy, for example vasoconstriction, placental ischemia and enhanced coagulation which occur frequently in human pregnancies are reported to be caused by genes associated with thrombophilia.

Currently five single nucleotide polymorphisms (SNPs) in inherited thromboembolism have been associated as risk factors for RPL and these are routinely used in fertility clinics aiming to understand the causes of miscarriages. These polymorphisms includes two types of *methylene-tetra-hydro-folate reductase* (*MTHFR*) base pair substitution; *MTHFR* 1298 and 677; *factor V leiden* variant, *PAI*-1 and *FII prothrombin* genetic variation (Barut et al., 2018).

In healthy pregnancies, *MTHFR* gene encodes for a catalytic enzyme which plays a role in folate metabolism. Patients with the nucleotide transition of either *MTHFR* C677T (cytosine to thymine) or A1298C (adenine to cytosine) lack the ability of producing the methylene tetra-hydro-folate reductase enzyme required for converting homocysteine into methionine, this results to increased level of homocysteine and excess blood clotting (Servy et al., 2018).

Patients heterozygous for *factor V Leiden* variant, a mutated form of human factor V protein encoded by the *FV* gene in thrombophilia, are reported to have increased risk of miscarriages at early gestational weeks compared to those without the gene (Kardi et al., 2018). The *FV* gene

point polymorphism at position 1691 causes activated protein C resistance and the inappropriate degradation of the produced factor V protein these results in excess fibrin secretion and blood clotting during pregnancy (Jusić et al., 2018). The *plasminogen activator inhibitor* (PAI-1) gene deficiency caused by nucleotide substitution of guanine (indels) at 4G/5G region of the *SERPINE* 1 gene have also been reported to be a risk factor for miscarriages in women with inherited thrombophilia (Chen, Nie, & Lu, 2015). *PAI*-1 gene encodes for a serine protease inhibitor enzyme which plays an important role in fibrinolysis during pregnancy, mutation on this gene is associated with reduced fibrinolytic activity and increased blood clotting during pregnancy.

Additionally, the *prothrombin* gene nucleotide substitution of guanine to adenine at position 20210 is reported to be another risk factor for RPL (Barut et al., 2018). The *prothrombin* gene mutation inhibits the conversion of fibrinogen to fibrin during fibrinolysis, which in turn, results to elevated pro-clotting protein during pregnancy. The accumulation of fibrinogen at early placental circulation in pregnant women might restrict fetal growth and increase blood clotting (Abu-Heija, 2014) . Therefore, it is possible that in idiopathic pregnancy loss, pregnant women homozygous or heterozygous for these thrombophilia associated genes are at higher risk of having recurrent miscarriages.

2.3 : Objectives of the study

The purpose of the second part of this thesis was to investigate the benefit of prophylactic treatments on the clinical pregnancy outcomes of women with polymorphisms associated with thrombophilia. The study group included women with previous miscarriages between 8 to 13 weeks of gestation.

2.4 : Significance of the study

The findings of the second part of this thesis would help to explain the relationship between thrombophilia polymorphisms and recurrent fetal losses. Furthermore, the administration of five different anticoagulant drugs would shed light into the most beneficial one to be used in women with polymorphisms associated with thrombophilia and RPL.

CHAPTER THREE

Material and Methods

Ethical approval was granted for this project by Near East University (YDU/2018/59-605).

PART 1: The effect of repeated controlled ovarian hyperstimulation cycles on the quantity and maturity of oocytes obtained, fertilization rate, day 3 embryo qualities and clinical pregnancy outcome

3.1 : Controlled Ovarian Hyperstimulation (COS) Protocol

The study group included a total 65 donors who underwent COS protocol with an interval of 120 days before new stimulation cycles where initiated. Exogenous gonadotropin releasing hormone (GnRH) antagonist was used to artificially suppress LH receptor, by inhibiting the activity of luteinizing hormone (LH) from the anterior pituitary gland of the hypothalamus *in vivo*. The pituitary glands of each donor were desensitized and 1mg firmagon was administered to donors at the luteal phase of the each cycle. The administered dose of firmagon was decreased to 0.5mg before human chronic gonadotropins (hCG) initiation three days after menstruation, follicular development were monitored on the 7^{th} day. In cases where there was no follicular activity more dose of human menopausal gonadotropins was administered alongside ultra-purified urinary FSH, further hCG dosage was administered according to the follicular response of each donor.

All oocytes were retrieved from donors by transvaginal aspiration of the ovulated ovum from the ovarian follicle 36 hours after stimulation by antagonist hCG. After aspiration, the follicular fluid containing the oocytes were analyzed by the embryologist and classified into immature MI and mature MII oocytes. The retrieved oocytes were visualized under a light microscope to verify if they were surrounded by the oocyte- cumulus complex essential for their nourishment outside the body. Only oocytes surrounded by cumulus complex were transferred into the culture media

designed to provide the required nutrients needed to sustain them before fertilization.

The attached cumulus complexes were cultured for 4 -hours post retrieval in order for them to reach maturation, each culture media containing the retrieved oocyte were incubated at 37°C to maintain normal body temperature *in vivo*.

The obtained oocytes were fertilized same day after retrieval and were referred to as day zero embryos. On day 3, the cleavage stage embryos continue to divide and replicate into sizes, all embryos were graded and scored using two criteria, (i) the number of divided cell inside the culture media and (ii) their appearance under higher power microscope. The score of appearance was maintained on a scale of 1- 6, according to ASRM and ESHRE guidelines (Medicine & Embryology, 2011). A normal day 3 embryo should contain 6 to 10 spherical shaped cells. Embryos, with 6 to 10 cleaved cells most likely produces viable blastocyst compared to embryos with less cell number (ALPHA Scientists In Reproductive Medicine & ESHRE Special Interest Group Embryology, 2011).

3.2 : Embryo Grading

G1 represents embryos with cells of equal size and no fragmentation, G2 represents embryos with cells of equal size and minor fragmentation, G3 are embryo with cells of equal size and moderate fragmentation, G4 represents embryos with cells of equal or unequal size with heavy fragmentation while arrest embryos are cells of unequal size with heavy fragmentation. According to ESHRE grading, G1 embryonic cells of equal size with no fragmentation, G2 embryonic cells of equal size with minor fragmentation and G3 embryonic of equal size with moderate fragmentation have the greatest potential of developing viable blastomeric cells, while grade 4 embryos of equal or unequal size with heavy fragmentation and embryonic arrested cells

of unequal size with heavy fragmentation have less potential of becoming healthy fetus (ALPHA Scientists In Reproductive Medicine & ESHRE Special Interest Group Embryology, 2011).

3.3 : Biostatistics

The biostatistics investigation was performed using IBM SPSS software 20th edition. In the first part of the research, all samples were separated into four different groups; A to D, all groups where not normality distributed. Therefore, non-parametric test was conducted on all variables between group A and B then C and D with a confidence interval of 95 and a valid alpha value of 0.05. The differences in the total number of oocytes obtained, oocytes maturity (MI and MII), fertilization rate, embryo grading and clinical pregnancy outcomes of donors grouped A to D were investigated by the Mann Whitney non-parametric test. P-values lower than 0.05 indicate a significance difference, while P-values of 0.05 or higher indicate no significance difference for all compared variables among groups.

PART 2: The effect of thrombophilia polymorphisms on reoccurring miscarriages and the effect of anticoagulant drugs

3.4 : DNA Extraction for Real Time Analysis

The study group included 62 women with recurrent pregnancy loss. The experiments were performed at Elite Hospital laboratory, Nicosia, North Cyprus. DNA isolation and purification was conducted using the QIAamp DNA mini kit (Qiagen). The samples were lysed enzymatically with no mechanical-homogenization required. For this analysis, 200µl of peripheral blood sample was isolated from the study group into the ethylenediaminetetra-acetic acid (EDTA) tubes with no leukocytes separation prior to the procedure. The EDTA tube serves as anticoagulant for reducing the risk of hemolysis in blood samples. The tubes containing the isolated blood sample were loaded with 200ml of AL lysis buffer and 20µl protease enzyme completely free from DNAse and RNAse enzyme into the T-shaker incubator machine and incubated for 10min. Then, 200µl of ethanol was added to the tubes and transferred into the QIAamp spin column machine.

3.5 : QIAamp Spin Column Isolation

The QIAamp Spin column purification involved a three step procedure using a standard micro-centrifuge designed to prevent cross contamination between samples. The samples were transferred into the QIAamp Mini spin column tube. For the first isolation step, 500ml of AW1 buffer was added to the tube containing the sample mix and centrifuge at full speed at 8000rpm. After which, the mini spin column tubes were placed in a clean 2ml collection tube. For the second spinning step, 500ml of AW2 buffer was added to the tube containing the sample mix and spun at 8000 rpm. The QIAamp mini spin column tube was placed in a 1.5ml centrifuge tube

and 200µl of AE buffer was added and incubated for 1 min, then centrifuge at 800rpm for 1 min to increase DNA yield. The condition of the buffer was frequently adjusted to allow DNA absorption onto the QIAamp silica membrane during the process.

3.6 : Master Cycler Realplex

All tubes containing the isolated DNA sample were transferred into 5 strips of 0.2 ml of Realplex Mx3005p tube and labeled. The *MTHFR* A1298C polymorphism was examined by using forward: 5′- CTT TGG GGA GGT GAA GGA CTA C-3′ and Reverse: 5′-CAC TTT GTG AGC ATT CCG GTT TG-3′ primers. For *MTHFR* C677T polymorphism, amplification was conducted with primers forward (F) 5′-TGA AGG AGA AGG TGT CTG CGG GA-3′ and reverse (R) 5′-AGG ACG GTG CGG TGA GAG TG-3′ primers. While 15 to 20 oligonucleotide probes was used for *factor V Leiden* G1619A 5'GGACAGGC(G/A)AGGAATAC-3'; *FII* 20210, 5'CTCTCAGC(G/A)AGCCTCAA-3'; *PAI-1* 4G/5G, 5-ACACGTGG(/G)GGAGTCAG-3 (Barut et al., 2018).

All the reaction volumes were set at 25µl and rotor (36/72 position) and loaded with 0.5µl Taq polymerase enzyme with no reference dye needed. The real time PCR amplification procedure included a prior melting phase for 2 min at 94 °C followed by 40 cycles of annealing for 3mins at 54°Cand elongation for 5mins at 72°C. The channel window of the real time PCR equipment was set at fret 1 source 470nm. The reaction filter was set for 520nm FAM and 605nm LC610. The detector of the machine was set at 610bp and the auto-calibration temperature was set at 54°C.

All polymorphisms, *MTHFR* 677 and 1298 polymorphisms, *factor V leiden* 1691, *Factor II* (*prothrombin* 20210) and *PAI 4G/5G* were genotyped using the real time polymerase chain

reactions. The isolated DNA was amplified in the presence of two probes complementary to the targeted sequence. The 5' prime was marked with a donor flurophore while the 3' prime carries the acceptor flourophore. During the analysis when there was hybridization between the probe and the targeted DNA, a signal was emitted with a specific wavelength. The difference in the melting temperature of the two possible hybrids produced during sample run allowed the samples to be identified as wild type (normal), homozygous (mutant) or heterozygous (carrier). The emitted signal during sample run is directly proportional to the PCR product. The widths of the peaks were set as 3 for all samples.

3.7 : Treatments

Thromboprophylactic doses were prescribed at 6-7 gestational weeks after fetal heart beat detection by ultrasound examination. All 62 patients were separately administrated 15mg dose of zinco c daily, 0.4ml dose enoxaparin (4000IU) daily, 75 mg dose of aspirin daily, 81mg dose of aspirin daily and 10 mg dose of folic acid daily. Treatments were discontinued at 36 gestational weeks prior to child birth.

3.8 : Biostatistics

In the second part of the research, the IBM SPSS software 23th edition was used for analyzing the data. The statistical significance between thrombophilia associated polymorphism, pregnancy loss and medication was investigated using the Pearson-chi square test with odd ratio, alpha 0.05 and 95% confidence intervals. Depending on the type of data, the Pearson-chi square test or fisher exact test results were used for statistical decision. The average ratio for age, number of pregnancy loss and clinical pregnancy outcome was investigated using the Mann Whitney.

Similar to the previous investigation, P-values < 0.05 were reported to be statistically significant while P-values>0.05 were non-significant.

CHAPTER FOUR

RESULT

PART 1: The effect of repeated controlled ovarian hyperstimulation cycles on the quantity and maturity of oocytes obtained, fertilization rates, quality of day 3 pre-implantation embryos and clinical pregnancy outcomes

A total of 65 patients who underwent multiple oocyte donation cycles were included in the first part of the thesis study. Donor's age range was between 18- 33 years. Donors who underwent less than three stimulation regimen were grouped as A, while donors with three or more stimulation cycles were grouped as B. Differences in total number and maturity of oocytes, fertilization rate, day 3 embryo qualities and clinical pregnancy outcomes were compared between groups A and B. There was a significant difference between the total number of oocytes retrieved in group A compared to group B, in such donors in group B had higher number of oocytes retrieved than those in group A, $P < 0.05$. Further difference was observed in the number of immature MI oocytes and mature MII oocytes obtained between groups A and B. Donors in group A had lower number of immature oocytes compared to donors in group B ($P< 0.05$). However, donors in group B had more mature oocytes obtained than donors in group A. The average ratios and boxplot representation of the total number of oocytes collected, MI and MII oocytes obtained are presented in table 4.1and figure 1a, 1b and 1c.

Table 4.1: Overall number of oocytes retrieved and the oocyte maturities

Variable	Stimulation Cycle	N	Mean ± Std	Median (min-max)	P-value	
Total number of oocytes	2 or less 3 or more	**45** **20**	70.76 ± 10.49 90.8 ± 51.71	20 (6 - 51) 74 (37-243)	**0.00**	S
Immature oocytes	2 or less 3 or more	**32** **20**	3.031 ± 1.84 11.60 ± 5.97	2.5 (1 - 9) 10.5 (3 – 25)	**0.00**	S
Mature oocytes	2 or less 3 or more	**45** **20**	18.67 ± 9.89 79.2 ± 48.01	16 (5 - 46) 62.5 (28- 225)	**0.00**	S

N= is the number of donor, A= Less than 3 Cycles, B = more than 3 Cycles, p-value < 0.05, S= significant difference.
Test* non-parametric test* (Mann Whitney)

Figure 1a: Boxplot representation of the total of oocytes retrieved for group A and B

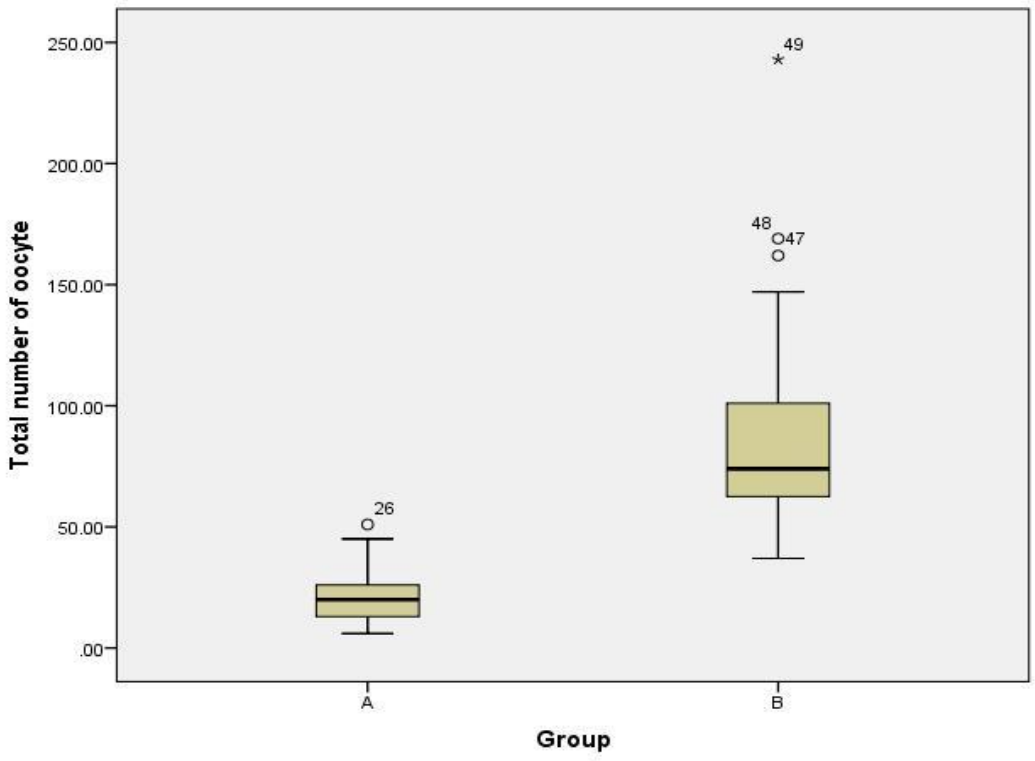


Figure 1b: Boxplot representation of immature oocytes obtained for group A and B

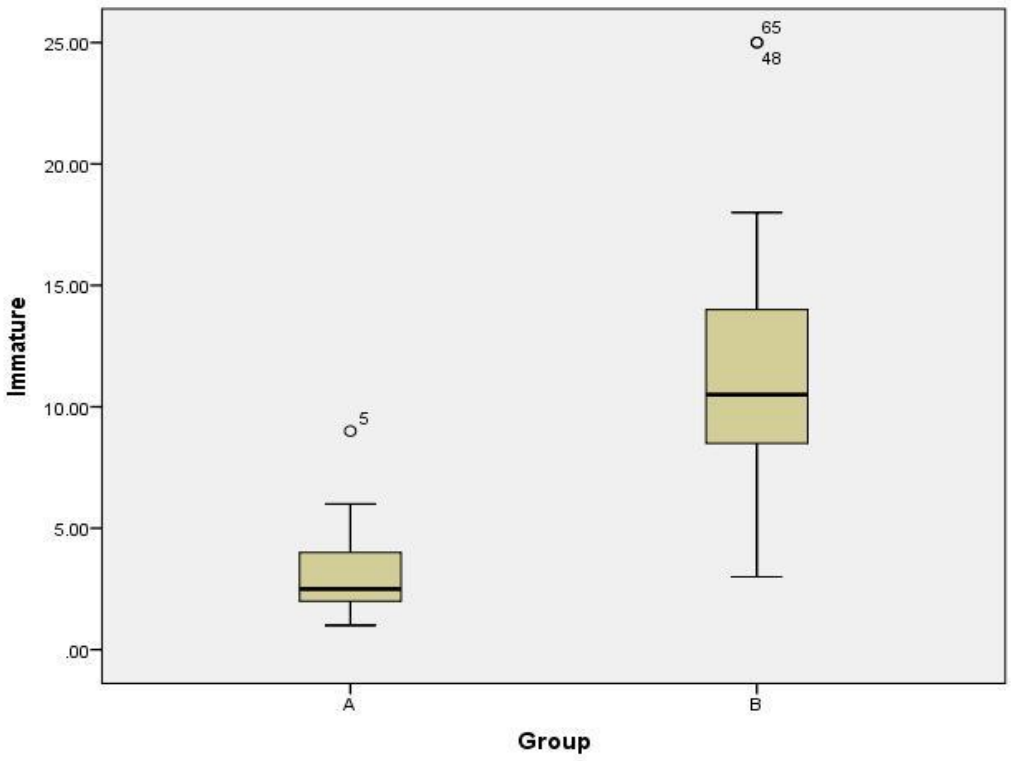
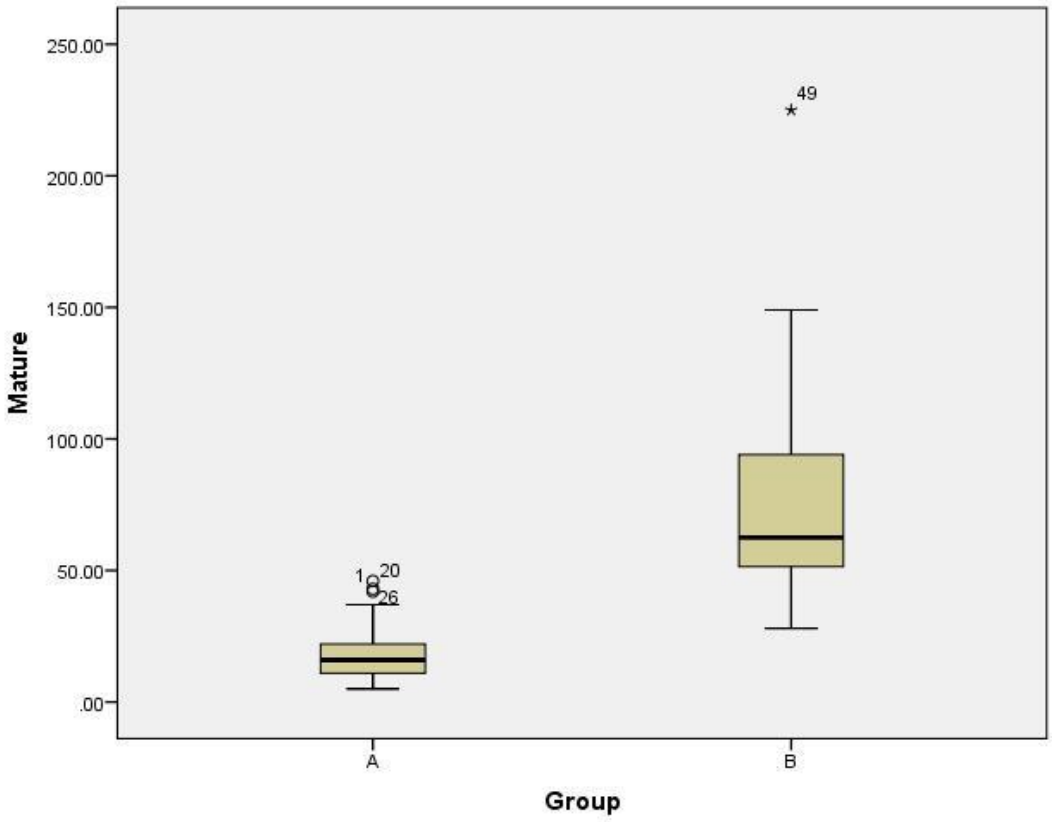


Figure 1c: Boxplot representation of mature oocytes obtained for group A and B.



There was statistical significant differences observed in the ratio of fertilized oocytes, G1-G3 embryo qualities and clinical pregnancy rates between donors in group A and donors in group B ($P<0.05$). Donors in group B had higher fertilization rate, more good quality embryos grade (G1-G3) and higher clinical pregnancy rates than donors in group A.

In contrast, no statistical differences were observed between the ratio of bad quality embryos (G4) and arrested embryos of donors in group A and B, ($P = 0.444$, 0.227; respectively). The

average ratio of fertilized oocytes, G1-G4 embryo quality grading and the pregnancy rate of donors in group A and B are presented in table 4.1.

Table 4.2: Fertilization rate, embryo grading (G1-Arrested) embryos and clinical pregnancy outcome of groups A and B

Variable	Stimulation cycle	N	Mean ± Std	Median (min to max)	P-value	
Fertilization rates	**2 or less** **3 or more**	45 20	12.89 ± 7.84 54.15 ± 33.83	11 (3- 37) 42 (22 -147)	**0.00**	**S**
G1 stage	**2 or less** **3 or more**	44 20	6.79 ± 5.14 26.25 ± 20.82	6 (1-6) 15 (7 -78)	**0.00**	**S**
G2 stage	**2 or less** **3 or more**	39 20	3.31 ± 2.09 10.8 ± 6.53	3 (1 - 11) 10 (2 - 25)	**0.00**	**S**
G3 stage	**2 or less** **3 or more**	28 20	3.14 ± 2.58 9.9 ± 7.14	2.5 (1 - 13) 8 (2- 28)	**0.00**	**S**
G4 stage	**2 or less** **3 or more**	8 12	2.75 ± 2.71 3.17 ± 3.13	1.5 (1- 8) 2 (1 - 12)	**0.444**	**NS**
Arrested embryos	**2 or less** **3 or more**	19 19	2.42 ± 1.30 4.37 ± 4.39	2 (1-5) 3 (1-17)	**0.227**	**NS**
Clinical pregnancy rates	**2 or less** **3 or more**	36 20	1.611 ± 0.67 5.0 ± 2.85	1 (1-4) 1 (1-14)	**0.00**	**S**

N= is the number of donors. Group A = 2 or less cycles, group B = 3 or more cycles NS = NO significant difference, S = significance differences, *Non-parametric Test* (Mann Whitney)

Further evaluation was performed according to the number of oocytes obtained per cycle. Donors with fifteen or less oocytes obtained were grouped as C, while donors with sixteen or more oocytes obtained were grouped as D. Similar to groups A and B, the quality of immature (MI) and mature (MII) oocytes, fertilization rate, day 3 embryo quality and clinical pregnancy rates

were investigated among donors in groups C and D. A significant difference was detected in the number of immature and mature oocytes retrieved, fertilized oocytes, day 3 (G1-G3) embryo quality and clinical pregnancy outcomes of donors in group C and D, P<0.05. It was observed that donors in group C had fewer immature oocytes compared to donors in group D. On the contrary, donors in group D had higher number of mature (MII) oocytes than donors in group C. Furthermore, donors in group D had increased number of fertilized oocytes than donors in group C. The general evaluation of MI and MII oocytes, fertilization rates, day 3embryo qualities and clinical pregnancy rates of group C and D are presented in table 4.3.

Table 4.3: General evaluation according to the number of oocytes collected

Variable	Number of oocytes obtained	N	Mean ± Std	(min to max)	P-Values	
Mature oocytes	**<=15 Oocytes**	20	10.65 ± 2.62	10 (5-15)	**0.00**	**S**
	=>16 Oocytes	45	49.13 ± 46.16	37 (14 - 225)		
Immature oocytes	**<=15 Oocytes**	12	2.17 ± 1.40	2 (1-5)	**0.00**	**S**
	=>16 Oocytes	40	7.58 ± 5.98	5.5 (1-25)		
Fertilization rates	**<=15 Oocytes**	20	7.5 ± 2.37	8 (3-12)	**0.00**	**S**
	=>16 Oocytes	45	3.62 ± 29.97	25 (7- 147)		

N= is the number of donors, Group C=15 or less, group D = 16 or more, P-value < 0.05, S= significant difference
***Non parametric Test *Mann Whitney Test**

Furthermore, donors in group D, had significantly better embryo qualities (G1- G3) and increased clinical pregnancy rates than donors in group C (P<0.05). No statistical difference was observed in the quality of G4 and arrested embryos of donors in group C and D (P=0.061, 0.982,

respectively). The quality of the embryos obtained and clinical pregnancy rates from donors in group C and D is presented in table 4.4.

Table 4.4: General evaluated of factors using number of oocyte retrieved per donors

Variable compared	Oocytes obtained	N	Mean ± Std	Median (min to max)	P-Values	
G1	**<= 15 Oocytes**	19	4.32 ± 2.47	4 (1-9)	**0.00**	**S**
	=>16 Oocytes	45	16.44 ± 16.88	10 (1-78)		
G2	**<=15 Oocytes**	15	2.13 ± 0.834	2 (1 -4)	**0.00**	**S**
	<= 16 Oocytes	44	7.11 ± 5.77	5.5 (1-25)		
G3	**<= 15 Oocytes**	8	1.88 ± 1.13	5.5 (1-25)	**0.00**	**S**
	=> 16 Oocytes	40	6.75 ± 6.24	4 (1-28)		
G4	**<= 15 Oocytes**	4	1.25 ± 0.5	1 (1-2)	**0.061**	**NS**
	=> 15 Oocytes	16	3.44 ± 3.09	2 (1-12)		
Arrested embryos	**<= 15 Oocytes**	5	2.8 ± 1.79	3 (1-5)	**0.982**	**NS**
	=> 16 Oocytes	33	3.45 ± 3.55	2 (1 -17)		
Clinical pregnancy rates	**<= 15 Oocytes**	12	1.42 ± 0.67	1(1-3)	**0.03**	**S**
	=> 15 Oocytes	44	3.2 ± 2.58	2(1-14)		

N = is the number of donor, group C= 15 or less oocyte, group D= 16 or more oocytes, NS= No significant difference, S= significant differences. *Non-parametric* "Mann Whitney test"

PART 2: The effect of thrombophilia polymorphisms on recurrent miscarriages and the effect of anti-coagulant drugs

The study group of the second part of this thesis included 62 women who were experiencing miscarriages in the first trimester of pregnancy. The clinical pregnancy outcomes were investigated according to the genotypes of thrombophilia polymorphisms and the benefits of five different prophylactic medications. Patients' ages were between 30-50 years. Out of sixty two women, 28/62 (42.2%) had one previous miscarriage, 21/62 (33.9%) women had two previous miscarriages, 11/62 (17.7%) women had 3 previous miscarriages while 2/62 (3.2%) had four previous miscarriages until 13 weeks of gestation.

In terms of age association with recurrent fetal loss, no significant association was found (P = 0.744). The average ratio for age, miscarriages and clinical pregnancy outcomes is presented in table 4.6.

Table 4.5: The average ratios for age and number of pregnancy loss

Parameter	Clinical Pregnancy Outcome	N	Mean Age	P-Value
Patients Age	**Positive** **Negative**	48 14	31.1 32.86	**0.744**
Number of pregnancy Loss	**Positive** **Negative**	48 14	29.89 37.04	**0.16**

N= is the number of patient

The genotypes of each patient were grouped into wild type, heterozygous or homozygous for *MTHFR* 1298 and 677, *FVL* 1691, *PAI-1* 4G/5G and *FII* 20210, respectively. The statistical distribution of thrombophilia polymorphisms analyzed is shown in Table 4.7.

Table 4.6: Frequency analysis of thrombophilia gene polymorphisms

Genotypes	N (%)
***MTHFR* 1298 A>C**	
AA (wild type)	25 (40.3%)
AC	22 (35.5%)
CC	15 (24.2%)
***MTHFR* 677 C>T**	
CC (wild type)	28 (45.2%)
CT	28 (45.2%)
TT	6 (9.7%)
***Factor V Lieden* 1691 G>A**	
GG (wild type)	54 (87.1%)
GA	4 (6.5%)
AA	4 (6.5%)
***PAI-1* G/G gene**	
5G/5G (wild type)	44 (71%)
4G/5G	12 (19.4%)
4G/4G	6 (9.7%)
***FII* (*prothrombin*) 20210 G>A**	
GG (wild type)	59 (95.2%)
GA	3 (4.8%)
AA	0

The effect of five different prophylactic drug doses was compared between clinical pregnancy outcomes of patients. Patients were grouped into four categories. Positive group consisted of patients with positive pregnancy and live birth, while negative group consisted of patients with early or late miscarriages with no live birth. Patients who used medication were grouped into

one group, while patients who did not use medication were grouped into another. The clinical pregnancy outcomes were compared for different genotypes of *MTHFR* 1298 and 677, *FVL* A1691G, *PAI-1* 4G/5G and *FII* 20210 in association with different anticoagulant treatment.

There was no statistical difference in the clinical pregnancy outcomes of patients with different *MTHFR* 1298 genotypes (AA, AC and CC) in the presence or absence of zinco c or enoxaparin treatments (P = 0.606, 0.274, 0.081 for zinco c treatment; and P = 0.653, 0.323, 1 for enoxaparin; respectively). Additionally, no difference was found in the clinical pregnancy outcomes of patients with *MTHFR* 1298AA (wild type) genotype in the presence or absence of 75mg dose of aspirin (P =1.00). However, significant statistical difference was detected in the clinical pregnancy outcomes of patients with *MTHFR* A1298C genotype treated with 75mg of aspirin when compared to those without treatment (P = 0.043). Patients with *MTHFR* A1298C who were treated with 75mg of aspirin had higher rate of negative pregnancy results than positive rates compared to *MTHFR* A1298C patients with no treatment. Statistical analysis was not performed for *MTHFR* C1298C homozygous patients, since all patients received 75mg of aspirin.

On the other hand, no significant difference was found in the clinical pregnancy outcomes of patients with *MTHFR* 1298 AA, AC and CC genotypes in the presence or absence of 81mg of aspirin or folic acid treatment (P = 0.645, 0.309, 0.569 for 81mg aspirin treatment; and P = 0.137, 0.548, 1 for folic acid treatment; respectively).

Furthermore, a significant association was detected in the clinical pregnancy outcomes of patients with *MTHFR* C677C genotypes and zinco c treatment (P = 0.038). Patients with *MTHFR* C677C (wild type) genotypes who were treated with zinco c had significantly higher positive pregnancy and live birth rates compared to those with *MTHFR* C677C genotypes who

did not receive zinco c administration. However, no statistical differences were found for the clinical pregnancy outcomes of patients with *MTHFR* C677T and T677T genotypes treated with zinco c compared to patients who did not receive the treatment (P = 0.172, 1; respectively).

In addition, there was no significant differences observed in the clinical pregnancy outcomes of patients with *MTHFR* 677 genotypes (CC, CT and TT) in the presence or absence of either enoxaparin or 75mg dose of aspirin treatments (P = 0.678, 0.467, 0.736 for enoxaparin; and P = 0.143, 0.286, 1,00 for 75mg for aspirin treatments; respectively). Likewise, no statistical difference was detected in the clinical pregnancy outcomes of *MTHFR* 677 CC, CT genotypes in the presence or absence of 81mg aspirin or folic acid treatments (P = 0.569, 0.651 for 81mg aspirin treatment; and P = 1, 0.067, 0.421; for folic acid; respectively). As reported, there was no analysis for patients with *MTHFR* 677 TT genotypes for 81mg aspirin administration, since all these patients received this treatment.

No statistical difference was observed in the clinical pregnancy outcomes of different *FVL* 1691 (GG and GA) genotypes in the presence or absence of either zinco c or enoxaparin treatment (P= 0.06, 1.000 for zinco c; and P = 0.736, 1.00, 1.00; respectively). As reported, there was no statistical analysis performed for *FVL* 1691 AA homozygous cases because no patients received zinco c treatment. Additionally, no significant difference was observed in the pregnancy outcomes of *FVL* 1691 (GG, GA and AA) genotypes treated with either 75mg aspirin or 81mg aspirin when compared to patients who did not receive treatments (P = 0.102 for 75mg aspirin; and P = 1.00, 1.00,0.333; respectively). There was no statistical analysis performed for *FVL* 1691 heterozygous and homozygous genotypes, in the presence of 75mg dose of aspirin. No statistical difference was found in the clinical pregnancy outcomes of *FVL* 1691 GG, GA and AA genotypes in the presence or absence of folic acid treatment (P = 0.421, 1, 1; respectively).

There was no statistical difference detected in the clinical pregnancy outcomes of patients with different *PAI-1* genotypes (5G/5G, 4G/5G and 4G/4G) in the presence or absence of zinco c or enoxaparin treatments (P = 0.135, 0.167 and 1.00 for zinco c treatment; and P = 0.746, 1.00, 0.333 for enoxaparin treatment; respectively). Additionally, no statistical difference was observed in the clinical pregnancy outcomes of patients with *PAI-1* 5G/5G genotype, upon treatment with 75mg of aspirin when compared to those without medication (P = 0.102). There was no statistical analysis reported for *PAI-1* 4G/5G and 4G/4G genotypes since none of the patients received 75mg dose of aspirin. In addition, no statistical difference was detected between the clinical pregnancy outcomes of PAI-1 5G/5G, 4G/5G and 4G/4G genotypes upon treatment with 81mg dose of aspirin when compared to *PAI-1* 5G/5G, 4G/5G and 4G/4G genotypes without 81mg dose of aspirin treatments (P = 1.00, 1.00, 1.00; respectively). Likewise, no difference was detected in the pregnancy outcomes of patients with *PAI-1* 5G/5G, 4G/5G and 4G/4G genotypes treated with folic acid compared to no treatment group (P = 0.698, 1.00, 1.00; respectively).

Lastly, no statistical difference was found in the clinical pregnancy outcomes of patients with *FII* 20210 GG and GA genotypes in the presence or absence of either zinco c or enoxaparin treatment (P = 0.06, 0.0395, for zinco c treatment; and P = 1.00, 1.00, for enoxaparin; respectively). Also, no statistical difference was detected in the clinical pregnancy outcomes of patients with *FII* 20210 GG and GA genotypes in the presence or absence of either 75 mg dose of aspirin or 81mg dose of aspirin treatment (1.00, 0.333, for 75mg aspirin treatment and P = 1.00, 1.00 for 81mg of aspirin treatment; respectively). Similarly, no statistical association was observed for folic acid treatment of patients with *FII* 20210 GG and GA genotypes (P = 1.00, 1.00; respectively). The number of patients with *FII* 20210 homozygote genotype was too small

to be included in any statistical analysis. Tables 4.7 a to 4.7 e show the evaluation of all treatments for the clinical pregnancy outcomes of patients with different thrombophilia associated gene genotypes.

Table 4.7a: Evaluation of pregnancy outcomes of patients with different thrombophilia associated gene genotypes in treatments with zinco c administration

Polymorphisms	**Genotypes**	**Pregnancy Outcome**	**Patients not treated with zinco C n (%)**	**Patients treated with zinco C n (%)**	**P-Value**
***MTHFR* 1298**	**AA**	Positive Negative	4 (21.1%) 2 (33.3%)	15 (78.9%) 4 (66.7%)	**0.606**
	AA	Positive Negative	4(23.5%) 3 (60%)	13(76.5%) 2 (40%)	**0.274**
	CC	Positive Negative	1 (8.3%) 2 (66.7%)	11 (91.7%) 1 (33.3%)	**0.081**
***MTHFR* 677**	**CC**	Positive Negative	4 (16.7%) 3 (75.0%)	20 (83.3%) 1 (25%)	**0.038**
	CT	Positive Negative	4 (20%) 4 (50%)	20 (83.3%) 1 (25%)	**0.172**
	TT	Positive Negative	1 (25%) 0 (0%)	3 (75%) 2 (100%)	**1**
***FVL* 1691**	**GG**	Positive Negative	8 (18.6%) 7 (63.6%)	35 (81.4%) 4 (36.4%)	**0.06**
	GA	Positive Negative	1(33.3%) 0 (0%)	2 (66.7%) 1 (100%)	**1**
	AA	Positive Negative	None None	1 (100%) 1 (100%)	**no analysis**
***PAI-1* G/G**	**4G/4G**	Positive Negative	7(29.1%) 6 (50%)	25 (78.1) 6 (50%)	**0.135**
	4G/5G	Positive Negative	1 (9.1%) 1 (100%)	10(90.9%) 0 (0%)	**0.167**
	5G/5G	Positive Negative	1 (20%) 0 (0%)	4 (80%) 1 (100%)	**1**
***FII* 20210**	**AA**	Positive Negative	8(17.4%) 6(46.2%)	38(82.6%) 7 (53.8%)	**0.06**
	GA	Positive Negative	1 (50%) 1 (100%)	1 (50%) 0 (0%)	**1**

Table 4.7b: Evaluation of pregnancy outcomes of patients with different thrombophilia associated genes in treatment with enoxaparin administration

Polymorphisms	Genotypes	Pregnancy Outcome	Patients not treated with enoxaparin n (%)	Patients treated with enoxaparin n (%)	P-value
***MTHFR* 1298**	**AA**	Positive Negative	12 (63.2%) 3 (50%)	7(36.8%) 3 (50%)	**0.653**
	AC	Positive Negative	8 (47.1%) 4 (80%)	9(52.9%) 1 (20%)	**0.323**
	CC	Positive Negative	5 (41.7%) 1 (33.3%)	7 (58%) 2(66.7%)	**1**
***MTHFR* 677**	**CC**	Positive Negative	14 (58.3%) 3 (75%)	10(41.7%) 1 (25%)	**1**
	CT	Positive Negative	9 (45%) 5 (62.5%)	11 (55%) 3(37.5%)	**0.678**
	TT	Positive Negative	2 (50%) 0 (0%)	2(50%) 2 (100%)	**0.467**
***FVL* 1691**	**GG**	Positive Negative	23 (53.5%) 7 (63.6%)	20(46.5%) 4 (36.4%)	**0.736**
	GA	Positive Negative	1 (33.3%) 0 (0%)	2 (66.7%) 1 (100%)	**1**
	AA	Positive Negative	1 (50%) 1 (50%)	1 (50%) 1 (50%)	**1**
***PAI-1* G/G**	**4G/4G**	Positive Negative	18 (56.3%) 6 (50%)	14 (43.8%) 6 (50%)	**0.746**
	4G/5G	Positive Negative	6 (54.5%) 1 (100%)	5 (45.5%) 0 (0%)	**1**
	5G/5G	Positive Negative	1 (20%) 1 (100%)	4 (80%) 0 (0%)	**0.333**
***FII* 20210**	**GG**	Positive Negative	24 (52.2%) 7 (53.8%)	22(47.8%) 6 (46.2%)	**1**
	GA	Positive Negative	1 (50%) 1 (100%)	1 (50%) 0 (0%)	**1**

Table 4.7c: Evaluation of pregnancy outcomes of patients with different thrombophilia associated gene genotypes in treatments with 75mg dose of aspirin

Polymorphisms	Genotypes	Clinical Pregnancy Outcome	Patients not treated with Aspirin 75mg n(%)	Patients treated with Aspirin 75mg n(%)	P-value
***MTHFR* 1298**	**AA**	Positive Negative	18(94.7%) 6 (100%)	1 (5.3%) 0 (0%)	**1**
	AC	Positive Negative	17 (100%) 3 (60%)	0 (0%) 2 (40%)	**0.043**
	CC	Positive Negative	None None	12 (100%) 3 (100%)	**no analysis**
MTHFR 677	**CC**	Positive Negative	24 (100%) 3 (75%)	0(0%) 1 (25%)	**0.143**
	CT	Positive Negative	20 (100%) 7 (87.5%)	0 (0%) 1 (12.5%)	**0.286**
	TT	Positive Negative	3(75%) 2 (100%)	1 (12.5%) 0 (0%)	**1**
***FVL* 1619**	**GG**	Positive Negative	42(97.7%) 9 (81.8%)	1 (2.3%) 2 (18.2%)	**0.102**
	GA	Positive Negative	None None	3 (100%) 1 (100%)	**no analysis**
	AA	Positive Negative	None None	2 (100%) 2(100%)	**no analysis**
PAI-1	**4G/4G**	Positive Negative	31(96.9%) 10(83.3%)	1 (3.1%) 2 (16.7%)	**0.176**
	4G/5G	Positive Negative	11 (100%) 1 (100%)	None None	**no analysis**
	5G/5G	Positive Negative	5(100%) 1 (100%)	None None	**no analysis**
***FII* 20210**	**GG**	Positive Negative	45(97.8%) 12(92.3%)	1 (22%) 1(7.7%)	**0.395**
	GA	Positive Negative	2(100%) 0(0%)	0 (0%) 1(100%)	**0.333**

Table 4.7d: Evaluation of pregnancy outcomes of patients with different thrombophilia associated gene genotypes in treatments with 81mg dose of aspirin

Polymorphisms	Genotype	Clinical pregnancy outcome	Patients not treated with Aspirin 81 n(%)	Patients treated with Aspirin 81 n(%)	P-value
***MTHFR* 1298**	**AA**	Positive Negative	10(52.6%) 2 (33.3%)	9(47.4%) 4(66.7%)	**0.645**
	AC	Positive Negative	5 (29.4%) 3(60%)	12(70.6%) 2(40%)	**0.309**
	CC	Positive Negative	7(58.3%) 1(33.3%)	5(47.1%) 2 (66.7%)	**0.569**
***MTHFR* 677**	**CC**	Positive Negative	13(54.2%) 1(25%)	11(45.8%) 3 (75%)	**0.569**
	CT	Positive Negative	5 (25%) 3(37.5%)	15 (75%) 5 (62.5%)	**0.651**
	TT	Positive Negative	None None	4 (100%) 2 (100%)	**No Analysis**
***FVL* 1691**	**GG**	Positive Negative	18(49.1%) 5(45.5%)	25(58.1%) 6 (54.5%)	**1**
	GA	Positive Negative	2(66.7%) 1(100%)	1 (33.3%) 0 (0%)	**1**
	AA	Positive Negative	2(100%) 0(0%)	0 (0%) 2 (100%)	**0.333**
PAI-1	**4G/4G**	Positive Negative	16 (100%) 6 (100%)	16 (100%) 6 (100%)	**1**
	4G/5G	Positive Negative	4 (36.4%) 0 (0%)	1 (63.6%) 1 (100%)	**1**
	5G/5G	Positive Negative	2(40%) 0 (0%)	3 (60%) 1 (100%)	**1**
***FII* 20210**	**GG**	Positive Negative	21(45.7%) 5 (38.5%)	25(54.3%) 8 (61.5%)	**0.757**
	GA	Positive Negative	1(50%) 1(100%)	1 (50%) 0 (0%)	**1**

Table 4.7e: Evaluation of pregnancy outcomes of patients with different thrombophilia gene genotypes in treatments with folic acids administration

Polymorphisms	Genotypes	Clinical pregnancy outcome	Patients not treated with Folic acid n(%)	Patients treated with Folic acid n(%)	P-value
***MTHFR* 1298**	**AA**	Positive Negative	12 (63.2%) 6 (100%)	7 (36.8%) 0 (0%)	**0.137**
	AC	Positive Negative	14(82.4%) 3(60%)	3 (17.6%) 2 (40%)	**0.548**
	CC	Positive Negative	9 (75%) 3(100%)	3 (25%) 0 (0%)	**1**
***MTHFR* 677**	**CC**	Positive Negative	19 (79.2%) 4 (100%)	5 (20.8%) 0 (100%)	**1**
	CT	Positive Negative	16 (80%) 6 (75%)	4 (20%) 2 (25%)	**1**
	TT	Positive Negative	0 (0%) 2 (100%)	4(100%) 0(0%)	**0.067**
***FVL* 1691**	**GG**	Positive Negative	32 (74.4%) 10 (90.9%)	11(25.6%) 1(9.1%)	**0.421**
	GA	Positive Negative	2(66.7%) 1(100%)	1 (33.3%) 0 (0%)	**1**
	AA	Positive Negative	1(50%) 1(50%)	1(50%) 1(50%)	**1**
PAI-1	**4G/4G**	Positive Negative	23 (79.1%) 10(83.3%)	9(28.1%) 2 (16.7%)	**0.698**
	4G/5G	Positive Negative	9 (81.8%) 1(100%)	2(18.2%) 0(0%)	**1**
	5G/5G	Positive Negative	3 (60%) 1(100%)	2(40%) 0(0%)	**1**
***FII* 20210**	**GG**	Positive Negative	34(73.9%) 12(92.3%)	12(26.1%) 1(7.7%)	**0.26**
	GA	Positive Negative	1 (50%) 0(0%)	1(50%) 1(100%)	**1**

CHAPTER FIVE

DISCUSSION

PART 1: The effect of repeated ovarian hyperstimulation cycles on the quantity and maturity of oocytes obtained, fertilization rates, quality of day 3 pre-implantation embryos and clinical pregnancy outcomes

The first part of the thesis was designed to investigate the effect of multiple ovarian stimulation cycles on the quality and quantity of oocytes obtained, fertilization rate, embryo qualities and clinical pregnancy outcomes of young healthy donors between the ages of 18-33 years. To date, there are limited studies on the effects of multiple ovarian hyperstimulation cycles on oocyte quality or quantity and further embryonic development.

The results of this investigation showed that there was significantly lower number of immature oocytes (MI) retrieved from oocyte donors stimulated two or less times (group A) when compared to donors stimulated three or more times (group B). However, donors with three or more stimulation cycles (group B) had significantly higher number of mature oocytes (MII) retrieved than donors stimulated two or less times (group A). Furthermore, donors undergoing three or more COS cycles (group B) had higher number of fertilized oocytes with better developmental qualities and higher clinical pregnancy rates than donors with two or less stimulation cycles (group A). Alternatively, donors in groups (A and B) maintained similar ratios of bad quality embryos with heavy fragmentation and embryos arrested at the cleavage stage (G4 and arrest).

Further investigation showed that donors with maximum of 15 oocytes (group C) had significantly fewer number of immature (MI) oocytes obtained compared to donors with more

than fifteen oocytes retrieved (group D). On the contrary, donors in group D had more mature oocytes obtained compared to donors in group C. In addition, the number of fertilized oocytes was significantly higher in group D donors compared to donors in group C. Likewise, donors in group D had better quality embryos and increased clinical pregnancy rates than donors in group C. Similar to groups A and B, there was no difference observed in the ratio of bad quality embryos and arrested embryos from donors in groups (C and D).

According to Caligara et al. (2001), each donor have fixed quota of recruited ovarian follicle pre-selected by the ovaries which gets partly excluded from atresia due to high dose of gonadotropin administration during multiple COS cycles (Caligara et al., 2001). Therefore, higher doses of GnRH was suggested to positively affect ovarian response by providing an over-ridding mechanism in which more follicles escape atresia (Gougeon, 1986). This may support the findings of this study, since more number of immature and mature oocytes was obtained from donors with three or more stimulation cycles. Furthermore, the quality of pre-implantation embryos was not lowered by the number of COS cycles that has great implications on the clinical applications of multiple hyperstimulation procedures.

Furthermore, Baart and colleagues (2007) supported the findings of this study. They reported that all patients maintained the same quantity of oocytes, fertilization rates, clinical pregnancy rates and chromosomal segregation during meiosis in women undergoing either milder or conventional stimulation protocols, indicating that stimulation protocols may not change the outcome of each cycle (Baart et al., 2007). However, the rate of spontaneous abortion was higher in donors with milder stimulations compared to donors with conventional cycles. On the other hand, the estimated proportion of chromosomal abnormalities increased in patients who

underwent conventional stimulation cycles which may be due to ovarian ageing as a result of advanced maternal age (Baart et al., 2007).

Although, such studies are very scarce, repeated COS cycle does not seem to affect ovarian response to exogenous gonadotropins, since the quality of oocytes obtained and their pre-implantation developmental potentials was also not affected by successive stimulation cycles. However, the effect of multiple COS on the health of donor's undergoing multiple hyperstimulation cycles for IVF procedures still needs to be assessed. One of the advantages of this study was that only young and healthy donors with no history of infertility or ovulation disorder were included, as often hypothesized to be the reason for low oocyte quality, embryo quality and pregnancy rates, thus donors become less sensitive to COS with advance maternal ageing leading to poor IVF treatment outcome (Kim et al., 2013).

Additionally, aside the known health complications associated with multiple COS cycle, such as ovarian hyperstimulation syndrome (OHSS), premature ovarian failure and polycystic ovarian syndrome (PCOS), debate is still ongoing on whether or not artificial gonadotropin administration, alongside interminable ovulation treatments used to improve follicular growth during IVF treatments increase donors' risk of developing ovarian or breast carcinoma (Jain et al ., 2005). However, these evaluations were not in the scope of this thesis. Therefore, this suggest that further studies involving repeated COS cycles and IVF treatment follow up should not be narrowed down to factors evaluated in this study. Rather, a valid oocyte donation database should be established in order to evaluate any short and long time risks associated with multiple COS cycles on donor's health and aftermaths their offsprings.

The finding of this study indicates that multiple stimulations do not negatively affect or impair ovarian response in terms of quality and quantity of oocytes retrieved, fertilization rate, good embryo qualities (G1-G3) or clinical pregnancy outcomes rather it improves IVF treatment outcome with the same proportion of bad quality embryos and arrested embryos.

5.2 : Conclusion

In summary, the first part of this thesis revealed that the quantity and maturity of oocytes, fertilization rates, embryo qualities (Grade 1- Grade 3) and clinical pregnancy rates were higher in donors who underwent three or more stimulations cycles compared to donors who underwent two or less COS cycles. Also, donors with more than 15 oocytes obtained had significantly higher quality of oocytes, fertilization rates, embryo qualities (grade 1- grade 3) and clinical pregnancy outcomes compared to donors with fifteen or less oocytes obtained. These results suggest that multiple stimulation cycles do not have any adverse effect or impairment on ovarian response in terms of quantity and quality of oocytes retrieved, fertilized oocytes, embryo qualities, implantation and pregnancy rates. Therefore, further extrapolation of this data may imply that donors could go through multiple COS cycles without any negative effect on the factors evaluated in these studies, though the short- and long-term effects on donors' health should be evaluated for future purposes.

PART 2: The effect of thrombophilia polymorphisms on recurrent miscarriages and the effect of anti-coagulant drugs

In the second part of the thesis, the genes associated with thrombophilia were genotyped in 62 women with recurrent fetal losses and the effect of five different prophylactic medications on the clinical pregnancy rates were investigated. A large number of studies have investigated the association between thrombophilia genetic polymorphisms and recurrent pregnancy loss. However, to date contradictory results have been published. Zhu et al (2018) reported no statistical association between *MTHFR* (C677T and A1298C) polymorphisms and RPL (Zhu et al., 2018). A number of studies supported this finding, in such no association was reported between *MTHFR* C677T and A1298C polymorphisms and habitual abortion in women with PCOS (Szafarowska et al., 2016), idiopathic RPL in Korean women (Hwang et al., 2017), *MTHFR* A1298C polymorphism and RPL in Chinese women (Chen et al., 2016), in East Asians and mixed subgroups (Cao et al., 2013).

On the other hand, other studies have reported an association between RPL and *MTHFR* polymorphisms. A significant association was reported between *MTHFR* C677T (Xu et al., 2019 and Rai, 2016) and A1298C (Xu et al., 2019) polymorphisms and unexplained RPL, as well as habitual abortion in Syrian women (Al-Achkar et al., 2017). *MTHFR* C677T genotype was also associated with RPL among Asians but not Caucasians (Wu et al., 2012). Moreover, results from previously published meta-analysis showed that there is a significant association between parental *MTHFR* A1298C and C677T polymorphisms and RPL (Yang et al., 2016). The statistical association of fetal *MTHFR* A1298C polymorphism in women with RPL was also reported (Yang et al., 2016). Similarly, *MTHFR* 1298 (AC and CC) polymorphisms were associated with spontaneously aborted embryos as well as women with RPL (Nair et al., 2013).

Barut et al. (2018) showed a correlation between heterozygous *FVL*, *Prothrombin* and *PAI-1* 4G/5G polymorphisms and RPL in Turkish (Barut et al., 2018) and in Iranian women (Kamali et al., 2018). The association of *FVL* G1691A polymorphisms and RPL was also reported in Bosnian women (Jusić et al., 2018) and early habitual abortion in heterogeneous population (Sergi et al., 2015). Furthermore, *FVL* 1691 G/A variation was associated with pre-eclampsia (Ahmed et al., 2019). Similarly, *PAI-1* 4G/5G genotype was shown to be a genetic risk factor for RPL in Czech and Bulgarian women (Adler et al., 2018) and in heterogeneous populations (Shakarami et al., 2015 and Chen et al., 2015). In addition, Gao et al (2015) reported a significant association for *Prothrombin* G20210A mutation and fetal losses in both primary and secondary RPL in European women (Gao & Tao, 2015) and unexplained RPL in Isfahan province (Kardi et al., 2018).

Even though there is inconsistent data on the association of polymorphisms involved in thrombophilia, such as *MTHFR* polymorphisms, there is a handful of studies, including meta-analysis, supporting the significant association of these polymorphisms with RPL (Yang et al., 2016, Rai, 2016 and Nair et al., 2013). Therefore, the second part of this thesis aimed to investigate the association of these polymorphisms with pregnancy loss.

Currently, in RPL cases where pregnancy complications related to thrombophilia such as placental deficiencies, hypercoagulability and lower rate of fibrinolysis are common. The use of prophylactic treatments against thrombophilia becomes necessary in order to increase the live birth rates in women with RPL (Stefanski et al., 2018). Also, several studies regarding the benefit of anti-coagulant drugs in preventing recurrent fetal losses in women with prior miscarriages and inherited thrombophilia polymorphisms were either inconsistent or inconclusive. Schleussuer et al. (2015) reported increased rates of intrauterine growth restrictions

in five women and eleven cases of placental deficiency with three cases of intra-uterine fetal death in women treated with low molecular weight heparin (LMWH) doses compared to those without treatment. However, no difference was detected in the live birth rates of patients with heparin treatments compared to patients without the treatment (Schleussner et al., 2015). While, in women with acquired thrombophilia, lower dose of aspirin combined with heparin was reported to be an effective treatment for recurrent miscarriages in women with antiphospholipid syndrome (Goel et al., 2006).

In this study, the live birth rates of patients with *MTHFR* 1298 and 677, *FVL* 1691, *PAI-1* G/G and *FII* 20210 genotypes treated with either enoxaparin 0.4ml (LMWH), 75mg dose of aspirin, 81mg dose of aspirin or 10mg of folic acids did not show any significant difference compared to patients who did not receive any form of treatment. Previously published data supported this finding, in such meta-analysis the clinical impact of anti-coagulant treatments against thrombophilia showed that treatment with LMWH (enoxaparin) had no benefit in women experiencing recurrent pregnancy loss (>10 weeks). They reported no evidence for the benefit of either aspirin combined with LMWH or aspirin without LMWH (Skeith et al., 2016). Similar findings were also reported by Areia et al. (2016), in such the live birth rates and pregnancy outcomes were very similar in patients with hereditary thrombophilia who received only aspirin compared to aspirin and LMWH combined (Areia et al., 2016). Furthermore, no beneficial effect of anti-coagulant drugs was reported for the treatment of RPL in women with *FVL* and *prothrombin* polymorphisms (Kaandorp et al., 2010).

Badawy and colleagues (2008) evaluated the benefit of folic acid in combination with LMWH and the use of only folic acid in 340 women with inherited thrombophilia and habitual abortion (Badawy et al., 2008). Folic acid doses were discontinued at 14 weeks of gestation while LMWH

doses were discontinued at 34 weeks of gestation. Both early and late pregnancy losses were reported to be significantly lower in patients treated with folic acid combined with LMWH doses compared to those treated with only folic acid dose (Badawy et al., 2008). Although, the administration of LMWH was reported to have improved live birth rates in some patients, the routine use of LMWH treatment did not improve late pregnancy complications in women with *MTHFR* polymorphism (Cetin et al., 2017). The benefit of folic acid and 5-methylenetetrahydrofolate treatments were reported to be similar in patients with idiopathic recurrent miscarriages with respect to *MTHFR* C677T and A1298T polymorphisms (Hekmatdoost et al., 2015). Furthermore, the use of LMWH (enoxaparin) and aspirin was reported to be an effective therapy for C677T *MTHFR*-heterozygous genotypes with recurrent miscarriages (Merviel et al., 2017). However, the use of LMHW was reported as potential treatment for women with RPL and inherited thrombophilia polymorphisms with poor obstetric history (Mutlu et al., 2015).

The results of this study showed that treatment with 15mg dose of zinco c significantly improved pregnancy and live birth rates by preventing possible miscarriages in patients with *MTHFR* C677T genotypes when compared to *MTHFR* C677T who did not receive zinco c treatments. Although, there have not been many research studies on the benefits of zinco c treatment for the prevention of recurrent pregnancy loss in women with inherited thrombophilia, Ahad and colleagues (2013) placed emphasis on the essential role of zinc in cell division, hormonal function, blood coagulation as well as menstrual disorders in women and infertility in men (Zare et al., 2013).

5.3 : Conclusion

The second part of this thesis indicates that zinco c treatment can be used for preventing recurrent fetal losses in women with *MTHFR* C677C wild type genotypes. Also, treatment with 75mg aspirin led to increase rate of negative pregnancy outcome than live birth in *MTHFR* A1298C genotypes. To date, there are limited number of studies performed to evaluate the efficacy and safety of anti-coagulant medications, such as aspirin and heparin, in women with recurrent miscarriages.

One of the main limitations of these studies is the lack of the control group. This is further complicated with different genotypes of polymorphisms involved in thrombophilia. Therefore, this study forms the basis of future studies to shed light in the clinical use of anti-coagulant medications.

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