T regulatory cells in follicular fluid in women with fertility problems

University of Crete
Faculty of Medicine

Graduate Program
Molecular basis of human disease
Graduate Student: Lykakis Ioannis

Ο ρόλος των Τ ρυθμιστών κυττάρων στο ωοθηλακιακό υγρό γυναικών με πρόβλημα υπογονιμότητας

Πανεπιστήμιο Κρήτης
Τμήμα Ιατρικής

Μεταπτυχιακό Πρόγραμμα
Μοριακή και κυτταρική βάση των ανθρωπίνων νοσημάτων
Μεταπτυχιακός φοιτητής Λυκάκης Ιωάννης

Εισαγωγή

Η υπογονιμότητα αποτελεί διαταραχή που εμφανίζεται με αρκετά υψηλή συχνότητα και αφορά και στα δύο φύλα, ανευρίσκεται δε στο 25% των ζευγαριών. Ο γυναικείος παράγοντας αποτελεί το πιο σύνηθες αίτιο και σχετίζεται με γυναικολογικές διαταραχές όπως σύνδρομο πολυκυστικών ωοθηκών και ενδομητρίωση. Τα CD4+ CD25+ Τ ρυθμιστικά κύτταρα αποτελούν έναν υποπληθυσμό των Τ κυττάρων και είναι υπεύθυνα για την διαμόρφωση του ανοσιακού συστήματος. Κατά την διάρκεια της εγκυμοσύνης παρατηρείται συστηματική επέκταση των κυττάρων αυτών προλαμβάνοντας έτσι την αποβολή του εμβρύου. Αρκετές έρευνες έχουν αποδείξει ότι η υπογονιμότητα σχετίζεται με μειωμένα επίπεδα Τ ρυθμιστικών κυττάτων. Στόχος αυτής της μελέτης είναι να διερευνήσουμε τα επίπεδα των Τ ρυθμιστικών κυττάτων στο ωοθηλακιακό υγρό γυναικών με υπογονιμότητα. Για το συγκεκριμένο στόχο συλλέχθησαν 74 δείγματα και χωρίστηκαν σε τρεις ομάδες. Η πρώτη ομάδα (24 δείγματα) αποτελούνταν από γυναίκες με πολυκιστικό σύνδρομο , η δεύτερη ομάδα (20 δείγματα) από γυναίκες με ανεξήγητη υπογονιμότητα και η τρίτη (30 δείγμτα) από γυναίκες στις οποίες υπεύθυνος ήταν ο αντρικός παράγοντας. Για την αναγνώριση των Τ ρυθμιστικών κυττάρων πραγματοποιήθηκε κυτταρομετρία ροής. Τα επίπεδα των κυττάρων που ήταν θετικά σε CD4+ ήταν στατιστικώς σημαντικά αυξημένα σε γυναίκες με σύνδρομο πολυκυστικών ωοθηκών σε σύγκριση με τις γυναικές που είχαν ανεξήγητη υπογονιμότητα και υπογονονιμότητα που οφειλόταν στον ανδρικό παράγοντα. Επίσης τα επίπεδα των ενεργοποιημένων Τ ρυθμιστικών κυττάρων ήταν στατιστικώς σημαντικά μειωμένα στις γυναίκες με σύνδρομο πολυκυστικών ωοθηκών σε σύγκριση με τις γυναικές που είχαν ανεξήγητη υπογονιμότητα και υπογονονιμότητα που οφειλόταν στον ανδρικό παράγοντα . Τα αποτελέσμτα αυτά υποδηλώνουν ότι τα μειωμένα επίπεδα Τ ρυθμιστικών πιθανώς να σχετίζονται με την υπογονιμότητα στις γυναίκες με πολυκυστικό σύνδρομο.

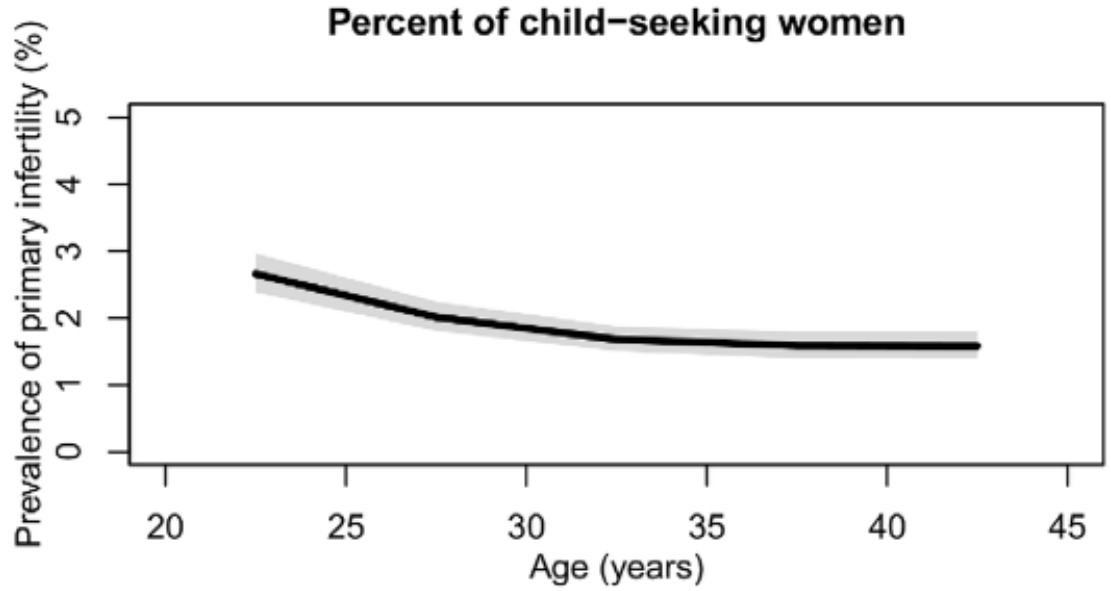
Abstract

Infertility is a common disorder that involves both genders and it is found in 25% of couples. Female factor infertility which is the most common is associated with gynecological disorders such as polycystic ovary syndrome (PCOS) and endometriosis. $CD4^+$ $CD25^+$ regulatory T cells (Treg) is a subpopulation of T cells responsible for the modulation of immune system. A systematic expansion of Treg is observed during pregnancy and prevents fetal rejection. A lot of studies show the association of infertility with reduced levels of Treg cells. The aim of this research is to investigate the levels of Tregulatory cells in follicular fluid in infertility women. 74 samples were collected and divided in three group . The first group (24 samples) was women with polycystic ovary syndrome , the second (20 samples) was women with unexplained infertility and the third (30 samples) was women with male factor infertility. Flow cytometry was performed in order to identified the levels of Tregulatory cells. The levels of CD4+posititve cells were significant higher in PCOS women comparing with unexplained infertility women and male factor, also the levels of activated Tregulatory cells are significant decreased comparing with male factor infertility. These results indicate that decreased levels of activated Tregutory cells may be associated with fertility problems in PCOS women.

Introduction

Infertility is a common gynecological disorder. It involves both genders rather than each sex alone and it is defined by the failure to conceive a woman less than 35 years after 12 months of contraception free intercourse and 6 months in woman older than 35 years of contraception free intercourse [1,2].
A lot of studies demonstrate that 80-90% of couples will conceive in the first year after contraception free intercourse [3] . After six months without

contraception the couple may think the possibility of infertility [4] .The prevalence of infertility is very difficult to determined but it is estimated that 25% of the couples have fertility problems[5]. Two percent of child-seeking women from 20 to 44 years old are unable to have a child birth and the prevalence of secondary infertility(women with a prior child birth) is related increased with the woman's age (Table.1) Also this study demonstrated that the levels of infertility were similar in last twenty years (from 1990 to 2010) [6]



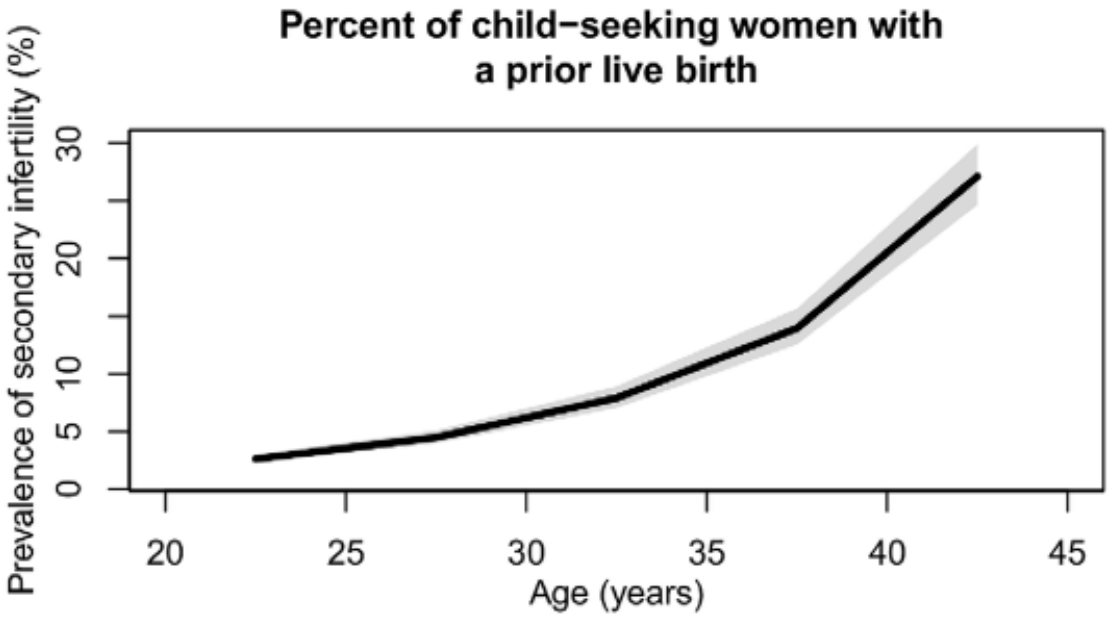


Table 1. prevalence of primary and secondary infertility in child-seeking women according to age

In developed countries, female infertility is responsible for 37% of infertile couples ,in 35% both partners have infertility problems , 8% the male factor is responsible for and 5% the cause of infertility was not found (Table 2)[7]

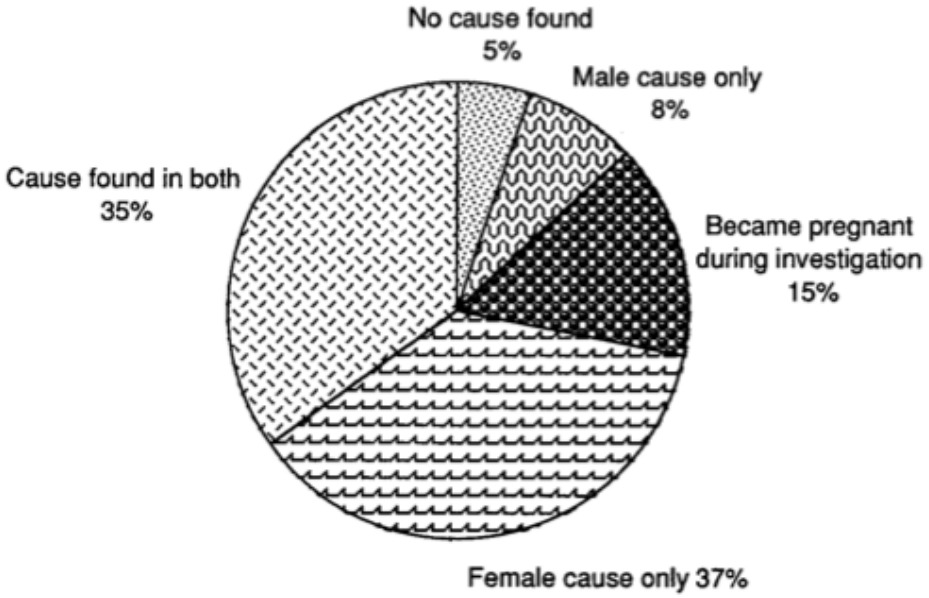


Table 2 distribution of causes of infertility by partner in developed countries

The male factor infertility is divided into four main categories (Table .3)[8]:

1. Testicular disease 30-40%
2. Post-testicular defects 10-20%
3. Hypothalamic pituitary disease – 1-2 %
4. Idiopathic – 40-50%

Table 3 . Causes of male infertility

Hypothalamic-pituitary disorders (GnRH; LH and FSH deficiency)
Congenital disorders
Congenital GnRH deficiency (Kallmann syndrome)
Hemochromatosis
Multiorgan genetic disorders (Prader-Willi syndrome, Laurence-Moon-Beidl syndrome)
Acquired disorders
Pituitary and hypothalamic tumors (macroadenoma, craniopharyngioma)
Infiltrative disorders (sarcoidosis, histiocytosis, tuberculosis, fungal infections)
Trauma, postsurgery, postirradiation
Vascular (infarction, aneurysm)
Hormonal (hyperprolactinemia, androgen excess, estrogen excess, cortisol excess)
Drugs (opioids and psychotropic drugs, GnRH agonists or antagonists)
Systemic disorders
Chronic illnesses
Nutritional deficiencies
Obesity
Primary gonadal disorders
Congenital disorders
Klinefelter's syndrome (XXY) and its variants (XXY/XY; XXXY)
Cryptorchidism
Myotonic dystrophy
Functional prepubertal castrate syndrome (congenital anorchia)
Varicocele
Androgen insensitivity syndromes
5-alpha-reductase deficiency
Y chromosome deletions

Acquired disorders
Viral orchitis (mumps, echovirus, arbovirus)
Granulomatous orchitis (leprosy, tuberculosis)
Epididymo-orchitis (gonorrhea, chlamydia)
Drugs (eg, alkylating agents, alcohol, marijuana, antiandrogens, ketoconazole, spironolactone)
Ionizing radiation
Environmental toxins (eg, dibromochloropropane, carbon disulfide, cadmium, lead, mercury)
Hyperthermia
Immunologic disorders, including polyglandular autoimmune disease
Trauma
Torsion
Castration
Systemic illness (eg renal failure, hepatic cirrhosis, cancer, sickle cell disease, amyloidosis, vasculitis)
Disorders of sperm transport
Epididymal dysfunction (drugs, infection)
Abnormalities of the vas deferens (congenital absence, Young's syndrome, infection, vasectomy)
Ejaculatory dysfunction (spinal cord disease, autonomic dysfunction, premature ejaculation)
Unexplained male factor infertility

The main causes by anatomic location of the female factor infertility are (Table 4) :

a) Ovulatory disorders 24,8%
b) Pelvic adhesion 12,4%
c) Acquired tubal abnormalities 11,2%
d) Bilateral tubal occlusion 10,6%
e) Endometriosis 5,7%
f) Hyperprolactinaemia 6,7%

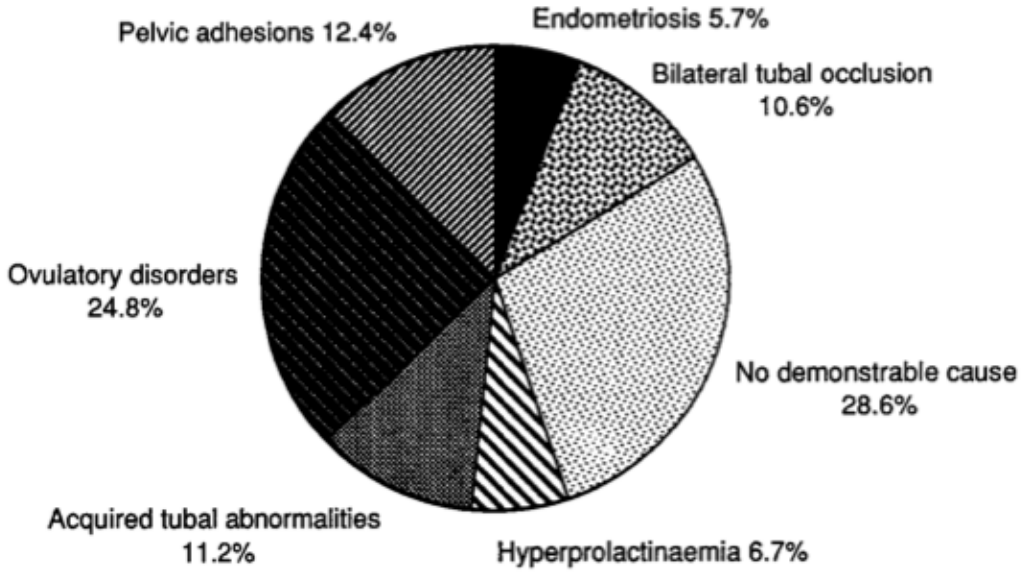


Table 4 distribution of most common specific causes of infertility in women in developed countries

Ovulatory disorders are the main cause of female infertility. There is a dysfunction in ovulation which includes the irregular and infrequent menses (oligovulation) or the absence of ovulation (anovulation). Anovulation is a disorder in which the eggs are not developed or released from the follicles. The main symptom of anovulation is the absence of menses. The causes of anovulation include the primary hypothalamic – pituitary dysfunction, medications and other disorders(Table.5) [9] The World Health Organization has classified anovulation into three main classes in order to treat each disorder according to the underline endocrine dysfunction(Table 6)[10]

Table 5 causes of anovulation

Primary hypothalamic-pituitary dysfunction
Kallman's syndrome
Idiopathic hypogonadotropic hypogonadism
Tumors, trauma, or radiation of the hypothalamic or pituitary area
Sheehan's syndrome
Empty sella syndrome
Pituitary adenoma or other pituitary tumors
Lymphocytic hypophysitis (autoimmune diseases)
Lactational amenorrhea
Stress
Eating disorders
Intense exercise
Immaturity at onset of menarche or perimenopausal decline
Other disorders
Polycystic ovary syndrome
Hyperthyroidism or hypothyroidism
Hormone producing tumors (adrenal, ovarian)
Chronic liver or renal disease
Cushing's disease
Congenital adrenal hyperplasia

Premature ovarian failure, which may be autoimmune, genetic, surgical, idiopathic or radiation
Turner syndrome
Androgen insensitivity syndrome
Medications
Oral contraceptives
Progestins
Antidepressant and antipsychotic drugs
Corticosteroids
Chemotherapeutic agents

Table 6 World Health Organization classification of anovulation

WHO class 1: Hypogonadotropic hypogonadal anovulation (hypothalamic amenorrhea)
These women have low or low-normal serum follicle-stimulating hormone (FSH) concentrations and low serum estradiol concentrations due to decreased hypothalamic secretion of gonadotropin-releasing hormone (GnRH) or pituitary unresponsiveness to GnRH.
WHO class 2: Normogonadotropic normoestrogenic anovulation
These women may secrete normal amounts of gonadotropins and estrogens. However, FSH secretion during the follicular phase of the cycle is subnormal. This group includes women with polycystic ovary syndrome (PCOS). Some ovulate occasionally, especially those with oligomenorrhea.
WHO class 3: Hypergonadotropic hypoestrogenic anovulation
The primary causes are premature ovarian failure (absence of ovarian follicles due to early menopause) and ovarian resistance (follicular form).
Hyperprolactinemic anovulation
These women are anovulatory because hyperprolactinemia inhibits gonadotropin and therefore estrogen secretion; they may have regular anovulatory cycles, but most have oligomenorrhea or amenorrhea. Their serum gonadotropin concentrations are usually normal.

Pelvic adhesions/tubal occlusion can impact fertility because they block the transportation of the oocytes to the fallopian tubes. The main reason of pelvic adhesions is the pelvic inflammation caused by viral , fungal, bacterial and parasitic infections . Another causes are previous pelvic surgery, inflammatory bowel disease , intra-abdominal infections such as peritonitis, appendicitis and severe endometriosis.[11]

Uterus abnormalities are another significant factor which can lead to infertility. Uterine leiomyoma is the most common benign tumor of smooth muscles . A lot of studies demonstrate that only submucosa and intramular myomas are implicated with infertility, and after myomectomy the pregnancy rates are significant increased[12]. Another uterine anomalies such as mullerian duct anomalies , endometrial polyps could decrease the reproductive outcome [13].

Cervix produces mucus which allows the sperm to travel from the vagina through the uterus[14], cervical abnormalities such as stenosis could impair fertility[15].

Moreover there are a lot of acquired factors that are responsible for female infertility. The age of the woman is an important factor, as the age of the woman is increased the quality of the oocyte is decreased and it’s more difficult to get pregnant (Table 7) [16]

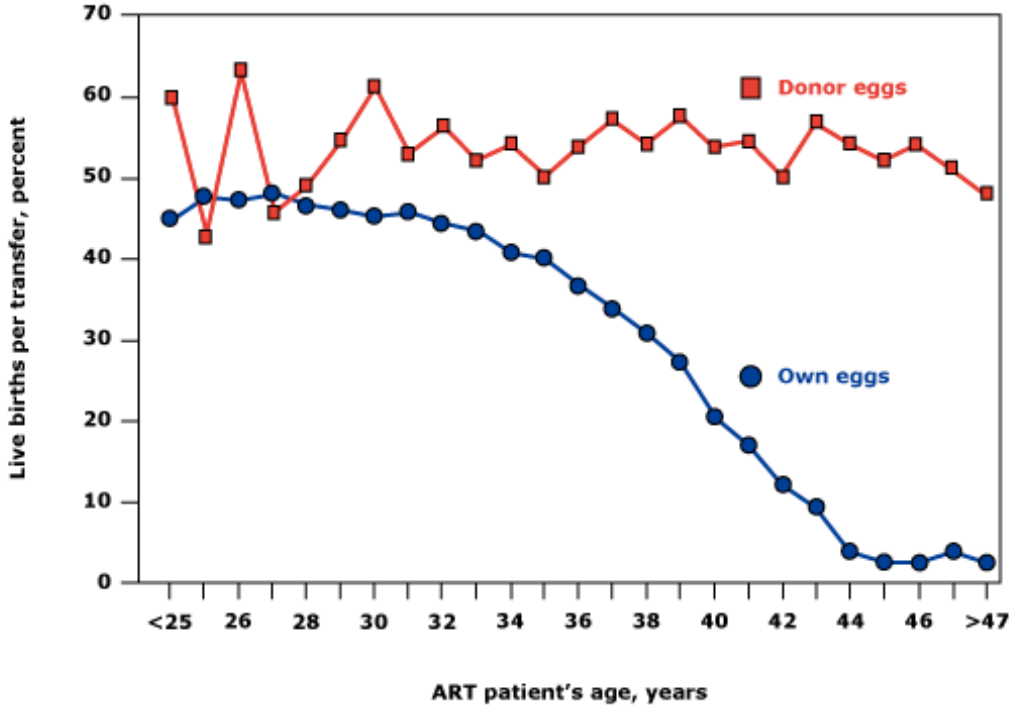


Table 7 The above figure compares percentages of transfers resulting in live births for ART cycles using fresh embryos from donor eggs with those for ART cycles using a woman's own eggs, among women of different ages. The likelihood of a fertilized egg implanting is related to the age of the woman who produced the egg. Thus, the percentage of transfers resulting in live births for cycles using embryos from women's own eggs declines as women get older. In contrast, since egg donors are typically in their 20s or early 30s, the percentage of transfers resulting in live births for cycles using embryos from donor eggs remained consistently high at above 40 percent

Reproduced from: Centers for Disease Control and Prevention, American Society for Reproductive Medicine, Society for Assisted Reproductive Technology. 2006 Assisted Reproductive Technology Success Rates: National Summary and Fertility Clinic Reports, Atlanta: Centers for Disease Control and Prevention, 2008.

Other important factor implicating in infertility is tobacco smoke. Tobacco smoke can harmful the ovaries and influence the balance of estrogens. Also is associated with earlier menopause[17], cigarette smoking is a target for every stage of reproductive function (folliculogenesis, embryo transport , endometrial receptivity, endometrial angiogenesis, uterine blood flow and the uterine myometrium) [18] .Smokers have 60% infertility problems than non-

smokers[19]
Eating disorders are correlated with fertility problems. Obese woman produce more estrogens than the non-obese and it's more impossible to get pregnant [20]. On the other hand women with little body fat mass have lower levels of estorgens and disruption of the menstrual cycle [20]. Chemotherapeutical drugs such as alkylating agents have high risk of gonadotoxicity . They cause destruction of oocytes, follicular depletion, cortical fibrosis and ovarian blood-vessel damage [21].
Finally they have been a lot of gene mutation that are related with female infertility (Table 8) [22]

Table 8.Genes wherein mutation causes female infertility

Gene	Encoded protein	Effect of deficiency
BMP15	Bone morphogenetic protein 15	Hypergonadotrophic ovarian failure (POF4)
BMPR1B	Bone morphogenetic protein receptor 1B	Ovarian dysfunction, hypergonadotrophic hypogonadism and acromesomelic chondrodysplasia
CBX2; M33	Chromobox protein homolog 2 ; Drosophila polycomb class	Autosomal 46,XY, male-to-female sex reversal (phenotypically perfect females)
CHD7	Chromodomain-helicase-DNA-binding protein 7	CHARGE syndrome and Kallmann syndrome (KAL5)
DIAPH2	Diaphanous homolog 2	Hypergonadotrophic, premature ovarian failure (POF2A)
FGF8	Fibroblast growth factor 8	Normosmic hypogonadotrophic hypogonadism and K allmann syndrome (KAL6)
FGFR1	Fibroblast growth factor receptor 1	Kallmann syndrome (KAL2)
FSHR	FSH receptor	Hypergonadotrophic hypogonadism and ovarian hyperstimulation syndrome
FSHB	Follitropin subunit beta	Deficiency of follicle-stimulating hormone, primary amenorrhoea and infertility
FOXL	Forkhead box L2	Isolated premature ovarian failure (POF3) associated

2		with BPES type I; FOXL2 402C --> G mutations associated with human granulosa cell tumours
FMR1	Fragile X mental retardation	Premature ovarian failure (POF1) associated with premutations
GNRH 1	Gonadotropin releasing hormone	Normosmic hypogonadotrophic hypogonadism
GNRH R	GnRH receptor	Hypogonadotrophic hypogonadism
KAL1	Kallmann syndrome	Hypogonadotrophic hypogonadism and insomnia, X-linked Kallmann syndrome (KAL1)
KISS1 R GP R54	KISS1 receptor	Hypogonadotrophic hypogonadism
LHB	Luteinizing hormone beta polypeptide	
LHCG R	LH/choriogonadotrophin receptor	Hypergonadotrophic hypogonadism (luteinizing hormone resistance)
DAX1	Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1	X-linked congenital adrenal hypoplasia with hypogonadotrophic hypogonadism; dosage-sensitive male-to-female sex reversal
NR5A 1; SF1	Steroidogenic factor 1	46,XY male-to-female sex reversal and streak gonads and congenital lipoid adrenal hyperplasia; 46,XX gonadal dysgenesis and 46,XX primary ovarian insufficiency
POF1 B	Premature ovarian failure 1B	Hypergonadotrophic, primary amenorrhea (POF2B)
PROK 2	Prokineticin	Normosmic hypogonadotrophic hypogonadism and Kallmann syndrome (KAL4)
PROK R2	Prokineticin receptor 2	Kallmann syndrome (KAL3)
RSPO 1	R-spondin family, member 1	46,XX, female-to-male sex reversal (individuals contain testes)
SRY	Sex-determining region Y	Mutations lead to 46,XY females; translocations lead to 46,XX males
SOX9	SRY-related HMB-box gene 9	Autosomal 46,XY male-to-female sex reversal (campomelic dysplasia)
TAC3	Tachykinin 3	Normosmic hypogonadotrophic hypogonadism

TACR 3	Tachykinin receptor 3	Normosmic hypogonadotrophic hypogonadism

Polycystic ovary syndrome is one of the most common cause of infertility in women [23]. The disease was first described by Stein and Leventha and they correlated amenorrhea with polycystic ovaries [24] , today we believe that is a systematic syndrome which is characterized by the following features:

A) menstrual disorders such as oligorrhea or amenorrhea
B) elevated levels of androgenic hormones
C) polycystic ovary
D) obesity and insulin resistance

Polycystic ovary syndrome is difficult to diagnosed because of its heterogeneity they set the following criteria:

National Institute of Health criteria (1990)
Evidence of anovulation or oligo-ovulation
Evidence of clinical or biochemical hyperandrogenism (either)
Clinical: hirsutism, acne, or male pattern balding
Biochemical: high serum androgen concentration

Rotterdam criteria by the European Society of Human Reproduction and Embryology
Two out of three following findings are required for diagnosis:
Evidence of anovulation or oligo-ovulation
Evidence of clinical or biochemical hyperandrogenism (either)
Clinical: hirsutism, acne, or male pattern balding
Biochemical: high serum androgen concentration
A polycystic ovary (by ultrasound)

Androgen Excess and PCOS Society criteria (2009)
Evidence of ovarian dysfunction (either)
Evidence of anovulation or oligo-ovulation
A polycystic ovary (by ultrasound)
Evidence of clinical or biochemical hyperandrogenism (either)
Clinical: hirsutism, acne, or male pattern balding
Biochemical: high serum androgen concentration

Pathophysiology of polycystic ovary syndrome
The exact pathophysiology is not well defined although they have been found a lot of abnormalities that play a major role in the pathogenesis.

Hypothalamic-pituitary abnormalities.

In PCOS elevated levels of luteinizing hormone (LH) and normal levels of follicle-stimulating hormone (FSH) were found in more than the half patients[25,26] so the LH:FSH ratio could be up to 2,5[26]. Also the levels of gonadotropin- releasing hormone (GnRH) are elevated and it affects the

secretions of gonadotropins. It is unclear whether this is the primary or the secondary effect. There are studies showing that the elevated GnRH levels leads to abnormal secretion of gonadotropins [27,28] and other indicating that GnRH abnormality is a secondary effect [29].

The levels of androgens in ovaries are higher than the normal and that results in growth of ovarian follicles and hyperplasia of stromal and thecal cells. The high levels of androgens are responsible for anovulation and polycystic appearance of the ovaries [30]. Also up to 70% of all patients have elevated levels of androgenic hormones androstenedione , DHEA-S and testosterone in the circulation[29]. Androstenedione is secreted by stromal and thecal cells in ovaries and it is stimulated by LH and converted to estradiol by an FSH dependent- aromatase . The high ration of LH : FSH results in elevated secretion of androstenedione , which is not aromatized because the levels of FSH are normal low levels [29].

Polycystic ovary syndrome and insulin resistance

Insulin resistance co-exists very often with PCOS. 25% of adolescents with PCOS have metabolic syndrome [31] and 10% of all women with PCOS will have diabetes mellitus type 2 [32]. Hyperinsulinemia stimulates insulin-like growth factor-I (IGF-I) signal transduction pathway in the ovarian thecal cell and leads to elevated levels of androgens because blocks the negative feedback of LH [33] Hyperinsulenemia is responsible for excessively androgen production[33]

In polycystic ovary syndrome treatment medication is based to symptoms each woman presents. There are four main target a) regular menstrual cycle and endometrial protection b) treatment of hirsutism or acne c) restoration of infertility d)normal levels of insulin in circulation

Oral contraceptives are used for regulation of the menstrual cycle. They decrease the androgen levels and protect the endometrium. The endometrial protection is succeeded by normal ovulation (woman with chronic anovulation have increased risk of endometrial cancer) and progesterone which antagonists endometrial proliferation (protection against endometrial cancer) [34]. Oral contraceptives pills are effective in reducing hirsuitsm in most of the patients, when hirsuitsm persists an anti-androgenic drug such as spironolactone is added

[35]. Weight loss in overweight women with PCOS is very useful because restores the menstrual cycle to normal and decreases the levels of androgens in circulation [36]. In the end infertility is a major problems in this disorder, the initial approach to treat infertility is described below [37].

Step	Intervention	Cost	Risk of multiple gestation pregnancy
1	Weight loss (if baseline weight is elevated)	Low	Not increased
2	Clomiphene*	Low	Modest increase in risk
3	If DHEAS >2 mcg/mL Clomiphene plus glucocorticoid	Low	Modest increase in risk
4	FSH injections	Resource intensive	Markedly increased
5	Ovarian surgery	Resource intensive	Not increased
6	In vitro fertilization	Resource intensive	Potentially increased but controllable (eg, single embryo transfer)

Endometriosis is a common gynecological disorder that is characterized by the growth of hormone-responsive endometrial tissue outside the uterine cavity. The most common sites in decreasing order of frequency are the ovaries, vagina, cervix, or uterosacral ligaments or in the rectovaginal septum . More unusual sites are laparotomy scars, pleura, lung, diaphragm, kidney, spleen, gallbladder, nasal mucosa, spinal canal, stomach, breast and they can be responsible for cyclic hemoptysis and catamenial seizures [38,39] . Endometriosis is associated with local inflammation and the major symptoms are the pelvic pain and infertility (30-40% of women), other related symptoms could be dysmenorrheal, dyspareunia, dysuria and dyshezia. The pathophysiology of the diseases remains unknown, many theories have been proposed in order to explain this disorder. These theories do not necessarily exclude each other probably because endometriosis is likely to be multifactorial and to involve an interplay between several factors[40] The most popular hypotheses are retrograde menstruation and implantation of endometrial fragments into the peritoneal cavity, proposed by Sampson [41] in 1927. The theory of retrogrademenstruation and implantation is supported by the common observance of reflux flow and intraperitoneal spillage of viable endometrial tissue in ovulating women during menstruation [42]. The incidence of endometriosis is also increased in cases of anatomical menstrual outflow obstruction that predispose to retrograde flow [42]. Furthermore, ablation of the eutopic (i.e., intrauterine) endometrium in some women with endometriosis dramatically reduced the risk of recurrence [43].

Endometriosis is classified into four stages :

1.minimal endometriosis (isolated ectopic tissue wihout adhesions)
2. mild endometriosis (superficial ectopic tissue less than 5cm in ovaries and peritoneum without adhesions)
3.moderate (superficial -invasive ectopic tissue and adhesions in peritoneum or ovaries
4.severe (large endometriomas and extensive adhesions)

Endometriosis is related with infertility because can lead to anatomical changes , adhesions and production of substances such as growth factors ,cytokines which affect the normal ovulation, fertilization and implantation [44,45]. Oral contraceptives pills , progestin and gonadotropin-releasing hormone (GnRH) agonists are used as medical therapy for endometriosis but they do not improve

pregnancy rates [46]. These drugs delay fertility instead of increasing pregnancy rates [46]. Therefore, *in vitro* fertilization (IVF) should be considered in woman who want to get pregnant . In advance stages of endometriosis surgical treatment is suggested , it restores anatomical anomalies , removes endometriomas and adhesions. Also there are studies which show that surgical treatment improves pregnancy rates in advanced stages of endometriosis compared to medications or no-treatment [47].

Unexplained infertility is the failure to conceive a woman less than 35 years after 12 months of contraception free intercourse and 6 months in woman older than 35 years of contraception free intercourse [2] in the absence of definable cause .It is responsible for 15% of all infertility cases [48]. There are many studies that are trying to explain unexplained infertility, but the cause appears to be multifactorial, it depends on both partners which combined reduce fertility rate (e.g woman up to 35 years old, man with low normal semen) [49].In some cases it has been reported that changes in follicle development , in ovulation and the luteal phase are responsible for [50]. In other cases male semen analysis is low normal [51]. Finally problems with implantation and egg transport have been shown that are responsible for [52].

Treatment of unexplained infertility is empiric because there is not a definable cause [53] . A lot of epidemiological studies indicate that cigarette smoking , drinking alcohol , caffeine and obesity are related with fertility problems[54]. The child-seeking woman should achieve a normal body weight, should quit smoking, reduce caffeine intake to no more 250mg daily (two cups of coffee) and reduce alcohol intake to no-more than four drinks per week [54].

Intrauterine insemination ICU is required when the other interventions fail to result in pregnancy. It involves the placement of washed sperm into the uterine cavity around the time of ovulation. It is performed with natural evaluation or with ovulation induction using clomiphene citrate, or injectable gonadotropins . Pregnancies rates in ICU plus ovulation induction are significant higher than ICU alone [55].

In vintro fertilization (IVF) is suggested when the other treatment are failed. It offers the highest success rates compared to other but it’s more costly [56].

$CD4^+$ $CD25^+$ regulatory T cells (Treg) is a subpopulation of T cells responsible for the modulation of immune system , maintenance of tolerance to self-antigens and protection from autoimmunity [57,58]. T reg are produced in thymus and express the transcription factor forkhead box P3 (FOXP3) which is necessary for their development and their function [59]. They derived from thymus and export into the periphery and activated by specific antigens [59].

Also $CD4^+$ $CD25^+$ regulatory T cells can be according to cell surface

expression of CD62L, both population express FOXP3 but the $CD4^+$ $CD25^+$

$CD62L^{high}$ have better suppressive functions comparing with $CD4^+$ $CD25^+$

$CD62L^{low}$ [70]

In pregnancy female's immune system must tolerate the paternal alloantigens . There is a regulatory mechanism that permits the avoidance of fetal rejection [60]. A systematic expansion of Treg is observed during pregnancy at the very early stages [61,62]. Treg cells are specific to paternally derived cells [63,64] and protect fetus rejection by the woman's immune system [64]. Numerous of studies show that infertility is associated with reduced levels of Treg and reduced expression of FOXP3 [65]. Also decreased levels of Treg and expression of FOXP3 are found in women with miscarriages [66]
There are studies that show T regs association with a lot of gynecological

disorders. Women with endometriosis have decreased levels of $CD4^+$ $CD25^+$ FOXP3 regulatory T cells (Treg) in peripheral blood compared to healthy [67]

and increased levels of $CD4^+$ $CD25^+$ FOXP3 regulatory T cells (Treg) in

peritoneal fluid[67]. Also the levels of $CD4^+$ $CD25^+$ FOXP3 regulatory T cells (Treg) in the endometrium is significant decreased during proliferative and early secretary phase of the menstrual phase [68]. It is believed that elevated levels of FOXP3+ cells during the proliferative phase may be required for the induction of immune tolerance and successful embryo implantation [69]. All the above show that endometriosis may be associated with disruption in Treg population [67].

There are no data that show the role of Tregulatory cells in Polycystic ovary

syndrome. Also there is no research which investigates if there are Treg cells in the follicular fluid in women with fertility problems. Are there Treg cells in follicular fluid? If there are will they be activated and will the number be decreased in women with fertility problems??

This study focuses on investigation of T reg cells in follicular fluid. We want to see if there are Treg cells in follicular fluid , measure the number Treg cells and the activation of Treg in women with fertility problems and compare with

control women .

Materials and Methods

Patients

There were three group of patients and 74 samples were totally collected. The first group (24 samples) was women with polycystic ovary syndrome , the second (20 samples) was women with unexplained infertility and the third (30 samples) was women in which the male factor was responsible for.

Collection and preparation of follicular fluid

Patients received a long GnRH-agonist protocol whereby busereline 0,1mg Gonapeptyl Fleming was administered daily from three weeks before the beginning of the stimulation protocol until the moment of ovum pick-up (OPU). When at least the follicles where >17mm, 10000IU hCG (Pregnyl) was administered . Oocytes were retrieved 37 hours later by means of ultrasound guided follicular aspiration. To prevent contamination with blodd or flushing medium, only follicular fluid from the first punctured follicle was collected. Venous blood samples were taken immediately following the assisted reproductive technology (ART) procedure.

Follicular fluid was collected from each volunteer. In a fifteen millimeters falcon tube, five millimeters Ficoll Hypaque solution and ten millimeters of

follicular fluid was added. The falcon tube was centrifuged at 1600rpm for 30 minutes. The cells collected from interphase were washed in PBS and centrifuged at 1800rpm for 10 min. The cells that there were collected suspended to an antibody staining buffer for flow cytometry analysis

Monoclonal antibodies

We used peridinin chlorophyll protein(PCP)-conjugated anti-CD4 (BD.Bioscience) phycoerythrin (PE)-conjugated anti-CD25(Beckman Coulter) and with fluorescein isothiocyanate (FITC)- conjugated anti-CD62L mouse antihuman(BD.Bioscience) monoclonal antibodies.

Flow cytometry analysis

The isolated cells as described above were diluted with 0,3 millimeters of PBS/FBS 5% in a 2 millimeters falcon tube . After there were stained with peridinin chlorophyll protein(PCP)-conjugated anti-CD4, with phycoerythrin (PE)-conjugated anti-CD25 and with fluorescein isothiocyanate (FITC)-conjugated anti-CD62L monoclonal antibodies. The solution was incubated for 20 minutes. Afterwards PBS/FBS 5% solution was added and the falcon tubes were centrifuged at 1800rpm for 10 minutes. The collected cells was washed with 0,5 millimeters of PBS/FBS 5% . The stained samples were analyzed using Beckman Coulter cytometer and flowjo software analysis.

Statistical analysis

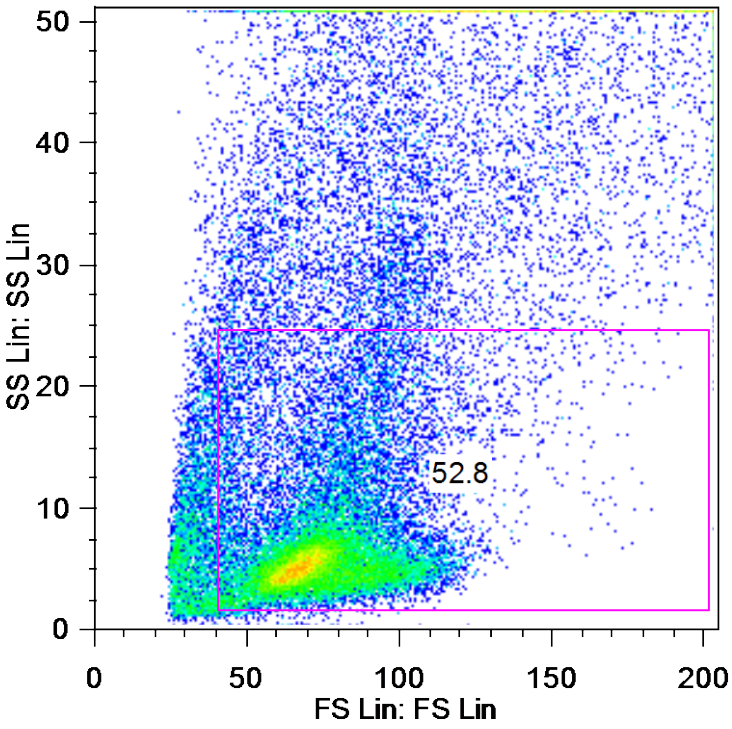
Statistical analysis was carried out using graph pad prism 5. We compared CD4+positive samples in PCOS women with unexplained infertility women and male factor infertility using chi-square test. Also we compared the percentage number of CD4+ CD25+ and the percentage number of CD4+ CD25+ CD62Lhigh in PCOS women, unexplained infertility women with male factor infertility women using one-way ANOVA test.

Results

Increased number of CD4+ positive cells in women with polycystic ovarian syndrome.

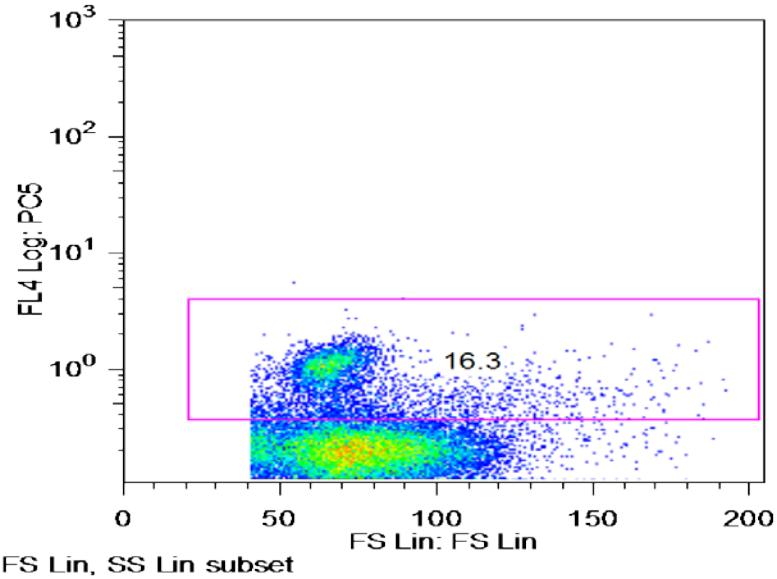
FACS analysis in follicular fluid was performed in all samples as described above. We used three groups of patients, first group was women with PCOS, second group women with unexplained factor and third women in which the male factor was responsible for fertility problem. In some samples we observed the absence of CD4+ cells (fig1). In first group (women with PCOS) 20 samples were positive to CD4+ cells and 4 negative to CD4+ cells , in the second group (unexplained infertility) 11 samples were positive to CD4+ cells and 9 samples negative to CD4+ cells and in the third group (male factor infertility) 18 samples positive to CD4+ cells and 12 negative to CD4+ cells (fig2)

1.1a.



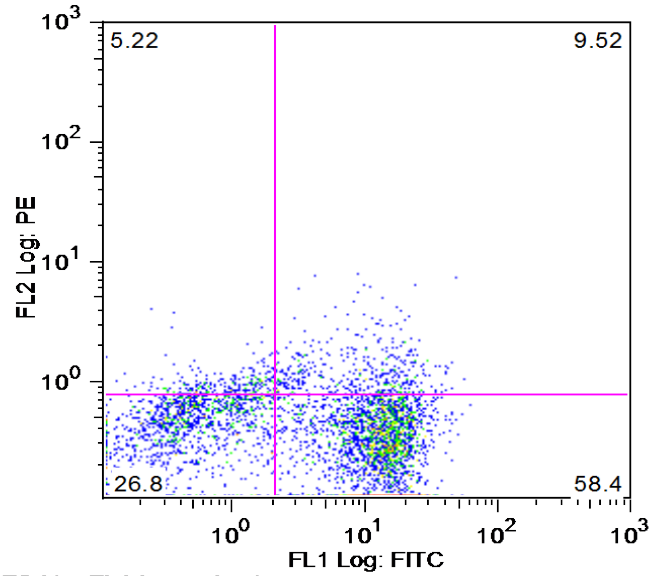
Ungated
FOLLICULLAR FLUID CD4PC5 CD25PE CD62LFITC 00034014 1436 795.LMD
Event Count: 50000

1.1b



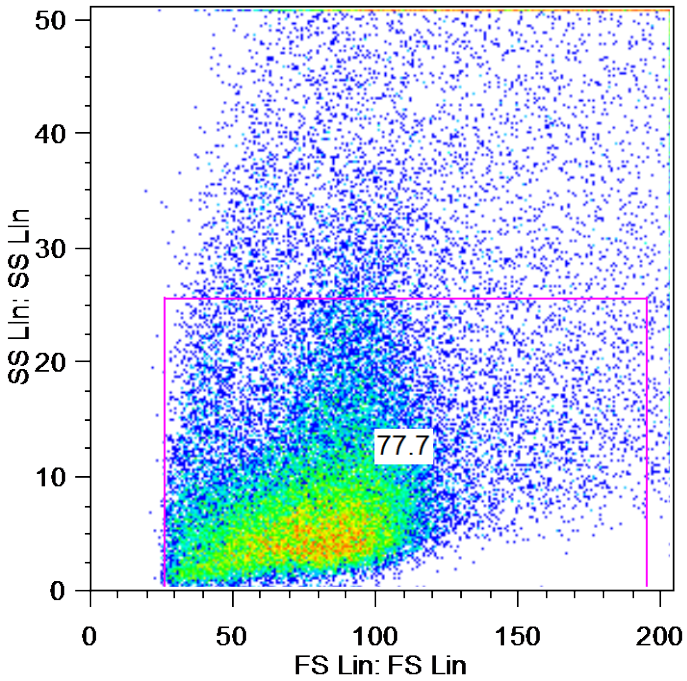
FS Lin, SS Lin subset
FOLLICULLAR FLUID CD4PC5 CD25PE CD62LFITC 00034014 1436 795.LMD
Event Count: 26416

1.1c



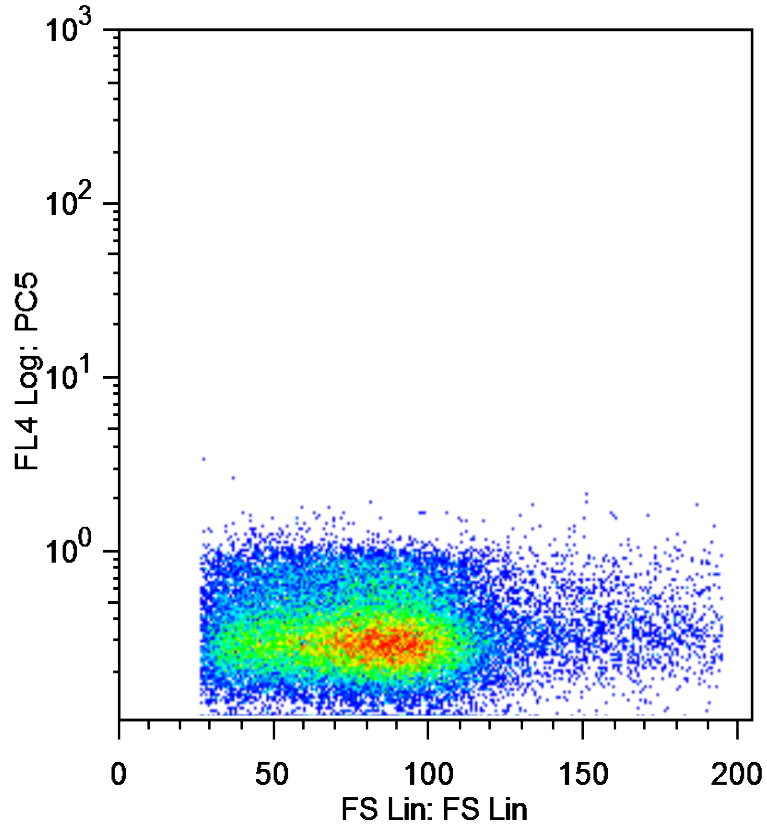
FS Lin, FL4 Log subset
FOLLICULLAR FLUID CD4PC5 CD25PE CD62LFITC 00034014 1436 795.LMD
Event Count: 4308

1.2a



Ungated
FOLLICULAR FLUID CD4PC5 CD25PE CD62LFITC 00035150 1425 902.LMD
Event Count: 50000

1.2b



FS Lin, SS Lin subset
FOLLICULAR FLUID CD4PC5 CD25PE CD62LFITC 00035150 1425 902.LMD
Event Count: 38841

Figure 1. : 1.1 representative analysis of a polycystic ovary syndrome follicular fluid sample. 1.1a shows the number of alive cells 1.1b show the CD4+ positive cells and 1.1c activated Tregualtory cells 1.2 representative analysis of an unexplained infertility sample. 1.2a shows the number of alive cells , 1.2b there are not CD4+ cells found.
(PC5)-conjugated anti-CD4 .PE-conjugated anti-CD25 and with FITC- conjugated anti-CD62L mouse antihuman monoclonal antibodies

Figure 2. 2.a the number of CD4+positive and CD4+negative samples in three categories PCOS, unexplained infertility and male factor 2.b the percentage of CD4+positive and CD4+negative samples in three categories

2.a

	PCOS	unexplained infertility	male factor
CD4+ positive	**20**	**11**	**18**
CD4+ negative	**4**	**9**	**12**
TOTAL	**24**	**20**	**30**

2.b	**PCOS**	**unexplained infertility**	**male factor**
CD4+ positive	**83,3%**	**55%**	**60%**
CD4+ negative	**17,7%**	**45%**	**40%**
TOTAL	**100%**	**100%**	**100%**

The percentage of CD4+ positive cells was significant higher in woman with polycystic ovary syndrome comparing with woman with unexplained infertility(p<0,001) and male factor infertility(p=0,005). Also the difference between unexplained infertility and male factor was not statistical significant. (fig.3)

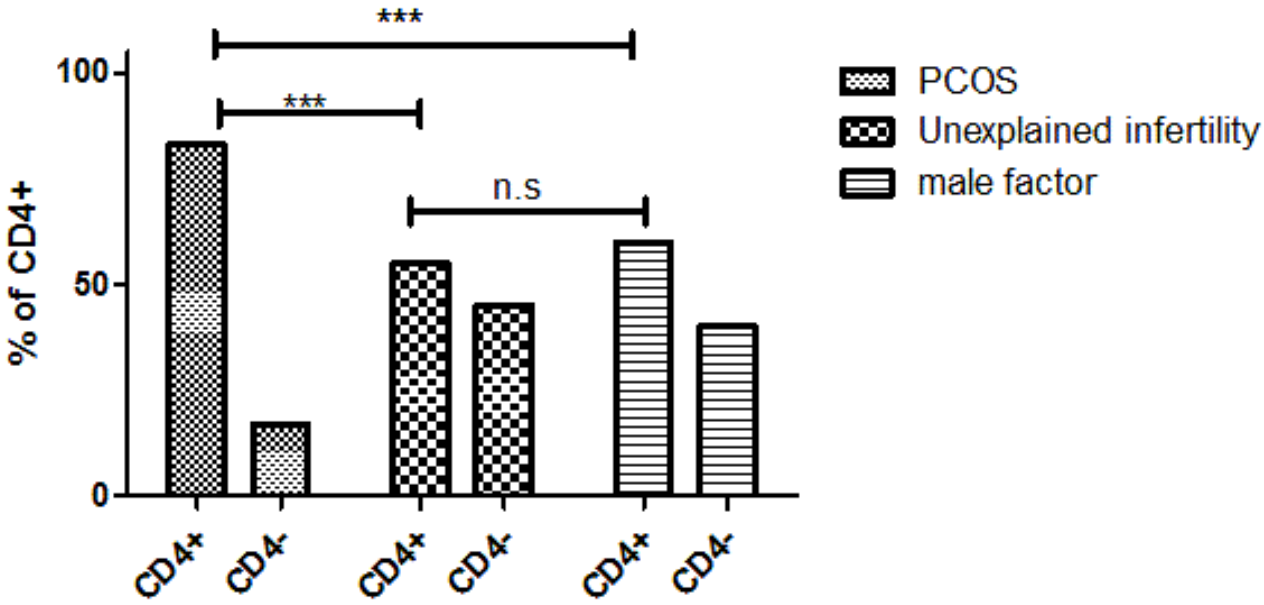


figure 3. percentage of CD4+ positive cells in three groups. The number of CD4 positive cells was significant higher in woman with polycystic ovary syndrome comparing with the unexplained infertility(p<0,001) and male factor (p=0,005). No difference between unexplained infertility and male facto. Differences between groups were analyzed by chi-square test

Analysis of $CD4^+$ $CD25^+$

In CD4+ positive samples, we calculate the number of $CD4^+$ $CD25^+$ cells. The male factor group was used as a control because the fertility problem was not associated with the women. We compared the percentage of CD4+ CD25+ cells in PCOS women(first group) with the control(male factor infertility)and women with unexplained infertility with the control(fig4). The results were not statistical significant $p>0,05$. PCOS women and women with unexplained infertility problems had similar numbers of CD4+CD25+ in follicular fluid comparing with the control

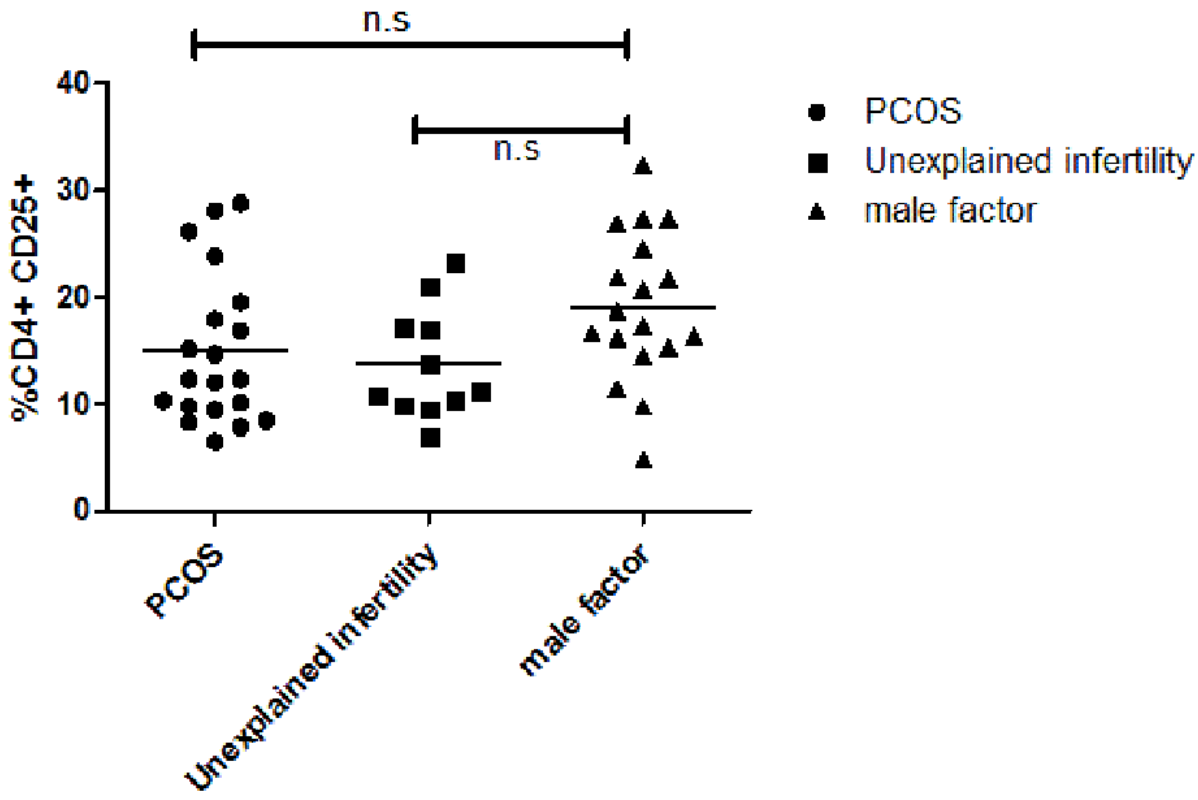


figure 4**.** the percentage of CD4+ CD25+ cells in three group. PCOS and unexplained infertility women was not statistical significant comparing with the control. Differences between groups were analyzed by one-way ANOVA test.

Analysis of $CD4^+$ $CD25^+$ $CD62L^{high}$

We next analyzed the number of $CD4^+$ $CD25^+$ $CD62L^{high}$ in three groups..

We compared the percentage of Treg activated cells in PCOS women with control and unexplained infertility woman with the control. In PCOS women there was a significant decrease ($p<0,05$) in the number of activated T regulatory cells comparing to the control (male factor infertility). In women with unexplained infertility the difference was not statistical significant (fig 5)

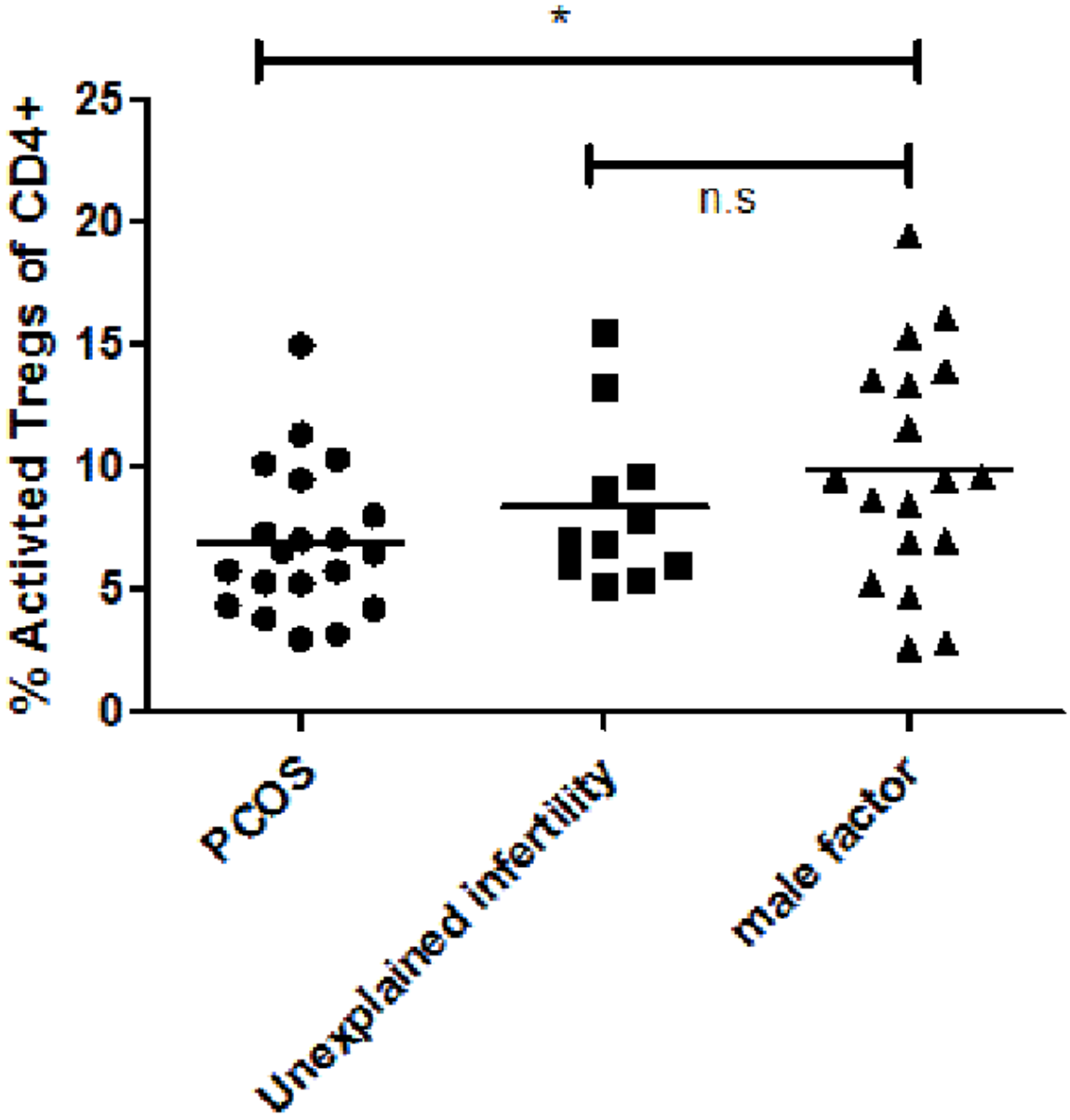


figure 5. the percentage of Treg activated cells in three group. Women with PCOS have significant decreased number of activated Tregulatory cells ($p<0,05$) comparing with the male factor group and woman with unexplained infertility the decreased levels of activated

Tregs were not statistical significant. Differences between groups were analyzed by one-way ANOVA test.

Discussion

In our study we found that a) the number of CD4+ positive cells in follicular fluid is significant higher in women with PCOS comparing with unexplained infertility and male factor infertility b) CD4+ CD25+ was not statistical significant between three group and c) the number of CD4+ CD25+ CD62L+ is significant decreased in PCOS comparing with male factor infertility.
The results of our study show that CD4+ positive cells are mostly found in follicular fluid in women with polycystic ovary syndrome comparing with unexplained infertility women and male factor infertility. There are not other data to compare with, but CD4+ positivity may be associated with the pathophysiology of the disease.
Also there numerous of studies which show that the low circulating levels of CD4+ CD25+ FOXP3 cells are associated with miscarriages and repeated implantation failure[71]. Other studies show the correlation of low circulation levels of CD4+ CD25+ FOXP3 with endometriosis [67]. We examined in this study the levels of CD4+ CD25+ and CD4+ CD25+ CD62L+(activated Treg) in follicular fluid in order to see if there differences. We observered that the levels of CD4+ CD25+ was not significant different in women with PCOS and unexplained infertility comparing with women with male factor infertility. These results indicate that women with fertility problems (PCOS, unexplained infertility) have similar levels of Treg cells in follicular fluid with the healthy. The activation of Treg cells in PCOS women was significant decreased comparing with male factor infertility and in unexplained infertility was not significant differ. This result indicates that in PCOS women the reduced

activation of Treg cells in follicular fluid may be associated with fertility problems.
The observation of the number of activated Treg cells in follicular fluid could be used as a prognostic factor for fertility in PCOS, or it can be as a possible therapeutic target.
Conclusions
Further studies are needed to examine if there is a correlation between low levels of activated Treg cells in follicular fluid and other disorders which cause fertility problems (e.g endometriosis) or in women with repeated implantation failures and miscarriages .

References

1) Maheshwari, A. (2008). Human Reproduction. pp. 538–542. Text "Effect of female age on the diagnostic categories of infertility" ignored

2) Practice Committee of the American Society for Reproductive Medicine. Definitions of infertility and recurrent pregnancy loss. Fertil Steril 2008; 90:560.

3) GUTTMACHER AF. Factors affecting normal expectancy of conception. J Am Med Assoc 1956; 161:855.

4) Gnoth C, Godehardt D, Godehardt E, et al. Time to pregnancy: results of the German prospective study and impact on the management of infertility. Hum Reprod 2003; 18:1959.

5) http://www.who.int/reproductivehealth/topics/infertility/burden/en/index.html

6) National, Regional, and Global Trends in Infertility Prevalence Since 1990: A Systematic Analysis of 277 Health Surveys, Maya N. Mascarenhas et al plos medicine 2012

7) WHO Technical Report Series. Recent Advances in Medically Assisted Conception Number 820, 1992

8) World Health Organization. Towards more objectivity in diagnosis and management of male infertility. Int J Androl 1987; 7(Suppl):1.

9) www.uptodate.com female infertility causes

10) World Health Organization classification of anovulation

11) Pathophysiology of pelvic adhesions. Modern trends in preventing infertility. Drollette CM, Badawy SZ. J Reprod Med. 1992 Feb;37(2):107-21;

12) Pritts EA. Fibroids and infertility: a systematic review of the evidence. Obstet Gynecol Surv 2001; 56:483.

13) Homer HA, Li TC, Cooke ID. The septate uterus: a review of management and reproductive outcome. Fertil Steril 2000

14) Hatcher RA, Trussell J, Stewart F, Nelson AL, Cates W, Guest F, Kowal D. Contraceptive Technology. 18th Edition, 2004 Ardent Media Inc, New York

15) Embryo transfer in case of stenosed and/or forbidding cervix Ohl J et.al Gynecol Obstet Fertil. 2009

16) Centers for Disease Control and Prevention, American Society for Reproductive Medicine, Society for Assisted Reproductive Technology. 2006 Assisted Reproductive Technology Success Rates: National Summary and Fertility Clinic Reports, Atlanta: Centers for Disease Control and Prevention, 2008.

17) Cooper GS, Baird DD, Hulka BS, Weinberg CR, Savitz DA, Hughes CL Jr. Follicle-stimulating hormone concentrations in relation to active and passive smoking. Obstet Gynecol 1995;85:407–411

18) Dechanet C, Anahory T, Mathieu Daude JC, Quantin X, Reyftmann L, Hamamah S, Hedon B, Dechaud H (2011). "Effects of cigarette smoking on reproduction". Hum. Reprod. Update 17 (1): 76–95.

19) Regulated fertility services: a commissioning aid - June 2009, from the Department of Health UK

20) Nelson LR, Bulun SE (September 2001). "Estrogen production and action". J. Am. Acad. Dermatol. 45 (3 Suppl): S116–24

21) Chemotherapy and fertility Zeev Blumenfeld et.al Best Practice & Research Clinical Obstetrics & Gynaecology June 2012

22) Fauser, B. C. J. M.; Diedrich, K.; Bouchard, P.; Dominguez, F.; Matzuk, M.; Franks, S.; Hamamah, S.; Simon, C. et al. (2011). "Contemporary genetic technologies and female reproduction". Human Reproduction Update 17 (6): 829–847.

23) Azziz R, Woods KS, Reyna R, et al. The prevalence and features of the polycystic ovary syndrome in an unselected population. J Clin Endocrinol Metab 2004; 89:2745.

24) Stein, IF. Amenorrhea associated with bilateral polycystic ovaries. Am J Obstet Gynecol 1935; 29:181.

25) Mortensen M, Ehrmann DA, Littlejohn E, Rosenfield RL. Asymptomatic volunteers with a polycystic ovary are a functionally distinct but heterogeneous population. J Clin Endocrinol Metab 2009; 94:1579.

26) Taylor AE, McCourt B, Martin KA, et al. Determinants of abnormal gonadotropin secretion in clinically defined women with polycystic ovary syndrome. J Clin Endocrinol Metab 1997; 82:2248.

27) Venturoli S, Porcu E, Fabri R, et al. Longitudinal evalua- tions of different gonadotropin pulsatile patterns in anovulatory cycles of young girls. J Clin Endocrinol Metab 1992; 74:836–841.

28) Zumoff B, Freeman R, Coupey S, Saenger P, Markowitz M, Kream J. A chronobiologic abnormality in luteinizing hor- mone secretion in teenage girls with the polycystic ovary syndrome. N Engl J Med 1983; 309:1206–1209.

29) POLYCYSTIC OVARY SYNDROME (PCOS) pathogenesis and treatment review CLEVELAND CLINIC JOURNAL OF MEDICINE VOLUME 70 • NUMBER 1 JANUARY 2003. 31.

30) Hirshfeld-Cytron J, Barnes RB, Ehrmann DA, et al. Characterization of functionally typical and atypical types of polycystic ovary syndrome. J Clin Endocrinol Metab 2009; 94:1587.

31) Coviello AD, Legro RS, Dunaif A. Adolescent girls with polycystic ovary syndrome have an increased risk of the metabolic syndrome associated with increasing androgen levels independent of obesity and insulin resistance. J Clin Endocrinol Metab 2006; 91:492.

32) Ehrmann DA, Barnes RB, Rosenfield RL, et al. Prevalence of impaired glucose tolerance and diabetes in women with polycystic ovary syndrome. Diabetes Care 1999; 22:141.

33)Flier JS, Moller DE, Moses AC, et al. Insulin-mediated pseudoacromegaly: clinical and biochemical characterization of a syndrome of selective insulin resistance. J Clin Endocrinol Metab 1993; 76:1533.

34)Cancer risk and PCOS. Dumesic DA, et.al review Steroids. 2013

35)Martin KA, Chang RJ, Ehrmann DA, et al. Evaluation and treatment of hirsutism in premenopausal women: an endocrine society clinical practice guideline. J Clin Endocrinol Metab 2008; 93:1105.

36)Bates GW, Whitworth NS. Effect of body weight reduction on plasma androgens in obese, infertile women. Fertil Steril 1982; 38:406.

37)Courtesy of Robert L Barbieri, MD.

38)Markham SM, Carpenter SE, Rock JA. Extrapelvic endometriosis. Obstet Gynecol Clin North Am. Mar 1989;16(1):193-219. [Medline].

39)Jubanyik KJ, Comite F. Extrapelvic endometriosis. Obstet Gynecol Clin North Am. Jun 1997;24(2):411-40

40)Fauser, B. C. J. M.; Diedrich, K.; Bouchard, P.; Dominguez, F.; Matzuk, M.; Franks, S.; Hamamah, S.; Simon, C. et al (2011). "Contemporary genetic technologies and female reproduction". Human Reproduction Update 17 (6): 829–847.

41)Sampson JA. 1927. Metastatic or embolic endometriosis, due to the menstrual dissemination of endometrial tissue into the venous circulation. Am. J. Pathol. 3:93–110

42)Halme J, Hammond MG, Hulka JF, Raj SG, Talbert LM. 1984. Retrograde menstruation in healthy women and in patients with endometriosis. Obstet. Gynecol. 64:151–54

43) Bulletti C, DeZiegler D, Stefanetti M, Cicinelli E, Pelosi E, Flamigni C. 2001. Endometriosis: absence of recurrence in patients after endometrial ablation. Hum. Reprod. 16:2676–79

44)Speroff L, Glass RH, Kase NG (1999). Clinical Gynecologic Endocrinology and Infertility (6th ed.). Lippincott Willimas Wilkins. p. 1057

45) Cahill DJ. What is the optimal medical management of infertility and minor endometriosis? Analysis and future prospects. Hum Reprod 2002; 17:1135.

46) Gonadotrophin-releasing hormone antagonists for assisted conception: a Cochrane Review Hughes et al Reproductive BioMedicine 2007

47) HUGHES, E.G. et al. 1993. A quantitative overview of con- trolled trials in endometriosis-associated infertility. Fertil. Steril. 59: 963–970.

48) Collins JA, Crosignani PG. Unexplained infertility: a review of diagnosis, prognosis, treatment efficacy and management. Int J Gynaecol Obstet 1992; 39:267.

49) www.uptodate.com unexplained infertility

50) Blacker CM, Ginsburg KA, Leach RE, et al. Unexplained infertility: evaluation of the luteal phase; results of the National Center for Infertility Research at Michigan. Fertil Steril 1997; 67:437.

51) Guzick DS, Carson SA, Coutifaris C, et al. Efficacy of superovulation and intrauterine insemination in the treatment of infertility. National Cooperative Reproductive Medicine Network. N Engl J Med 1999; 340:177.

52) Pandian Z, Bhattacharya S, Vale L, Templeton A. In vitro fertilisation for unexplained subfertility. Cochrane Database Syst Rev 2005; :CD003357.

53) The Practice Committee of the American Society for Reproductive Medicine. Effectiveness and treatment for unexplained infertility. Fertil Steril. 2006;86(5 suppl):S111-S114.

54) Barbieri RL. The initial fertility consultation: rec- ommendations concerning cigarette smoking, body mass index, and alcohol and caffeine con- sumption. Am J Obstet Gynecol. 2001;185:1168- 1173.

55) Guzick DS, Carson SA, Coutifaris C, et al. Effi- cacy of superovulation and intrauterine insemi- nation in the treatment of infertility. National Cooperative Reproductive Medicine Network. N Engl J Med. 1999;340:177-183.

56)Diagnosis and Treatment of Unexplained Infertility Alexander Quaas et.al review REVIEWS IN OBSTETRICS & GYNECOLOGY 2008

57)Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M: Immunological self-tolerance maintained by activated T-cells expressing IL-2 receptor alpha- chains (CD25). Breakdown of a single mechanism of self-tolerance causes various auto-immune diseases. J Immunol 1995; 155:1151–1164.

58) Kingsley CL, Karim M, Bushell AR, Wood K: CD25+CD4+ regulatory T cells prevent graft rejection: CTLA4- and IL-10-dependent immunoregulation of alloresponses. J Immunol 2002; 168:1080–1086.

59)A physiological role for inducible FOXP3^{+} TREG cells^{B} Lessons from women with reproductive failure. Lourdes Arruvito et.al Clinical Immunology 2010

60)Medawar PB: Some immunological and endocrinological problems raised by the evolution of viviparity in vertebrates. Symp Soc Exp Biol 1953; 7:320–338.

61)Somerset DA, Zheng Y, Kilby MD, Sansom DM, Drayson MT: Normal human pregnancy is associated with an elevation in the immune suppressive CD25+ CD4+ regulatory T-cell subset. Immunology 2004; 112:38–43.

62) Mjo¨sberg J, Svensson J, Johansson E, Hellstro¨m L, Casas R, Jenmalm MC, Boij R, Matthiesen L, Jo¨nsson JI, Berg G, Ernerudh J: Systemic reduction of functionally suppressive CD4dimCD25highFoxp3+ Tregs in human second trimester pregnancy is induced by progesterone and 17beta-estradiol. J Immunol 2009; 183:759–769.

63)Mjo¨sberg J, Berg G, Ernerudh J, Ekerfelt C: CD4+ CD25+ regulatory T cells in human pregnancy: development of a Treg-MLC-ELISPOT suppression assay and indications of paternal specific Tregs. Immunology 2007; 120:456–466.

64) Tilburgs T, Roelen DL, van der Mast BJ, de Groot-Swings GM, Kleijburg C, Scherjon SA, Claas FH: Evidence for a selective migration of fetus-specific CD4+CD25bright regulatory T cells from the peripheral blood to the decidua in human pregnancy. J Immunol 2008; 180:5737–5745.

65)Jasper MJ, Tremellen KP, Robertson SA: Primary unexplained infertility is associated with reduced expression of the T-regulatory cell transcription factor Foxp3 in endometrial

tissue. Mol Hum Reprod 2006; 12:301–308.

66) The $CD4^{+}$ $CD25^{bright}$ regulatory T cells and CTLA-4 expression in peripheral and decidual lymphocytes are down-regulated in human miscarriage Li-Ping Jin et.al Clinical Immunology (2010)

67) $CD4^{1}$ $CD25^{1}$ $FOXP3^{1}$ regulatory T cells in peripheral blood and peritoneal fluid of patients with endometriosis Joanna Olkowska-Truchanowicz et.al Hum. Reprod. Advance Access published September 27, 2012

68) Regulatory T cells and other leukocytes in the pathogenesis of endometriosis M. Berbic et. al Journal of Reproductive Immunology 2011

69) Arruvito, L., Sanz, M., Banham, A.H., Fainboim, L., 2007. Expansion of cd4+cd25+and FOXP3+ regulatory t cells during the follicular phase of the menstrual cycle: implications for human reproduction. J. Immunol. 178, 2572–2578.

70) $CD4^{+}$ $CD25^{+}$ $CD62^{+}$ T-Regulatory Cell Subset Has Optimal Suppressive and Proliferative Potential Fu.Yopp et.al American journal of transpatation 2004

71) Women with Multiple Implantation Failures and Recurrent Pregnancy Losses have Increased Peripheral Blood T Cell Activation Kwang Moon Yang et.al American Journal of Reproductive Immunology 63 (2010)