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# Tumor necrosis factor alpha and milk fat globule-epidermal growth factor 8: Novel biomarkers to predict implantation failure and pregnancy loss

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## ABSTRACT

### Objective

To determinate whether implantation failure (IF) and recurrent pregnancy loss (RPL) can be predicted in serum prior to in vitro fertilization (IVF)?.

### Design

Multicentric prospective controlled pilot clinical study from January 2016 to January 2020.

### Material and methods

Thirty women ages 21-35 years were recruited from 3 groups: fertile controls (C), unexplained IF (following 3 failed good quality embryo transfers), and RPL (at least 2 unexplained first trimester miscarriages) in their natural cycle in which serum tumor necrosis factor (TNFα) and milk fat globule-epidermal growth factor 8 (MFG-E8) estradiol (E2) and progesterone (P4) levels were quantified in the early proliferative (cycle day 2) and secretory phases (urinary luteinizing hormone (LH)+7 days). Additionally, an endometrial biopsy was obtained on urinary LH+7 days for MFG-E8 and TNF α protein and gene expression analysis.

### Results

Ten women were assigned to each group. No statistical differences were found in age, body mass index, antimullerian hormone, baseline follicle stimulating hormone and baseline antral follicle count among cohorts. Mean serum E2 and P4 levels were similar among all groups in both the proliferative and secretory phases: E2 proliferative (C 69.19±26.64 pg/ml, IF 64.19±32.56 pg/ml, RPL proliferative 57.44±38.51; p= 0.55), E2 secretory (C 164.10±52.57 pg/ml, IF 172.57±121, RPL 173.81±.97.35; p=0.25), P4 proliferative (C 0.45±0.15 ng/ml, IF 0.45±0.19 ng/ml, RPL 0.53±0.18 ng/ml; p=0.85), P4 secretory (C 7.42±4.06 ng/ml, IF 7.8±4.56 ng/ml, RPL 8.05±4.38 ng/ml; p= 0.74). Mean serum TNFα levels were significantly higher in both, the proliferative and secretory phases for the RPL group (proliferative RPL 9.98±4.47 pg/ml, IF 4.73±2.56 pg/ml, C 3.42±1.01 pg/ml; p=0.001 vs secretory RPL 8.67±4.45 pg/ml, C 3.35±0.94 pg/ml, IF 3.85±1.01 pg/ml; p= 0.03). Mean serum MFG-E8 levels were significantly higher in the IF group during the proliferative phase (IF 373±201 pg/ml, RPL 201±115 pg/ml, C 225.58±109.73pg/ml; p=0.03), but not in the secretory phase (IF 237±101 pg/ml, RPL 189±116 pg/ml, C 199.41±112.43 pg/ml; p=0.15). Endometrial MFG-E8 mRNA levels were significantly lower in the IF and RPL group compared to C (p=0.03). TNFα mRNA levels were not statistically significant among groups (p=0.12).

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**Conclusions**

TNFα and MFG-E8 serum levels may serve as serum markers to predict IF and RPL.

**KEYWORDS**

Tumor necrosis factor (TNFα) and milk fat globule-epidermal growth factor 8 (MFG-E8), implantation failure (IF), recurrent pregnancy loss (RPL).

## MANUSCRIPT

### Introduction

Successful implantation is dependent on both endometrial receptivity and the development of good quality embryos with implantation potential[1]. The uterus plays an indispensable role in the initiation and termination of the pregnancy. As host to the embryo, it is crucial to maintain a homeostatic relationship between the endometrium and the embryo. Endometrial receptivity in humans can be defined as a temporal maturation of the epithelium (primed by progesterone and estrogen) during which the trophectoderm attaches and invades the stroma[1]. Several cellular, hormonal and molecular pathways are involved in this orchestra. A synchronous embryo and endometrial development are indispensable[1-3].

Implantation begins when the trophectoderm cells contact the uterine wall, also known as apposition (the first stage of implantation). This stage is followed by adhesion in which the contact of the trophectoderm with the uterine epithelium increases. Finally, in the third stage, the syncytiotrophoblast and cytotrophoblasts penetrate and invade the vasculature and myometrium. In response to the implanted embryo, the uterine stroma undergoes decidualization[4]. Little is known as to the cellular and molecular changes that define the window of implantation of the human endometrium. Understanding the molecular events underlying the development and maintenance of a receptive endometrium is fundamental if we are to further improve the success of embryo implantation during in vitro fertilization (IVF) therapy.

In normal pregnancy, the trophoblast invades the endometrial layers releasing soluble mediators (such as tumor necrosis factor alpha, TNFα) into the maternal circulation, leading to a low-level physiological inflammatory response that is a characteristic feature of normal trophoblast adhesion and controlled embryonic invasion. On the other hand, exaggerated inflammation due to excessive levels of TNFα has been associated with clinical miscarriages and an up-regulation of inflammatory factors, such as interleukins (IL) 10, IL-8, IL-6. A disrupt equilibrium in these factors may account for the failure in implantation (IF)[5,6].

The endometrial development is controlled by sex steroids, which regulate the secretion of growth factors and cytokines and the establishment of the window of implantation. Among these factors, a novel gene/protein, milk fat globule-epidermal growth factor 8 (MFG-E8). In extra-uterine tissues, this secreted protein has been reported to have functions in apoptosis control, neovascularization, cell remodeling, and immunomodulation. Recent studies have shown that MGF-E8 is up-regulated over 2-fold during the receptive phase in the endometrium[7,8]. Also, it is highly expressed in human chorionic villi at all trimesters of gestation and it is up regulated in vitro by Human chorionic gonadotrophin (hCG )of trophoblast origin[9]. MFG-E8 and its integrin receptor participate in trophoblast adhesion in an in vitro model of human implantation[10,11]. Furthermore, it has been demonstrated that endometrial MFG-E8 gene expression, is significantly up-regulated by TNFα[12]. Also, MFG-E8 protein secretion has been associated with microvesicles (MV) from human endometrial epithelial cells and demonstrated that TNFα significantly up-regulated MFG-E8 expression in the secreted MV[13].

These in vitro data therefore strongly suggest that MFG-E8, either soluble and/or in MV, can be used as a detectable biomarker from serum under excessive inflammatory conditions. However, no studies published to date address the possible association between MFG-E8 excess or deficiency and IF, or its relationship to TNFα secretion.

We hypothesize that TNFα and MFG-E8 cooperatively maintain the integrity of the normal endometrium, and that in patients with IF, or recurrent pregnancy loss (RPL) of unexplained origin, excessive TNFα increases the maternal shedding of MFG-E8, disrupting the normal protective effect of this protein, resulting in damage of the endometrial epithelium and impairing trophoblast invasion. We propose that TNFα is up-regulated in serum of women with implantation defects, and this causes perturbation of MFG-E8 secretion. The basis for this hypothesis is found by precedent in human tissue and murine models as well as by way of our largely unpublished preliminary data.

## Materials and Methods

*Study design and patient populations*

This is a multicenter, prospective controlled clinical pilot study, from December 2015 through January 2020 included three groups of patients between 18 and 35 years of age. Fertile controls (C): women who participated in the donor egg program as egg donors with regular menstrual cycles, previously confirmed ovulation, and who were of proven fertility (n=10), patients with unexplained IF: patients who have failed implantation following 2 or more frozen embryo transfers of good quality blastocysts (n=10), and patients with recurrent unexplained first trimester miscarriages: at least 2 consecutive miscarriages under 10 weeks of gestation of unexplained origin, after spontaneous or IVF conceptions (n=10).

Patients with history of uterine surgery, abnormal uterine cavity (fibroids, endometrial polyps, adhesions, adenomyosis and congenital uterine abnormalities), hydrosalpinx, diminished ovarian reserve, harboring chromosomal rearrangements, thrombophilia, or autoimmune diseases were excluded.

*Intervention*

Participants were asked to come in their natural cycle in which serum MFG-E8, TNFα, estradiol (E2) and progesterone (P4) levels were quantified in the early proliferative (cycle day 2) and secretory phases (urinary LH+7 days). Additionally, an endometrial biopsy was obtained on urinary LH+7 days for MFG-E8 and TNF α protein and gene expression analysis. A clinician performed the endometrial biopsy procedures using a pipelle, a plastic biopsy catheter approximately 3 mm in diameter (e.g., Pipelle de Cornier, Laboratoire CCD, France). Participants were advised to attend with a full bladder and to take pain medication before the procedure, according to clinic protocols. The procedure was carried out as described previously[14]. If it was not possible to insert the pipelle into the uterus, a tenaculum, local anesthetic, and cervical dilatation were permitted. All women provided written informed consent.

The following kits were used: ELISA Kit for Milk Fat Globule EGF Factor 8 from USCN Life Science Inc, with the cat. No. E91286Hu-96 tests, and TNFα, Life Technology Inc., No. KHC3011 to measure MFG-E8 and TNFα respectively. A preliminary study was performed under IRB approval (EVMS IRB# 14-05-WC-0078) to validate the technical usefulness of these commercial kits. Immulite Immunoassay System (Siemens, NY) was used to measure estradiol and progesterone. Manufacturer's recommendations were followed to perform the tests. Positive and negative controls as well as serial dilutions were tested. A standard curve was created by plotting the mean optical densities (OD). Samples were run in duplicates. Endometrial biopsies were placed in sterile normal saline and immediately processed for mRNA (quantitative RT-PCR) and protein extraction (Western blot) for MFG-E8 and TNFα, in order to correlate their levels of endometrial expression with the serum levels of these biomarkers.

*Outcome measures*

The primary outcome was serum and endometrial MFG-E8 and TNFα. Secondary outcome measures included serum estradiol and progesterone levels.

*Statistical analysis*

Continuous data was reported as mean+-SD with Clopper-Pearson binomial 95% confidence intervals (95% CI). Groups were compared using ANOVA for continuous normally distributed data and Kruskal-Wallis when the conditions of normality were not met. Categorical data was analyzed using Fisher exact or Chi squared tests as appropriate. Statistical analyses were performed using SAS version 9.4 (SAS institute Inc., Cary, NC, USA). All p-values were two-sided and were considered significant if less than 0.05.

*Regulatory approval*

This study was approved by EVMS IRB committee (#15-01-FB-004).

## RESULTS

Ten women were assigned into each group. Patient demographic and cycle characteristics are described in **Table 1**. No statistical differences were found in age, body mass index (BMI), antimullerian hormone (AMH), baseline follicle stimulating hormone (FSH) among cohorts.

Mean serum E2 and P4 levels were similar among all groups in both the proliferative and secretory phases: E2 proliferative (C 69.19±26.64 pg/ml, IF 64.19±32.56 pg/ml, RPL proliferative 57.44±38.51; p= 0.55), E2 secretory (C 164.10±52.57 pg/ml, IF 172.57±121, RPL 173.81±.97.35; p=0.25), P4 proliferative (C 0.45±0.15 ng/ml, IF 0.45±0.19 ng/ml, RPL 0.53±0.18 ng/ml; p=0.85), P4 secretory (C 7.42±4.06 ng/ml, IF 7.8±4.56 ng/ml, RPL 8.05±4.38 ng/ml; p= 0.74).

Mean serum TNFα levels were significantly higher in both, the proliferative and secretory phases for the RPL group (proliferative RPL 9.98±4.47 pg/ml, IF 4.73±2.56 pg/ml, C 3.42±1.01 pg/ml; p=0.001 vs secretory RPL 8.67±4.45 pg/ml, C 3.35±0.94 pg/ml, IF 3.85±1.01 pg/ml; p= 0.03).

Mean serum MFG-E8 levels were significantly higher in the IF group during the proliferative phase (IF 373±201 pg/ml, RPL 201±115 pg/ml, C 225.58±109.73pg/ml; p=0.03), but not in the secretory phase (IF 237±101 pg/ml, RPL 189±116 pg/ml, C 199.41±112.43 pg/ml; p=0.15).

| | Controls | | Implantation failure | | Recurrent pregnancy loss | | P value |
|---|---|---|---|---|---|---|---|
| | N=10 | | N=10 | | N=10 | | |
| | Mean | SD | Mean | SD | Mean | SD | |
| **Patient age (years)** | 26.8 | 4.1 | 27.2 | 6.1 | 27.4 | 4.8 | 0.44 |
| **BMI (kg/m$^2$)** | 21.6 | 4.2 | 22.9 | 2.3 | 22 | 3.5 | 0.53 |
| **Baseline FSH (IU/mL)** | 6.4 | 3.1 | 6.7 | 4.2 | 6.5 | 4.8 | 0.68 |
| **Antimullerian hormone (ng/ml)** | 3.1 | 2.4 | 3.2 | 2.3 | 3.2 | 3 | 0.35 |
| **Baseline Antral Follicle count** | 16.3 | 5.1 | 14.7 | 5 | 15.8 | 4.8 | 0.70 |

Demographic characteristics among cohorts

**Table 1**

**Note**: Data presented as mean, percentages and standard deviation, unless stated otherwise.

**Abbreviations**: BMI, body mass index; FSH, follicle-stimulating hormone. Significance established at p < .05.

Endometrial MFG-E8 mRNA levels were significantly lower in the IF and RPL group compared to C (p=0.03). TNFα mRNA levels were not statistically significant among groups (p=0.12).

## DISCUSSION

To our knowledge, this is the first study to determine whether IF and RPL can be predicted in serum prior IVF by measuring MFG-E8 and TNFα. In a normal pregnancy, mediators such as TNFα are released creating a physiological inflammatory response. However, an exaggerated release of TNFα has been associated with IF and recurrent pregnancy loss (RPL). Recent studies demonstrated that TNFα up-regulates the expression of inflammatory factors such as MFG-E8[11]. MFG-E8 is known to modulate implantation by acting at various levels of the trophoblast and endometrial compartments[12]. Hence an overexpression of this protein may result in apoptosis, endometrial damage, and impaired implantation. Our results showed that mean serum MFG-E8 levels were significantly higher in the IF group during the proliferative phase but not in the secretory phase and that.

TNF-α, an important pro-inflammatory and pro-apoptotic cytokine, may have both physiological and pathological roles in endometrial homeostasis. Numerous studies have shown that TNF-α induces cells to undergo apoptosis and have suggested that local TNF-α production is critically involved in placental trophoblast invasion[15]. During normal implantation, the inflammation caused by TNF-α may improve embryo implantation and assist with endometrial repair in response to injury[16,17]. However, TNF-α levels could be deregulated in certain pathological conditions, such as when maternal and fetal vascular perfusion are reduced. Our results demonstrated that mean serum TNFα levels were significantly higher in both the proliferative and secretory phases for the RPL group.

Serum estradiol levels have not been reported to discriminate between fertile and infertile patients. Our results are in accordance with these findings showing that serum estradiol levels did not significantly differ between groups. And as expected, mean serum progesterone levels were significantly higher in the secretory phase compared to the proliferative phase in all groups.

This new concept could lead to the discovery of novel mechanisms and holds strong potential for diagnostic and therapeutic alternatives. The findings may have a significant clinical impact, providing the basis for the potential therapeutic use of MFG-E8 and TNFα antagonists[18-21]. Recent studies have shown that MFG-E8 offers therapeutic benefits by mitigating inflammation and tissue injury after hemorrhagic stroke and aiding in the healing of injured intestinal mucosa[21,22]. Additionally, TNFα inhibitors have been demonstrated to significantly increase IVF success rates in infertile patients. By understanding the physiology and pathophysiology underlying implantation, we can continue to develop innovative research ideas to improve IVF outcomes and prevent IF and RPL.

## CONCLUSION

These novels differentially expressed serum and endometrial markers may provide information on the physiology of implantation and could generate the basis for non-invasive diagnostic tools and therapeutic use of MFG-E8/TNFα antagonists in women with IF and RPL.

## FUNDING

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## CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

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