Study of a protein based angiogenic profile in endometrial tissue of women undergoing assisted reproductive techniques - A pilot study

Background: Infertility is a common condition nowadays affecting about 10 to 15 percent of reproductive-aged couples. It is defined as the failure to achieve pregnancy after 12 months or more of regular unprotected intercourses. Infertility treatment is a complex process influenced by numerous factors. Recent advances in assisted reproduction techniques (ART) have provided effective treatment for infertile couples. A large number of independed factors seem to be associated with the success outcome of ART. The aim of this study is to detect the role of angiogenenic factors in endometrial tissue of women who underwent ART.

Methods: Eight samples of endometrial tissue from women who underwent ovarian stimulation were analyzed using the Proteome ProfilerTM Human Angiogenesis Array Kit, screening for the presence of 55 soluble angiogenesis-related factors. For the analysis of array's results, PCA procedure and Mann-Whitney U test ware used.

Results: A protein profile based on the expression of a subset of 7 factors could separate the 8 women in 2 groups. Among the group of the 4 patients with the higher expression of the 7 combined factors, 2 pregnancies were observed, while in the other group with the lower expression only a biochemical pregnancy was observed. These findings showed that the expression of angiogenic factors is strongly collerated with pregnancy.

Conclusion: In conclusion, we developed an "angiogenic profile" for patients who underwent ovarian stimulation, based on the combination of 7 angiogenic factors, which can be used, after appropriate validation, as a prognostic marker for ART outcome.

Keywords: Angiogenesis, endometrial, fertility, profile, prognostic, arrays.

Sofia Paraskevi Trachana[1], Eleftherios Pilalis[2], Depy Mavrogianni[1], Georgios Patrikios[1], Manousos Makridakis[3], Aristotelis Chatziioannou[2], Peter Drakakis[1] and Dimitrios Loutradis[1]

[1]First Department of Obstetrics and Gynecology, Medical School, National and Kapodistrian University of Athens, Alexandra General Hospital, Athens, Greece

[2]Enios Applications P.C., Al. Pantou 25, 17671 Kallithea, Greece

[3]Biotechnology Division, Biomedical Research Foundation, Academy of Athens (BRFAA), Athens, Greece

**Author for correspondence:*

sp.voula@yahoo.com

Introduction

Usually deficiency in reproductive system is known as a disease called infertility, and has a negative concept for the vast majority of people. Infertility is a common condition in our days affecting about 10 to 15 percent of reproductive-aged couples [1-4]. It is defined as the failure to achieve a successful pregnancy after 12 months or more of regular and unprotected intercourses [5]. Although the prevalence of infertility is believed to have remained relatively stable during the past 40 years, the demand for infertility evaluation and treatment has increased [6]. Infertility may be caused by male or female factors or both of them. Usually, after 24 months with appropriate treatment 5-10% of couples achieve pregnancy. Sometimes no specific reason can be found for the patients and the treatment has no successful result. Fortunately, recent advances in assisted reproduction techniques (ART) have provided effective tools for diagnosis and treatment of infertile couples. ART has been used for more than 20 years, reporting an increasing number of cycles treated and an increasing pregnancy rate [7].

Although a number of genetic, immunologic, infection, endocrine and other factors affect fertility, identifying the exact cause of infertility is very important. A large number of independent factors seem to be associated with the success outcome of ART. The crucial role of implantation as a major factor of ART failure has been highlighted in many studies. Synchronization of the availability of good quality oocytes and adequate endometrial maturation are very important for successful implantation [8]. Due to the fact that endometrial maturation varies considerably in each patient, an adequate endometrial

maturation and improved uterine receptivity seem to be the reason for improved pregnancy rates [9]. Characteristics of endometrial tissue such as thickness, volume, blood flow and vessel's architecture, have been associated with its receptivity [10], while other studies support the role of oxytocin receptors in the quality of endometrium, due to the potential interaction of oxytocin receptors with the hormones included in the ovulation induction regiments [11].

Several clinical studies suggest the importance of implantation in a narrow window of uterine receptivity, between 6 and 8 days after ovulation. This brief and precise period, called "implantation window", lasts less than 48 h and coincides with the formation of large and smooth projections, called "pinopodes", on the apical membranes of the endometrial epithelial cells [12]. Fully developed pinopodes existed for 1 day only which may correspond to the short period of optimal endometrial receptivity, and may suggest the optimal date for successful embryo transfer in ART patients [13]. Finally, other studies have mentioned the role of endometrial secretion cytokines in IVF (in vitro fertilization). Factors, such as tumor necrosis (TNF-a), interleukins (IL-1β, IL-6, IL-4), interferon (IFN-γ) and monocyte chemoattractant protein (MCP) in high concentration, are associated with recurrent implantation failure [14,15].

In recent years many studies have focused on the research of new prognostic and predictive factors for the effectiveness of ART. The major role of angiogenesis in embryo implantation and thus, probably in the maintenance of gestation, is under consideration. Implantation and development of a human embryo requires an increased level of angiogenesis. Various growth factors have been associated with placental angiogenesis and embryonic development. Angiogenic factors like Endothelin, Angiogenin, VEGF, and others, have been associated with success outcomes of ART [16].

The aim of the present study was to detect the expression of 55 angiogenic factors in the endometrial tissue of women who underwent ovarian stimulation for ART. We attempted to identify an "angiogenic profile" based on 7 out of 55 angiogenic factors which seems to be associated with success outcomes of ART.

Methods

■ Patients

Eight (8) women undergoing ovarian stimulation for Assisted Reproduction Techniques, participated in this observational, single-institution study. All participants met the criteria mentioned in **(TABLE 1)**. Endometrial tissue was prospectively collected but retrospectively analyzed. All patients were treated at the 1st Dept of Obstetrics and Gynecology – Subunit of Reproductive Endocrinology and Infertility (University of Athens, Alexandra General Hospital, Athens, Greece). The ovarian induction protocols, was as in present studies described [8,9].

The study protocol had met appropriate Institutional Review Board approval (1st Department of Obstetrics and Gynecology, Alexandra General Hospital, Athens, Greece) and written informed Rconsent was given by all subjects for the collection and study of the endometrial tissue. The study was conducted according to the principles expressed in the declaration of Helsinki.

■ Endometrial tissue collection

Patients' endometrial tissue was collected with the technique of "pipelle biopsy" on the day of oocyte retrieval. All patients previously underwent ovarian stimulation, while all attempts with natural cycle were excluded.

■ Protein extraction from tissue

According to the protocol [17], each tissue was stored and cooled successively to -20 º C for 1-2 hours and then transferred to -80 º C where it remained until it was to be used. The frozen tissue was first weighed, diced into pieces and further homogenized with mechanical bleder. After being thawed in RIPA buffer (3 ml of pre-cold RIPA buffer per gram of tissue), containing Protease Inhibitor and Phosphatase Inhibitor it was incubated on ice for 30 minutes. Finally, it was transferred to microcentrifuge tubes and was submitted in consecutive centrifugations (2 times, in 10000g and 4ºC, for 10 min each time) to obtain the product for analysis.

■ Array based detection of angiogenic factors

The Proteome Profiler™ Human Angiogenesis Array Kit, (RnD Systems, USA

Supplementary TABLE 1: Supplementary TABLE 1: Protein array data for all the 55 factors.

			patients					
Protein Name	**u781**	**u782**	**u783**	**u784**	**u925**	**u926**	**u927**	**u928**
Positive Control	390.42	396.00	398.85	350.06	357.30	383.93	363.27	348.36
Activin A	0.38	0.27	0.72	1.15	-0.15	0.88	0.01	0.34
ADAMTS-1	1.11	0.73	9.54	2.85	20.90	6.02	0.86	0.71
Angiogenin	195.93	345.58	428.90	412.43	395.53	463.00	361.27	329.17
Angiopoietin - 1	6.44	2.14	31.91	29.84	78.24	87.75	2.75	1.05
Angiopoietin - 2	110.33	10.70	201.64	125.56	280.20	233.71	5.17	3.36
Angiostatin / Plasminogen	5.56	1.68	20.24	4.67	89.17	79.18	0.95	1.71
Amphiregulin	1.40	0.52	-3.10	2.11	12.72	8.61	0.01	0.22
Artemin	5.00	1.30	10.57	1.54	50.32	35.95	0.31	0.52
Positive Control	388.75	355.04	333.42	350.78	384.56	345.30	350.76	350.73
Coagulation Factor III	217.88	252.24	305.60	184.30	411.47	427.85	223.51	174.89
CXCL16	2.06	1.43	44.90	21.16	65.29	81.12	1.17	0.91
DPPIV	133.97	75.26	347.52	232.16	242.98	382.63	54.66	95.38
EGF	1.47	1.04	13.75	4.84	26.45	12.68	0.95	0.54
EG - VEGF	2.94	2.65	227.32	6.78	144.15	42.51	1.84	1.03
Endoglin	4.63	2.02	235.90	63.94	213.57	296.37	2.14	3.61
Endostatin / Collagen XVIII	111.73	84.66	228.70	110.41	272.49	266.86	50.00	36.91
Endothelin - 1	267.70	160.66	209.75	297.27	316.48	360.23	104.74	75.91
FGF acidic	4.91	1.90	22.86	41.73	231.87	381.12	1.71	0.76
FGF basic	5.14	36.59	263.76	259.01	245.16	193.81	35.70	283.39
FGF - 4	1.18	0.99	1.97	1.77	32.84	2.33	0.34	0.44
FGF - 7	1.60	0.81	53.25	32.00	83.00	2.71	0.92	1.40
GDNF	1.38	0.77	4.02	1.37	50.74	14.74	0.67	1.09
GM - CSF	1.20	1.07	13.66	5.15	42.34	23.24	1.26	0.68
HB - EGF	4.44	2.18	75.52	14.09	73.50	93.03	2.04	0.68
HGF	2.37	2.72	204.44	137.81	78.89	143.75	5.01	3.68
IGFBP - 1	24.88	2.60	150.01	68.86	173.70	140.88	1.88	1.30
IGFBP - 2	12.48	85.36	336.73	299.70	346.76	398.40	63.50	119.15
IGFBP - 3	54.20	27.50	202.47	218.86	315.16	423.10	45.03	8.43
IL - 1β	2.20	0.39	3.91	2.70	24.41	26.86	0.56	0.50
IL - 8	1.22	1.53	267.19	293.65	15.66	254.26	1.72	2.77
LAP (TGF - β1)	3.92	1.51	38.18	29.53	63.98	22.81	1.70	1.68
Leptin	1.22	0.76	3.74	2.90	33.88	3.35	0.35	0.49
MCP - 1	2.07	1.24	81.37	272.19	51.53	92.87	0.69	2.03
MIP - 1α	0.17	0.54	1.93	1.04	27.46	9.21	0.50	0.40
MMP-8	16.03	78.81	306.56	208.75	185.25	349.30	82.71	34.92
MMP-9	16.38	46.29	306.10	202.63	188.65	419.16	71.07	0.00
NRG1 - β1	2.58	2.40	84.07	51.15	50.55	80.41	2.08	0.63
Pentraxin 3 (PTX3)	1.83	0.56	49.21	13.98	42.42	59.53	0.52	0.60
PD-ECGF	37.81	3.83	111.64	20.01	160.23	176.90	2.20	2.97
PDGF-AA	2.28	0.78	-2.63	6.29	38.63	60.50	1.69	1.03
PDGF-AB/PDGF-BB	1.10	0.41	4.96	11.16	19.46	12.58	0.80	1.20
Persephin	54.88	4.14	36.78	14.09	125.63	141.21	1.88	1.58
Platelet Factor 4 (PF4)	275.98	239.73	359.32	346.85	365.87	354.10	220.00	233.78
P/GF	7.60	3.20	35.06	50.13	112.77	91.29	3.22	3.14
Prolactin	2.79	0.80	40.44	12.67	69.27	34.59	0.19	0.44
Serpin B5	0.39	0.86	1.31	1.79	26.00	4.40	0.42	0.47
serpin E1	6.39	28.44	299.88	242.24	180.69	81.50	28.42	85.66
Serpin F1	202.90	168.52	223.58	138.63	299.23	402.53	136.49	0.09
TIMP - 1	98.72	287.49	462.55	468.91	348.67	504.09	297.04	296.67
TIMP- 4	2.32	45.53	19.04	10.91	56.78	55.18	1.39	0.71
Thrombospondin - 1	2.57	0.99	27.24	28.06	66.78	136.47	0.40	0.32
Thrombospondin - 2	0.94	0.24	0.67	1.06	11.80	7.02	0.24	0.29
uPA	73.29	191.27	324.01	403.37	271.84	389.57	303.73	251.05

Vasohibin	2.43	0.63	0.64	0.79	27.53	12.53	0.49	0.45
VEGF	19.37	5.92	107.70	100.39	98.80	96.84	4.55	2.79
VEGF - C	0.78	0.28	0.07	0.53	16.78	1.71	0.01	0.14
Positive Control	288.81	316.94	335.71	367.14	326.12	338.75	353.96	336.67

TABLE 1: Inclusion criteria for the participating patients.

1	Age ≤ 45 years old.
2	Written informed consent should be signed.
3	Normal hormonal profile.
4	The patients should not have any genetic disorders.
5	Factors such as the protocol to be used for ovarian stimulation, and past attempts with ART, are not affect the participation of the patients in the study.

Catalog Number: ARRY 007) [18], was applied to screen for the presence of 55 soluble angiogenesis related factors present in the patients' endometrial tissue according to the manufacturer's instructions.

Briefly, fifty – five antibodies specific to relevant angiogenic factors (shown in **TABLE 2**) are attached onto a cellulose membrane fixed in duplicate.

One (1) ml of the sample is incubated with 500 μl of a relevant buffer and 15 microliters of a mixture of biotynilated antibodies for 1 hour. A special plate is provided where the kit's antibody coated membrane is also incubated for 1 hour with a blocking buffer in order to avoid subsequent non-specific binding of antibodies to proteins of interest. The blocking buffer is then removed and the membrane was incubated with the sample-antibody mixture (prepared in previous step) for 16 hours at 4 ° C. The following day, the mixture of supernatant-antibody-buffer is drained off the membrane which is subsequently washed 3 times with Wash Buffer for 10 minutes at a time and then the membrane is incubated with streptavidin-HRP for 30 minutes.

Another wash is performed and finally the membrane is incubated with a chemiluminescent detection reagent. Protein targets were detected by enhanced chemiluminescence.

This way the proteins associated with angiogenesis are detected, present in endometrial tissue and reflect an angiogenic profile for each patient **(FIGURE 1)**.

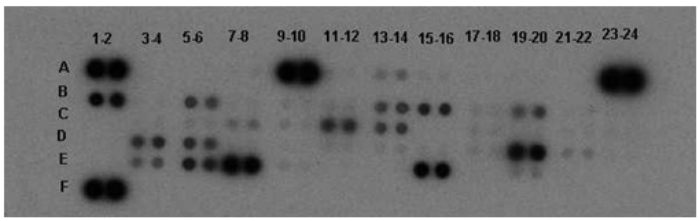
Films were scanned at a GS-800 imaging densitometer (Bio Rad) in transmission mode and the images were analyzed using the Quantity One 4.4.1 Software package (Bio Rad).

TABLE 2: The fifty five angiogenesis related factors used.

Protein Name		
Activin A	FGF-7	PDGF-AB/PDGF-BB
ADAMTS-1	GDNF	Persephin
Angiogenin	GM-CSF	Platelet Factor 4 (PF4)
Angiopoietin-1	HB-EGF	PlGF
Angiopoietin-2	HGF	Prolactin
Angiostatin/ Plasminogen	IGFBP-1	Serpin B5
Amphiregulin	IGFBP-2	Serpin E1
Artemin	IGFBP-3	Serpin F1
FGF-4	IL-1β	TIMP-1
Coagulation Factor III	IL-8	TIMP-4
CXCL16	LAP (TGF-β1)	Thrombospondin-1
DPPIV	Leptin	Thrombospondin-2
EGF	MCP-1	uPA
EG-VEGF	MIP-1α	Vasohibin
Endoglin	MMP-8	VEGF
Endostatin/ Collagen XVIII	MMP-9	VEGF -C
Endothelin-1	NRG1-β1	PDGF-AA
FGF acidic	Pentraxin 3 (PTX3)	
FGF basic	PD-ECGF	

■ Principal component analysis (PCA) and statistical analysis

For the analysis of array's results, PCA was performed. PCA is a statistical procedure that uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of linearly uncorrelated variables called principal components. The first principal component has the largest possible variance (that is, accounts for as much of the variability in the data as possible). For the statistical analysis, the non-parametric Mann-Whitney and Fisher's exact tests were used.



	1-2	3-4	5-6	7-8	9-10	11-12	13-14	15-16	17-18	19-20	21-22	23-24
A	POS		Activin A	ADAMTS-1	Angiogenin	Angiopoietin-1	Angiopoietin-2	Angiostatin /Plasminogen	Amphiregulin	Artemin		POS
B	Coagulation Factor III	CXCL16	DPPIV	EGF	EG-VEGF	Endoglin	Endostatin / Collagen XVIII	Endothelin-1	FGF-acidic	FGF-basic	FGF-4	FGF-7
C	GDNF	GM-CSF	HB-EGF	HGF	IGFBP-1	IGFBP-2	IGFBP-3	IL-1β	IL-8	LAP (TGF-β1)	Leptin	MCP-1
D	MIP-1α	MMP-8	MMP-9	NRG1-β1	PTX3	PD-ECGF	PDGF-AA	PDGF-AB/ PDGF-BB	Persephin	PF4	PIGF	Prolactin
E	Serpin B5	Serpin E1	Serpin F1	TIMP-1	TIMP-4	Thrombo-spondin 1	Thrombo-spondin 2	uPA	Vasohibin	VEGF	VEGF-C	
F	POS											NEG

FIGURE 1. Angiogenic profile using Protein Arrays.

Demonstration of the angiogenic profile determined in the endometrial tissue of a participant patient (u927), using the Proteome Profiler Angiogenesis Array kit. Each spot corresponds to an angiogenic factor shown in the Table below.

Results

■ Patients

From February 2014 to February 2015 samples of endometrial tissue from 8 patients who underwent ovarian stimulation, were obtained. Their characteristics are shown in **Table 3**. Six out of 8 women had female infertility factor while in 2 cases the factor was male. All patients received treatment for ovarian stimulation. No ovarian hyperstimulation was observed and no cycle was canceled for any reason.

TABLE 3: Baseline characteristics of patients.

Characteristics	N (%)
Age (median, range)	35,5 (29-43)
Years of infertility (median, age)	3,875 (2-8)
Number of previous attempts (median, range)	2,125 (2-7)
Hormonological profil (median, range)	
FSH (IU/L)	7,3 (3,2-13,99)
LH (IU/L)	5,54 (2,9-12,4)
E2 (pg/mL)	39,96 (13-63)
PRL (ng/mL)	17,3 (5,65-31,8)
TSH (mIU/L)	2,2 (1,26-4,27)
Protocols	
GnRH antagonist	4 (50%)
GnRH agonist	4 (50%)
Endometrial thickness on day of oocyte retrieval (median,range) mm	10,15 (7-12)
Pregnancy	
Pregnancy	2 (25%)
Biochemical pregnancy	1 (12,5%)
No pregnancy	5 (62,5%)
Extrauterine pregnancy	0

■ Results of Principal component analysis (PCA)

PCA was performed (R package FactoMineR,) [19] in order to evaluate the contribution of each sample to the variance of the dataset, as well as to inspect the correlation among samples. **FIGURE 2** shows the projection of samples to the first two principal components, which together account for 86.79% of the total variance. From the dataset of the 55 angiogenic factors, seven factors seem to be clearly distinguished from the others, due to their higher expression and their optimal combination **(TABLE 4)**. It is very important to mention that this method is mainly based on the results derived from the combination of the factors. For example, other factors have high expression too, but they are not included in the subset of the seven since their combination with the other factors is not optimal for separating the patients into groups.

All protein array data for the 55 factors are included in Supplementary **TABLE 1**. Based on the expression and the optimal combination of the 7 dominant factors, the samples form two groups with correlated expressions. This was confirmed by the image of **FIGURE 3**

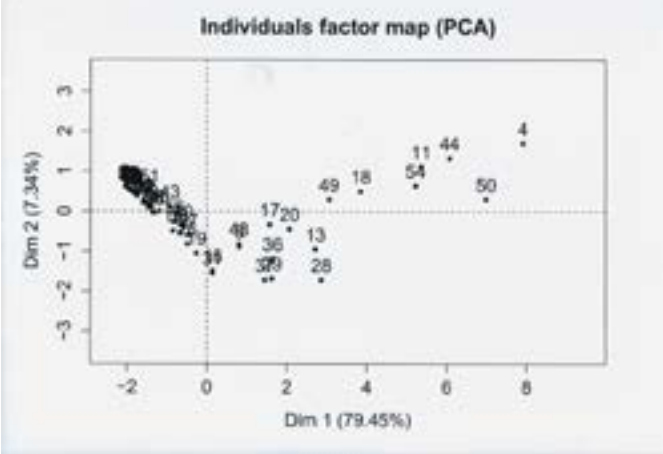


FIGURE 2: Principal component analysis.

Figure 2 depicts the expression of the 55 angiogenic factors and their projection to the first two principal components. Seven factors (number 4, 50, 44,11,54,18 and 49) (TABLE 4) seems to be distinguished from the others due to their higher expression.

TABLE 4: The subset of 7 factors distinguished and their corresponding pathways apart from angiogenesis

Number and Factor	Pathways
4= Angiogenin	cell proliferation related pathways
50= Coagulation Factor III	apoptosis signaling and thrombotic phenotype of cancer patients
44= Endothelin - 1	cell proliferation and apoptosis related pathways
11= Platelet Factor 4 (PF4)	Involved in cancer related thrombosis
54= Serpin F1	deterring cancer cell proliferation by inducing p53
18= TIMP - 1	cancer cell survival pathways
49 = uPA	suppress penetration of tumor cells in malignancies.

(euclidian distance, R package gplots) which shows that the dataset can be separated in two clusters (Cluster 1: u927, u782, u781, u928 and Cluster 2: u925, u926, u783, u784).

As it shows in **FIGURE 3**, the 8 patients are clearly separated into 2 subsets. The first subset includes patients u927, u782, u781, and u928. In this group one biochemical pregnancy was achieved in patient u782. The second cluster includes patients u925, u784, u926 and u783. In this subset, there were two pregnancies in patients u926 and u783.

The differences in the expression levels of the 55 factors among the patients are depicted in the HeatMap of **FIGURE 4**. For each patient, the expression level of each factor was compared to the median expression among all 8 patients.

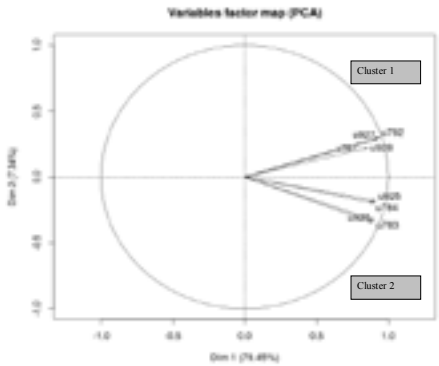


FIGURE 3: Separation of the 8 samples in two groups.

The separation of the 8 samples in two groups was based on the expression of the 7 dominant factors. Patients u927, u782, u781, and u928 are included in the first group, among them one biochemical pregnancy was achieved in patient u782. The second cluster includes Patients u925, u784, u926 and u783 are included in the second cluster in which two pregnancies in patients' u926 and u783 were achieved.

The visualization of the separation of the two groups is almost ideal since the two clusters are clearly separated and the highest expression level of the factors in the second cluster is obvious.

Statistical Analysis

The non-parametric Mann – Whitney U test, was used in order to estimate if there is any significant difference in angiogenic factors - both for all 55 factors (**TABLE 5a** – placed at the end of the document) and for the 7 dominant factors (**TABLE 5b** - placed at the end of the

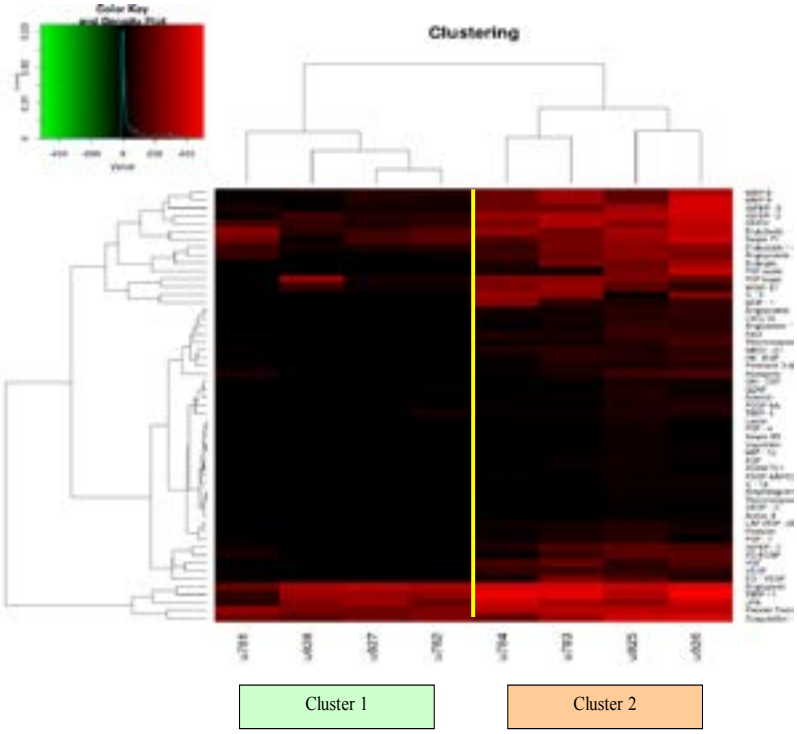


FIGURE 4: HeatMap of expression level of the 55 angiogenic factors, including the dominant 7. Relative expression levels of the 55 angiogenic factors. Expression values are displayed according to the colour scale, in which red expression above median expression and green represents below median expression. The two clusters are clearly separated. In the second cluster the expression level is obviously higher than in the first cluster. Intrauterine pregnancy achieved in patients' u926 and u783, who have very high expression level.

TABLE 5a: Mann – Whitney U test for all the 55 angiogenic factors.

	pregnancy	N	Mean	Std. Deviation	Std. Error Mean	p-value
Activin A	No	5	0,26	0,34	0,15	0,180
	yes	3	0,77	0,45	0,26	
ADAMTS-1	No	5	6,62	8,82	3,94	0,881
	Yes	3	3,20	2,66	1,54	
Angiogenin	No	5	342,16	89,85	40,18	0,297
	Yes	3	407,00	58,90	34,00	
Angiopoietin - 1	No	5	24,08	32,75	14,65	0,655
	Yes	3	39,91	43,68	25,22	
Angiopoietin - 2	No	5	120,14	121,67	54,41	0,655
	Yes	3	123,32	111,52	64,39	
Angiostatin / Plasminogen	No	5	23,53	37,51	16,78	0,881
	Yes	3	28,51	43,91	25,35	
Amphiregulin	No	5	2,25	6,08	2,72	0,297
	Yes	3	3,75	4,29	2,47	
Artemin	No	5	13,34	21,09	9,43	0,881
	Yes	3	12,93	19,94	11,51	
Coagulation Factor III	No	5	266,67	93,74	41,92	0,655
	Yes	3	288,13	125,68	72,56	
CXCL16	No	5	22,87	30,29	13,55	0,456
	Yes	3	34,57	41,50	23,96	

	No	5	174,90	119,26	53,33	0,655
DPPIV	Yes	3	230,02	153,70	88,74	
EGF	No	5	8,63	11,40	5,10	0,881
	Yes	3	6,19	5,94	3,43	
EG - VEGF	No	5	75,46	104,88	46,90	0,881
	Yes	3	17,31	21,92	12,65	
Endoglin	No	5	91,97	121,46	54,32	0,881
	Yes	3	120,78	155,19	89,60	
Endostatin / Collagen XVIII	No	5	139,97	106,00	47,41	0,881
	Yes	3	153,98	98,60	56,93	
Endothelin - 1	No	5	194,92	103,19	46,15	0,297
	Yes	3	272,72	102,02	58,90	
FGF acidic	No	5	52,42	100,71	45,04	0,297
	Yes	3	141,58	208,40	120,32	
FGF basic	No	5	166,63	134,59	60,19	0,881
	Yes	3	163,14	114,34	66,01	
FGF - 4	No	5	7,35	14,26	6,38	0,655
	Yes	3	1,70	0,67	0,39	
FGF - 7	No	5	28,03	38,08	17,03	0,655
	Yes	3	11,84	17,48	10,09	
GDNF	No	5	11,58	21,93	9,81	0,881
	Yes	3	5,63	7,90	4,56	
GM - CSF	No	5	11,83	17,91	8,01	0,881
	Yes	3	9,82	11,80	6,81	
HB - EGF	No	5	31,24	39,53	17,68	0,456
	Yes	3	36,43	49,37	28,51	
HGF	No	5	58,88	87,65	39,20	0,655
	Yes	3	94,76	79,76	46,05	
IGFBP - 1	No	5	70,35	84,48	37,78	0,881
	Yes	3	70,78	69,16	39,93	
IGFBP - 2	No	5	175,72	156,22	69,86	0,456
	Yes	3	261,15	160,04	92,40	
IGFBP - 3	No	5	125,06	129,58	57,95	0,456
	Yes	3	223,15	197,83	114,22	
IL - 1β	No	5	6,32	10,21	4,57	0,881
	Yes	3	9,98	14,66	8,46	
IL - 8	No	5	57,71	117,25	52,44	0,456
	Yes	3	183,15	158,51	91,52	
LAP (TGF - β1)	No	5	21,89	28,18	12,60	0,655
	Yes	3	17,95	14,63	8,45	
Leptin	No	5	7,94	14,57	6,51	0,881
	Yes	3	2,34	1,38	0,80	
MCP - 1	No	5	27,54	37,06	16,57	0,297
	Yes	3	122,10	137,82	79,57	
MIP - 1α	No	5	6,09	11,97	5,35	0,456
	Yes	3	3,60	4,87	2,81	
MMP-8	No	5	125,09	120,80	54,02	0,297
	Yes	3	212,29	135,28	78,10	
MMP-9	No	5	116,44	129,24	57,80	0,297
	Yes	3	222,69	187,24	108,10	
NRG1 - β1	No	5	27,98	37,81	16,91	0,456
	Yes	3	44,65	39,41	22,75	
	No	5	18,92	24,68	11,04	0,655

Pentraxin 3 (PTX3)	Yes	3	24,69	30,91	17,85	
PD-ECGF	No	5	62,97	70,27	31,43	0,655
	Yes	3	66,91	95,59	55,19	
PDGF-AA	No	5	8,20	17,12	7,66	0,456
	Yes	3	22,52	33,00	19,05	
PDGF-AB/PDGF-BB	No	5	5,50	7,99	3,57	0,881
	Yes	3	8,05	6,65	3,84	
Persephin	No	5	44,15	51,01	22,81	0,655
	Yes	3	53,15	76,43	44,13	
Platelet Factor 4 (PF4)	No	5	290,99	68,58	30,67	0,881
	Yes	3	313,56	64,04	36,97	
PlGF	No	5	32,36	46,88	20,96	0,655
	Yes	3	48,21	44,08	25,45	
Prolactin	No	5	22,63	31,15	13,93	0,881
	Yes	3	16,02	17,14	9,90	
Serpin B5	No	5	5,72	11,34	5,07	0,297
	Yes	3	2,35	1,84	1,06	
serpin E1	No	5	120,21	120,95	54,09	0,881
	Yes	3	117,39	111,33	64,28	
Serpin F1	No	5	172,46	112,49	50,31	0,655
	Yes	3	236,56	144,51	83,43	
TIMP - 1	No	5	300,73	131,65	58,87	0,297
	Yes	3	420,16	116,24	67,11	
TIMP- 4	No	5	16,05	24,01	10,74	0,297
	Yes	3	37,21	23,28	13,44	
Thrombospondin - 1	No	5	19,46	28,79	12,87	0,297
	Yes	3	55,17	71,69	41,39	
Thrombospondin - 2	No	5	2,79	5,05	2,26	0,764
	Yes	3	2,77	3,70	2,14	
uPA	No	5	244,78	99,92	44,68	0,297
	Yes	3	328,07	118,67	68,52	
Vasohibin	No	5	6,31	11,89	5,32	0,655
	Yes	3	4,65	6,82	3,94	
VEGF	No	5	46,64	52,17	23,33	0,655
	Yes	3	67,72	53,55	30,92	
VEGF - C	No	5	3,56	7,40	3,31	0,456
	Yes	3	0,84	0,76	0,44	

TABLE 5b: Mann – Whitney U test for the 7 dominant angiogenic factors.

Factor	pregnancy	N	Mean	Std. Deviation	Std. Error Mean	p-value
Angiogenin	no	5	342,16	89,85	40,18	0,297
	yes	3	407,00	58,90	34,00	
Coagulation Factor III	no	5	266,67	93,74	41,92	0,655
	yes	3	288,13	125,68	72,56	
Endothelin - 1	no	5	194,92	103,19	46,15	0,297
	yes	3	272,72	102,02	58,90	
Platelet Factor 4 (PF4)	no	5	290,99	68,58	30,67	0,881
	yes	3	313,56	64,04	36,97	
Serpin F1	no	5	172,46	112,49	50,31	0,655
	yes	3	236,56	144,51	83,43	
TIMP - 1	no	5	300,73	131,65	58,87	0,297
	yes	3	420,16	116,24	67,11	
uPA	no	5	244,78	99,92	44,68	0,297
	yes	3	328,07	118,67	68,52	

document), between the patients who achieve pregnancy (including the biochemical one) and the others. The results showed no significant difference between the 2 groups.

Discussion

In the current study, we investigated the contribution and the importance of a set of 55 angiogenic factors in the achievement or not of achieving pregnancy after ovarian stimulation. The methodology which was used is based on protein arrays and has produced promising prognostic tools in other studies, mainly related to carcinogenesis, although different biological features were exploited [20-23]. The basic biological material used in the study was the endometrial tissue which was collected from the patients the day of oocyte retrieval after ovarian stimulation. Endometrial tissue contains protein components which reliably reflect the biology of the endometrium. The inclusion of pro- and anti-angiogenic factors is in concert to the in vivo mechanisms of the angiogenic activity, which is the result of the balance between these two groups in the microenviroment of the endometrium, ensuring the cyclic variation during the menstrual cycle [24].

A total of 55 angiogenic factors have been examined and it demonstated that a combination of seven factors **(TABLE 4)**, constitute an "angiogenic profile" which have been shown a strongest expression especially when pregnancy occurred. In literature rare similar studies have detected and correlated a higher pregnancy rate with the expression of specific individual angiogenic factors. In our study, for first time we examined 55 angiogenic factors simultaneously.

Angiogenin, is a pro-angiogenic growth factor which has mainly been studied in carcinogenesis. It is associated with cell apoptosis and survival through the p53 pathway [25]. Recent studies have highlighted the presence not only of angiogenin but also of VEGF (vascular endothelial growth factor) and bFGF in follicular fluid. The concentration of angiogenin in follicular fluid, have been associated with the maturity of the oocyte, attributed to the reduction of cell apoptosis which promote oocyte maturation [26]. Furthermore, other studies support that angiogenin has a pivotal role in ovarian hyperstimulation syndrome and in early gestation maintenance [27].

Endothelin (ET-1) is a polypeptide produced and released by the endothelial cells [28]. It's concentration in amniotic fluid is 10 to 100 times higher than in plasma [29]. Endothelin has been found in endometrium, amniotic fluid and amniotic membranes both in humans and animals [30]. Its role in human reproduction is not clear yet, although some studies support that that the presence of endothelin both in endometrium and embryo increase the chance of successful implantation and pregnancy [31]. Similar results related to the pregnancies have been described for the angiogenic factor Serpin-F1. Serpin, belongs to the inhibitors of proteolytic enzymes and it's concentration in endometrial tissue have been associated with the implantation of the trophoblast, the maintenance of pregnancy and the fetal growth [32,33].

Some other angiogenic factors have also been associated with pregnancy and included in the final seven factors that compose the "angiogenic profile" of the endometrium. Platelet factor 4 (PF4) and Coagulation factor III, are produced by endometrial tissue and correlate with the recruitment of macrophages in the circular monthly endometrial remodeling, the interaction between endometrium and trophoblast, the implantation and the maintenance of pregnancy [33,34]. On the other hand, uPA (urokinase plasminogen activator) and TIMP-1 (tissue inhibitor of metalloproteinase-1), have been associated with "implantation window". The factor uPA, stimulates the production of specific enzymes by the emdometrial tissue, which promote the interaction between the endometrium and trophoblast and favor implantation [35,36]. TIMP-1, was found to be over expressed in endometrial period corresponding to the "implantation window" and promotes the production of enzymes that increase the receptivity of endometrium [37-39]. Many other factors such as VEGF, HB-EGF, FGF, and EG-VEGF were studied in our research and despite the fact that they are not included in the "angiogenic profile", are strongly correlated with pregnancy [39-47].

Technical issues such as local endometrial injury for tissue obtaining are unlikely to have affected our results. Endometrial injury to improve implantation for women undergoing assisted reproductive techniques has attracted a

lot of attention recently and has rapidly become incorporated into clinical practice. Some studies demonstrate that endometrial scratching performed either during the spontaneous, preceding cycle, or during the IVF itself, significantly improve the rate of implantation, clinical pregnancies, and live births. These observations suggest that mechanical injury of the endometrium may enhance uterine receptivity by provoking the immune system to generate an inflammatory reaction. Recent findings suggest that a Th1 inflammatory response is necessary for the acquisition of uterine receptivity, while Th2-humoral inflammation is required for pregnancy maintenance. Other studies support that endometrial injury on the day of oocyte retrieval is associated with a reduction of clinical and ongoing pregnancy rates. Nevertheless, there are still no reliable researches to support these controversial views and for this reason we believe that our results have been not affected by the technique [48-63].

There are certain limitations associated with this report. The number of patients included was relatively low but this was due to the fact that the biological material that was used in the study was very difficult to be collected as we used endometrial tissue obtained in the day of oocyte retrieval. Furthermore, a commercially available kit was used because our intention was to leverage angiogenic profiling for eventual and widespread use in everyday practice. It is possible that other factors might also be useful but their study would complicate the practicability of this method. In spite of the limited number of patients, the results are remarkable as "angiogenic profile" of the 7 factors can not only separate the patients into 2 clusters but also classify the pregnancies in the same group, which means that a combination of angiogenic factors may contribute more than others to pregnancy achievement.

Conclusion

To the best of our knowledge, this is the first time that 55 angiogenic factors are studied simultaneously in endometrial tissue of women who undergoing ART, and are associated with pregnancy success. With the appropriate validation a combination of angiogenic factors could be used as predictive tool for ART outcome.

List of Abbreviations

ART : assisted reproduction techniques

IVF : in vitro fertilization

TNF-a: Tumor necrosis factor – a

MCP: monocyte chemoattractant protein

PCA: Principal component analysis

FSH: Follicle stimulation hormone

LH: Luteinizing hormone

E2: Estradiol

PRL: Prolactin

TSH: Thyroid Stimulating Hormone

VEGF: vascular endothelial growth factor

ET-1: Endothelin

PF4: Platelet factor 4

uPA: urokinase plasminogen activator

TIMP-1: tissue inhibitor of metalloproteinase-1

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Ethics Approval and Consent to Participate

The study protocol had met appropriate Institutional Review Board approval (1st Department of Obstetrics and Gynecology, Alexandra General Hospital, Athens, Greece) and written informed consent was given by all subjects for the collection and study of the endometrial tissue. The study was conducted according to the principles expressed in the declaration of Helsinki.

Conflict of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Study Design: DL, S-P T, DM.

Acquisition of data: S-P T, GP

Analyzed the data: S-P T, EP, MM

Manuscript drafting: S-P T

Critically revision of Manuscript: DM, AC, GP, PD, S-P T.

Final approval: DL.

ALL authors read and approved the final manuscript

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