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Research Article

Serum Proteomic Profile of Women Undergoing Assisted Reproduction Technology and its Relation to the Outcome

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Abstract

Infertility is a major public health issue estimated to affect between 8% and 12% of couples globally. Despite the continuous improvements in the assisted reproduction technology (ART) field the success rates still remain modest. Proteomics can identify and quantify overall proteins within a cell, tissue or an organism. As such they can identify alterations in the expression of key proteins (biomarkers) which could then be utilized for prediction of the outcome of an ART cycle, for prevention of an unsuccessful attempt and for monitoring of the cycle in a personalized manner in order to achieve the desirable outcome. Our study examined the serum proteomic profile of women undergoing ART cycle and its relation to the ART outcome. A total of 62 differentially expressed proteins were identified. In our study we found that Vitamin D, Complement C3, Complement C4 and APOA1 may play a crucial role in the in vitro fertilization (IVF) outcome.

Keywords: Proteomics, Assisted Reproduction Technology, In Vitro Fertilization, Vitamin D, Complement C3, Complement C4, APOA1.

Introduction

Infertility is a major public health issue estimated to affect between 8% and 12% of couples globally (1). The actual definition of infertility is the inability to achieve a pregnancy after 12 months or more of regular unprotected sexual intercourse. Infertility can either be primary, when no pregnancy has been achieved before by a person , or secondary when at least one prior pregnancy has been achieved (2). However, the rapidly developing field of assisted reproduction technology (ART) has been an enormous asset for people struggling with infertility. Currently, ART is practiced in over one hundred countries and ART cycles have increased from around 140,000 in 1991 to over 3.2 million in 2018. Approximately 670,000 infants were born in 2018 from ART practices, while around 60 million ART cycles have been performed since the birth of the first IVF baby, Louise Brown in 1978, resulting in approximately 10 million infants (3).

Despite the continuous improvements in the ART field the success rates still remain modest, with only 25%-30% of the cycles resulting in a live birth (4). Therefore, various methods are applied in order for higher success rates to be achieved. The recent developed field of proteomics could be considered a promising addition to the armamentarium of tools available to combat lower success rates and as a result global infertility. Proteomics can identify and quantify overall proteins within a cell, tissue, or an organism. As such they can identify alterations in the expression of key proteins (biomarkers) which could then be utilized for the prediction of the outcome of an IVF cycle, they could prevent an unsuccessful attempt and monitor the cycle in a personalized manner in order to achieve the desirable outcome. This approach is the main scope of predictive, preventive, and personalized medicine (5,6).

Materials and Methods

Study Group and collection

The population under study consisted of 22 women undergoing an ART cycle in the IVF University Clinic of Alexandra Hospital in Athens, Greece which was working in collaboration with Embryoland IVF Clinic, Athens, Greece. The study was approved by the 574/21.09.2018 of the Greek National Authority of Assisted Reproduction and all the women included in the study provided informed consent. From each woman four total blood samples were collected, one earlier than the beginning of the fertility medication, one at the time of embryo transfer (ET) day, one 6 days after ET and one 12 days after ET. Out of the 22 women, 15 achieved implantation and/or pregnancy (successful cycles) and 7 did not (unsuccessful cycles). The inclusion criteria were as follows: (i) woman's age between 28 and 42 years; (ii) absence of uterine or endometrial conditions (myomas, polyps, malformations, endometritis, thin endometrium); (iii) absence of hydrosalpinx; (v) no infectious risk; (vi) transfer on day 5 of 1–2 fresh embryos, at least one being of good quality; and (vii) fresh own oocyte cycles without preimplantation genetic diagnosis or testicular biopsy. The ART cycle was performed either with IVF or intracytoplasmic sperm injection (ICSI) method, depending on the infertility cause. The demographic and clinical characteristics of the study population are detailed in Table 1**.** The indications for IVF were male factor (50%), female (32%), and unexplained infertility (23%).

Table 1. Demographic and Clinical characteristics of the recruited patients undergoing ART program.

Sample	Race	Age	Infertility cause	Number of attempts	Number of oocytes retrieved	Sperm (millions)	Fertilization method	Pregnancy	Outcome
1	Caucasian	33	Oligoasthenospermia	1	14	5	ICSI	-	-
2	Caucasian	36	Unovulation	3	2	100	ICSI	+	1 infant
3	Caucasian	31	Unexplained	1	11	29	ICSI + IVF	+	Miscarriage
4	Caucasian	38	Unexplained	1	4	137	ICSI	+	Biochemical
5	Caucasian	37	Unovulation	3	4	84	ICSI	+	1 infant

6	Caucasian	31	Blocked tubes/ asthenospermia	1	5	40	ICSI	+	1 Infant
7	Caucasian	36	Severe asthenorspermia	5	6	Donor sperm	IVF	+	1 Infant
8	Caucasian	42	Unexplained	1	8	32	ICSI	-	-
9	Caucasian	35	Asthenospermia	1	11	18	ICSI	-	-
10	Caucasian	36	Oligospermia	3	7	1,7	ICSI	+	1 Infant
11	Caucasian	35	Tubal factor	1	5	80	ICSI	+	2 Infants
12	Caucasian	32	Bad oocyte quality	1	3	112	IVF	-	-
13	Caucasian	30	Unexplained	2	7	2	ICSI	-	-
14	Caucasian	32	PCOS	4	4	13	ICSI	+	Miscarriage
15	Caucasian	37	Unexplained	1	7	17	ICSI	+	1 Infant
16	Caucasian	37	Asthenospermia	3	2	39	ICSI	+	1 Infant
17	Caucasian	35	Asthenospermia	1	11	58	ICSI	+	1 Infant
18	Caucasian	35	Asthenospermia	1	4	41	ICSI	+	1 Infant
19	Caucasian	33	Asthenospermia	1	3	18	ICSI	+	1 Infant
20	Caucasian	40	Asthenospermia	1	3	35	ICSI	-	-
21	Other	28	Asthenospermia	1	10	2	ICSI	+	1 Infant
22	Caucasian	42	Bad oocyte quality	1	1	32	ICSI	-	-

Sample collection

Our standard IVF protocol has been described previously. Briefly, it consists of a conventional antagonist protocol, ovarian stimulation being performed with only recombinant follicle-stimulating hormone (rFSH). Ovulation was triggered with recombinant human chorionic gonadotropin (rec hCG) (Ovitrelle, Merck) when ≥ 3 follicles ≥ 18.5 mm were observed. Oocyte retrieval was scheduled 36 h after triggering ovulation. After IVF/ICSI was performed, embryos were frozen. The luteal phase was supported by progesterone 200 mg every 8 h, starting from day 13-14 where the endometrium was ≥ 8mm. The hormone was administered vaginally. In all the artificial cycles 6mg/day of estradiol was also administered orally from the 3rd day of menstruation.

Sample preparation

In total, four blood samples were collected from each patient. More precisely, the first sample was collected prior to the beginning of the ART program, the second at the day of ET, the third 6 days post ET and the fourth 12 days post ET. Following centrifugation at 3000g for 5 minutes, serum was collected and stored at -80˚C until use. The protein concentration was determined with a Bioanalyzer system (Agilent Technologies Inc., Waldbornn, Germany), using Protein 200 plus kit (Agilent Technologies Inc.).

Two-dimensional gel electrophoresis (2-DE)

Two-dimensional gel electrophoresis (2-DE) was performed as previously described (7). The gel images were scanned in a GS-800 Calibrated Densitometer (Bio-Rad, Hercules, CA, USA). The images were stored in digital format for further analysis.

Protein Visualization and Image analysis

PD-Quest v8.o image processing software (Bio-Rad) was used for the detection, alignment, matching and quantification of the protein spots, in all the gels that were analyzed, according to the manufacturer's instructions. In order to verify the accuracy of matching, manual inspection of the spots was done. Protein expression was quantified by spot volume. After subtraction of the background values, normalization of the protein spots was carried out according to the total quantity of the valid spots. Optical density (O.D.) level (%) of each protein from the 4 groups of the quartets was determined separately and calculated as the sum of the percentage volume of all spots from the gels containing the same protein. The protein spot selection for MS identification was based on the O.D. alterations observed between the four groups.

Protein identification by MALDI -TOF-MS

For the analysis of the serum proteins, MALDI-TOF-MS analysis was performed as previously described (7). The matching of peptides as well as the protein searches were performed automatically with the use of MASCOT Server 2 (Matrix Science). The peptide masses were compared with the theoretical peptide masses of all available proteins from the Homo sapiens in the SWISS -PROT and TremBL databases.

Stringent criteria were used for protein identification with a maximum allowed mass error of 25 ppm and a minimum of four matching peptides. Probability score with $p < 0.05$ was used as the criterion for affirmative protein identification. Monoisotopic masses were used, and one missed trypsin cleavage site was calculated for proteolytic products. Search parameters included potential residue mass modification for carbamidomethylation and oxidation. Redundancy of proteins that appeared in the database under different names and accession numbers was eliminated. If more than one protein was identified under one spot, the single protein member with the highest protein score was singled out from the multiprotein family. MASCOT search score is −10 log (p), where p is the probability that an observed match is a random event. Scores >50 indicate identity or extensive homology at the $p<0.05$ level.

Verification methods

Four proteins were further measured in the same serum samples using automated methods. The methods have previously been described (8,9). More specifically, vitamin D was measured with automated chemiluminescence immunoassay (CLIA) method on Atellica® IM Analyzer (Siemens Healthineers Tarrytown, NY 10591 USA), while Complement C3, Complement C4 and APOA1 proteins were measured with automated immunoturbidimetric assay on Atellica® CH Analyzer (Siemens Healthineers Tarrytown, NY 10591 USA).

Statistical analysis

In order to ensure confidence in our experiments, we used an experimental design based on quadruple 2-DE gels per patient and separate preparations per experiment. Comparisons were performed between samples (pre-IVF medical treatment, ET day, 6 days post ET, 12 days post ET of pregnant and non-pregnant individuals). Mean densitometric values of all spots corresponding to a specific protein from each group were first checked for normal distribution using unequal variances. Data with normally distributed densitometric values were exported to Microsoft Excel 2019 software (Microsoft Corp., Redmond, WA, USA) and compared with the two pair t-test assuming unequal variance. Means of spot intensities for proteins with non-normally distributed values were compared for statistical significance with the Mann-Whitney nonparametric test (GraphPad Instant 3 software: GraphPad Software Inc., La Jolla, CA, USA). Statistical significance (alpha-level) was defined as $p<0.05$.

Statistical significance of protein quantification by CLIA method and immunoturbidimetric assay was performed by the use of student t-test of the quantity values of each protein. Mean values of the samples from the four different days (pre-IVF treatment, ET, 6 days post ET, 12 days post ET) were compared between successful and unsuccessful cycles by the t-test. Statistical significance was defined as $p<0.05$.

Results

Figure 1 shows the types of comparisons that our study focused on. Figure 2 represents a quartet of the 2DE-gels of a pregnant and a non-pregnant woman. Table 2 represents the differentially expressed proteins that were found in our study. Functional relationships analysis of the differentially expressed proteins was performed using the STRING v.10 database (Search Tool for the Retrieval of Interacting Genes/Proteins, http://string-db.org). Figure 3 and 4 show the functional relationships of the differentially expressed proteins in pregnant and non-pregnant women. The PANTHER database was used to reveal the biological function of each identified protein which are represented in figure 5 and 6 (http://panther.appliedbiosystems.com/). APOA1, Complement C3, Complement C4, and Vitamin D blood serum levels, after verification methods were performed, are represented in figures 7-10. Figures 11-14 each represent the mean levels of each protein at the four different days in pregnant and non-pregnant individuals together.

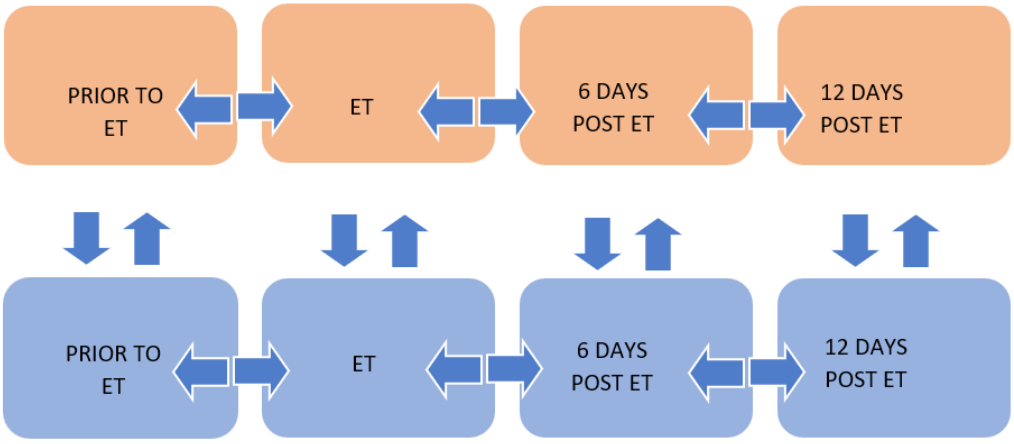

Figure 1. Types of comparisons of the differential protein expression in serum samples of women who achieved pregnancy (orange color) and women that did not achieve pregnancy (blue color) after an IVF cycle. ET: Embryo Transfer.

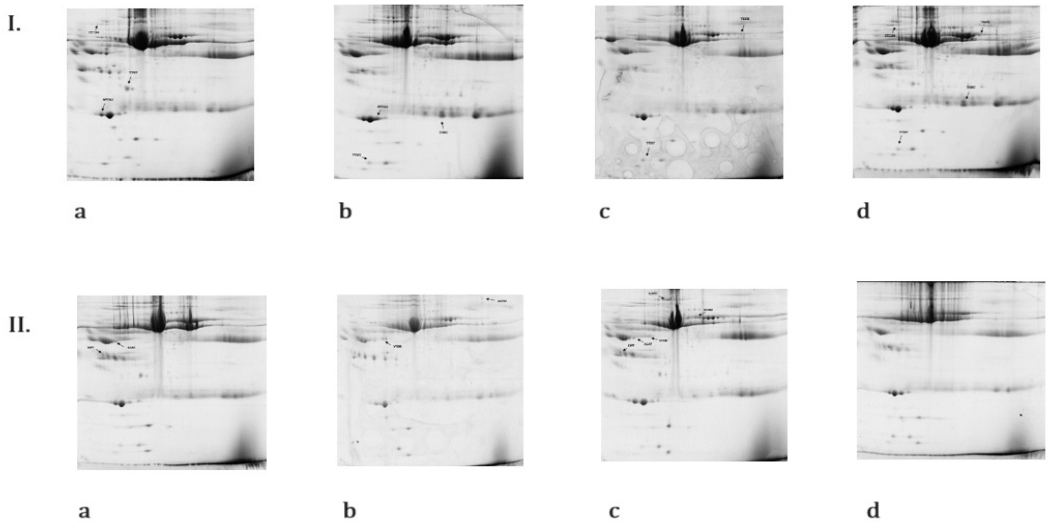

Figure 2. 2-DE Gel images with differentially expressed spots. I: A quartet of samples in the first row are the gels from a pregnant woman: a. prior to IVF treatment, b. on ET day, c. 6 days post ET and d. 12 days post IVF. II: Second row represents a quartet of the samples of a non-pregnant woman: a. prior to IVF treatment, b. on ET day, c. 6 days post ET and d. 12 days post IVF.

Table 2. Differentially expressed proteins (DEPs).

Full protein name	Abbreviation	Mascot Score	Coverage	Protein MW	pI
Afamin	AFAM	52	7	70963.00	5.60
Albumin	ALBU	55	14	71317.00	5.90
Alpha-1-antitrypsin	A1AT	76	16	46878.00	5.30
Alpha-1B-glycoprotein	A1BG	89	31	54790.00	5.50
Alpha-2-macroglobulin	A2MG	59	13	164613	6.02
Alpha-ketoglutarate-dependent dioxygenase alkB homolog 2	ALKB2	46	30	29475	10.28
Angiotensinogen	ANGT	102	26	53406.00	5.90
Antithrombin-III	ANT3	129	35	53025.00	6.30
Apolipoprotein A-I	APOA1	246	62	30759.00	5.50
Apolipoprotein A-IV	APOA4	134	26	45371.00	5.20
Apolipoprotein E	APOE	130	38	36246.00	5.50
Beta-2-glycoprotein 1	APOH	61	25	39584	9.49
CD5 antigen-like	CD5L	200	57	39603.00	5.20
Ceruloplasmin	CERU	140	18	122983.00	5.40
Complement C1q subcomponent subunit B	C1QB	89	34	26933.00	9.60
Complement C1r subcomponent	C1R	83	17	81606.00	5.80
Complement C1s subcomponent	C1S	265	39	78174.00	4.70
Complement C3	CO3	194	16	188569.00	6.00
Complement C4-A	CO4A	71	11	194261.00	6.70
Complement component C6	CO6	107	18	108367.00	6.40
Complement factor B	CFAB	55	18	86847	6.74
Complement factor H	CFAH	96	15	143680.00	6.20
G patch domain and ankyrin repeat-containing protein 1	GPAN1	51	22	39518	9.36
Glucosamine-6-phosphate isomerase 2	GNPI2	54	22	31293	6.50

Growth/differentiation factor 2	GDF2	50	15	47861	6.03
Haptoglobin	HPT	85	25	45861.00	6.10
Hemoglobin subunit beta	HBB	224	84	16102.00	6.90
Hemoglobin subunit delta	HBD	87	39	16159	9.06
Hemopexin	HEMO	164	40	52385.00	6.60
Ig gamma-1 chain C region	IGHG1	64	28	36596.00	9.40
Ig kappa chain C region	IGKC	87	86	11773.00	5.50
Ig mu chain C region	IGHM	76	16	49960.00	6.40
Ig mu heavy chain disease protein	MUCB	65	17	43543	4.99
Immunoglobulin gamma-1 heavy chain	IGG1	64	14	49925.00	9.70
Immunoglobulin heavy constant gamma 1	IGHG1	52	13	45861.00	6.10
Immunoglobulin kappa constant	IGKC	50	47	11929.00	6.10
Immunoglobulin lambda constant 1	IGLC1	51	46	11512.00	9.20
Kelch-like protein 23	KLH23	51	16	65079	5.31
Keratin, type I cytoskeletal 10	K1C10	110	26	59020.00	5.00
Keratin, type I cytoskeletal 9	K1C9	74	19	62255.00	5.00
Keratin, type II cytoskeletal 1	K2C1	60	15	66170	8.82
Keratin, type II cytoskeletal 2 epidermal	K22E	59	16	65678	8.85
Kininogen-1	KNG1	140	25	72996.00	6.40
Leucine-rich alpha-2-glycoprotein	A2GL	55	12	38382.00	6.50
Parvalbumin alpha	PRVA	61	56	12051	4.84
Perilipin-4	PLIN4	55	17	135204	9.73
Plasminogen	PLMN	77	19	93247.00	7.30
Protein AMBP	AMBP	109	26	39886.00	5.90
Prothrombin	THRB	126	28	71475.00	5.60
Retinol-binding protein 4	RET4	86	42	23337.00	5.70

S-adenosylmethionine decarboxylase proenzyme	DCAM	52	29	38714.00	5.60
Serotransferrin	TRFE	183	26	79294.00	7.00
Serum albumin	ALBU	92	16	71317.00	5.90
Serum amyloid P-component	SAMP	68	24	25485.00	6.10
Serum paraoxonase/arylesterase 1	PON1	83	31	39877	4.96
Small ubiquitin-related modifier 3	SUMO3	51	33	11687.00	5.20
Transthyretin	TTHY	96	63	15991.00	5.40
Tyrosine-protein kinase STYK1	STYK1	65	28	48116	7.82
Uncharacterized protein C11orf70	CK070	55	38	31296.00	6.40
Vitamin D-binding protein	VTDB	75	28	54480.00	5.20
Vitronectin	VTNC	61	18	55069	5.46
Zinc finger protein 273	ZN273	53	16	66868	10.54

A total of 62 DEPs were identified. MASCOT score>50 indicate identity or extensive homology at the $p<0.05$ level.

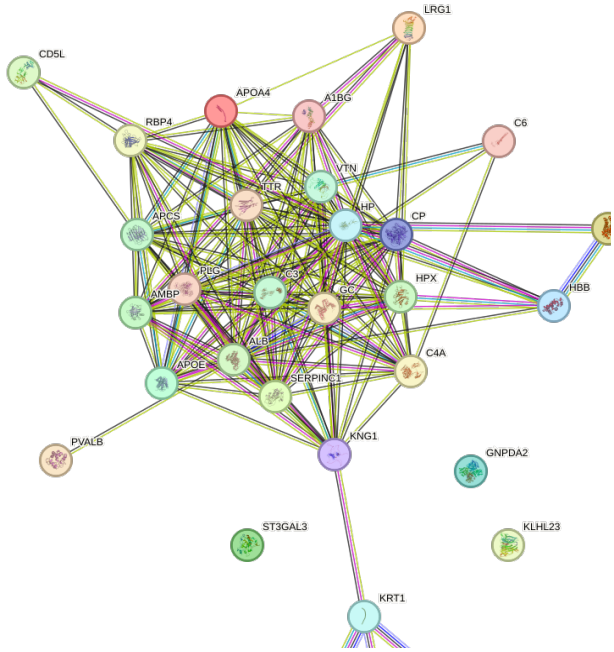

Figure 3. STRING protein network in pregnant women.

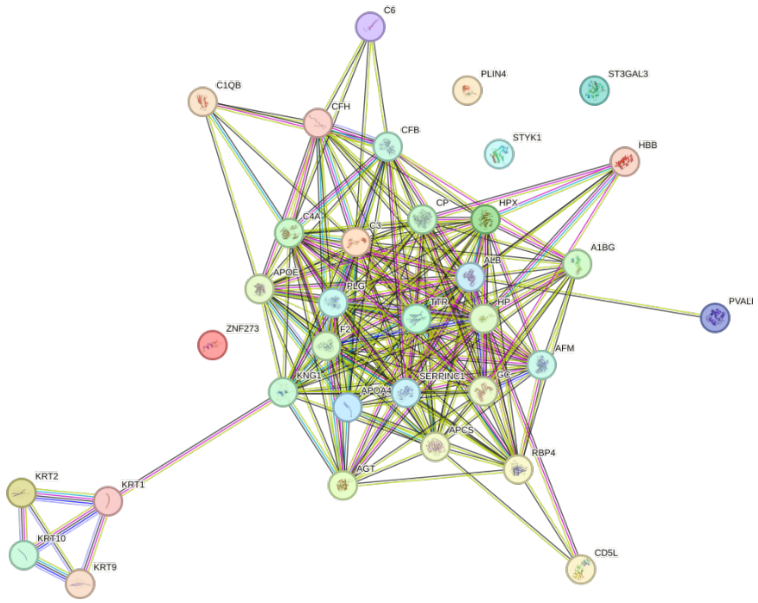

Figure 4. STRING protein network in non-pregnant women.

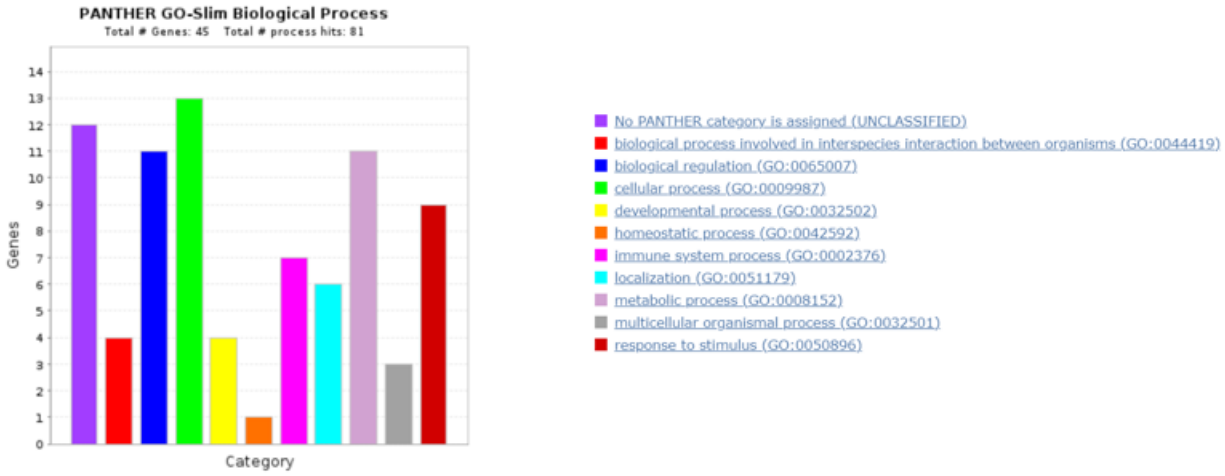

Figure 5. PANTHER Analysis. Biological processes of proteins found in pregnant women.

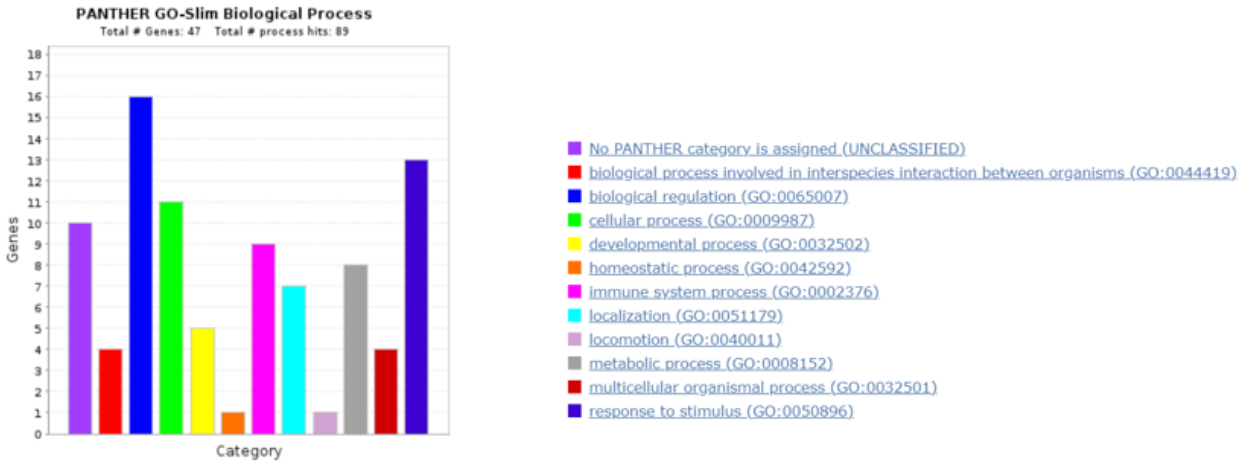

Figure 6. PANTHER Analysis. Biological processes of proteins found in non-pregnant women.

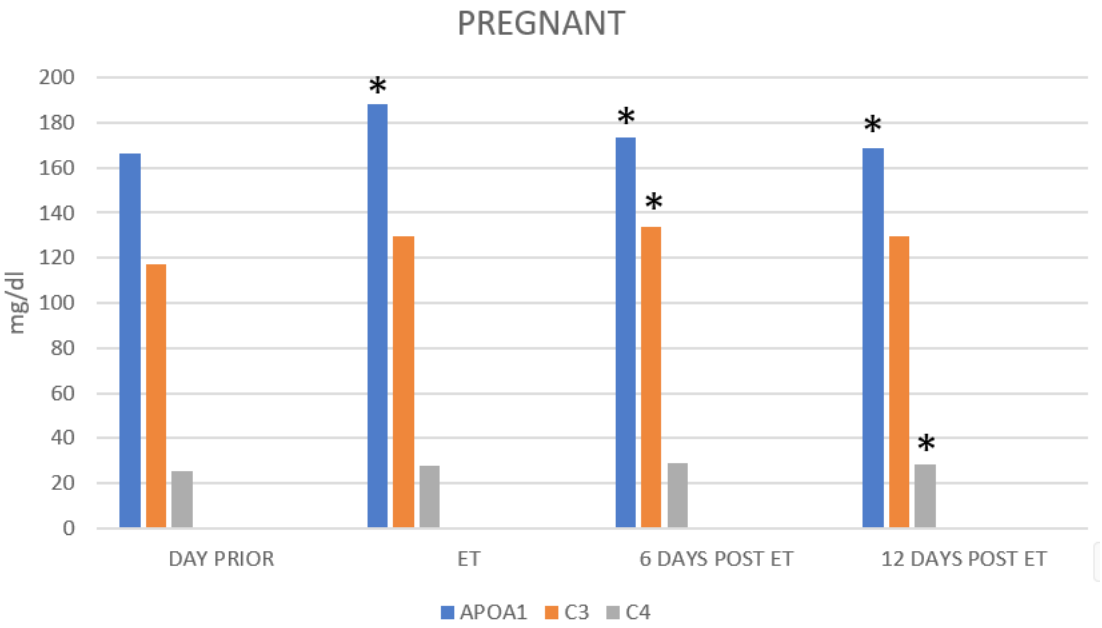

Figure 7. Quantification of APOA1, C3 and C4 proteins in successful IVF cycles using Immunoturbidimetric Assay. * p<0,05

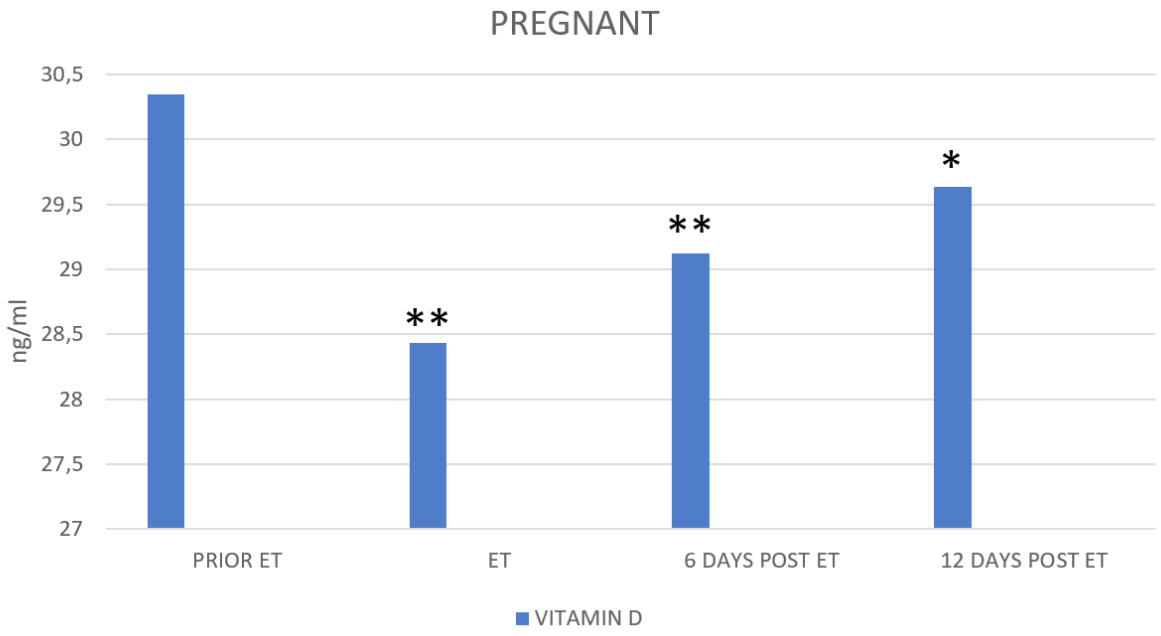

Figure 8. Quantification of Vitamin D in successful IVF cycles using Chemiluminescence Immunoassay (CLIA). *p<0,05 **p<0,005

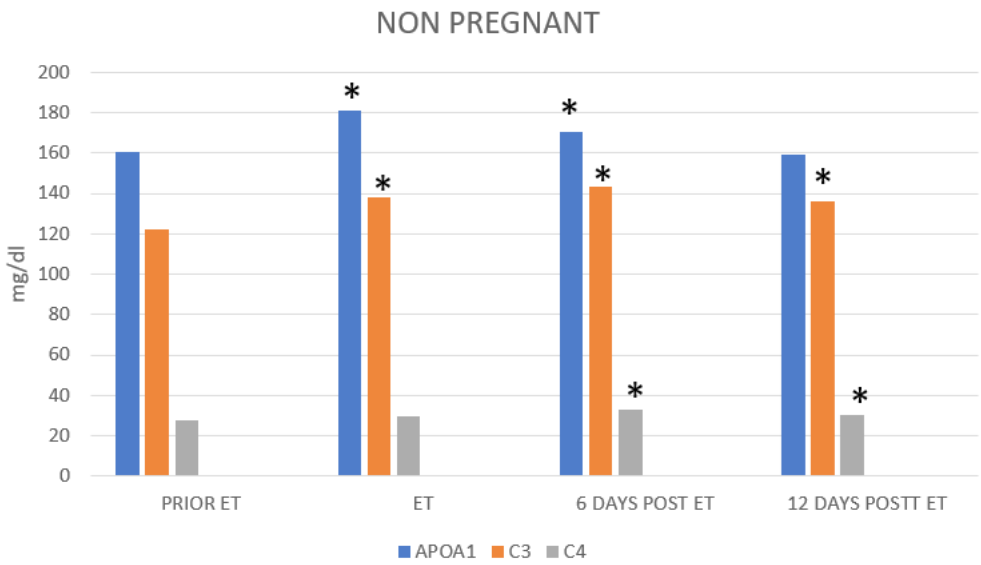

Figure 9. Quantification of APOA1, C3 and C4 proteins in unsuccessful IVF cycles using Immunoturbidimetric Assay. * p<0,05

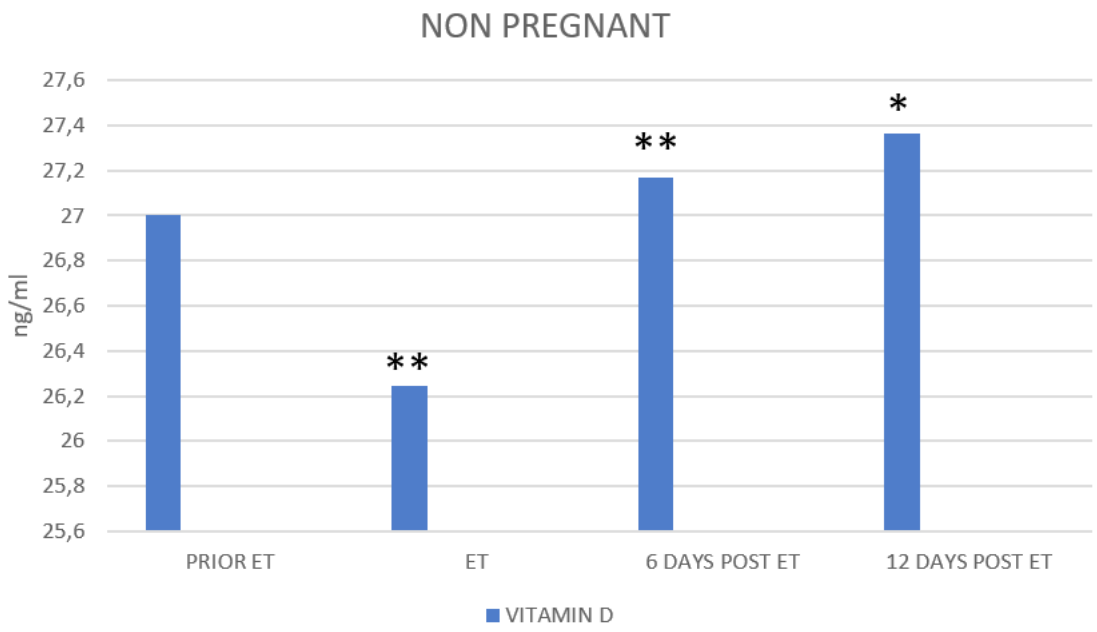

Figure 10. Quantification of Vitamin D in unsuccessful IVF cycles using Chemiluminescence Immunoassay (CLIA). * p<0,05 **p<0,005

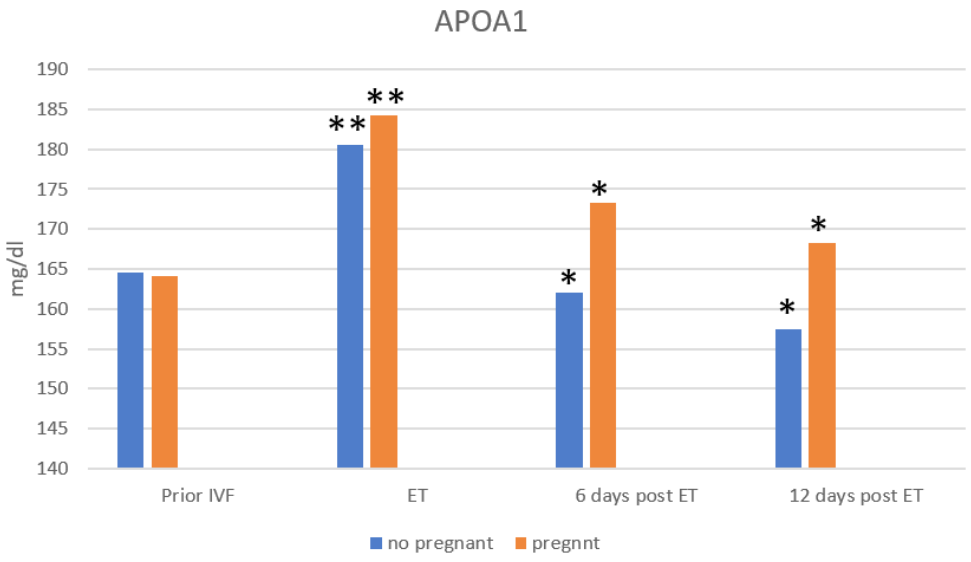

Figure 11. APOA1 mean levels in blood serum prior to IVF, at ET day, 6 days post ET and 12 days post ET in pregnant and non-pregnant woman. * p<0,05 **p<0,005

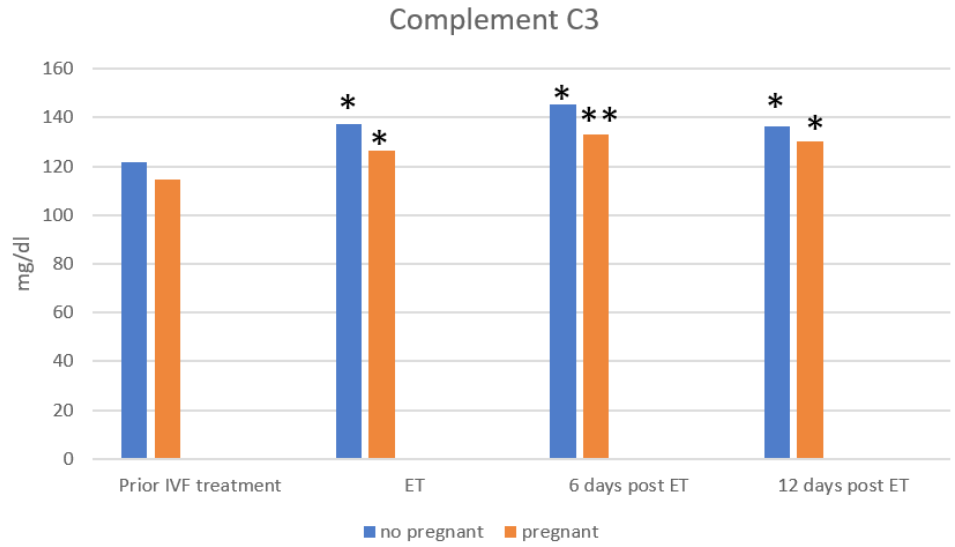

Figure 12. Complement C3 mean levels in blood serum prior to IVF, at ET day, 6 days post ET and 12 days post ET in pregnant and non-pregnant woman. * p<0,05 **p<0,005

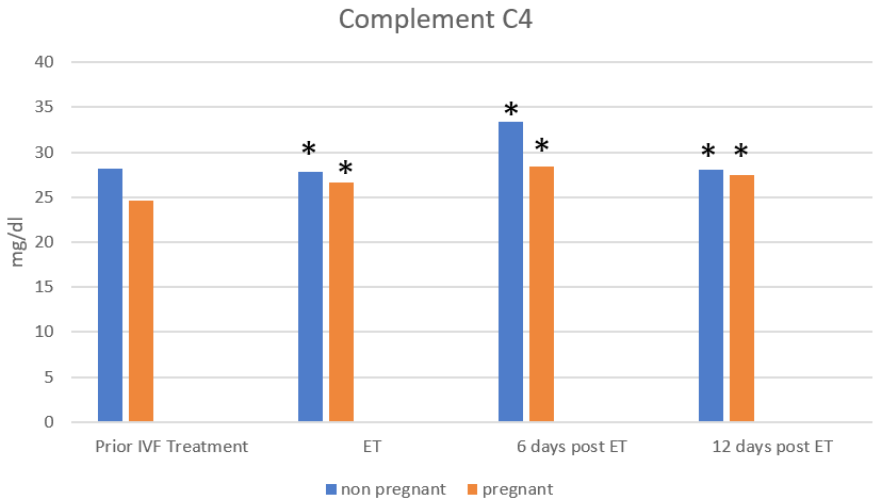

Figure 13. Complement C4 mean levels in blood serum prior to IVF, at ET day, 6 days post ET and 12 days post ET in pregnant and non-pregnant woman. * p<0,05

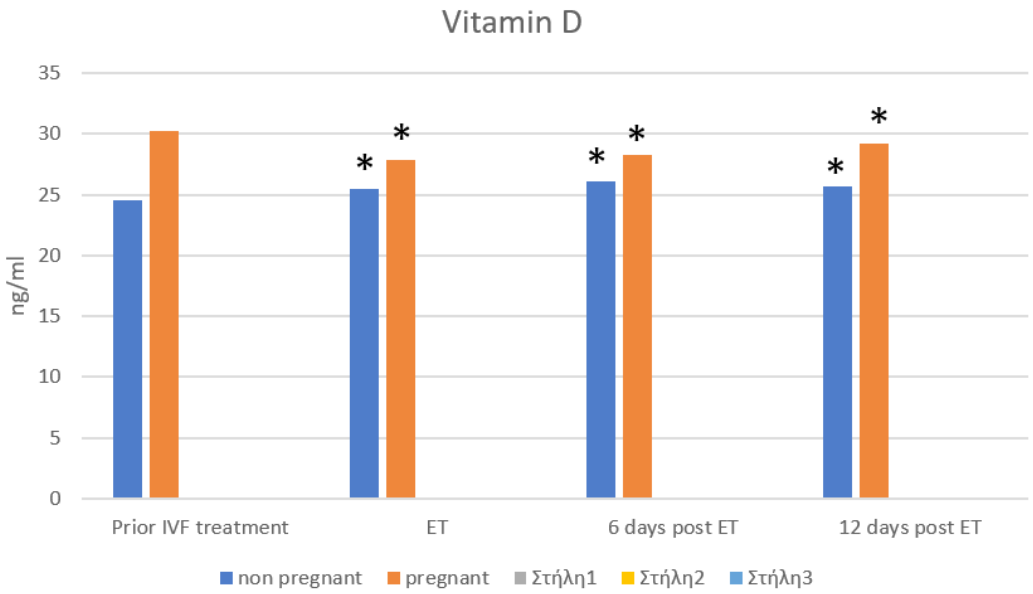

Figure 14. Vitamin D mean levels in blood serum prior to IVF, at ET day, 6 days post ET and 12 days post ET in pregnant and non-pregnant woman. * p<0,05.

Discussion

Proteomics could be a really beneficial tool for the fertility field not only in assisting the comprehension of the mechanisms behind female fertility and infertility, but also in contributing to the increase of IVF success rates. Proteomic biomarkers utility can be versatile in these fields. Proteomics could be utilized for predicting an IVF outcome, for preventing an unsuccessful IVF attempt, and for monitoring an IVF cycle in a personalized manner in order to achieve the desirable outcome. Proteomics in the IVF field could also potentially lead to the identification of therapeutic targets, increasing the possibility of a successful IVF cycle. Specifically, these therapeutic targets could target the implantation procedure of the embryo when given prior to the embryo transfer, or could increase the achievement of a healthy delivery when being prescribed after the implantation has occurred. Despite the advancements in fertility, infertility, as well as IVF field, there is still a need for further investigation in order to bridge the knowledge gap which still remains in these fields. Until now, no specific proteomic biomarkers have been identified. This hindrance in identifying a biomarker may be due to the limited studies in relation to the high number of proteins that can be identified, as well as due to the heterogeneity of the studies that have already been performed. More precisely, there is a heterogeneity in the sample used (follicular fluid, serum, endometrial tissue, endometrial fluid, and urine), the mass spectrometer that is used, as well as the conditions under which the experiments are conducted. Therefore, there is difficulty in the comparison of the results. Moreover, many of these studies are done in an invasive manner, when for example endometrial tissue is used. Sample collection in these cases could be difficult, and this could represent another limitation. Therefore, it is important to increase the number of studies done in a noninvasive manner (follicular fluid aspirate, uterine fluid, etc.). Another important point to mention is that single cell proteomics are still not fully developed. As a result, oocyte proteomics remain far behind in research. Lastly, proteomic technology not only has a high cost, as it is a newly developed field, but also its techniques are multi-step and thus may be more complicated.

In the current study, the protein composition of serum of women undergoing IVF procedure was investigated in four different days of the IVF treatment. Specifically, serum proteome was studied prior to the beginning of the medical treatment, at the day of ET, six days and twelve days after ET. The content of the protein composition of serum was analyzed and characterized based on Protein-Protein interactions (PPIs) and pathway analysis. In addition, quantitative measurements were performed to determine the concentrations of those proteins which may play

an important role in the fertility and in the outcome of the IVF procedure. Among the proteins that were identified in our study, APOA1, Complement C3, Complement C4 were chosen to be verified in the same blood serum samples. In our study, we also identified vitamin D binding protein which was the reason why we also measured vitamin D levels in the serum samples.

Vitamin D and its relation to the IVF outcome has already been studied in other research groups. Hayder et al., who compared preconception serum levels of 25-hydroxyvitamin D (25(OH)D) of women who achieved pregnancy and women that did not achieve pregnancy after IVF treatment, concluded that vitamin D levels were statistically higher in those who achieved pregnancy and that 25(OH)D level was a significant predictor of the IVF outcome (10). A systematic review and meta-analysis done by Meng et al., showed that moderate daily dosing of vitamin D supplementation could be beneficial for the clinical pregnancy rate of fertile woman. However, a larger size and high quality randomized controlled trials are necessary to optimize the parameters of vitamin D supplementation to help more infertile couples (11). Furthermore, a study by Wu et al., showed that women with higher vitamin D levels (>20ng/ml) had significantly higher number of mature oocytes as well as blastocyst formation rate than those with lower rates of vitamin D (<20ng/ml) (12). Li et al., studied the urine proteome of woman and found a correlation between vitamin D and the ovarian reserve. Specifically, he found that individuals with diminished ovarian reserve had lower levels of vitamin D binding protein (VDBP) in their urine (13). Ciepiela et al. also found that higher levels of vitamin D in the serum of woman is associated with clinical pregnancy after IVF procedure but lower levels of vitamin D in the follicular fluid is associated with high-quality oocytes (14). However, a study done by Luo et al., who divided patients undergoing first IVF/ICSI cycle into three groups, vitamin D deficiency (25OH-D<10ng/ml) group, vitamin D deficiency group (10ng/ml≤25OH-D<20ng/ml) and non-vitamin D deficiency group (25OH-D ≥20ng/ml) found no correlation between vitamin D serum levels and pregnancy outcome or embryo quality (15). No association was also found between Vitamin D level and ovarian reserve in the study of Ota et al., (16). In our study preconception vitamin D level was higher in pregnant woman 6 and 12 days after ET.

In our study we found an abundance of proteins of the immune system in the non-pregnant serum including proteins of the complement cascade as well as immunoglobins. In the study of Sun et al., increased levels of complement C4-B were found in follicular fluid of woman who had recurrent spontaneous abortion (17). Koshak et al., found that in the serum of females with unexplained infertility there were increased levels of Immunoglobins as well as increased levels of proteins of the complement cascade (18).

A study performed by Kurdi et al., who analyzed protein profiles of follicular fluid from woman undergoing ART procedure, showed that APOA1 levels were significantly lower in the proteome of the follicular fluid in women who managed to achieve pregnancy (19). That finding together with the results of the present study indicates that APOA1 may play a crucial role in the outcome of an IVF cycle which must further be investigated in a higher cohort of patients.

Conclusions

Currently no specific biomarker has been discovered in the IVF field which could predict the IVF outcome, help prevent an unsuccessful IVF attempt or help monitor the IVF cycle in a personalized manner. By identifying proteomic biomarkers of good-quality oocytes or optimal timing of endometrial receptivity, emotional and physiological stress of the individual undergoing IVF procedure could be prevented. In addition, the cost of the IVF procedure could be reduced, as the desirable outcome could be achieved with less IVF cycles. In total, it could lead to higher IVF success rates.

Conflict of Interest

The Authors declare that they have no potential conflicts of interest.

Author contributions

G.T.: conceptualization and supervision; G.T. and P.D.: scientifically responsible; P.D., N.T., A.N., D.L.: thesis supervisory committee of V.K.; N.K.: sample collection; G.T., V.K., E.B., S.P., K.V.: methodology development; V.K.: writing the original draft; G.T., V.K.: preparation of tables and figures; G.T., P.D.: reviewing and editing the final draft.

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