

# Upregulation of *ICAM-1* and *HLA-G* Genes Expression in Cumulus Cells of Infertile Women with Poor Response to Ovarian Stimulation with a Healthy Lifestyle

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## Research

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## Abstract

**Purpose:** Diagnosis of women with poor ovarian response (POR) to exogenous gonadotropin stimulation is a challenge for reproductive specialists. This study aimed to investigate the expression profile for *ICAM-1* and *HLA-G* genes in the cumulus cells of infertile women with POR based on their healthy lifestyle.

**Methods:** Eighty women under the age of 35 were enrolled and divided into two groups: 1- POR without a healthy lifestyle (n=40) and POR with a healthy lifestyle (n=40). The *ICAM-1* and *HLA-G* genes expression was compared by the quantitative PCR between the groups. The ICAM-1 and HLA-G protein levels were analyzed by the western blotting technique, and the methylation pattern was investigated by the methylation-specific PCR.

**Results:** The qRT-PCR assay showed that *ICAM-1* and *HLA-G* genes were differentially expressed between the two groups studied. *ICAM-1*, *HLA-G* genes, and proteins expressions in POR with healthy lifestyle were upregulated compared to the second group ( $P < 0.05$ ). *ICAM-1* and *HLA-G* DNA methylation status in the POR with a healthy lifestyle were decreased compared to the other group ( $P < 0.05$ ). The oocyte quality and clinical pregnancy ratio were significantly higher in the POR with a healthy lifestyle women than in the other ones ( $P < 0.05$ ).

**Conclusion:** The upregulation of *ICAM-1* and *HLA-G* in POR women is probably related to lifestyle. Lifestyle may affect the methylation pattern, protein levels and alteration of gene expression profiles in the cumulus cells of POR women. Additionally, lifestyle may be considered a marker for ovulation, oocyte maturation, preimplantation and clinical pregnancy process.

## Introduction

The term poor response to ovarian stimulation (POR) typically refers to woman with a reduced ovarian reserve or poor ovarian response to exogenous gonadotropin stimulation [1]. In fact, the POR or the cycle without ovulation is a cycle in which the ovary is unable to release the oocyte. As a result, ovulation does not occur. Despite the efforts to optimize the definition of POR, unfortunately there is still limited knowledge about the POR pathophysiology, and the etiology hinders the practical solutions to manage the condition [2]. Indeed, the diagnosis of anovulation is not easy. Contrary to popular belief, women with anovulation have more or less a normal menstrual cycle and usually for the first time, they find their own problem when they want to become pregnant [3]. Recently, POSEIDON criteria have been used to diagnose POR, including age, Anti Müllerian Hormone (AMH) dose, Antral Follicle Count (AFC), and oocyte number [4]. The consequential complications of POR are intrinsic ovarian resistance to gonadotropin stimulation, reduced ovarian reserve, ovulation and oocyte maturation disorder [5]. Reduced ovarian reserve is defined as the impaired fertility ability leading to infertility. In fact, excessive use of plastics objects in the human life has been proposed as one of the causes of creating POR in women [6]. Indeed, excessive use of plastics to store hot food and drinks can cause various diseases. Polycarbonate is a type of plastic containing Bisphenol A (BPA) [7]. Over time, when plastic is heated or exposed to acidic or alkaline substances, it breaks down and BPA attaches to food and drink, so that it can enter the body [8]. BPA, by mimicking sex hormones, can disrupt the endocrine system, hormones and hormonal signaling pathways [9]. BPA even in small amounts may interfere with the ovulation, ovarian response, oocyte maturation and pregnancy [10]. In fact, lifestyle plays a key role in regulating ovarian response, oocyte maturation and genes expression. Gene's expression alteration in POR patients is a condition damaging oocyte developmental, resulting in reduced rates of fertilization, embryonic development, and implantation [11]. The role of gene's expression alteration in the pathophysiology and etiology of POR is uncertain, but studies support the hypothesis that nutrition and lifestyle play a crucial role in regulating the response of intrinsic ovarian resistance to gonadotropin stimulation, reduced ovarian reserve, oocyte maturation, implantation and gene expression [12].

ICAM-1, known as CD54, plays a pivotal role during oocyte maturation, and it is a protein encoded by the *ICAM1* gene. The responsibility of *ICAM-1* is encoding a cell surface glycoprotein usually expressed on endothelial cells and cells of the immune system. As mentioned, ICAM-1 plays a central role in the ovulation process [13]. Indeed, the main components of this ovulation process are the disruption of the extracellular matrix (ECM) at the follicular peak and the change in follicular vessels [14]. In these events, ICAM-1 participates by secreting proteases and active inhibitors. Indeed, biochemical markers of the oocyte

maturation and ovulation are highly important. Upregulation of *ICAM-1* in immature oocytes as well as downregulation of *ICAM-1* in mature oocytes has been observed.

Human leukocyte antigen-G (HLA-G) is regarded to play a vital role in oocyte maturation and implantation of embryos. HLA-G can be effective in controlling trophoblast invasion and maintaining a local immunosuppressive state. The expression and distribution of *HLA-G* is on human spermatogenic cells, primary and secondary oocytes, and preimplantation embryos. Furthermore, the *HLA-G* production is related to the good quality of cumulus cells (CCs) in oocytes [15].

In the present study, we aimed to assess the genes expression, proteins level and methylation status of *ICAM-1* and *HLA-G* in the cumulus cells of infertile POR patients based on their lifestyle following ovarian stimulation with a gonadotropin releasing hormone (GnRH) antagonist protocol.

## Materials And Methods

### Patient selection

In the present study, eighty women under the age of 35 and BMI 18-25 Kg /m<sup>2</sup>, who participated in an intracytoplasmic sperm injection (ICSI) program, were selected. The patients who were diagnosed by the Poseidon group1 subgroup 1b that included; [patients <35 years old with adequate ovarian reserve parameters (AFC>5; AMH>1.2 ng/ml) and with an unexpected poor or suboptimal ovarian response (subgroup 1b: 4-9 oocytes retrieved] (4). The patients were divided into two groups: without a healthy lifestyle (n=40, infertile women with poor response (POR) to ovarian stimulation history), and with a healthy lifestyle (n=40, infertile women with poor response (POR) to ovarian stimulation history). The healthy lifestyle group included women who did not use plastic containers for hot food, hot drink and foodstuffs store. However, the group without a healthy lifestyle included women who used excessive plastic containers to store hot food, hot drink and foodstuffs. Information on a healthy lifestyle was obtained using a validated food frequency questionnaire. The main exclusion criteria were women affected by endometriosis, and women with a history of uterus and ovaries operation. Furthermore, in this study, at the request of the patients, the treatment IVF/ICSI cycle was canceled, while they had a low number of follicles or lacked growth of follicles. The Ethics Committee at Royan Institute, Iran approved this prospective case-control study (No. IR.ACECR.ROYAN.REC.1394.150). All participants gave informed consent prior to inclusion in the study. We ensured the confidentiality of the patients' identities by data anonymization during analysis. This research did not affect the treatment of patients.

### Antagonist stimulation protocol

In this study, Controlled of ovarian stimulation (COS) was controlled from the third day of the cycle. The patients received regular, daily subcutaneous (SC) injections of recombinant follicle stimulating hormone (rFSH, Gonal-F, Serono, Switzerland). The first dose of rFSH for each patient was started according to sonographic monitoring, and AFC, estradiol (E2) level, and AMH for each patient were evaluated. In the stage of growing follicles >12 mm, the patients received SC injections of a GnRH antagonist, cetrorelix (Cetrotide®, Merck Serono, Germany). The protocol consisted of daily Cetrotide® SC injections until the criteria for human chorionic gonadotropin (hCG) administration were met. When more than 3 follicles reached diameters of at least 18 mm and E2 levels of 1000-4000 pg/mL. When at least three follicles reached diameters of ≥18 mm as well as E2 levels of 1000-4000 pg/mL, each patient received an intramuscular (IM) injection of 10000 IU of hCG (Pregnyl®, Organon, Netherlands) or SC injection of 250 µg Ovidrel (Merck Serono, Germany).

### Isolation of cumulus cells

Following follicular puncture, the cumulus-oocyte complexes (COCs) were collected and washed at least 3-5 times in G-IVFTM medium (Vitrolife, Sweden) to remove blood and excess cells. Immediately after washing, the COCs were moved to a CO<sub>2</sub> incubator at 37°C for 2 hours in G-IVFTM medium. Cumulus cells were denuded from COCs by 80 IU of hyaluronidase, (Sigma, USA) [16]. After oocyte denudation, a pellet of cumulus cells was washed with phosphate-buffered saline (PBS), and RNA stabilizer reagent buffer was added for further analyses. Cumulus cells were immediately transferred to liquid nitrogen for snap freeze, and then they were stored at -80°C. The cumulus cells were denuded from metaphase II gametes (MII). In the IVF

laboratory, MII gametes were fertilized by the ICSI process within 10 minutes after denudation, and they were incubated until the embryo transfer was processed. Fertilization was evaluated (16-20 hours after ICSI). According to our IVF laboratory standards process, embryos were graded by embryologist specialists at the pronuclear (16-20 hours) and cleavage stages (48-72 hours) [17]. Then, the embryologist selected 1 or 2 embryos for transfer. For successful embryo transfer, they considered the embryo grades, age of the patients, and previous ART cycles. The quality of the embryos at the cleavage stage was classified based on these criteria: [The excellent quality of embryo should be ( $\geq 4$  cells or  $\geq 8$  cells and  $<10$  % fragmentation), the good quality of embryo should be ( $\geq 4$  cells or  $\geq 8$  cells and 10-20% fragmentation), and the poor quality of embryo should be ( $<4$  cells or  $<8$  cells and  $>20$  % fragmentation)] [18].

### **RNA extraction, cDNA synthesis and qPCR**

Total RNA was extracted by a Trizol (TRI; Sigma-Aldrich, US) and treated with RNase-free DNaseI according to the manufacturer's instructions. RNA concentration and purity were quantified using a Nanodrop2000 Spectrophotometer (Thermo, USA). Reverse transcription was performed at 25°C for 10 min, 42°C for 1h and then 70°C for 10 min. For this reaction, cDNA was synthesized using the Revert Aid™ H-Minus First Strand cDNA synthesis (Fermentas, German) by the random Hexa nucleotides primer (0.2 $\mu$ g/ $\mu$ l) in a solution (20 $\mu$ l) containing 4 $\mu$ l 5x reaction buffer, 1 $\mu$ l Ribonuclease inhibitor (20U/ $\mu$ l), 2 $\mu$ l dNTP mix (10mM) and RNase-DNase free water.

### **Real-Time PCR quantification**

*ICAM-1* and *HLA-G* were chosen as target genes, and 18srRNA was utilized as a normalizer for each sample. Primers were designed by the Primer Express 3.0 software for the real-time PCR. Table 1 presents the primer sequences. The real-time PCR was performed in a Step OnePlus™ instrument (Applied Biosystems, USA). The reaction contained 2 $\mu$ l cDNA, 10 $\mu$ l of Power SYBR Green master mix (TAKARA, Japan) and 1 $\mu$ l (of 500 nM primer) of forward and reverse primers. The reactions were performed in duplicate. The thermal cycling condition for the amplification was 95°C for 10 min, followed by 40 cycles of 95°C for 15s and 60° for 10 min. The Ct data were determined using default threshold settings. The  $2^{-\Delta\Delta CT}$  method was used to analyze the relative expression level of target genes (Livak&Schmittgen, 2001). Melt curve analysis was performed to determine the specificity of the real-time PCR assay. A t-test was used to investigate whether the differences between *ICAM-1* and *HLA-G* genes expression were calculated by the  $2^{-\Delta\Delta CT}$  method.

Table 1: Real time gene expression and MSP primers sequences

Primers for real time gene expression	sequences	Product size	
F- <i>ICAM1</i>	5'-GCAATGTGCAAGAAGATAGCCA-3'	105 bp	
R- <i>ICAM1</i>	5'-GGGCAAGACCTCAGGTCATGT-3'		
F- <i>HLA-G</i>	5'-AGCTGTGGTGGTGCCTTC-3'	106 bp	
R- <i>HLA-G</i>	5'-GGGCAGGGAAGACTGCTT-3'		
Primers for MSP	sequences	Product size	CpG Island Info
F- <i>ICAM1-M</i>	CGCGATTTTTTTGGTTTTTC	169 bp	
R- <i>ICAM1-M</i>	TATTTACTTAACCACCGCCTATACG		chr19:10,269,612-10,271,289
F- <i>ICAM1-UM</i>	GTTGTGTGATTTTTTTGGTTTTTT	171 bp	
R- <i>ICAM1-UM</i>	TTTACTTAACCACCACCTATACATA		
F- <i>HLA G-M</i>	CGTAGGTATATTGTTTATATTCGCG	185 bp	
R- <i>HLA G-M</i>	TACCTAAAAAAACCCCAAACG		
F- <i>HLA G-UM</i>	TGTAGGTATATTGTTTATATTTGTGG	186 bp	chr6:29827777-29828817
R- <i>HLA G-UM</i>	CTACCTAAAAAAACCCCAAACAC		

## Protein expression level

### Western blot analysis

The total protein was extracted from equal amounts of cumulus cells in all samples using lysis buffer (7M urea, 2M thiourea, 4% CHAPS [w/v], 75 mM DTT, 1% ampholyte [w/v], and 40 mM Tris-HCl), and then the protein concentration was evaluated by bicinchoninic acid assay (Thermo Scientific, Rockford). Additionally, 40 µg of protein for each sample was used onto a discontinuous 12% SDS-polyacrylamide gel and exposed to vertical electrophoresis. Afterward, the proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules). Subsequently, with 3% bovine serum albumin (Sigma-Aldrich) as well as 1% of nonfat dry milk (Amersham, GE Healthcare Life Sciences, Little Chalfont, UK), the membranes blocked process was performed. The time required for membranes blocked process is 2 hours at RT. In this stage, the membranes were incubated for overnight at 4°C with primary antibodies against human ICAM1 (G-5) (1:1,000, Cat. No.: SC-8439; SantaCruz, Madeira), HLA-G (4H84) (1:1,000, Cat.No.: sc-21799; SantaCruz, Madeira) and β-actin (1:1,000, Cat. No.: A2228; Sigma-Aldrich). In the next stage, the membranes secondary antibodies were used. Indeed, for 1.5 hours in RT, the membranes were exposed to horseradish peroxidase-conjugated secondary antibodies. The identification of ICAM1 and β-actin proteins was gained by goat antirabbit IgG (1:5,000, Cat. No.: ab6112; Abcam, Cambridge, UK), and antimouse IgG (1:10,000, Cat.No.: 7076; Cell Signaling Technology, Danvers) immunoglobulins, respectively (Fig. 1). Chemiluminescence detection system (Amersham) was used for protein visualization. The ImageJ software version 1.50i (US National Institutes of Health, Bethesda) was used to quantify protein bands intensity on the PVDF paper. The changes in ICAM-1 and HLA-G level were normalized against β-actin as a housekeeping protein and then calculated regarding infertile women with a poor response to ovarian stimulation without a healthy lifestyle.

### Methylation-specific polymerase chain reaction (MSP)

DNA isolation and bisulfite conversion

Genomic DNA was extracted from COCs by the QIAamp DNA Micro Kit (QIAGEN, Netherlands) according to the manufacturer's instructions. DNA quantity and quality were measured using a Nanodrop 2000 Spectrophotometer (Thermo, USA). Genomic DNA was treated by bisulfite using the EZ DNA Methylation-Gold (Zymo research, Germany) according to the manufacturer's instructions. After conversion, DNA was eluted in buffer (Qiagen) to a final concentration of 30 ng/ $\mu$ l.

### Methylation-specific PCR (MSP)

The DNA methylation status of *ICAM-1* and *HLA-G* genes in COCs samples was evaluated by the methylation-specific PCR (MSP). For MSP, we had to use specific primer pairs for both methylated and unmethylated promoter sequences. The primers were designed using the Meth primer and GeneRunner software. Each MSP reaction was performed in a total volume of 25  $\mu$ L. One microliter of sodium bisulfite converted DNA was added into a 24  $\mu$ L reaction mixture containing 0.5 U of hot start Gold Taq Polymerase (Promega, USA), 5  $\mu$  L of the 10  $\times$  PCR buffer, 2.0  $\mu$  L of  $MgCl_2$  (50 mmol/L), 0.5  $\mu$  L of dNTP (10 mmol/L; Fermentas) and 1  $\mu$  L of the corresponding forward and reverse primers (10  $\mu$  mol/L dH<sub>2</sub>O up to final volume of 25  $\mu$  L). Sodium bisulfite treated DNA was amplified in two separate MSP reactions, one with a set of primers specific for methylated, and the one for unmethylated *ICAM-1* and *HLA-G* promoters sequences (Table 1). For MSP positive control, we used fully methylated DNA (100%) and unmethylated DNA (0%), which the fully methylated DNA was made of Msssmethylase (NEB, Ipswich, MA, USA) using human placental genomic DNA (gDNA; Sigma Aldrich), and the unmethylated DNA was made of human placental genomic DNA (gDNA; Sigma Aldrich). To check the MSP method sensitivity, we used the fully methylated and unmethylated DNA with different serial dilutions: the ratio 1% to 99%, 5% to 95%, 10% to 90%, 20% to 80%, 35% to 65%, 60% to 40%, and 100% to 0% Methylated DNA was combined with unmethylated DNA, respectively. Thermo cycling conditions were as follows: 95  $^{\circ}$ C for 5 min followed by 40 cycles of 95  $^{\circ}$ C for 30 s, 63  $^{\circ}$ C for 60 s and 72  $^{\circ}$ C for 60 s, with a final extension of 72  $^{\circ}$ C for 4 min. MSP products for methylated and unmethylated *ICAM-1* and *HLA-G* promoters were run on 2% agarose gels containing 40 mM Tris-acetate/1.0 mM EDTA (pH = 8) and were visualized by ethidium bromide staining (Fig2). Methylation genes pattern visualization was performed using the enhanced chemiluminescence detection system (Amersham). The intensity of methylation genes bands on the agarose gel was quantified using the ImageJ software version 1.50i (US National Institutes of Health, Bethesda).

### Data analysis

Relative gene expressions were calculated by the  $2^{-\Delta\Delta Ct}$  method. The normalized  $\Delta Ct$  value of each sample was calculated using reference genes. In this study, categorical variables were presented as number (%) and continuous variables as mean  $\pm$  SD. The independent t-test was used to assess the mean differences in demographic and clinical characteristics between the groups. Chi-square analysis was used for qualitative data. Univariate and backward multiple linear regression, including all variables, were used to evaluate the association between *ICAM-1* and *HLA-G* genes, proteins, methylation and some demographic and clinical variables. Statistical analyses were conducted using the IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp., Armonk, NY, USA). All statistical tests were 2-tailed, and a  $P < 0.05$  was considered statistically significant.

## Results

Table 2 presents the participants' demographic and clinical characteristics. The mean (SD) number of oocytes was statistically significantly higher in the healthy lifestyle group (Group1) than in the group without a healthy lifestyle (Group 2), ( $3.83 \pm 0.99$  vs.  $3.27 \pm 0.64$ ,  $p = 0.004$ ). The number of the MII retrieved oocyte was statistically significantly larger in Group 1 than in Group 2 ( $2.83 \pm 0.99$  vs.  $2.35 \pm 0.66$ ,  $p = 0.013$ ).

Table 2  
Clinical parameters for without healthy lifestyle and with healthy lifestyle of POR patients

(n = 80)	Without healthy lifestyle	With healthy lifestyle	P-value
Age (year)	29.60 ± 3.77	29.66 ± 4.26	0.948
Marriage duration (year)	4.15 ± 0.89	4.39 ± 1.24	0.322
Infertility duration (year)	3.23 ± 0.80	3.59 ± 1.16	0.109
AMH	1.30 ± 0.22	1.39 ± 0.31	0.123
AFC	5.95 ± 1.04	6.49 ± 1.38	0.051
no. Oocyte	3.27 ± 0.64	3.83 ± 0.99	0.004*
no. MII	2.35 ± 0.66	2.83 ± 0.99	0.013*
Oocyte quality	2.50 ± 0.93	3.51 ± 1.05	0.000*
Total embryo	1.30 ± 0.56	1.93 ± 0.26	0.000*
no. clinical Pregnancy	1.80 ± 0.41	1.70 ± 0.46	0.040
<i>ICAM1</i> Gene (Fold change)	1.14 ± 0.65	14.1 ± 4.52	0.000*
<i>HLA-G</i> Gene (Fold change)	1.17 ± 0.63	14.11 ± 4.52	0.000*
<i>ICAM1</i> DNA methylation (Fold change)	1.20 ± 0.57	15.13 ± 4.28	0.000*
<i>HLA-G</i> DNA methylation (Fold change)	1.25 ± 0.53	15.21 ± 4.33	0.000*
<i>ICAM1</i> Protein (Fold change)	0.98 ± 0.07	1.22 ± 0.12	0.000*
<i>HLA-G</i> Protein (Fold change)	0.98 ± 0.07	1.22 ± 0.12	0.000*
<b>Continuous variables are presented as mean ± SD. *P &lt; 0.05 was considered significant.</b>			
AFC: antral follicular count AMH: anti Müllerian hormone , MII: Retrieved oocytes were classified into metaphase II			

The clinical pregnancy rate (fetal heart detection by ultrasound) was significantly different between the two groups. Indeed, the clinical pregnancy rate was higher in the healthy lifestyle group than in Group 2 (1.80 ± 0.41 vs. 1.70 ± 0.46, 0.040). There was a statistically significant difference in the clinical variables of total embryo number and oocyte quality between the groups. The number of total embryo and oocyte quality was statistically significantly higher in Group 1 than in Group 2 (oocyte quality: 3.51 ± 1.05 vs. 2.50 ± 0.93, p=0.000; total embryo: 1.93 ± 0.26 vs. 1.30 ± 0.56, p = 0.000). In addition, a marginally significant difference was observed in the AFC level between the two groups (p = 0.051). There was no statistically significant difference in age, marriage duration, infertility duration, and AMH level.

As Fig [3, (a,b)] shows, the relative expression of *ICAM1* and *HLA-G* genes and proteins was significantly increased in Group 1 (p = 0.000) than in Group 2.

A notable increase in *ICAM1* and *HLA-G* DNA methylation was observed in Group 2 and in Group 1 (p = 0.000), (Fig. 3, c).

Based on the univariate analysis (Table 3, 4), oocyte number (p = 0.03), and oocyte quality, total embryo, and type of group (p = 0.000) in the groups studied (women with and without a healthy lifestyle) were significantly associated with *ICAM1* and *HLA-G* gene expression and DNA methylation. Variables of oocyte number, and oocyte quality, total embryo, MII number and type of group were significantly associated (Table 2) with *ICAM1* and *HLA-G* proteins expression in the groups studied.

Table 3

Univariate regression analysis for factors associated with ICAM1 and HLA-G Genes, proteins and DNA methylation

	<i>ICAM1</i> Gene			<i>HLA-G</i> Gene			ICAM1 Protein			HLA-G Protein		
	B	SE	P value	B	SE	P value	B	SE	P value	B	SE	P value
Age	-0.105	0.204	0.609	0.108	0.204	0.598	-0.005	0.004	0.277	-0.004	0.004	0.322
AMH	3.11	3.014	0.305	3.09	3.01	0.307	0.50	0.065	0.444	0.044	0.065	0.499
AFC	1.15	0.65	0.079	1.14	0.64	0.081	0.024	0.014	0.088	0.022	0.014	0.108
no .Oocyte	1.99	0.903	0.030	1.98	0.901	0.031	0.058	0.019	0.003	0.056	0.019	0.004
no. MII	1.66	0.91	0.073	1.66	0.91	0.073	0.051	0.019	0.009	0.049	0.019	0.013
Oocyte quality	2.62	0.67	0.000	2.62	0.67	0.000	0.052	0.015	0.001	0.050	0.015	0.001
Total embryo	7.34	1.28	0.000	7.32	1.28	0.000	0.149	0.028	0.000	0.147	0.028	0.000
no .Pregnancy	-0.91	1.89	0.631	-0.903	1.88	0.632	-0.022	0.040	0.589	-0.017	0.040	0.674
Group (Without healthy lifestylevs. With healthy lifestyle)	-12.96	0.722	0.000	-12.96	0.721	0.000	-0.242	0.022	0.000	-0.240	0.022	0.000
<b>B; Unstandardized coefficient, SE; Standard error, AFC: antral follicular count, AMH: Anti Müllerian hormone</b>												

Table 4

Multivariate regression analysis for factors associated with *ICAM1* and *HLA-G* Genes and proteins

	<i>ICAM1</i> Gene			<i>HLA-G</i> Gene			ICAM1 Protein			HLA-G Protein		
	B	SE	P value	B	SE	P value	B	SE	P value	B	SE	P value
AMH							-0.148	0.061	0.018	-0.149	0.062	0.018
no. MII							0.048	0.019	0.016	0.046	0.020	0.021
Group (Without healthy lifestylevs. With healthy lifestyle)	-12.96	0.722	0.000	-12.96	0.721	0.000	-0.233	0.022	0.000	-0.231	0.022	0.000
<b>B; Unstandardized coefficient, SE; Standard error, AMH: anti Müllerian hormone</b>												

After adjusting all variables, based on the multiple linear regression model results, the AMH level had negatively significant association with the ICAM1 and HLA-G proteins expression ( $p = 0.018$ ); for each unit increase in the AMH level, the expected oocyte quality was decreased by 0.14. The multiple linear regression model indicated that the number of MII was positively associated with the ICAM1 and HLA-G proteins expression ( $p = 0.021$ ); for each unit increase in AMH, the expected ICAM1 and HLA-G proteins expression was increased by 0.04. This multivariate model revealed in groups 1 and 2 was negatively

associated with *ICAM1* and *HLA-G* genes. Although the *ICAM1* and *HLA-G* protein expression in women without a healthy lifestyle was decreased by nearly 13 times than in women with a healthy lifestyle ( $p = 0.000$ ), the expected *ICAM1* and *HLA-G* protein expression was decreased by nearly 0.23 times in women without a healthy lifestyle than in women with a healthy lifestyle ( $p = 0.000$ ) (Table 4). The rest of variables included in the univariate model (Table 3) were not significantly associated with oocyte quality in multiple models.

## Discussion

In the present study, our finding showed that *ICAM-1* and *HLA-G* transcripts and proteins in POR with healthy lifestyle were upregulated compared to the POR without a healthy lifestyle. In addition, the *ICAM-1* and *HLA-G* DNA methylation status in the POR with a healthy lifestyle was hypomethylated compared to the POR without a healthy lifestyle. In fact, lifestyle habit can affect the transcript and proteins profile alterations in cumulus cells from POR patients. Furthermore, it may affect the methylation status in the cumulus cells of POR patients. Moreover, based on our data, the groups without a healthy lifestyle showed significantly lower numbers of good quality oocytes and clinical pregnancy rate compared to the group with a healthy lifestyle. As a result, lifestyle habit can affect the ovulation, oocyte maturation and clinical pregnancy rate in POR patients. Oocyte quality is one of the major limiting factors for the success of the ART cycle in POR patients [19]. Oocyte maturation is achieved during folliculogenesis. Folliculogenesis is the maturation of the ovarian follicle. Folliculogenesis needs communication between the oocyte and surrounding somatic cells [20]. One of the kinds of somatic cells is cumulus cells. Cumulus cells play a potential role in achieving oocyte development [21]. Based on a recent study, some genes are expressed in the cumulus cells that can be considered genetic markers for the association between oocyte and embryo quality. Probably, developmental signals from cumulus cells are transferred to the oocyte via gap junctions and pathways, which can affect the oocyte maturation [22]. The present study aimed to determine the novel biochemical marker to evaluate the oocyte quality and clinical pregnancy rate in the ART treatment of POR patients, and our data showed that lifestyle might be considered a biochemical marker for oocyte maturation increasing the clinical pregnancy rate. One of the consequent complications of POR is oocyte maturation disorder [23]. Nowadays, overuse of plastic objects to store hot food and hot drinks has caused numerous health problems in humans like reproduction disorders, including POR [24]. In fact, plastic objects made of polycarbonate and polycarbonates contain BPA [25]. According to the recent study, BPA may affect the infertility related gene expression that is closely associated with reproductive, but this association interaction mechanism is unclear. Owing to heat, acidic or alkaline substances, polycarbonate will break and BPA can attach to food and drink, and it can enter the body [26]. BPA can act like sex hormones, so that it can disrupt the endocrine system and hormonal signaling pathways. BPA in small amounts may interfere in the reproduction related gene expression, and it can interfere in the function of ovary, uterus and other reproductive organs [27]. Pednekar et al. [28] reported that infertile women showed significantly higher plasma concentrations of BPA. As a result, lifestyle habit plays a vital role in the regulating of ovarian response, oocyte maturation, reproductive process and genes expression. In this study, we evaluated the expression profile for *ICAM-1* and *HLA-G* genes in the cumulus cells of infertile women with poor response to ovarian stimulation (POR) based on their healthy lifestyle. The probable effect of plastic objects usage was observed on the genes, proteins, DNA methylation pattern, oocyte quality and clinical pregnancy rate in these women. According to other studies, altered gene expression of CCs may play a role in women with POR pathogenesis [29, 30]. Therefore, assessment of genes expression is valuable to understand the etiology of infertile women with POR patients, since oocyte maturation dysfunction is one of the disorders in POR patients [31]. Recently, it has been shown that alternation of *ICAM1* and *HLA-G* genes can affect the quality and quantity of oocytes [32]. Our findings revealed that a major contributor to oocyte maturity disorder and infertility in the POR patients without a healthy lifestyle can be a reduction in *ICAM1* and *HLA-G* transcript and proteins expression as well as hypermethylation of DNA in *ICAM1* and *HLA-G*. According to recent studies, comparison of POR patients to healthy, normal ovulatory fertile women with a history of male infertility history has shown differential expression of *ICAM-1* genes [33].

The *ICAM-1* gene expression pattern indicated that it is important in oocyte maturity and grading. It appeared that dysregulation of *ICAM-1* expression could be associated with defective oocyte maturation and grading [34]. The studies demonstrated that *ICAM-1* was a probable key effector of the oocyte maturation. Although the sICAM-1 release was very high in immature oocytes, it was decreased in mature oocytes. They regarded sICAM-1 as a biochemical marker for oocyte

maturation and grading [35]. The ICAM-1 appears to play a critical role in oocyte development, while ICAM-1 levels play the role of a predictive marker in oocyte maturation and quality. Borgtti and Rizzo [36] proposed sICAM-1 as a biochemical marker for oocyte maturation and grading. Their findings supported a significant correlation between *ICAM-1* gene and protein expression and oocyte maturation. A recent study has shown that *ICAM-1* is expressed on leukocytes, epithelial and endothelial cells [37]. Furthermore, HLA-G plays a vital role in the oocyte maturation and preimplantation of embryos. Jurisicova et al. detected HLA-G mRNA expression by preimplantation human embryos [38]. They demonstrated the presence of HLA-G mRNA and protein in the blastocysts tested [39]. Yao et al. [40] revealed the expression of the HLA-G protein by human preimplantation embryos with an increasing ratio of positive embryos with developmental stage. Kaneko [41] indicated that the low expression of HLA-G in cumulus cells was associated with lower oocyte quality, poor fertilization, and reduced embryo development [42]. Rooij van reported the low level of HLA-G in the cumulus cells of POR patients [43]. The gene expression alternation in the cumulus cells of women with POR after dehydroepiandrosterone (DHEA) supplementation can affect the oocyte maturation [44]. According to recent study, oocyte quality decreases with increasing age [45]. Our findings indicated that age was directly associated with oocyte quality and aging. Furthermore, another study showed that younger POR patients' quality of oocyte and grading were better than older patients' [46]. Our study also highlighted the role of age as one of the components in oocyte maturation. The present study emphasized the association of lifestyle with the *ICAM1* and *HLA-G* genes expression alteration and oocyte quality as an opinion for the deeper understanding of the etiology of infertile women with POR patients.

## Conclusion

The current study was the first single center lifestyle program of POR patients in Iran. We found that upregulation of *ICAM1* and *HLA-G* in POR appeared to be related to nutrition and lifestyle. The nutrition and lifestyle may be effective on the methylation pattern, expression profile and proteins level in cumulus cells from POR. Moreover, nutrition and lifestyle may act as a biochemical marker for ovulation and the oocyte maturation process.

## Abbreviations

POR  
Poor Ovarian Response  
AMH  
Anti Müllerian Hormone  
AFC  
Antra Follicle Count  
BPA  
BisphenolA  
ECM  
Extracellular Matrix  
HLA-G  
Human Leukocyte Antigen-G  
CCs  
Cumulus Cells  
GnRH  
Gonadotropin Releasing Hormone  
ICSI  
Intracytoplasmic Sperm Injection  
COS  
Controlled Of Ovarian Stimulation  
SC

Subcutaneous  
RFSH  
Recombinant Follicle Stimulating Hormone  
E2  
Estradiol  
HCG  
Human Chorionic Gonadotropin  
IM  
Intramuscular  
CoCs  
Cumulus-Oocyte Complexes  
PBS  
Phosphate-Buffered Saline  
MII  
Metaphase II Gametes  
MSP  
Methylation-Specific Polymerase Chain Reaction  
DHEA  
Dehydroepiandrosterone

## **Declarations**

### **Funding**

Not available

### **Conflicts of interest/Competing interests**

Authors have NO conflict of interest

### **Availability of data and material**

They are available on the request

### **Code availability**

None

### **Authors' contributions**

ZN and AM: conceptualization of project, ZN and SA: data analyses, SA: collection and laboratory work, MK: data collection

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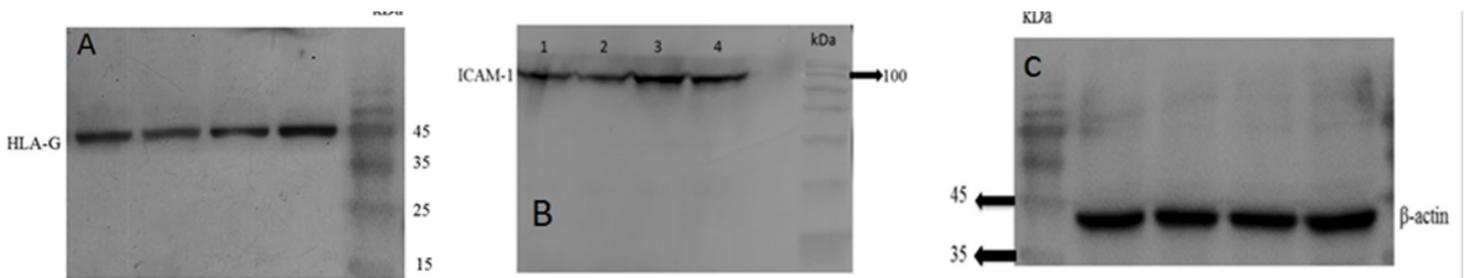
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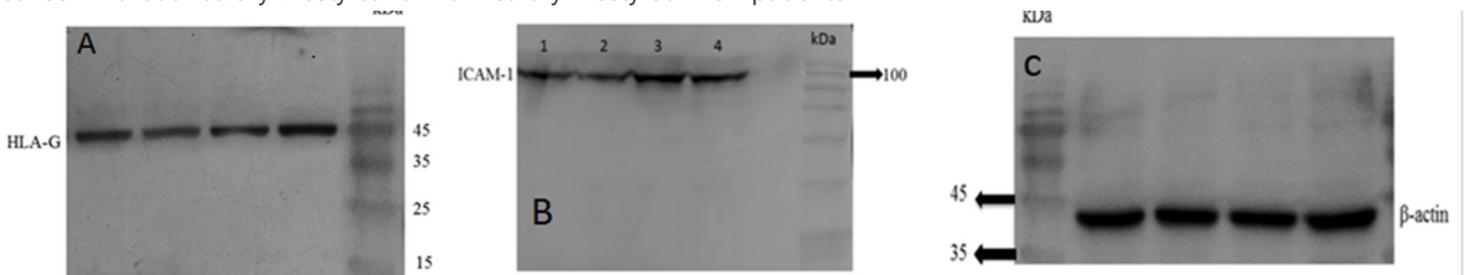
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## Figures



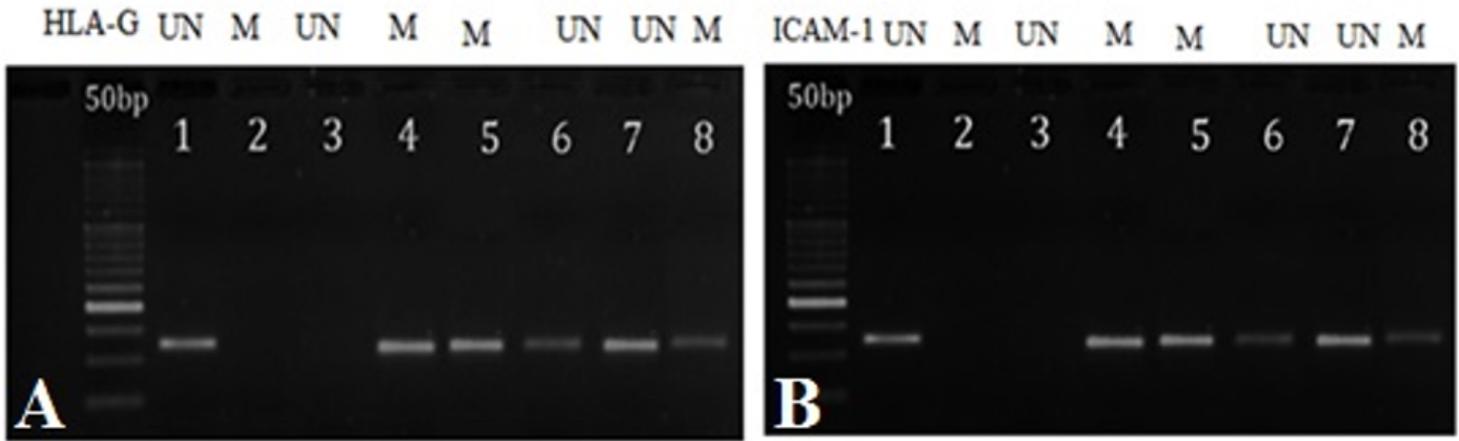
**Figure 1**

Comparison of (A) HLA-G(43 kDa), (B) ICAM-1 (100 kDa) and (C)  $\beta$ -actin (42 kDa). Protein levels in western blot analysis between without healthy lifestyle and with healthy lifestyle of POR patients.



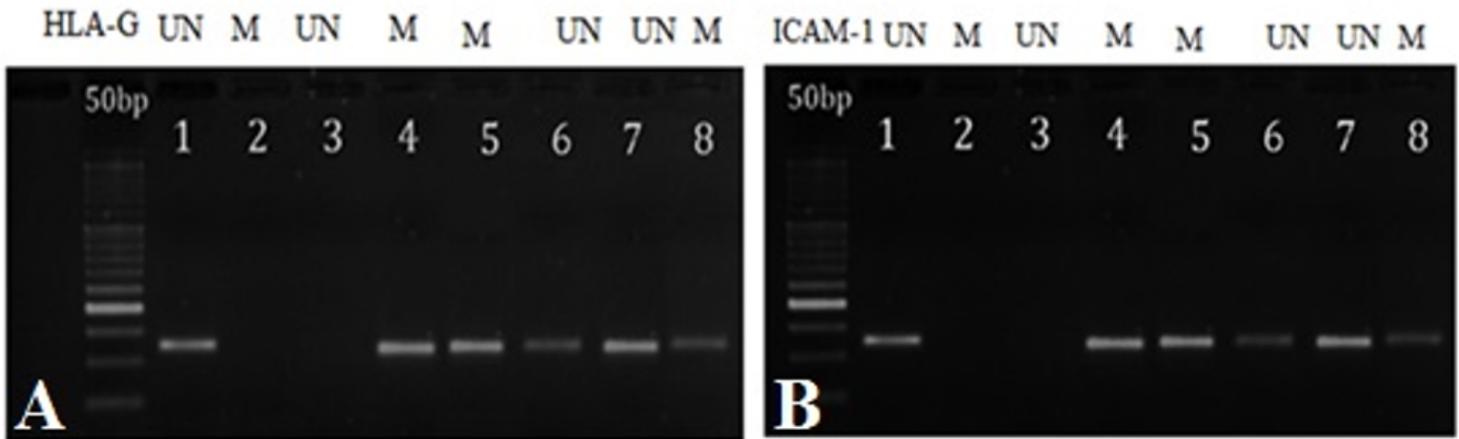
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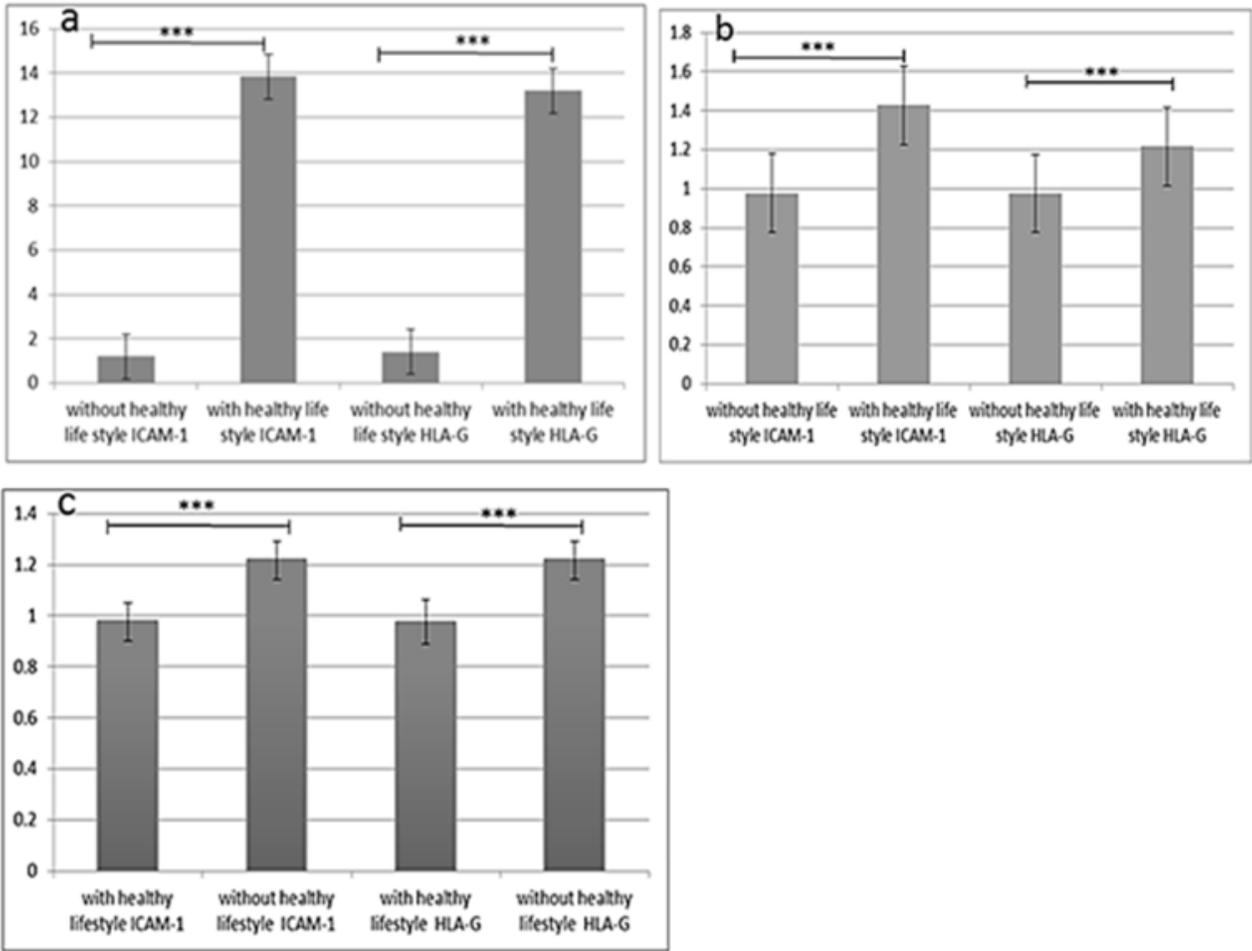
**Figure 2**

Methylation profiling of (A) HLA-G,(B) ICAM-1by methylation-specific PCR (MSP). Results of MSP on(A) HLA-G,(B) ICAM-1 promoters from gDNA isolated from without healthy lifestyleand with healthy lifestyleof POR patients. (M=methylated primer;UN=unmethylated primer).The 1 and 2 bands are for MSP positive control of fully methylated gDNA (100%). The 3 and 4 bands are for MSP positive control ofunmethylated placental gDNA, The 5 and 6 bands are for without healthy lifestyle. The 7 and 8 bands are for healthy lifestyle of POR patients.



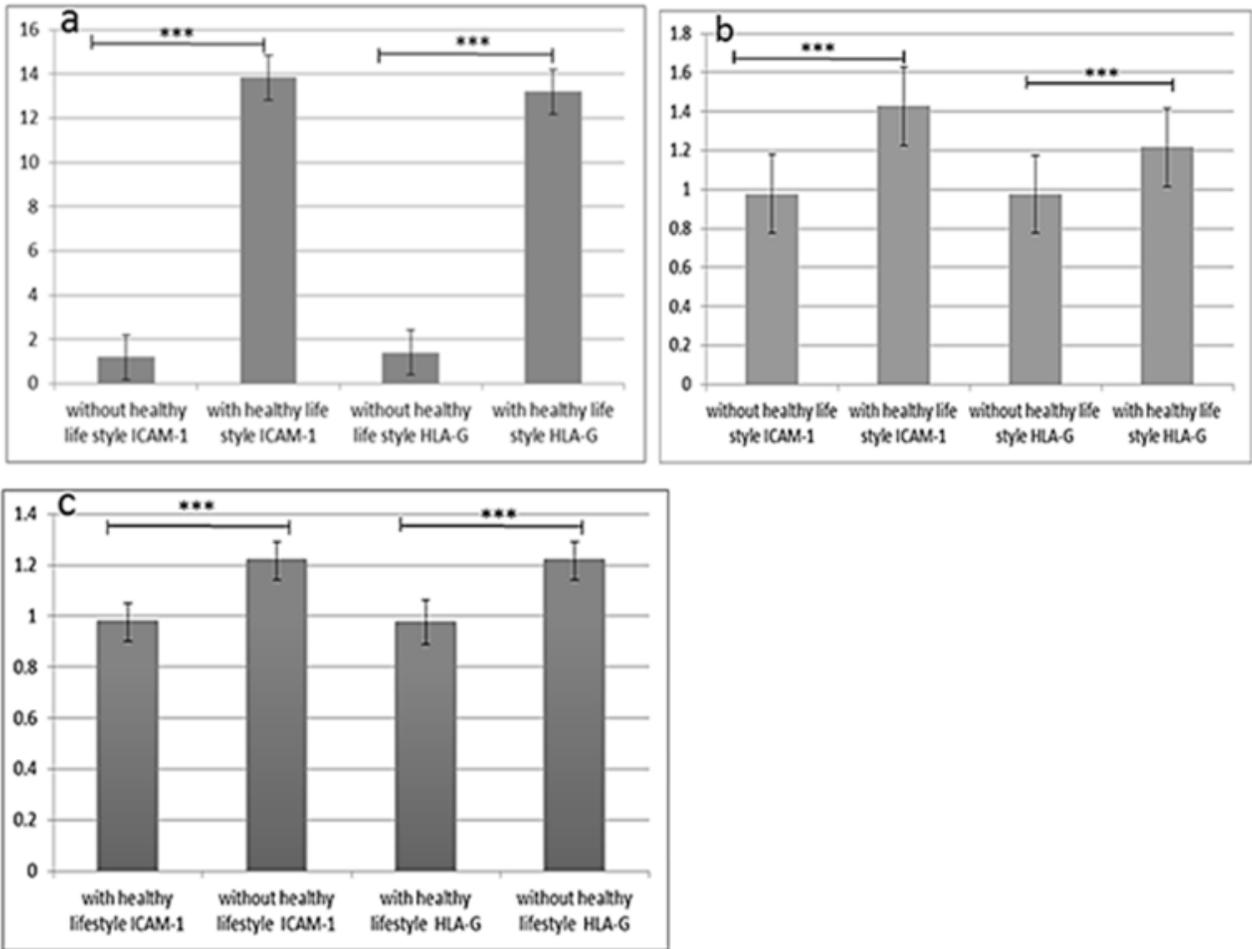
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**Figure 3**

a) Comparison of ICAM-1 and HLA-G genes expression of Real time Q PCR analysis b) ICAM-1 and HLA-G proteins expression and, c) ICAM-1 and HLA-G DNA methylation between without healthy lifestyle and with healthy lifestyle of POR patients.



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