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Cell-free mtDNA level and its biomarker potency for ART outcome are different in follicular fluid of PCOS and non-PCOS women

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1 **Cell-free mtDNA level and biomarker potency for ART**
2 **outcome are different in follicular fluid of PCOS and non-**
3 **PCOS women**

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21

22 **Abbreviations**

23 CfDNA: cell-free DNA

24 FF cf-mtDNA: follicular fluid cell-free mitochondrial DNA

25 PCOS: polycystic ovary syndrome

26 ART: assisted reproductive techniques

27 MGCs: mural granulosa cells

28 FF: follicular fluid

29 ICSI: intracytoplasmic sperm injection

30 COC: cumulus oocyte complex

31 RCN: relative copy number

32 DAMP: damage-associated molecular pattern

33 ROS: reactive oxygen species

34 **Abstract**

35 **Introduction:** Lack of reliable biomarkers for estimating the outcome is one of the current
36 gaps in ART. In this study, we assessed whether cell-free mitochondrial DNA within the follicular
37 fluid (FF cf-mtDNA) of PCOS patients has biomarker applicability or not. Furthermore, probable
38 involved mechanisms in the FF cf-mtDNA pathway were evaluated.

39 **Methods:** The level of FF cf-mtDNA was compared between 50 PCOS patients and 50 women
40 without any certain reproductive disorder, and analyzed for correlations with ART outcome. The
41 associations between levels of FF cf-mtDNA and *TFAM*, *POLG*, and *RNase H1* genes
42 expression in mural granulosa cells (MGCs), as well as IL-6, and TNF α in follicular fluid (FF)
43 were assessed.

44 **Results:** We identified that FF cf-mtDNA level was significantly lower in PCOS women and
45 was accompanied by a reduction in the expression of mtDNA biogenesis genes in MGCs of the
46 patients. Although a significant association between FF cf-mtDNA level and ART outcome was
47 observed in the control group, no correlation could be proved in the PCOS group. Moreover, the
48 expression level of TFAM was negatively associated, while amounts of IL-6 and TNF α were
49 positively correlated with FF cf-mtDNA level in both groups.

50 **Conclusion:** PCOS patients present a lower FF cf-mtDNA level in comparison with non-PCOS
51 women. FF cf-mtDNA has biomarker applicability for ART outcome in women without any
52 certain reproductive disorder, but not for those with PCOS. It seems that mtDNA packaging
53 dysfunction results in elevated FF cf-mtDNA, and subsequent effects are triggered by increasing
54 the inflammatory cytokines.

55 **Keywords:** Biomarker; Cell-free mitochondrial DNA; Follicular fluid; PCOS; ART.

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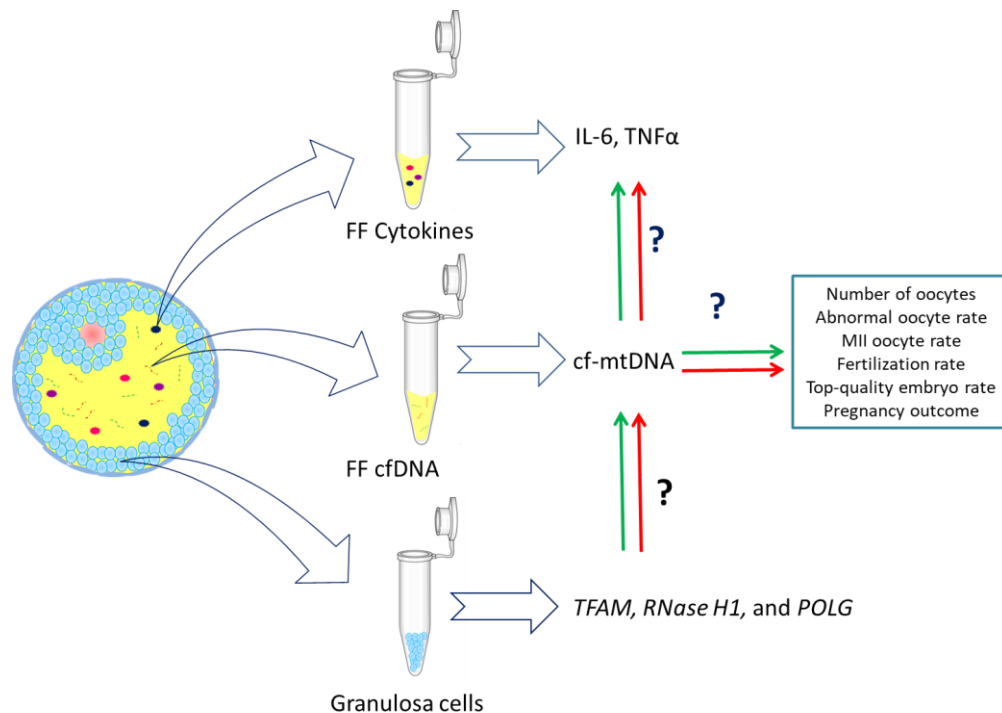
1. Introduction

58 Although the field of ART has developed in recent years, many challenges remain, such as a
59 lack of efficient methods for laboratory analyses and prediction of ART outcome. The current
60 strategy for selection of top-quality embryos, which is based on morphological criteria, is neither
61 completely reliable nor reflective of the molecular characteristics of the embryo [1, 2]. On the
62 other hand, genetic assessment of the embryo requires the biopsy procedure, an invasive
63 method that cannot be applied routinely for all cases [3]. To solve this problem, recent studies
64 have shifted to identifying molecular biomarkers, which are more reliable than the mere
65 conventional assessments [4].

66 CfDNA is a class of biomarkers that has opened new doors for the use of non-invasive
67 biomarkers in body fluids [5]. It consists of both nuclear and mitochondrial DNA, which are
68 released actively, as a homeostatic mechanism, or passively through necrotic and apoptotic
69 events [6, 7]. The cfDNA level varies in different disorders, thus providing practical information
70 in pathological situations [8, 9]. Several helpful studies have evaluated cfDNA level and proved
71 associations with ART outcome [1, 2, 10-12]. Nonetheless, there are some controversies and
72 gaps, such as insufficient research on FF cf-mtDNA, and its biomarker potential in distinct
73 female reproductive diseases [13]. Although mitochondria play a vital role in female reproductive
74 potential, due to the highly variable nature of mtDNA, it is not clear whether FF cf-mtDNA can
75 provide a reliable biomarker for ART outcome in women with different reproductive status [14,
76 15]. By all accounts, FF cf-mtDNA biomarker potential has not been assessed specifically in
77 PCOS patients regarding ART outcome.

78 To test its applicability as a biomarker, we compared the cf-mtDNA level within the FF pools of
79 PCOS patients with women without any certain reproductive disorder, in addition to investigating
80 its impact on ART outcome. Moreover, to discover the probable involved mechanisms, we
81 assessed two pathways: 1) expression levels of mtDNA biogenesis genes (polymerase gamma
82 (POLG), mitochondrial transcription factor A (TFAM), and ribonuclease H1 (RNase H1)) [16], in
83 MGCs of the patients to find out if FF cf-mtDNA level has any association with mtDNA
84 biogenesis events, and 2) FF levels of inflammatory cytokines (interleukin-6 (IL-6), and tumor
85 necrosis factor- α (TNF- α)), which can vary as the result of mitochondrial dysfunction [17-19]
86 (summarized in Fig 1).

87



88

89 **Fig. 1. Schematic figure of the study.** Green and red arrows refer to correlations in control
 90 and PCOS groups, respectively.

91

92 2. Materials and Methods

93 2.1. Patients' characteristics

94 All patients were selected from ICSI candidates at the Infertility Department of Shariati Hospital
 95 (Tehran, Iran). 50 PCOS patients and 50 women without any certain reproductive disorder (as
 96 the control group) were included in the study. The Ethics committee of Tehran University of
 97 Medical Sciences approved the study (IR.TUMS.MEDICINE.REC.1397.197). All participants
 98 signed the informed consent. PCOS disorder was diagnosed by Rotterdam criteria [20], and the
 99 control group was women with unexplained or tubal/cervical factor infertility. Patients with
 100 obesity, pelvic disorders (e.g., endometriosis, and fibroid), endocrine diseases (e.g.,
 101 hyperprolactinemia, Cushing syndrome, and hyper/hypothyroidism), other reproductive
 102 disorders (e.g., recurrent implantation failure and recurrent miscarriage), as well as severe male
 103 factor infertility were excluded from the study. The patients' age and BMI were between 22-39
 104 and 19-28, respectively. Other characteristics are listed in Table 2.

105

106 2.2. Controlled ovarian stimulation

107 All patients received an antagonist regime for controlled ovarian stimulation. Briefly, from day 3
 108 of the menstrual cycle, 150-300 IU/day of recombinant follicle-stimulating hormone (r-FSH)

109 (Gonal-F, Merck Serono SA, Switzerland) was prescribed. The optimal dosage was adjusted
110 according to the ovarian response and estradiol concentration. Ovaries were monitored, and at
111 the time that at least two follicles with the size of 14-15 mm were observed, 0.25 mg/day
112 cetorelix acetate Cetrotide (Merck Serono SA, Switzerland), as the gonadotropin-releasing
113 hormone (GnRH) antagonist was administered. Once ≥ 2 follicles reached a diameter of 18 mm,
114 Cetrotide consumption was discontinued, while 10,000 IU human chorionic gonadotropin (hCG)
115 (Ovitrelle, Merck Serono SA, Switzerland) was administered. After 36 h, oocytes were retrieved
116 via transvaginal ultrasound-guided aspiration.

117

118 **2.3. ICSI and ART outcome**

119 About 2 h after the surgery, COCs were denuded by hyaluronidase enzyme. Oocytes were
120 assessed under the microscope for quality and maturity [21]. Irregular oocytes regarding their
121 size, shape, and homogeneity of the ooplasm, as well as size of the perivitelline space and
122 thickness of the zona pellucida, were considered abnormal. Furthermore, they were categorized
123 as germinal vesicle (GV), metaphase I (MI), and metaphase II (MII) with respect to the maturity
124 stage. Mature oocytes underwent ICSI (day 0), and were incubated at 37^oc in 89% N₂, 6% CO₂,
125 and 5% O₂. Fertilization was confirmed if 2 pronuclei and extrusion of the 2nd polar body were
126 observed 18-20 h after the microinjection. On the first and second days, embryos were
127 monitored for cleavage status. The quality of the day 3 embryos was recorded by the
128 morphological criteria [22]. Grades 1 and 2 which refer to less than 20% fragmentation, and 6-8
129 regular blastomeres were defined as top-quality embryos. After embryo transfer, clinical
130 pregnancy was determined by observation of the gestational sac on ultrasound in the following
131 4 weeks.

132 ART outcome was defined as follows:

133 Abnormal oocyte rate: number of abnormal oocytes/total number of oocytes, MII oocyte rate:
134 number of MII oocytes/total number of oocytes, fertilization rate: number of fertilized
135 oocytes/total number of microinjected oocytes, top-quality embryo rate: number of grade 1-2
136 embryos/total number of embryos. All rates were reported in percentage.

137

138 **2.4. Sample preparation**

139 On the day of oocyte retrieval, follicles were aspirated without flushing and blood contamination.
140 All FFs from each patient were pooled and centrifuged at 3000g for 15 min. The volume of 15 ml
141 from the supernatant was aliquoted and stored at -80^oc for cfDNA extraction and cytokines
142 measurement.

143 To isolate MGCs, the FF-derived pellet was transferred onto 5 ml of Ficoll-Paque PREMIUM
144 density gradient media (GE Healthcare Bio-Sciences, Sweden) and centrifuged at 400g for 20
145 min. The ring-like layer was collected and washed for 5 min, at 600g. Cells were resuspended in

146 1 ml of phosphate-buffered saline (PBS) (Sigma, Germany) with 1% bovine serum albumin
 147 (BSA) (Sigma, Germany) and passed through a 40 µm cell strainer (BD Biosciences, CA, USA).
 148 Finally, the MGCs were collected by back-wash of the strainer with PBS containing 1% BSA and
 149 evaluated for viability. Subsequently, cells were processed for RNA extraction.

150

151 2.5. CfDNA extraction and quantification

152 A volume of 300 µl from each pool of FF was processed for cfDNA extraction by QIAamp DNA
 153 Mini kit (QIAGEN, Germany), according to the manufacturer's instructions. The extracted
 154 cfDNAs were eluted in 50 µl of elution buffer and quantified by NanoDrop Spectrophotometer
 155 (Thermo Scientific, USA) for concentrations.

156 For cf-mtDNA quantification, primers of *ND1* (as mitochondrial DNA gene), and *β globin* (as
 157 nuclear reference gene) were designed by AlleleID 6 software (Table 1) and synthesized by
 158 Metabion company (Metabion, Germany).

159 To evaluate the cf-mtDNA/cf-nDNA ratio, quantitative polymerase chain reaction (qPCR) was
 160 performed. Briefly, 4 µl of extracted cfDNA, 2 µl of 10 µM primers (forward and reverse), and
 161 12.5 µl of RealQ Plus 2x Master Mix Green (Ampliqon, Denmark) were mixed, and total volume
 162 was brought to 25 µl by DEPC water. The reaction was run by StepOne Real-Time PCR System
 163 with the following cycling conditions: 95°C for 15 min, followed by 40 cycles of 95°C for 20 sec,
 164 and 60°C for 1 min. Melting curve was also performed by 15 sec at 95°C, 1 min at 60°C, and 15
 165 sec at 95°C. All reactions were performed in duplicate. Finally, data was collected and FF cf-
 166 mtDNA RCN was calculated by $2x2^{\Delta CT}$ equation [23].

167

Gene name	Primers sequences (5'→3')	Amplicon size (bp)
TFAM NM_001270782.1	F: GAGGTGGTTTTTCATCTGTCTTG R: CAACGCTGGGCAATTCTTC	147
RNase H1 NM_002936.5	F: GGTATAACTAACTGGGTTCAAGG R: CTCCTTCTCTGGCTAATCTGTC	193
POLG NM_002693.2	F: GGAACCTTGCCCCACATTG R: GGCTGGTCCAAGAGTAACG	97
HPRT1 NM_000194.3	F: CGTCGTGATTAGTGATGATGAAC R: CAGAGGGCTACAATGTGATGG	177
ND1 NC_012920.1	F: CCCTAAAACCCGCCACATCT R: GAGCGATGGTGAGAGCTAAGGT	69
β globin NC_000011.10	F: ACCTCAAGGGCACCTTTGC R: AAAACATCAAGCGTCCCATAGAC	101

168

169 **Table 1. The sequence of primers that were used for qRT-PCR and qPCR**

170

171 **2.6. RNA extraction and quantification**

172 Total RNA of MGCs from each patient was extracted by Total RNA Prep kit (BIOFACT, South
173 Korea), according to the manufacturer's protocol. Extracted RNAs were eluted in 40 µl of elution
174 buffer. RNAs' quantity and quality were assessed by NanoDrop Spectrophotometer and
175 electrophoresis, respectively. Complementary DNA (cDNA) was synthesized for 500 ng of RNA
176 by 2X RT-PCR Pre-Mix kit (BIOFACT, South Korea), using oligo (dT) primer, and stored at -80°C
177 for subsequent assays.

178 Exon-exon spanning primers for *POLG*, *TFAM*, *RNase H1*, and Hypoxanthine
179 phosphoribosyltransferase 1 (*HPRT1*) (as the endogenous reference gene) were designed
180 (Table 1) and synthesized as mentioned above. They were checked by PCR to prove they do
181 not amplify DNA as the template.

182 Finally, quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed
183 in a reaction volume of 25 µl containing 12.5 µl RealQ Plus 2x Master Mix Green (Ampliqon,
184 Denmark), 2µl of 10 µM forward and reverse primers, 1 µl of cDNA, and 9.5 µl DEPC water. The
185 thermal cycle program was 95°C for 15 min, followed by 35 cycles of 95°C for 20 sec, and 60°C for
186 1 min on StepOne Real-Time PCR System. Melting curve was run precisely as described in the
187 previous section. All reactions were performed in duplicate. Data was collected, and the relative
188 expression level was calculated by $2^{-\Delta CT}$ method.

189

190 **2.7. Cytokine measurement**

191 A volume of 10 µl from the FF pool of each patient was added to 90 µl of dilution buffers of IL-6
192 and TNFα ELISA kits (Sino Biological, China). The next steps were taken according to the
193 manufacturer's protocol, and optical density (OD) was determined at 450 nm by a microplate
194 reader machine (BioTek, USA). All procedures were implemented in triplicate, and the standard
195 sample was considered for each set of the assay. The final concentrations were calculated
196 according to the standard sample.

197

198 **2.8. Statistical analysis**

199 Statistical analyses were performed by version 22.0 of SPSS software. Graphs were designed
200 by Graphpad Prism, version 8.4.2. Briefly, the normality of the data was assessed by Shapiro-
201 Wilk test. Comparison of all variables between control and PCOS groups was carried out by
202 Mann-Whitney test. Linear regression was used for analysis of the effect of patients'
203 characteristics on FF cf-mtDNA RCN, as well as FF cf-mtDNA impact on ART outcome.
204 Kruskal-Wallis and Mann-Whitney tests were applied for comparison of FF cf-mtDNA RCN
205 between distinguished defined categories in control and PCOS groups, respectively. Receiving
206 Operator Curve (ROC) was carried out to estimate the predictive value of FF cf-mtDNA RCN for
207 pregnancy outcome. Moreover, associations between levels of FF cf-mtDNA and cytokines, as

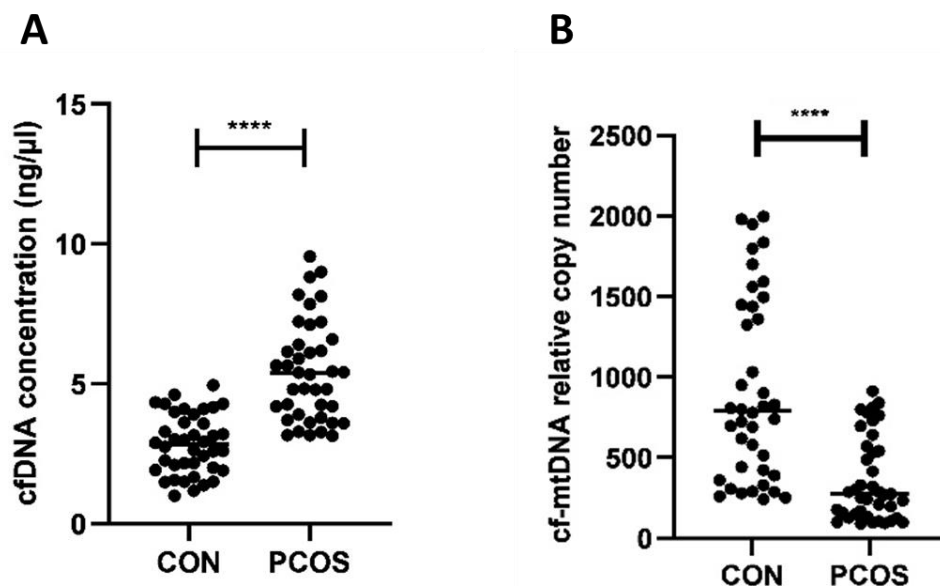
208 well as relative gene expression levels were analyzed by Spearman correlation test. Results
209 were assumed to be significant if $p \leq 0.05$.

210

211 3. Results

212 3.1. Patients' characteristics in relation to FF cf-mtDNA RCN

213 Although cfDNA concentration in PCOS patients was significantly higher than in the control
214 group (4.45 ± 1.8 ng/ μ l, and 2.84 ± 1.07 ng/ μ l, respectively, $p < 0.0001$), cf-mtDNA RCN was
215 significantly lower in PCOS patients (362.51 ± 260.57 in PCOS versus 921.34 ± 568.95 in
216 control group, $p < 0.0001$) (Fig. 2). Thus, due to the distinguished FF cf-mtDNA profile in PCOS
217 patients, all the following analyses were performed separately within the two groups.



218

219 **Fig. 2. Comparison of cfDNA concentration (A), and FF cf-mtDNA RCN (B) between**
220 **control (CON) and PCOS groups. **** $P < 0.0001$.**

221

222 According to the linear regression results, none of the patients' characteristics (Age, BMI,
223 infertility length, FSH, LH, and E2) had influence on FF cf-mtDNA RCN in either group (Table 2).
224 Moreover, there was no association between cfDNA concentration and cf-mtDNA RCN in either
225 group (data not shown).

226

227

228

Variable	Mean ± SD	Min-Max	β ± SE	p-value
Age (year)				
Control	30.45 ± 4.56	22-38	0.13 ± 20.4	0.4
PCOS	29.4 ± 4.9	22-39	-0.18 ± 8.47	0.25
BMI (kg/m ²)				
Control	23.93 ± 2.08	19.8-27.32	-0.18 ± 43.6	0.26
PCOS	24.21 ± 1.74	20.82-27.38	0.04 ± 24.23	0.8
Infertility length (years)				
Control	4.87 ± 2.65	1-11	0.22 ± 33.95	0.17
PCOS	4.8 ± 2.46	1-10	-0.002 ± 17.17	0.99
AMH (ng/ml)				
Control	2.82 ± 0.85	1.4-4.9	-0.02 ± 107.98	0.89
PCOS	7.16 ± 1.93	3.8-11.2	0.12 ± 21.71	0.45
FSH (IU/L)				
Control	7.19 ± 3.98	2.7-18.32	-0.18 ± 22.8	0.26
PCOS	6.5 ± 3.45	3.5-15.1	-0.08 ± 12.21	0.61
LH (IU/L)				
Control	6.4 ± 3.27	2.6-15.9	-0.16 ± 28.87	0.32
PCOS	14.53 ± 3.9	8.5-22.6	-0.07 ± 10.83	0.67
E2 (pg/ml)				
Control	54.05 ± 35.18	10.4-121.2	0.19 ± 2.57	0.23
PCOS	51.8 ± 26.96	9.61-100.5	-0.03 ± 1.57	0.86

229

230 **Table 2. Associations between FF cf-mtDNA RCN and patients' characteristics.** Data is
231 presented as mean ± standard deviation (SD), regression coefficient ± standard error (β ± SE).
232 Body mass index (BMI), anti-Müllerian hormone (AMH), follicle-stimulating hormone (FSH),
233 luteinizing hormone (LH), 17β-estradiol (E2).

234

235 3.2. Impact of FF cf-mtDNA RCN on ART outcome

236 Regarding the retrieved oocytes, the higher number of oocytes was related to FF pools with
237 lower cf-mtDNA RCN, in both PCOS (p = 0.031) and control (p = 0.021) groups. Among
238 retrieved oocytes from each patient, only in PCOS women, FF cf-mtDNA RCN had an inverse
239 effect on MII oocyte rate (p = 0.01). Neither abnormal oocyte rate nor fertilization rate were
240 affected by FF cf-mtDNA RCN in both groups (Table 3).

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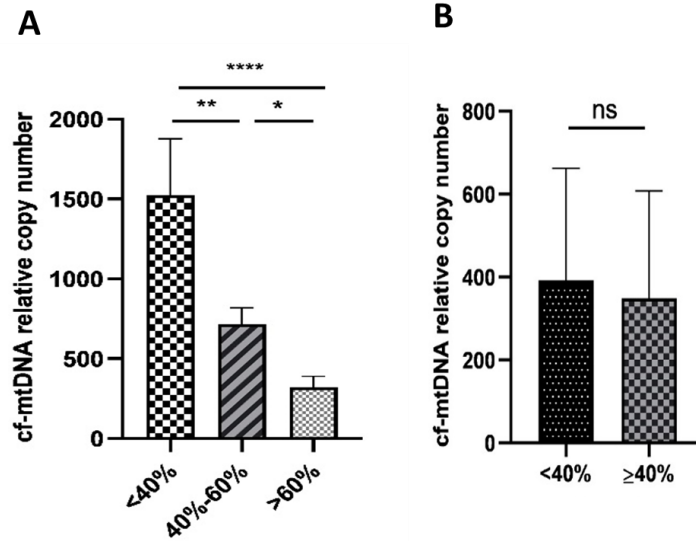
Variable	Mean ± SD	β ± SE	p-value
Number of oocytes			
Control	14.5 ± 6.02	-0.36 ± 0.002	0.021
PCOS	22.18 ± 5.4	-0.34 ± 0.003	0.031
MII oocyte rate (%)			
Control	71.9 ± 12.65	-0.11 ± 0.004	0.51
PCOS	69 ± 11.04	-0.39 ± 0.006	0.01
Abnormal oocyte rate (%)			
Control	7.72 ± 8.29	0.27 ± 0.002	0.1
PCOS	6.94 ± 6.93	-0.02 ± 0.004	0.9
Fertilization rate (%)			
Control	67.63 ± 9.78	-0.18 ± 0.003	0.26
PCOS	65.18 ± 7.36	-0.07 ± 0.005	0.65
Top-quality embryo rate (%)			
Control	51.9 ± 18.2	-0.84 ± 0.003	<0.0001
PCOS	50.1 ± 13.35	-0.22 ± 0.008	0.16

247

248 **Table 3. Correlations between FF cf-mtDNA RCN and ART outcome.** Data is presented as
249 mean ± standard deviation (SD), regression coefficient ± standard error (β ± SE). Metaphase II
250 (MII).

251

252 Linear regression was utilized in the investigation with regard to the impact of FF cf-mtDNA
253 RCN on day 3 embryo quality. The results demonstrated that by elevating the FF cf-mtDNA
254 RCN, the top-quality embryo rate was reduced among individuals in the control group (p <
255 0.0001) (Table 3). Furthermore, based on the rates of top-quality embryos, we divided the
256 control group into three categories: patients who had low (< 40%), moderate (40-60%), and high
257 (> 60%) rates of top-quality embryos. Analysis indicated that FF cf-mtDNA RCN was
258 dramatically reduced through low- to high-rate groups (Fig. 3-A). In contrast, in PCOS patients,
259 no significant association was observed between FF cf-mtDNA RCN and top-quality embryo
260 rate. In detail, according to the linear regression analysis, FF cf-mtDNA RCN had no significant
261 impact on top-quality embryo rate (p = 0.16) (Table 3). We also classified the PCOS patients
262 into two groups: patients with low (< 40%) and moderate/high (≥ 40%) rates for top-quality
263 embryos (due to the low number of patients that had more than 60% top-quality embryo, we
264 combined the moderate and high rates into a single category). Although FF cf-mtDNA RCN was
265 higher in the group of patients with the low number of top-quality embryos, the difference was
266 not significant (Fig. 3-B).

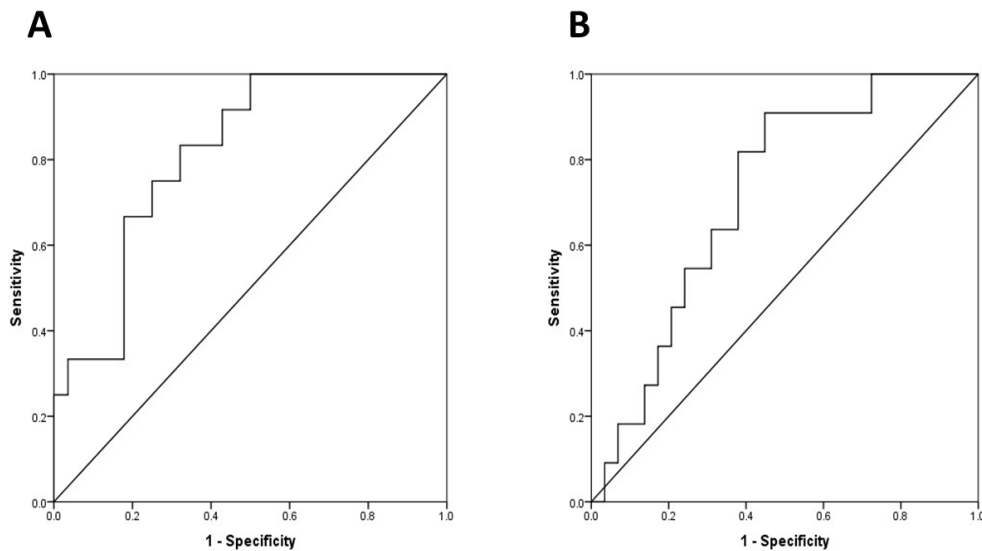


267

268 **Fig. 3. Comparison of FF cf-mtDNA RCN in different categories according to the rates of**
 269 **top-quality embryo in control (A) and PCOS (B) groups.** P < 0.05 was considered as a
 270 significant difference. *P = 0.036, **P=0.005, ****P <0.0001. Not significant (ns).

271

272 To evaluate the predictive value of FF cf-mtDNA RCN for clinical pregnancy outcome, ROC
 273 curve analysis was performed. In the control group, the area under the ROC curve was
 274 estimated at 0.81, with 83.33% specificity and 67.86% sensitivity, while in the PCOS group, the
 275 area was 0.71, with 81.8% and 55.2% for specificity and sensitivity, respectively (Table 4) (Fig.
 276 4).



277

278 **Fig. 4. Graphs of ROC curve analysis for prediction of clinical pregnancy outcome by FF**
 279 **cf-mtDNA RCN in control (A), and PCOS (B) groups.**

280

Groups	AuROC [95% CI]	Cut-off value	p-value	Sensitivity (%)	Specificity (%)
Control	0.813 [0.679-0.946]	≤760	0.002	67.86	83.33
PCOS	0.718 [0.555-0.88]	≤295	0.035	55.2	81.8

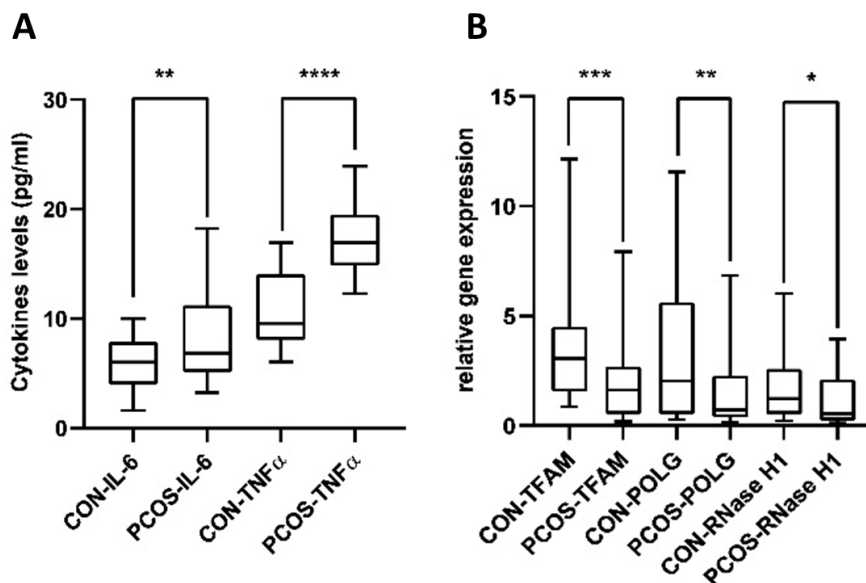
281

282 **Table 4. Predictive value of FF cf-mtDNA RCN for pregnancy outcome.** Data is presented
283 as area under the ROC curve (AuROC) [95% confidence interval].

284

285 3.3. Correlations between expression levels of mtDNA biogenesis 286 genes in MGCs and FF cf-mtDNA RCN

287 Expression levels of *TFAM* ($p = 0.0006$), *POLG* ($p = 0.008$), and *RNase H1* ($p = 0.014$) genes
288 were significantly declined in MGCs of PCOS patients in comparison with the control group (Fig.
289 5-B). Regarding correlations between these genes' expression levels and FF cf-mtDNA RCN in
290 each individual, results from Spearman analysis indicated only a significant negative association
291 between the levels of *TFAM* and FF cf-mtDNA in both groups ($r = -0.445$; $p = 0.004$, and $r = -$
292 0.338 ; $p = 0.033$, in control and PCOS patients, respectively).



293

294 **Fig. 5. Comparison of cytokines (A), and relative gene expression levels (B) between**
295 **control (CON) and PCOS groups.** *P = 0.014, **P = 0.008, ***P = 0.0006, ****P < 0.0001.
296 Interleukin-6 (IL-6), tumor necrosis factor-α (TNFα), mitochondrial transcription factor A (TFAM),
297 polymerase gamma (POLG), ribonuclease H1 (RNase H1).

298

299

3.4. Associations between FF cf-mtDNA RCN and inflammatory cytokines

When FF levels of the cytokines were compared between the two groups, PCOS patients had significantly higher levels of IL-6 (8.19 ± 3.95 pg/ml versus 5.73 ± 2.25 pg/ml; $p = 0.0089$), and TNF α (17.39 ± 3.1 pg/ml versus 10.6 ± 3.34 pg/ml, $p < 0.0001$) than the control group (Fig. 5-A). Spearman analysis also indicated significant positive correlations between the aforementioned cytokine levels and FF cf-mtDNA RCN in both groups ($r = 0.87$; $p < 0.0001$, and $r = 0.88$; $p < 0.0001$ for IL-6; as well $r = 0.91$; $p < 0.0001$, and $r = 0.49$; $p = 0.0013$ for TNF α , in control and PCOS groups, respectively) (Table 5).

Variable	Mean \pm SD	Min-Max	ρ	<i>p</i> -value
IL-6				
Control	5.73 ± 2.25	1.62-10.01	0.87	<0.0001
PCOS	8.19 ± 3.95	3.26-18.24	0.88	<0.0001
TNF α				
Control	10.6 ± 3.34	6.03-16.92	0.91	<0.0001
PCOS	17.39 ± 3.1	12.28-23.94	0.49	0.0013

Table 5. Correlations between levels of cytokines and FF cf-mtDNA RCN. Data is presented as mean \pm standard deviation (SD), and correlation coefficient (ρ) by Spearman analysis. Interleukin-6 (IL-6), tumor necrosis factor- α (TNF α).

4. Discussion

This study was designed to assess whether FF cf-mtDNA has an equal efficiency to be used as the biomarker of ART outcome in women with distinct reproductive potential, in addition to investigating the factors that can affect or be affected by FF cf-mtDNA, to determine the probable involved mechanisms. The results demonstrated that despite the higher FF cfDNA concentration in PCOS patients, FF cf-mtDNA RCN was significantly lower than in the control group, which consisted of women without any certain reproductive disorder. The reduced FF cf-mtDNA level in the PCOS group might be due to the low copy number of cellular mtDNA in the patients that has been reported by some studies [24-26]. Comparison of mtDNA biogenesis genes (*TFAM*, *POLG*, and *RNase H1*) between the groups, showed a significant reduction in expression levels of the mentioned genes in MGCs of the PCOS patients, which can prove the low mtDNA biogenesis events, and therefore the decrease in mtDNA reserve in follicular cells of the PCOS patients. All in all, due to the distinguished FF cf-mtDNA profile in PCOS and control groups, it was concluded that assessment of FF cf-mtDNA biomarker potential should be performed separately for each group.

330 Our results demonstrated that none of the patients' characteristics (e.g., age, BMI, and hormone
331 levels) had impact on FF cf-mtDNA RCN in either group. In this regard, FF cf-mtDNA seems to
332 provide a reliable biomarker that is not affected by individuals' characteristics. To evaluate its
333 biomarker potential, we compared the FF cf-mtDNA RCN of each patient with different factors in
334 ART. According to the results, cf-mtDNA RCN within the FF pools of each patient had an
335 inverse impact on the number of retrieved oocytes but did not influence either abnormal oocyte
336 rate or fertilization rate. Furthermore, only in PCOS patients, MII oocyte rate was negatively
337 affected by FF cf-mtDNA RCN. This can be justified as follows: during the maturation stage of
338 the oocyte, mitochondria as well as mtDNA play indispensable roles [27-29]. That being so,
339 perhaps owing to the low intracellular mtDNA reserve, as well as mitochondrial dysfunction as a
340 common characteristic in PCOS patients [26], any abundant stress/damage (which can be
341 diagnosed by elevated level of FF cf-mtDNA), can directly influence the oocytes' maturation.
342 Meanwhile, in women with sufficient mitochondria and mtDNA reserves, the maturation of the
343 oocytes cannot be impaired.

344 With respect to FF cf-mtDNA biomarker potential for embryo quality, results from the control
345 group revealed that women with the higher percentage of top-quality embryo displayed lower FF
346 cf-mtDNA RCN, and vice versa. But in the PCOS patients, no significant relationship was
347 observed. Since PCOS is a multifactorial disorder in which a wide spectrum of factors, such as
348 genetic, epigenetic, etc. are involved [30], it seems that FF cf-mtDNA alone cannot provide a
349 biomarker for embryo quality. Recently, Liu and colleagues reported no association between FF
350 cf-mtDNA RCN and day 3 embryo quality [2]. It must be noted that 60% of the couples in that
351 study had female factor infertility, but the types were not reported. Our results provide evidence
352 that women with distinguished reproductive disorders have different FF cf-mtDNA profiles that
353 have to be evaluated separately within their own groups. Furthermore, in some cases such as
354 PCOS, FF cf-mtDNA seemingly does not represent any association with day 3 embryo quality.
355 Hence, the heterogeneous population in the previous study [2] provides a logical explanation
356 why no correlation was found between FF cf-mtDNA RCN and day 3 embryo quality.

357 We also assessed the predictive value of FF cf-mtDNA RCN for pregnancy outcome. Although
358 both specificity and sensitivity were high in the control group, high specificity but low sensitivity
359 was obtained in PCOS patients. Thus, based on our results, FF cf-mtDNA seems to provide a
360 putative biomarker for ART outcome in women without any certain reproductive disorder, but
361 verification of its applicability in PCOS patients requires more investigation, especially with a
362 larger sample size.

363 To explore the probable involved mechanisms, we analyzed the correlations between FF cf-
364 mtDNA RCN and expression levels of mtDNA biogenesis genes in MGCs of the patients.
365 Among the three mentioned genes, only the expression level of *TFAM* was in a significant
366 negative association with FF cf-mtDNA RCN. *POLG*, and especially *RNase H1* have more
367 significant roles in mtDNA biogenesis rather than maintenance [31, 32]. But in addition to
368 biogenesis, *TFAM* is the essential factor for mtDNA packaging and formation of nucleoid
369 structures [33]. Due to the lack of histones, nucleoid structures are crucial for the protection of
370 mtDNA from damage such as oxidative stress [34]. In this regard, it seems reasonable that in
371 the patients with lower expression levels of *TFAM*, the FF cf-mtDNA RCN was raised.

372 Consistent with this, Xu and colleagues reported that there was a reduction in expression of
373 *TFAM* alongside the decrease and increase in levels of nucleoid and 8-hydroxydeoxyguanosine
374 (8-OHdG) (a product of DNA oxidation), respectively [35].

375 Data from cytokine analysis revealed that not only were the FF levels of IL-6 and TNF α higher in
376 PCOS patients, but they also had a significant positive correlation with FF cf-mtDNA RCN in
377 both groups. Elevated levels of the aforementioned cytokines in PCOS patients are the result of
378 the inflammatory phenotype of the disease that has been proved by other studies as well [36,
379 37]. Regarding the positive association with FF cf-mtDNA RCN, it should be stated that since
380 mitochondria historically originated from bacteria, extrusion of mtDNA to the cytosol or
381 extracellular environment results in recognition as endogenous DAMP by the immune system,
382 thus triggering the inflammatory responses through the secretion of cytokines, such as IL-6, IL-
383 8, TNF α , etc. [38]. Nonetheless, the consequence of inflammation is induction of cell
384 damage/death [21], which in the FF microenvironment can lead to impaired oocyte quality and
385 embryo developmental competence [44].

386 Taken together, to sum up the mechanisms involved, it seems that reduction of *TFAM*
387 expression level in follicular cells can make the mtDNA more susceptible to damage that might
388 lead to mtDNA break followed by extrusion to extracellular space. The cf-mtDNA in FF can be
389 recognized as DAMP, activating the inflammatory response. The induction of cell death/damage
390 by the inflammation can reduce the follicular cell population and reduce the support of the
391 oocyte during the folliculogenesis, thus impairing its developmental competence. However, in
392 PCOS patients, the oocyte developmental competence cannot be reliably reflected, since
393 mtDNA dysfunction is the sole part of the spectrum of the factors that are involved in the
394 pathology.

395 Certainly, our study had some limitations. For instance, since follicles with distinct diameters
396 represent different molecular profiles, assessment of FF cf-mtDNA level in individual follicles
397 would better clarify their potential as biomarker. Furthermore, the absolute copy number of FF
398 cf-mtDNA would be more practical. Regarding the impact of patients' characteristics on FF cf-
399 mtDNA level, we recommend an examination equipped with larger sample sizes to cover a
400 wider spectrum of characteristics. We also suggest exploring the FF cf-mtDNA biomarker
401 potential in other female reproductive disorders, such as endometriosis and primary ovarian
402 insufficiency. And for the last consideration, due to the highly variable nature of mtDNA, we
403 recommend using it as a supplemental biomarker, rather than a standalone one.

404

405 **5. Conclusion**

406 Due to the low FF cf-mtDNA level, which might be a result of the reduction in intracellular
407 mtDNA copy number, FF cf-mtDNA analyses in PCOS patients should be performed
408 independently. According to the current evidence, FF cf-mtDNA level can be recommended as a
409 biomarker of ART outcome alongside other methods in women without any certain reproductive
410 disorder, but its potential in PCOS patients requires more investigations. Furthermore, it seems
411 that the reduction of *TFAM* expression level and elevation of inflammatory cytokines are

412 respectively the upstream and downstream of FF cf-mtDNA mechanism of action to influence
413 the oocytes' developmental competence.

414

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418

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422

423 **Disclosure and conflicts of interest**

424 The authors declare they have no conflict of interest.

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