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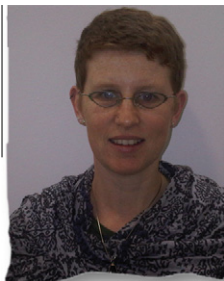
## ARTICLE

# Increased plasma cell-free DNA is associated with low pregnancy rates among women undergoing IVF—embryo transfer


Johanna Czamanski-Cohen <sup>b,\*</sup>, Orly Sarid <sup>b</sup>, Julie Cwikel <sup>b</sup>,  
Eitan Lunenfeld <sup>a,c</sup>, Amos Douvdevani <sup>d,c</sup>, Eliahu Levitas <sup>a,c</sup>, Iris Har-Vardi <sup>a,c</sup>

<sup>a</sup> Fertility and IVF Unit, Department of Ob/Gyn, Soroka University Medical Center, Beer Sheva 84105, Israel; <sup>b</sup> Center for Women's Health Studies and Promotion and The Spitzer Department of Social Work, Ben Gurion University of the Negev, Beer Sheva 84105, Israel; <sup>c</sup> Faculty of Health Sciences, Ben Gurion University of the Negev, Beer Sheva 84105, Israel; <sup>d</sup> Department of Clinical Biochemistry, Soroka University Medical Center, Beer Sheva 84105, Israel

\* Corresponding author. E-mail address: johannac@post.bgu.ac.il (J Czamanski-Cohen).



Johanna Czamanski-Cohen, MA, is a doctoral student in the Center for Women's Health Studies and Promotion, which is part of the Spitzer Department of Social Work at Ben Gurion University of the Negev. She is also an art therapist and a licensed professional counsellor in the State of Arizona. I have been conducting bio-behavioural research in the field of reproductive medicine for the past 4.5 years as well as utilizing cognitive behavioural interventions with women undergoing IVF.

**Abstract** This prospective repeated measures study was designed to examine the cell-free DNA (cfDNA) concentrations during ovarian stimulation and the relationship between cfDNA concentration and pregnancy rates in women undergoing IVF—embryo transfer. The study examined 37 women undergoing IVF treatment in an IVF unit in a university medical centre in southern Israel. cfDNA concentrations were measured by a direct fluorescence assay, pregnancy rates were identified by plasma  $\beta$  human chorionic gonadotrophin (HCG) concentrations and verified by vaginal ultrasound to determine gestational sac and fetal heart beats. Throughout the IVF cycle, at the three time points measured, the mean concentration of plasma cfDNA among all participants did not statistically significantly change. However, on the day of  $\beta$ HCG test in patients undergoing IVF—embryo transfer, plasma cfDNA concentrations were statistically significantly higher among women who did not conceive in comparison to those who conceived. Plasma cfDNA may reflect the presence of factors which interfere with embryo implantation. Further research is required to determine the usefulness of cfDNA as a biomarker of IVF outcome and to examine the underlying pathologies as potential sources for increased plasma cfDNA concentrations. 

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**KEYWORDS:** cell-free DNA, IVF—embryo transfer, pregnancy rate

## Introduction

IVF is known to be a costly and invasive method of promoting fertility, and is therefore usually used as a last resort after all other treatment options have been exhausted. Because of the taxing nature of IVF treatment, researchers have explored which factors are associated with IVF success and failure in an effort to find more cost-effective and accurate ways to promote IVF success, such as improved embryo selection.

Recent advances in research and practice have enabled molecular-level examination of embryo media in order to seek biomarkers that will improve the ability to predict embryo implantation potential and improve IVF treatment outcome. These methods, called 'omics' including genomics, transcriptomics, proteomics and metabolomics, focus on a variety of bodily fluids and tissues as well as embryonic culture media. These methods are still under investigation, but show a promising improvement and predictive value in assisted reproduction (Assou et al., 2010; Bedaiwy et al., 2010; Jarkovska et al., 2010; Katz-Jaffe and Mc Reynolds, 2011; Seli et al., 2010; Thouas and Gardner, 2010). The limitation of these methods is that they are very complex and need further breakthroughs to be implemented on a routine basis. For example, the complexity of analysing substances in embryo-growing media results from a miniscule amount of secreted molecules from few embryonic cells. All of the aforementioned methods relate to the secretion of substances from embryos to their culture media in order to predict implantation potential. This study analysed cell-free DNA (cfDNA) as a biomarker in the woman's bloodstream in order to see if this is associated with IVF outcome.

cfDNA is fragments of DNA released from the cell nucleus due to apoptotic or necrotic processes (Jahr et al., 2001). cfDNA has been found in the plasma of both healthy and diseased populations. Elevated concentrations have been identified in several medical conditions such as myocardial infarction (Destouni et al., 2009; Shimony et al., 2010), cancer (Gao et al., 2010; Kamat et al., 2010; Tokuhisa et al., 2007; Zhang et al., 2010), stroke (Arnalich et al., 2010), sepsis (García Moreira et al., 2009) and pre-eclampsia (Hahn et al., 2011). The methods of analysis are mainly real-time polymerase chain reaction (PCR) (Arnalich et al., 2010; Destouni et al., 2009; Kamat et al., 2010; Tokuhisa et al., 2007), quantitative PCR (Gao et al., 2010; García Moreira et al., 2009) and capillary electrophoresis (Hahn et al., 2011).

Fetal cfDNA in the bloodstream of pregnant women has also been found (Lázár et al., 2011). Current technology enables the differentiation of maternal and fetal cfDNA when the fetus is a male due to the presence of Y chromosomes. Increased fetal cfDNA is associated with pathologies of pregnancy such as pre-eclampsia. However, the mechanism for increased fetal cfDNA in the maternal bloodstream is yet to be fully understood (Lázár et al., 2011). One study looked at fetal cfDNA fragments in maternal plasma and found that shorter fragments (150–300 base pairs) did not increase after 9 weeks of gestation; however, longer fragments (500–760 base pairs) did increase throughout gestation (Vodicka et al., 2010). Plasma cfDNA concentrations have been found to be related to smoking status in pregnant women (Urato et al., 2008). However, findings from another

study showed that maternal smoking was not related to fetal cfDNA in maternal bloodstream or maternal plasma cfDNA concentrations (Lapaire et al., 2007).

The finding of embryo-toxic factors in the bloodstream of patients with repeated IVF failure and repeated spontaneous abortions led researchers to examine cfDNA concentrations in women undergoing IVF treatment utilizing capillary electrophoresis in serum taken 1 week after embryo transfer (Hart et al., 2005), but no relationship between cfDNA concentrations in sera and conception rates in IVF patients was found. Therefore, the current study examined cfDNA concentrations in the plasma of peripheral blood of women undergoing IVF as a possible predictive factor affecting conception rates in IVF—embryo transfer treatment.

A new simple and highly sensitive method of measuring plasma cfDNA (SYBR gold fluorescent-stained direct assay) has been recently developed (Shimony et al., 2010). However, the use of this novel assay to detect cfDNA has not been tested in patients undergoing IVF—embryo transfer. Thus the aims of the current study were (i) to examine the plasma cfDNA concentrations during ovarian stimulation in IVF and (ii) to examine the relationship between cfDNA concentrations and pregnancy rates in the plasma of women undergoing IVF—embryo transfer using this highly sensitive method.

## Materials and methods

A prospective design, with repeated measures, was chosen as appropriate for the aims of this study. Participants were recruited and data was collected between August 2009 and February 2011. The sample size for this paper was determined using OpenEpi software (Dean et al., 2010) based on the cfDNA concentrations reported in previous research (Shimony et al., 2010). The research protocol was approved by the ethics committee of the Soroka University Medical Centre in Beer-Sheva, Israel (research number 4693 approved on 4 April 2008). Women signed an informed consent after they agreed to participate in the study.

The study group comprised 37 women undergoing IVF—embryo transfer treatment at the Soroka University Medical Centre's IVF unit. The inclusion criteria were nulliparous women between the ages of 18–35 undergoing IVF—embryo transfer. The mean age of the participants was  $28 \pm 4$  years.

Patients undergoing IVF—embryo transfer were treated using standard ovarian stimulation protocols including down-regulation of the pituitary gland with a gonadotrophin-releasing hormone (GnRH) agonist (Decapeptyl 0.1; Ferring, Germany), followed by ovarian stimulation with exogenous FSH (Gonal-F; Merck-Serono, Switzerland; or Puregon; Schering-Plough, USA; or Menogon; Ferring). Oocyte retrieval was performed 36–38 h after the administration of 250 mg recombinant human chorionic gonadotrophin (HCG; Ovitrelle; Merck-Serono) when at least two or three follicles of 17–20 mm diameter were observed by ultrasound examination, and blood  $17\beta$ -oestradiol concentrations reached at least 150–200 pg/ml per follicle over 17 mm diameter. Embryos were transferred using an abdominal ultrasound-guided technique on the second or third day after oocyte retrieval, using a Soft-Trans Embryo

Transfer Catheter (K-Soft 5000; Cook, Ireland). Patients were instructed to start with luteal support using Estrofem (Novo Company, Copenhagen, Denmark) 4 mg/day and Endometrin (Ferring Pharmaceuticals, Parsippany, NJ, USA) 600 mg/day from the second day after oocyte retrieval until clinical pregnancy was determined.  $\beta$ HCG was determined 14 days after embryo transfer by  $\beta$ HCG assay ( $>7$  IU/l). Clinical pregnancy was defined by gestational sac and fetal heart beat at 6.5 weeks of pregnancy observed by vaginal ultrasound.

Hormone concentrations (oestrogen, prolactin, LH, FSH and thyroid-stimulating hormone) as well as number of oocytes retrieved, number of ovum fertilized, embryo quality and number of embryos implanted were obtained from patient records. Blood for analysis was taken during routine blood draws from the IVF patients including an additional 10 ml at three consecutive time points during the IVF cycle. The first time point (T1) was 2 weeks after GnRH agonist administration to ensure down-regulation of the pituitary gland at the expected low oestradiol concentrations. The second time point (T2) was during oocyte retrieval via the intravenous port placed for anaesthesia, prior to its administration. The third time point (T3) was during the routine blood test for  $\beta$ HCG, 2 weeks after embryo transfer.

Blood was collected in vials with sodium citrate or heparin, placed on ice for up to 1 h and 2 ml were transferred to tubes (BD, Plymouth, UK) for plasma separation and was then frozen in aliquots in liquid nitrogen (Nunc Brand Products, Denmark). After thawing, plasma was directly assayed for cfDNA without DNA extraction or amplification utilizing a method developed by Goldshtein et al. (2009), a rapid direct fluorescent assay for cfDNA quantification in biological fluids. Briefly, 10  $\mu$ l plasma was placed in each well of a 96-well plate along with 40  $\mu$ l SYBR Gold Nucleic Acid Gel Stain (Invitrogen, Paisley, UK). Each sample with a duplicate was read by a 96-well fluorometer (Spectrafluor Plus; Tecan, Durham, NC, USA) at 535 nm with excitation wavelength of 485 nm. The fluorescence of each sample was compared with a calibration curve. cfDNA concentrations were measured in ng/ml. A detailed description of this method can be found in Goldshtein et al. (2009). In short, the intra-day coefficient of variation was 16.0%, 7.9% and 4.8% in the low, elevated and high DNA ranges, respectively. Day-to-day coefficient of variation was 31.0%, 6.7% and 8.0% in the low, elevated and high DNA ranges, respectively.

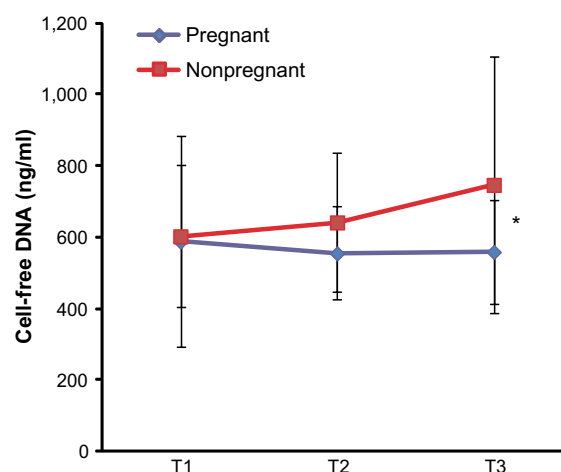
Data analysis was performed using the Statistical Package for Social Sciences (SPSS 17.0, Chicago, IL, USA). The level of significance for all tests was  $\leq 0.05$ . Within the group analysis, the mean cfDNA concentrations and mean cfDNA concentrations in comparison to smoking status (smoke, used to smoke but quit, and never smoked) were calculated and compared using one-way ANOVA. Student's t-test was used to compare mean cfDNA concentration, number of oocytes retrieved, oestrogen concentration at the time of pregnancy test and other hormonal concentration at baseline in pregnant and nonpregnant IVF patients. Spearman's correlation was used to examine the relationship between cfDNA concentration and implantation rate (number of embryos implanted/number of embryos transferred). The sensitivity, specificity and positive predictive value of the cfDNA concentrations was calculated using OpenEpi software (Dean et al., 2010).

## Results

Out of 37 women in the study, 16 (43%) conceived and 21 (57%) did not. Out of the 16 pregnancies, 12 babies were born (75%), one pregnancy was biochemical (6%) and three women (19%) miscarried. No difference was found in the mean ages of women who became pregnant ( $29.8 \pm 4.3$  years) and those who did not ( $27.9 \pm 4.1$  years).

The mean concentration of cfDNA in all participants was  $619 \pm 186$  ng/ml. At T1 (low oestradiol), the mean cfDNA concentration was  $597 \pm 243$  ng/ml; at T2 (oocyte retrieval), the mean cfDNA concentration was  $604 \pm 173$  ng/ml; and at T3 ( $\beta$ HCG),  $662 \pm 294$  ng/ml. No statistically significant difference was found in a within-group analysis of the three time points ( $F(2, 99) = 0.736$ ).

The cfDNA values were compared based on IVF results (conception/non-conception) (Figure 1). At T1, mean values of cfDNA among pregnant women, including women with a biochemical pregnancy and those who later aborted ( $588 \pm 295$  ng/ml), did not differ statistically significantly from the mean values of cfDNA of nonpregnant women ( $603 \pm 199$  ng/ml;  $t(31) = -1.71$ ). Also at T2, the mean value of cfDNA ( $555 \pm 130$  ng/ml) of pregnant women did not differ statistically significantly from cfDNA values of nonpregnant women ( $642 \pm 196$  ng/ml;  $t(34) = -1.5$ ). However, at T3, the mean values of cfDNA of nonpregnant women ( $747 \pm 358$  ng/ml) were statistically significantly higher than the mean values of cfDNA among pregnant women ( $559 \pm 144$  ng/ml;  $t(23.1) = -2.03$ ,  $P = 0.05$ ; Figure 1). The calculation of statistical power for these results show that with 95% two-sided confidence intervals (CI), power was 51%, and for 90% CI, power was 63%, due to the small sample size. No statistically significant difference in cfDNA concentrations was found for women who miscarried or had a biochemical pregnancy compared with those who had a live birth at any of the time points. Using a cut off of 750 ng/ml, based on the mean cfDNA concentration for nonpregnant



**Figure 1** Concentration of cell-free DNA in IVF pregnant ( $\beta$ HCG  $>7$  IU/l) versus nonpregnant women at three time points. T1 = 2 weeks after GnRH agonist administration; T2 = during oocyte retrieval prior to anaesthesia; T3 = during the routine blood test for  $\beta$ HCG, 2 weeks after embryo transfer.

women, the sensitivity of the measure of cfDNA concentration at T3 was found to be 39% with a specificity of 86%. Positive predictive value of high cfDNA concentration as a predictor of IVF failure was 78%.

A statistically significant relationship was found between oestrogen concentrations on the day of  $\beta$ HCG test and conception rates ( $t(2.5) = 34$ ,  $P = 0.016$ ) indicating that oestrogen is higher among women who conceived. All other laboratory parameters in pregnant versus nonpregnant women were not statistically significant (Table 1). In addition, no relationship was found between smoking status, implantation rate and mean plasma cfDNA concentration.

## Discussion

As far as is known, this study is the first to detect cfDNA plasma concentrations by a simple and easily applicable assay of plasma using SYBR Gold Nucleic Acid Gel Stain without further extraction or amplification of DNA among women undergoing IVF. The method presented here can detect cfDNA in a wide range of sizes, even including cfDNA as small as 12–18 base pairs (Goldshtein et al., 2009).

This study found that women who did not conceive during the IVF cycle had statistically significantly higher mean concentrations of cfDNA 14 days after embryo transfer, during the  $\beta$ HCG blood test, compared with women who did conceive. A previous study did not find a statistically significant difference in cfDNA concentrations comparing women who conceived in IVF treatment and those who did not (Hart et al., 2005). The difference between that study and the current study could be explained in two ways. First, the study by Hart et al. (2005) was conducted using capillary electrophoresis to measure cfDNA concentrations, a method which may be less sensitive in identifying fragments of cfDNA; as described, the minimal cfDNA molecular weight was 1 Kb. Second, the determination of cfDNA by Hart et al. (2005) was 1 week after embryo transfer, rather than 2 weeks in the current study.

cfDNA concentration at the time of  $\beta$ HCG test was highly specific (86%) with a positive predictive value of 78%, which means that high cfDNA concentrations (above a cut off of 750 ng/ml) are indicative of IVF failure 86% of the time. The sensitivity, however, was quite low (39%), indicating that having lower than pathological cfDNA concentrations does not promise IVF success. There are some women,

**Table 1** Hormonal and embryological characteristics.

| Characteristic                                      | No. of patients | Mean $\pm$ SD     |
|---|-----------------|-------------------|
| Baseline FSH (ng/ml)                                |                 |                   |
| Pregnant  | 12              | 13 $\pm$ 20.52    |
| Not pregnant  | 15              | 6 $\pm$ 3.03      |
| Baseline LH (ng/ml)                                 |                 |                   |
| Pregnant  | 9               | 6 $\pm$ 1.95      |
| Not pregnant  | 13              | 6 $\pm$ 3.29      |
| Baseline prolactin (ng/ml)                          |                 |                   |
| Pregnant  | 9               | 69 $\pm$ 155.52   |
| Not pregnant  | 12              | 75 $\pm$ 121.03   |
| Baseline oestradiol (ng/ml)                         |                 |                   |
| Pregnant  | 10              | 89 $\pm$ 90.24    |
| Not pregnant  | 13              | 70 $\pm$ 49.01    |
| Baseline TSH (ng/ml)                                |                 |                   |
| Pregnant  | 10              | 2 $\pm$ 0.76      |
| Not pregnant  | 12              | 2 $\pm$ 0.95      |
| Embryos transferred                                 |                 |                   |
| Pregnant  | 15              | 2 $\pm$ 0.59      |
| Not pregnant  | 22              | 2 $\pm$ 0.29      |
| Day of embryo transfer                              |                 |                   |
| Pregnant  | 14              | 2 $\pm$ 0.50      |
| Not pregnant  | 22              | 3 $\pm$ 0.49      |
| Oestradiol at $\beta$ HCG test (ng/ml) <sup>a</sup> |                 |                   |
| Pregnant  | 14              | 1927 $\pm$ 980.82 |
| Not pregnant  | 22              | 1254 $\pm$ 614.42 |
| Oocytes retrieved                                   |                 |                   |
| Pregnant  | 14              | 10 $\pm$ 4.12     |
| Not pregnant  | 22              | 10 $\pm$ 6.22     |
| Oocytes fertilized                                  |                 |                   |
| Pregnant  | 11              | 7 $\pm$ 3.88      |
| Not pregnant  | 21              | 6 $\pm$ 3.77      |

Pregnancy was defined as  $\beta$ HCG  $>7$  IU/L.

<sup>a</sup> $P = 0.016$ ; all other tests nonsignificant.



39% in this study, that have low cfDNA concentration and still do not conceive. This finding supports the fact that there are multiple variables affecting conception.

The question arising from these findings is whether there is an underlying pathology causing the elevated cfDNA as well as the infertility. Since cfDNA assays are not tissue specific, its origins cannot be accurately determined. However, elevated cfDNA concentrations are likely to be a consequence of increased apoptotic processes (Beck et al., 2009; Suzuki et al., 2008).

Evidence for the association between apoptotic processes and IVF outcome have been described in several studies. Liu and Li (2010) looked at the relationship between apoptosis in granulosa cells and IVF–embryo transfer success and speculated that oxidative stress in granulosa cells had an effect on IVF–embryo transfer failure, and also connected the higher apoptotic rate to lower oocyte quality. Díaz-Fontdevila et al. (2009) examined the apoptotic rate of cumulus cells in relation to infertility diagnosis and spermatozoa exposure and found that cumulus cells of women with a diagnosis of endometriosis and those exposed to spermatozoa had higher rates of apoptosis. Cumulus cells of embryos with low quality had higher apoptotic rates than those with high quality. One possible explanation for higher cfDNA concentrations in women who did not conceive is that cfDNA in the bloodstream originates from an embryo that has undergone apoptosis. However, since an embryo consists of only a few hundred cells at the time of the  $\beta$ HCG test, it is very unlikely that this accounts for the statistically significant difference in cfDNA concentrations between women who conceive compared with those who do not. To determine whether the cfDNA is maternal or embryonic in origin, DNA fragment fractionation and analysis should be used in future studies.

Since 1991, evidence has accumulated about the role of p53 in initiation of apoptosis (Yonish-Rouach et al., 1991). Furthermore, p53 has been previously identified as affecting fertility in general and implantation specifically (Kang et al., 2009). Another study strengthened this notion by finding elevated expression of p53 among women with recurrent pregnancy loss and more specifically recurrent IVF/intracytoplasmic sperm injection failure (Razieh Dehghani et al., 2009). Future research measuring p53 or parental genotypes within p53 and cfDNA concentrations in IVF patients is required to examine this hypothetical explanation.

Women who do not conceive during the IVF cycle suffer from an abrupt drop in oestrogen concentrations following implantation failure, as opposed to the pregnant women who maintain higher concentrations throughout pregnancy. It is plausible that the high concentrations of cfDNA in non-conceiving women are related to this oestrogen decline. In order to partially test this hypothesis, this study looked for correlations of plasma oestrogen concentrations and cfDNA concentrations at the  $\beta$ HCG time point; however, no relationship was identified. Another possible explanation for elevated plasma cfDNA concentrations would be endometrial shedding in women who did not conceive. However, a study that examined cfDNA concentrations throughout the menstrual cycle did not detect changes related to the normal menstrual cycle (Pölcher et al., 2010). Future research is required to explore if endometrial shedding and tissue

breakdown after IVF failure may result in higher cfDNA concentrations in plasma.

In addition, future research that includes genetic sequencing of DNA fragments to determine the origin of the cfDNA would be beneficial in identifying potential pathologies that affect fertility and prevent successful implantation post IVF treatment.

The findings of the current study need to be considered along with its limitations. The fact that the participating women were in the process of IVF treatment impeded this study's ability to conclude whether increased cfDNA is related to the infertility phenomenon or the IVF treatment itself. Further research looking at IVF populations during a nonstimulated menstrual cycle is required.

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