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Comparison of The Foetal Fraction (ff) of Cell-Free DNA in *In Vitro* Fertilisation Versus Natural Conception. Evaluation of ff with IVF Parameters.

Kostas K[1], Evangelia D[2], Depy M[3], Emmanuaela L[4], Ritsa B[4], Elli A[5], Nikos S[6], Dimitris L[7*]

[1]Athens Medical School, National and Kapodistrian University of Athens., Fertility Institute

[2]Department of Microbiology, Biopathology University of Athens, Medical School, Athens, National and Kapodistrian University of Athens,

[3]1st Department of Obstetrics and Gynaecology, "Alexandra" General Hospital, National and Kapodistrian University of Athens, Fertility Institute

[4]Fertility Institute

[5]Athens Medical School, National and Kapodistrian University of Athens, Fertility Institute

[6]Urology Department, School of Medicine, Ioannina University, Ioannina, Greece

[7]Athens Medical School, National and Kapodistrian University of Athens, Fertility Institute

***Corresponding author:** Dimitris L, Athens Medical School, National and Kapodistrian University of Athens, Greece, Fertility Institute, Tel: 0030 6932421747; Email: loutradi@otenet.gr

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Abstract

Introduction: As the offspring of assisted reproduction techniques (ART) have become a substantial proportion of the population, there has been increased attention on the safety of ART. Investigators have focused on identifying a tool that combines molecular or biological tests that could predict the outcome of IVF or ICSI and pregnancy development after ART-mediated embryo implementation.

Method: The aim of this study was to answer to the following questions. Is there a difference between Naturally Conceived (NC)and IVF pregnancies regarding foetal fraction (FF) of cfDNA in maternal age, birth weight, gender and gestational age?

Is there a difference between FF concentration regarding the parameters of IVF as possible predictive factors affecting the outcome of IVF?

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Results: The NC and IVF group were similar in terms of maternal age, BMI of the mother, gender, birth weight and gestational age. FF was not significantly different between NC and IVF groups. The results were similar after adjustment for maternal age via regression analysis. NC 10(3.8) vs 9(2.6) p 0.144. CF DNA was not associated with maternal age, birth weight, gender or gestational age in total sample or separately for the normal conception and IVF groups. No significant correlation was found for Cell Free DNA with IVF parameters.

Conclusion: The FF is an important factor for NIPT test accuracy. Several studies have found a reduction in FF for pregnancies following ART compared with natural conception, while other studies have presented no differences in the FF. All researchers agree on the importance of NIPT. However, knowledge on how the FF is affected in ART pregnancies compared with naturally conceived pregnancies is very limited.

In this study, no difference in FF for the IVF group compared with NC women was observed. The cffDNA concentrations in maternal serum do not appear to be affected in IVF conception. We suggest that FF is an independent factor compared with IVF parameters.

Keywords: ART Pregnancies, NIPT, Foetal Fraction (FF), Cell Free DNA, IVF-ICSI.

Introduction

As the offspring of assisted reproduction techniques (ART) have become a substantial proportion of the population, there has been increased attention on the safety of ART. Concern has been raised that children conceived by ART might be exposed to greater health risks than naturally conceived children. Ovulation-induction medications, the *in vitro* culture of embryos, vitrification, and the potential use of genetically and structurally abnormal sperm during intracytoplasmic sperm injection (ICSI) are independent risk factors. Recent advances in research and practice have enabled molecular-level examination of descendants of ART-mediated pregnancies [1,2].

The methods for diagnosing chromosome abnormalities and screening the viability of a transfer require embryo biopsy, a procedure that affects embryo quality and requires specialised skills. The principle of non-invasive chromosome screening (NICS) has recently been demonstrated; it is based on sequencing the genomic DNA detected in the culture medium from the embryo, avoiding the need for embryo biopsy and substantially increasing safety [3,4]. Invasive prenatal testing for ART is not accepted by expectant mothers because of the low but existing miscarriage rate due to the technique used.

Cell-free foetal DNA (cffDNA) is derived from the placenta and increases as the placenta grows [5,6]. The foetal fraction (FF) is the proportion of the maternal cell-free DNA (cfDNA) in a blood sample. A higher FF is associated with greater test sensitivity and positive predictive value (PPV) [7]. Current quantification demonstrates a median FF of ~11% at the time of testing [8]. While some laboratories do not report FF results, others report the test as failed if the FF is <4% [9]. The FF increases as gestational age advances, varies according to ethnicity, and is lower in women with a higher body mass index (BMI) and in pregnancies conceived by *in vitro* fertilisation (IVF) [10-13]. By using quantitative real-time polymerase chain reaction (PCR), high mean concentrations (6.2% of total plasma DNA) of foetal DNA were found in maternal plasma in early and late pregnancy [14].

cffDNA comprises fragments of DNA from the nucleus, a result of apoptotic or necrotic processes [15]. The plasma DNA concentration varies between 10 and 100 ng or 103 and 104 GE/mL [16,17].

The level of cffDNA has been determined in the bloodstream of pregnant women [18]. The technology enables the differentiation of maternal cfDNA and cffDNA when the foetus is male due to the presence of the Y chromosome. Increased cffDNA is associated with pathologies of pregnancy such as pre-eclampsia.

Investigators have focused on identifying a tool that combines molecular or biological tests that could predict the outcome of IVF or ICSI and pregnancy development after ART-mediated embryo implementation. Many tests are used in clinical practice to optimise treatment, including examining the level of follicle-stimulating hormone (FSH) and anti-Müllerian hormone (AMH), antral follicle count (AFC) by ultrasound, and ge-

netic determination of single nucleotide polymorphisms (SNPs) in genes such as follicle-stimulating hormone receptor (*FSHR*), anti-Müllerian hormone (*AMHR*), and the oestrogen receptor (*ESR*) [19,20]. The findings of these tests are crucial and the ultimate goal is to use routine diagnostic tests before IVF treatment to predict factors that are associated with IVF success or failure. This endeavour could identify more cost-effective and accurate ways to promote IVF success, such as improved embryo selection to drive a healthy delivery.

Kleijkers et al. [21] presented data suggesting that human culture media may have a profound influence on the phenotype of the offspring of ART. Two or three days of *in vitro* culture in two different commercially available culture media resulted in a 200 g difference in average birth weight. The difference in birth weight is paralleled by a difference in the kinetics and magnitude of human chorionic gonadotropin (hCG) increase in early pregnancy and a 500 g difference in body weight at 2 years of age. In addition, Orasanu et al. [22] observed that different culture media affect the initial hCG rise in ART-mediated pregnancies. One explanation for these observations is that different culture media lead to distinct methylation patterns in the placenta [23]. Such findings suggest that the media for human ART are not just an inert vehicle providing a nurturing environment for gametes and embryos. Rather, they may actually induce epigenetic changes with lifelong consequences for the health of the offspring. Researchers are investigating the effect of IVF conception on placental formation in the presence of several compounds originating from the culture media. Recent studies have confirmed the increased risk of placenta-related adverse pregnancy outcomes and the excess of imprinted disorders with abnormal methylation patterns after ART, which raises the issue of a potential ART-induced epigenetic risk [23].

Our group has examined the proteomic and metabolomic profiles of children born following ART compared with naturally conceived controls in search of epigenetic abnormalities [1,2]. The proteomic profile of children born after ISCI revealed an adverse cardiometabolic profile [2]. Moreover, plasma metabolomic profiling showed early indications for predisposition to latent insulin resistance in children conceived by ICSI [1]. Altogether, ART is likely to cause some epigenetic changes in the offspring, and these changes might be the molecular basis of complex traits and diseases.

If the FF is indeed lower in IVF conceptions, the expected consequence is a higher test failure rate. The current literature on the effect of IVF conception on cffDNA testing characteristics is limited and inconclusive. Costa et [26], Lambert-Messerlian et al. [25], and Pan et al. [24] showed no difference in the FF between the IVF and naturally conceived populations. On the other hand, Lee et al. [13] and Talbot et al. [27] demonstrated that the FF is significantly lower in IVF cases and that the test failure rate is higher compared with naturally conceived cases. In addition, the PPV of cffDNA testing is lower in singleton pregnancies conceived by IVF than those conceived spontaneously.

To investigate this discrepancy in the literature, we designed a case–control study. Our primary aim was to compare the FF and PPV of cffDNA testing in pregnancies conceived naturally and through IVF. Our secondary aim was to investigate whether there is a correlation between the FF and specific IVF parameters, including the hormonal profile, the ovulation induction protocol, and the embryologic profile. We recorded the maternal age, ethnicity, and BMI as well as the gestational age during noninvasive pregnancy testing (NIPT), aiming to assess the homogeneity of the sample in both groups. We sought to answer the following questions:

(i) Is there a difference between natural conception and ART (IVF/ICSI)-conceived pregnancy regarding the FF?

(ii) Is there a difference concerning the FF and maternal age, birth weight, offspring sex, and gestational age in the total sample and separately for the natural conception and IVF groups?

(iii) Is there a difference between the natural conception and IVF groups regarding maternal age divided into <35 and >35 years?

(iv) Is there a difference between the FF regarding the hormonal profile, maternal age, maternal BMI, the characteristics of ovarian stimulation, the number of oocytes, the maturation rate, the fertilisation rate, and the embryo quality as possible predictive factors affecting the outcome of IVF?

Materials and Methods

The study protocol was approved by the review boards of the Fertility Institute. All participants provided informed consent for their medical records to be used in the study and for cffDNA testing.

This cohort study comprised 31 women with singleton pregnancies who underwent cffDNA screening for trisomy 13, 18, and 21; sex determination; and FF. The women had undergone different reproductive modalities of in a private Unit Fertility Institute. The control group included 55 women who ex-

perienced natural conception. The population in this study was non-diabetic and non-smoking. For all women, anthropometric characteristics data such as age, weight, height, and BMI were recorded. For the IVF group, early follicular phase values of FSH, luteinising hormone (LH), prolactin (PRL), AMH, thyroid-stimulating hormone (TSH), and oestradiol (E2) within the preceding 6 months were recorded. The hCG value was also assessed twice before ultrasound examination at 7 weeks for confirmation of the heartbeat. In addition, the number of follicles during the monitoring, the number oocytes retrieved, the number of embryos, the characteristics of ovarian stimulation, the embryologic profile, the number of oocytes, the maturation rate, the fertilisation rate, the embryo quality, the cleavage rate, and the stage of the embryo on the day of embryo transfer were recorded for each participant in the study.

In both groups, cffDNA testing was performed at 13 weeks of gestation by using the Harmony Prenatal Test platform. Twenty millilitre samples of maternal blood were collected and sent to Ariosa Diagnostics, Inc. (San Jose, CA, USA) for analysis. The results were returned for pregnancy management and test characteristics were documented. Risk scores for aneuploidy were reported as percentages ranging from <0.01% to >99.9% or were inconclusive and no report was issued. The FF was reported as a percentage if >4%. For samples with a FF <4%, the laboratory did not generate a risk assessment.

Controlled Ovarian Hyperstimulation (COH)

COH was conducted according to the gonadotropin-releasing hormone (GnRH) agonist protocol, as described previously (Anagnostou et al., 2018). Briefly, patients <35 years old began a long stimulation protocol. On day 21 of the previous cycle, a baseline ultrasound scan was performed and buserelin acetate intranasal spray administration began at a dose of 100 μg five times per day. GnRH agonist administration was maintained until hCG administration began. The extent of ovarian suppression in all patients was evaluated by ultrasound scan and serum E2 levels (<40 pg/mL) before starting exogenous gonadotrophin administration (about 15 days after administering the spray). After performing a follow-up, hCG was given when at least two follicles were >18 mm and serum oestrogen levels were rising.

Oocytes were retrieved 36 h after the administration of 10,000 IU hCG. Follicular aspiration and oocyte retrieval were performed by transvaginal ultrasound–guided puncture. Patients >35 years old began a short-term protocol with buserelin (500 μg/day intranasal) on cycle day 2. Gonadotrophin administration began on day 3 at a dose of 200 IU of recombinant follicle-stimulating hormone (rFSH).

Plasma E2 levels were measured daily beginning 5 days after the start of the regimen until the day after hCG administration. The first scan was performed on day 7 and subsequent scans were performed every day until oocyte retrieval. The dose of rFSH was adjusted according to ovarian response 6 days after the onset of gonadotrophin administration. GnRH agonist administration was continued until 10,000 IU of hCG was injected intramuscularly. At the same time, the mean diameter of at least two leading follicles was >18 mm and serum E2 levels were rising.

Embryos were scored and chosen for transfer based on rapid cleavage, the absence of fragmentation, and the size of the blastomeres (good quality, A; poor quality, B) (Loutradis et al ,[28]. Biochemical pregnancy was defined as a positive biochemical pregnancy test 18 days after oocyte retrieval. Clinical pregnancy was defined as the presence of a gestational sac on ultrasound at 6 gestational weeks.

Statistical Analysis

Statistical analysis was performed with SPSS version 24, while the Sasieni algorithm (1997) and Hardy–Weinberg equilibrium were performed with an online calculator (available at http://ihg.gsf.de). The statistical methods used for the control of the statistical hypothesis were: independent samples t-test, two-proportion test (normal approximation), and parametric one-way analysis of variance (ANOVA). For qualitative data, the chi-squared test and Fisher's exact test were used. The non-parametric Mann–Whitney U test and the Kruskal–Wallis test were used when needed to compare continuous variables between different groups (when the normality assumption was not satisfied). Statistical significance was set at 0.05.

Results

Clinical Characteristics

Table 1 shows the clinical characteristic of both groups. The natural conception and IVF groups were similar in terms of age, weight, BMI, offspring sex, birth weight, and gestational age performed by NIPT.

		Group		P
		Natural conception (N = 55)	IVF (N = 31)	
		Mean (SD)	Mean (SD)	
Maternal age (years)		36.4 (3.1)	35.4 (3.8)	0.180+
Weight		62.3 (7.9)	59.7 (11.1)	0.242+
BMI		22.8 (3.0)	21.5 (3.7)	0.103+
BMI, N (%)				
	Underweight	1 (2.7)	3 (9.7)	0.405‡‡
	Normal	31 (83.8)	25 (80.6)	
	Overweight	4 (10.8)	1 (3.2)	
	Obese	1 (2.7)	2 (6.5)	
Offspring sex, N (%)				
	Male	24 (49.0)	11 (47.8)	0.927‡
	Female	25 (51.0)	12 (52.2)	
Birth weight		3097.1 (334.0)	3095.5 (421.5)	0.987+
Gestational age (weeks delivery)		38.5 (1)	38.0 (1.6)	0.101+

F+Student's t-test; ‡Pearson's chi-square test; ‡‡Fisher's exact test.

Abbreviations: BMI, body mass index; IVF, *in vitro* fertilisation; SD, standard deviation.

Table 1: Characteristics of the two study groups.

Comparison Of Ff Between The Natural Conception And Ivf Groups

The FF level was not significantly different between the natural conception and IVF groups. The results were similar after adjustment for maternal age via regression analysis (Table 2). The women were further categorised according to an FF cut-off of 6%. There was no difference between the natural conception and IVF groups based on this classification. Finally, the FF of the two groups was similar when maternal age was divided into <35 and >35 years.

a

	group		P		
	Normal conception	IVF			
	Mean (SD)	Mean (SD)		β (SE)++	P++
FF (%)	10 (3.8)	9 (2.6)	0.173+	-1.18 (0.80)	0.144

b

		Group				
		Normal conception		IVF		
		N	%	N	%	Fisher's exact test (P)
FF (%)	≤6	9	16.7	3	9.7	0.522
	>6	45	83.3	28	90.3	

c

				FF		
				Mean	SD	Student's t-test (P)
group	Normal conception	Age	≤35	10.4	4.6	0.823
	Normal conception		>35	10.1	3.4	
	IVF	Age	≤35	9.1	3.3	0.819
			>35	8.9	1.8	

F++comparison of FF between groups after adjustment for maternal age.

Abbreivations: FF, foetal fraction; IVF, *in vitro* fertilisation; standard error.

Table 2. Comparison of the FF between the two study groups. .a FF(%) in Natural Conception (NC) and IVF. b FF(%) >6 and <6 in NC vs IVF. c FF(%) in age >35vs<35

Clinical Characteristics Of The Total Sample And Separated Into The Natural Conception And Ivf Groups

There were no significant correlations between the FF and the maternal age, birth weight, offspring sex, or gestational age when considering the total sample or the natural conception and IVF groups separately (Table 3).

a.

			FF (%)					
			Total sample		Normal conception		IVF	
			Mean (SD)	P	Mean (SD)	P	Mean (SD)	P
Maternal age (years)								
	≤35		9.8 (4.0)	0.934++	10.4 (4.6)	0.823++	9.1 (3.3)	0.819++
	>35		9.7 (3.0)		10.1 (3.4)		8.9 (1.8)	
Maternal age (years), r+			0.08	0.466	0.05	0.730	0.08	0.671
Offspring sex								
	Male		9.6 (3.2)	0.409++	9.6 (3.3)	0.211++	9.5 (3.2)	0.551++
	Female		10.2 (3.7)		10.9 (4.1)		8.8 (2.3)	
Birth weight, r+			-0.11	0.380	-0.14	0.382	-0.07	0.758
Gestational age (weeks), r+			0.13	0.314	0.10	0.549	0.15	0.521
b								
				FF				
				Mean	SD	p		
Age (years)	≤35	group	Normal conception	10.4	4.6	0.355		
			IVF	9.1	3.3			
	>35	group	Normal conception	10.1	3.4	0.189		
			IVF	8.9	1.8			

F+Pearson's correlation coefficient; ++Student's t-test.

Abbreviations: FF, foetal fraction; IVF, *in vitro* fertilisation; SD, standard deviation.

Table 3. Correlation between the FF and maternal age, birth weight, offspring sex, and gestational age in the total sample and separately for the normal conception and IVF groups. a. FF(%) in total sample vs NC and IVF. b FF(%) in total sample in age >35 and <35 in NC and IVF group

The FF and Hormonal Profile of the IVF Group

In the IVF group, here were no significant correlations between the FF and the levels of hormones (βhCG, FSH, LH, PRL, TSH, and AMH). In addition, there were no significant correlations between the FF and the IVF parameters days of ovulation, E2 on the day of hCG administration, the number of embryos, and the morphological quality of embryos (Table 4). There were also no significant correlations between the βhCG change and the level of other hormones, the days of stimulation, E2 on the day of hCG administration, and the number of embryos (Table 5).

	(%) FF	
	r	P
βhCG change	-0.12+	0.512
A-βhCG	-0.28+	0.120
B-βhCG	-0.19+	0.312
FSH	-0.08++	0.659
LH	-0.25++	0.182
Prolactin	0.07+	0.728
TSH	-0.01++	0.937
Days of stimulation	0.10+	0.620
E2 on day of hCG	-0.13++	0.532
No embryos	-0.11++	0.541
Embryo quality	0.21+	0.269
AMH	0.01	0.975

+Spearman's correlation coefficient; ++Pearson's correlation coefficient.

Abbreivations: AMH, anti-Müllerian hormone; E2, oestradiol; FF, foetal fraction; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; LH, luteinising hormone; TSH, thyroid-stimulating hormone.

Table 4. Correlation between the FF and the level of hormones and IVF parameters

a.

	βhCG change	
	+r	P
FSH	0.10	0.580
LH	-0.30	0.096
Prolactin	0.28	0.132
TSH	0.10	0.576
Days of stimulation	0.00	0.988
E2 on day of hCG	0.21	0.295
No embryos	0.10	0.582
Embryo quality	-0.32	0.080
AMH	-0.11	0.574

b.

		FSH				P
		5–8 IU/L		>8 IU/L		
		Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)	
FF (%)		9.4 (3)	8.8 (7.7–11)	8.2 (1.7)	8.3 (6.6–9.5)	ns
βhCG Change		1108.2 (610.2)	960 (671.5–1652.5)	2459 (2816)	1150 (379–3315)	ns
E2 on day of hCG administration		2841.5 (1418.6)	2824 (1854.5–3768)	2649.1 (924.6)	2456 (2068–3492)	ns
E2 on day 7		1521.7 (1139.3)	1309 (503–2221)	1087.4 (469.3)	1015.5 (667–1375)	ns
Age (years)		34.3 (3.8)	34.5 (33–37)	37.3 (3)	37 (35–40)	**0.040**
Age, N (%)	≤35	13	81.3	3	18.8	**0.044**
	>35	7	46.7	8	53.3	
BMI		21.2 (4.4)	19.9 (19.1–21)	22 (2)	21.7 (20.7–23.2)	ns
BMI, N (%)	Underweight	3	100.0	0	0.0	ns
	Normal	15	60.0	10	40.0	
	Overweight	0	0.0	1	100.0	
	Obese	2	100.0	0	0.0	
Gestational age (weeks)		37.8 (1.5)	38.1 (37–39)	38.3 (1.9)	38.5 (37.4–39.7)	ns
Number of oocytes		8.3 (4.3)	10 (6–11)	8.9 (3.5)	9 (7–11)	ns

c.

		LH						P
		<5 IU/L		5–8 IU/L		>8 IU/L		
		Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)	
FF (%)		9.9 (1.7)	10.5 (8.8–11)	8.6 (2.9)	8.2 (6.9–9)	8.9 (2.9)	9.5 (7.3–10.5)	ns
βhCG change		2153.4 (2228.6)	1728.5 (957.5–2001.5)	1559.1 (1854)	960 (671.5–1651.5)	1005.6 (1158.4)	486 (330–1937)	ns
E2 on day of hCG administration		2424.3 (1592.4)	2212 (1336–2948)	2997.8 (1184.3)	2700 (2299–3452)	2633.9 (1086)	2068 (1938–3500)	ns
E2 on day 7		1332.7 (631.9)	1134 (828–1955)	1181.1 (762.9)	1054.5 (537.5–1571.5)	1647.3 (1409.6)	1138 (621–2976)	ns
Age (years)		34.6 (3.2)	33.5 (33–36.5)	35.3 (4.5)	36 (33–38)	36.3 (2.5)	36 (35–37)	ns
Age, N (%)	≤35	6	37.5	7	43.8	3	18.8	ns
	>35	2	13.3	9	60.0	4	26.7	
BMI		23.6 (6)	20.7 (19.4–28.2)	20.4 (2)	20.5 (19.2–21.4)	21.5 (2.7)	21.1 (19.6–23)	ns
BMI, N (%)	Under weight	0	0.0	2	66.7	1	33.3	ns
	Normal	6	24.0	14	56.0	5	20.0	
	Over weight	0	0.0	0	0.0	1	100.0	
	Obese	2	100.0	0	0.0	0	0.0	
Gestational age (weeks)		37.8 (1.6)	37 (36.9–39)	37.9 (1.5)	38.1 (37–39)	38.4 (2.1)	39 (38.4–39.4)	ns
Number of oocytes		7.4 (4.8)	7.5 (3.5–11)	8.6 (4.2)	9.5 (8–11)	9.6 (2.4)	10 (7–11)	ns

d.

		Prolactin				P
		<11 pg/mL		≥11 pg/mL		
		Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)	
FF (%)		8.6 (2.2)	8.4 (7.2–9.9)	9.3 (2.9)	9.1 (7.3–11)	ns
βhCG Change		732.5 (291.2)	720 (570–960)	2204.9 (2195.5)	1659 (910–2072)	**0.006**
E2 on day of hCG		2671.7 (1206.5)	2299 (1850–3452)	2837.7 (1292.2)	2948 (1938–3500)	ns
E2 on day 7		1058.2 (750.1)	814 (572–1309)	1541.2 (1028.9)	1375 (724–2058)	ns
Age (years)		35 (4.6)	36 (33–38)	35.6 (3.2)	35 (33–38)	ns
Age N (%)	≤35	6	37.5	10	62.5	ns
	>35	7	46.7	8	53.3	
BMI		20 (1.6)	20.2 (19.3–20.7)	22.6 (4.4)	21.1 (19.6–23.4)	ns
BMI N (%)	Underweight	2	66.7	1	33.3	ns
	Normal	11	44.0	14	56.0	
	Overweight	0	0.0	1	100.0	
	Obese	0	0.0	2	100.0	
Gestational age (weeks)		37.3 (1.8)	37 (36–39)	38.3 (1.5)	38.5 (38–39)	ns
Number of oocytes		7.9 (3.6)	9 (6–10)	8.9 (4.3)	9.5 (7–12)	ns

e.

		AMH				P
		≤3 ng/mL		>3 ng/mL		
		Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)	
FF%		9.2 (1.8)	8.9 (8.1–10.5)	8.8 (3.3)	8.2 (6.5–10.5)	ns
βhCG change		1960.1 (2395.3)	1005 (617–1866)	1238.2 (998.8)	950.5 (554.5–1795.5)	ns
E2 on day of hCG administration		2177 (683.9)	1938 (1839–2554)	3200.5 (1383.1)	3427 (2068–4192)	**0.036**
E2 on day 7		900.1 (445)	800 (572–1138)	1699.9 (1087.1)	1458 (724–2221)	ns
Age (years)		36.4 (4.4)	37 (33–40)	34.4 (2.9)	34.5 (33–36.5)	ns
Age N (%)	≤35	6	37.5	10	62.5	ns
	>35	9	60.0	6	40.0	
BMI		20.6 (2.3)	20.2 (19.1–21.1)	22.3 (4.6)	21 (19.8–23.1)	ns
BMI N (%)	Underweight	1	33.3	2	66.7	ns
	Normal	13	52.0	12	48.0	
	Overweight	1	100.0	0	0.0	
	Obese	0	0.0	2	100.0	
Gestational age (weeks)		38 (1.7)	38.4 (36.9–39)	38 (1.6)	38.3 (37–39)	ns
Number of oocytes		6.6 (3.9)	7 (1–10)	10.3 (3.2)	10.5 (9–12.5)	**0.006**

+Spearman's correlation coefficient.

Abbreviations: AMH, anti-Müllerian hormone; BMI, body mass index; E2, oestradiol; FF, foetal fraction; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; IQR, interquartile range; LH, luteinising hormone; SD, standard deviation; TSH, thyroid-stimulating hormone.

Table 5. Correlation between the βhCG, FSH, LH Prolactin, AMH change and the level of other hormones, days of stimulation, E2 on the day of hCG administration, the number of embryos, and the embryo quality and IVF parameters. a. βhCG b.FSH c.LH d.Prolactin e.AMH

Comparison of FSH, LH, PRL, And AMH with FF

For FSH, a level >8 IU/L was associated with patients >35 years old. A mean PRL level >11.0 pg/mL was associated with a higher mean βhCG level. A higher E2 level on the day of hCG administration and more oocytes collected when the AMH level was >3ng/mL. These results did not affect the FF.(Table 5)

One case of Down syndrome was recorded, in the IVF group. The mother had the following characteristics:

• 35 years old, weight 65 kg and height 1.68 m, with a 2.5-year period of infertility due to a tubal factor;

• FF of 4% and a PPV of 76.5%;

• first βhCG of 795IU/L and second βhCG of 1736IU/L

• FSH of 6.6IU/L, LH of 8.6IU/L, TSH of 2.68mIU/L, anti-TPO of <9IU/mL, anti-TG of <10 IU/mL, and AMH of 6.69 ng/mL;

• short protocol implemented with rFSH, 8 days of ovulation, amount of gonadotropins 1575IU, E2pg/ml on the day of hCG of 3299, pg/ml. Harvest 11 oocytes, 10 fertilised oocytes, two embryos transferred, on day 5 blastocysts.

Discussion

Although FF testing is considered a primary screening test, only a few studies have assessed its performance, especially in the group of patients submitted to ART. Maternal anxiety might in the population of women achieving pregnancy via ART might underlie their hesitancy to undergo this test. Moreover, in the contemporary literature there are contradictory results regarding the PPV of the FF in patients submitted to ART. Some groups have reported no significant contribution of method of conception [24-26], while others have observed a decreased FF in pregnancies conceived by IVF [13,27].

Age, ethnicity, BMI, and gestational age are critical components of FF testing. Hence, in this study we matched the participants in the natural conception and IVF groups for age, ethnicity (Caucasian), BMI, weeks of pregnancy when NIPT was performed, offspring sex, birth weight, and gestational age. According to our results, there were no significant differences between the natural conception and IVF groups in these clinical parameters (Table 1). The FF of the two study groups was not significantly different (Table 2). The results were similar after adjustment for maternal age via regression analysis. Furthermore, when categorised according to a cut-off point of 6% (Table 2), the FF was not significantly different between the natural conception and IVF groups. Moreover, the FF was not significantly different between the natural conception and IVF groups when the mothers were divided by age (>35 and <35 years). The FF was not associated with maternal age, birth weight, offspring sex, or gestational age in the total sample or separately for the natural conception and IVF groups (Table 3). While we have analysed a relatively homogenous population, the restrictive criteria have limited the sample size.

Lee et al. [13] and Talbot et al. [27] reported that the FF is significantly lower in pregnancies conceived by IVF than those conceived spontaneously. They suggested that a lower FF increased the test failure rate and decreased the PPV in IVF-mediated compared with spontaneous conceptions. These findings have implications for pre-test counselling provided to women conceiving by IVF. When comparing the demographic data of Lee et al. [13] and our current data, there are differences regarding age, BMI, and weight. For example, Lee et al. [13] reported different mean ages for the spontaneous conception and IVF groups (33.8 and 36.6 years, respectively) and differences in ethnicity (61.2% and 83.7% Caucasian, respectively). The heterogeneous sample in that study could explain the low FF in the IVF group. In the study conducted by Talbot et al. [27] the control group included high-risk pregnancies based on the combined first trimester screening, so these women had a high risk for trisomy 21. The authors found a reduction in the FF in pregnancies following fresh compared with frozen embryo transfer. They hypothesised that this reduction in the FF is due to the compromised placental formation following ovarian stimulation in fresh embryo transfers. This observation is in contrast to the data from Lee et al. [13], who did not observe any difference between fresh and frozen embryos regarding FF.

There is some concern about children conceived by ART and their exposure to greater health risks than naturally conceived children. Ovulation-induction medications, the *in vitro* culture of embryos, vitrification, and the potential use of genetically and structurally abnormal sperm during ICSI are independent risk factors. Recent advances in research and practice have enabled molecular-level examinations of descendants of ART-mediated pregnancies. Studies have confirmed the increased risk of placenta-related adverse pregnancy outcomes and an excess of imprinted disorders with abnormal methylation patterns after ART, which raises the issue of a potential ART-induced epigenetic risks [23]. This effect starts from the early development of the embryos in human culture media and may have a profound influence on the phenotype of the offspring [21].

We have investigated the proteomic and metabolomic profile of children born following ART compared with naturally conceived controls to identify epigenetic abnormalities [1,2]. We have found that ART likely causes some epigenetic changes in the offspring, which might be the molecular basis of complex traits and diseases. In this context, we examined the correlation between the FF and several parameters – hormones, maternal age, maternal BMI, type of gonadotrophins, characteristics of ovarian stimulation, embryologic profile, the number of oocytes, the maturation rate, the fertilisation rate, and the quality of embryos – to determine possible predictive factors affecting the outcome of IVF/ICSI. There were no significant correlations between the FF and the hormones. Women with FSH levels >8 IU/L were older and women >35 years old more often presented FSH levels >8 IU/L. Women with PRL >11 pg/mL also presented higher levels of βhCG. Women with AMH >3 ng/mL presented a significantly higher level of E2 on the day of hCG administration and more oocytes.

These results did not affect the FF. Indeed, the FF does not appear to have any association with the IVF profile and is therefore an independent factor concerning IVF parameters.

In the literature, authors have used cffDNA as an additional serum marker (e.g. Down syndrome screening) without adjustment in IVF pregnancies. IVF does not affect levels of cffDNA, which appears to be independent of traditional screening markers (e.g. hCG). Pan et al [24] showed that the cffDNA level in maternal serum seems not to be affected by IVF conception, and therefore may not need adjustment for pregnancies achieved by IVF compared with natural conceptions.

Lambert-Messerlian et al [25] observed that ART-mediated pregnancies and natural conceptions contribute similar levels of circulating cffDNA into the maternal circulation. Costa et al. [26] reported that examining cffDNA performed better than maternal serum screening in both spontaneous and ART-mediating pregnancies, thus decreasing the number of invasive procedures. However, these studies clearly present results that do not show an increase in circulating cffDNA in pregnancies achieved using ART, either in absolute levels or based on the FF. Our findings are consistent with the absence of increase in the amount of cffDNA in maternal plasma from pregnancies conceived by IVF compared with natural conception.

Even though Lee et al. [13] found a reduction in FF in patients submitted IVF, they reported that 97.6% of cffDNA tests in IVF pregnancies provided a result regarding trisomy 21, but the failure rate is higher, the FF is lower, and the PPV for trisomy 18 and 13 and Sex Chromosome Abnormality is decreased in IVF pregnancies compared with those conceived spontaneously. They recommend that these limitations should be taken into account during pre-test counselling in pregnant women who conceive via IVF.

Talbot et al. [27] showed a significant reduction in the FF in patients submitted to ART compared with patients who conceived naturally; the difference seemed to be more pronounced after fresh compared with frozen embryo transfer. Lee et al. [13] did not make this observation in frozen embryos, where the FF was similar in the fresh and frozen groups.

The FF is an important factor for NIPT test accuracy. Several studies have found a reduction in FF for pregnancies following ART compared with natural conception, while other studies have presented no differences in the FF. All researchers agree on the importance of NIPT. The most important issue is that even with a reduction in FF (97.6%), cffDNA tests for IVF pregnancies give accurate results regarding trisomy 21 [13]. However, knowledge on how the FF is affected in ART pregnancies compared with naturally conceived pregnancies is very limited.

Of course, ART-mediated pregnancies are different compared with natural conception for several reasons. The cause of infertility of the parents, the embryo culture media, and COH have been shown to influence the imprinting status of some genes [29]. Indeed, epigenetic changes during the preimplantation period could be a potential mechanism for altered growth, development, and metabolism of ART-conceived children. More specifically, concerns have been raised about the overall health of children born after IVF/ICSI, as this method has a greater risk for the introduction of a genetic errors by bypassing all intrinsic barriers for the fertilisation of abnormal gametes, thus eliminating sperm natural selection. The parameters for a successful NIPT result in natural conceptions are BMI, ethnicity, gestational age, maternal weight, and maternal height. On the other hand, in ART-mediated pregnancies, there are additional variables that play an important role in NIPT. Thus, we have to consider the culture media, the ovulation-induction protocol, and the stage of embryo transfer at day 3 or 5. Considering these factors, it seems very difficult to design a study with homogeneous material that would provide the true picture of the evaluation of NIPT in women who have undergone ART.

In conclusion, we found no difference in the FF for the natural conception and IVF groups. The FF in maternal serum does not appear to be affected in IVF conception. This is in agreement with other studies that have found no difference in the FF in maternal plasma from pregnancies conceived by IVF compared with natural conception. There were no correlations between the FF and IVF parameters. Thus, we suggest that the FF is an independent factor compared with IVF parameters.

Ethics Approval and Consent to Patriciate

The study received approval from the Fertility Institute board and all patients signed a consent form approved by the hospitals' ethical committee.

Consent for Publication

We provide a written consent form for publication.

Availability of Data and Materials

The authors confirm that the data supporting the findings of this research are available within the article.

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Conflict of Interest

The authors declare no conflict of interest, financial or otherwise.

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