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Follicular Fluid Cell-Free DNA as a Non-Invasive Biomarker of Pregnancy Outcome in IVF/ICSI Cycles: Influence of Follicle Size, Age, and BMI in a Nigerian Cohort

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ABSTRACT

Background: Cell-free DNA (cfDNA) in follicular fluid has emerged as a potential non-invasive biomarker of oocyte quality and implantation success in assisted reproductive technologies (ART). However, limited data exist regarding its clinical utility in African populations undergoing in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI).

Objectives: This study aimed to evaluate the association between follicular fluid cfDNA concentration, follicle size, and pregnancy outcomes in infertile women undergoing IVF/ICSI in Nigeria, and to assess the influence of age and body mass index (BMI).

Methods: A prospective observational study that was conducted at the IVF Centre, National Hospital Abuja. The study involved 105 infertile women aged 20-40 years. Follicular fluid samples were collected from follicles and categorized into < 16 mm, 16 – 20 mm, and >20 mm groups. cfDNA concentrations were measured using fluorometric assays. Patients were monitored for clinical pregnancy, and comparisons were made using independent t-test with significance set at $p \leq 0.05$.

Results: Women with positive pregnancy outcomes had significantly lower cfDNA levels across all follicle size groups. In the 16 – 20 mm category, cfDNA was 68.9 ± 9.7 ng/mL in the pregnant group versus 92.5 ± 11.1 ng/mL in the non-pregnant group ($p = 0.001$). Similar patterns were observed in the < 16 mm ($p = 0.012$) and >20 mm ($p = 0.005$) categories. Pregnant women also had slightly lower BMI and were younger across all groups.

Conclusion: High follicular fluid cfDNA is associated with poor pregnancy outcomes in IVF/ICSI. cfDNA may serve as a non-invasive biomarker of oocyte quality and ART success in Nigerian women.

Keywords: Follicular fluid, Cell free DNA (cfDNA), IVF, ICSI, Infertility, Pregnancy outcome, Follicle size, BMI, Biomarker, Nigeria.

INTRODUCTION

Infertility is a major public health challenge affecting an estimated number of persons or couples from 48.5 million to 186 million worldwide, according to the World Health Organization [1]. Beyond the biological implications, infertility in African societies carries devastating psychosocial consequences. In predominantly pronatalist cultures such as the sub-Saharan Africa where a woman's identity and social value are closely linked to childbearing, the inability to conceive can lead to domestic violence, marital instability, social isolation, and psychological trauma. Men are rarely blamed, the burden of infertility at most times falls to women, often regardless of the actual cause [2]. Despite advances in assisted reproductive technologies (ART) such as in-vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI), pregnancy and implantation rates in Africa remain suboptimal [3], especially in low-resource settings, identifying non-invasive biomarkers that predict oocytes competence and reproductive outcomes is essential for improving the efficiency of ART and tailoring treatment strategies [4]. In recent years, increasing attention has been given to the role of the follicular microenvironment in determining oocyte quality. Follicular fluid (FF), which is the microenvironment surrounding the developing oocyte contains biochemical signals that reflect the metabolic and cellular status of both the oocyte and its surrounding granulosa cells [5], making it valuable source for non-invasive biomarker discovery. Numerous molecules in FF including hormones (e.g., estradiol & progesterone), cytokines, oxidative stress markers, amino acids, and lipids have been studied in relation to oocyte competence and ART success [6]. However, many of these are not easily or cost effectively measurable in routine clinical practice. Among the more promising and increasingly accessible candidates is cell-free DNA (cfDNA).

Cell free DNA (cfDNA) refers to short DNA fragments that are released into extracellular fluids, such as blood plasma, urine, or follicular fluid, during apoptosis or necrosis [7]. In context of folliculogenesis, cfDNA primarily arises from granulosa cell turnover. Elevated cfDNA levels may indicate increased granulosa cell apoptosis, follicular atresia, or inflammation within the follicle, factors that can impair oocyte quality, fertilization potential, and embryo viability [8]. Studies have suggested that increased cfDNA in follicular fluid correlate with poor fertilization rates, lower embryo quality, and reduced implantation and pregnancy rates [9, 10]. For example, **Pan *et al.* [11]** demonstrated a clear inverse relationship between cfDNA levels in follicular

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fluid and embryo development potential, suggesting that cfDNA could serve as a real time biochemical indicator of follicular health.

While studies from developed countries suggest that higher follicular fluid cfDNA levels are associated with poor IVF outcomes, there is limited evidence from African populations, where different genetic, environmental, and infectious disease burden may affect cfDNA levels and their clinical interpretation. In particular, the impact of recurrent genital infections, subclinical inflammation, environmental toxins, and nutritional status, all of which are prevalent in sub-Saharan Africa where follicular cfDNA levels has not been fully explored. Additionally, parameters such as follicle size, maternal age, and body mass index (BMI) are known to influence ART outcomes and may interact with cfDNA dynamics. However, their combined relationship with cfDNA levels and pregnancy outcomes in African IVF/ICSI patients remains poorly characterized.

Follicle size, typically measured by transvaginal ultrasound on the day of oocyte retrieval, has traditionally served as a gross indicator of oocyte maturity. Follicles between 16-20 mm are generally considered optimal for retrieving matured oocytes. Yet, follicle size alone does not account for the functional or metabolic quality of the oocyte, nor does it consistently predict fertilization or implantation success [12]. This limitation emphasize the need to complement morphological assessments with molecular biomarkers like cfDNA. Maternal age is another well-established factor influencing reproductive success [13]. With increasing age, oocyte quality declines due to meiotic abnormality, mitochondrial dysfunction, and chromosomal abnormalities [14. 15]. Similarly, obesity (high BMI) is associated with hormonal imbalances, anovulation, reduced oocyte quality, and lower pregnancy rates. Both age and BMI could potentially influence cfDNA levels, either directly through their effect on granulosa cell health or indirectly via systematic inflammation and metabolic dysregulation [16].

These factors highlight the need for integrated approaches to predict ART outcomes. In low resource setting like Nigeria, identifying cost effective minimally invasive biomarkers is especially valuable. Fluorometric quantification of cfDNA is done by targeting fluorescent dyes that binds to double stranded DNA (dsDNA), the intensity of the fluorescent released is correlated to the level of DNA. Devices such as the Quantus or Qubit Fluorometer offers a practical, affordable, and scalable solution for measuring the concentration of cfDNA [17]. Unlike PCR-based approaches that require complex lab setups and amplification steps, these assays are simple, sensitive, and suitable for small sample volumes, making it ideal for clinical applications in developing countries. Despite the advantages, few studies have applied cfDNA analysis to ART populations in Nigeria or other African nations. With the unique demographic and clinical characteristics of this population, including higher prevalence of infection related infertility and delayed ART presentation, region specific research is essential. Understanding how cfDNA levels correlate with clinical pregnancy outcomes, and how they are modulated by follicle size, age, and BMI, could significantly enhance ART practices in Nigeria.

Therefore, this study was designed to evaluate follicular fluid cfDNA concentrations in relation to follicle size and pregnancy outcomes in infertile women undergoing IVF/ICSI at the National Hospital Abuja, Nigeria. The study also explored the influence of age and BMI on cfDNA levels and reproductive success. By generating evidence from a sub-Saharan African cohort, this work contributes to the global effort to identify reliable, affordable, and accessible biomarkers for improving assisted reproductive care.

MATERIALS AND METHODS

Study design and setting: This was a prospective observational study conducted at the IVF Centre of the National Hospital Abuja, Nigeria, between the periods from April 2023 to July 2024.

Study population: The study included 105 infertile women aged 20 – 40 years undergoing IVF/ICSI treatment.

Inclusion Criteria: Participant were eligible for the study if they met the following: Women aged 20–40 years. Infertile group were diagnosed with infertility of at least one year women with tubal factor, male factor, or unexplained infertility, normal ovarian reserve and undergoing IVF or ICSI treatment. Fertile women have normal ovarian reserve, defined by antral follicle count ≥ 5 and AMH ≥1.0 ng/mL. All participants must show the presence of at least one mature follicle ≥16 mm on the day of oocyte retrieval. Willing to provide informed consent for participation and follicular fluid sample collection.

Exclusion criteria: Patients who had any of the following: Polycystic ovary syndrome (PCOS) **&** endometriosis, or ovarian cysts. Patients with known endocrine disorders (e.g., uncontrolled thyroid dysfunction, diabetes mellitus **&** hyperprolactinemia). Evidence of pelvic or systemic infections**,** or recent antibiotic use that may alter follicular microenvironment. History of chemotherapy or pelvic radiation**.** Follicular fluid samples visibly contaminated with blood or insufficient for cfDNA analysis. Cycles where no oocytes were retrieved or no embryo transfer was performed**.**

Ovarian stimulation and oocyte retrieval: Controlled ovarian hyperstimulation was carried out using either a gonadotropin-releasing hormone (GnRH) agonist or antagonist protocol. Recombinant FSH or highly purified human menopausal gonadotropins in variable doses

between 150-450IU/ day were administered based on antral follicle count, age of patient and body weight. Follicular growth was monitored by transvaginal ultrasound, and final oocyte maturation was triggered using hCG or GnRH agonist when ≥ 2 follicles reach ≥ 18 mm in diameter. Oocyte retrieval was performed 34 – 36 hours post – trigger under ultrasound guidance.

Follicular fluid collection and classification: Follicular fluid (FF) was aspirated from selected individual follicles using separate tubes to avoid cross-contamination. Blood-stained samples were excluded as much as possible. Follicles were classified by diameter into three categories: < 16 mm, 16-20 mm, and > 20 mm based on ultrasound measurements. FF samples were centrifuged at 3000 rpm for 10 minutes at 4°C to remove cells and debris, then aliquot and stored at -80 °C until analysis.

cfDNA quantification: cfDNA concentrations in follicular fluid were quantified using the Qubit dsDNA High Sensitive (HS)'. Assay Kit (Thermo Fisher Scientific, USA) strictly following manufacturer's instructions. Measurements were performed in duplicate (to ensure accuracy and reproducibility) using the Qubit 4 Fluorometer. Final concentrations were expressed in nanograms per milliliter (ng/mL). Polymerase chain reaction (PCR) was not used as our study focused on quantifying total cfDNA content rather than analyzing its genetic sequence or origin.

Embryo transfer and pregnancy confirmation: Oocytes were fertilized via IVF or ICSI based on semen parameters. Embryos were assessed and graded on day 3 or 5, and up to three embryos were transferred based on individual clinical parameters. Luteal phase support was provided using vaginal micronized progesterone, Serum β-hCG was measured 14 days post-transfer. Clinical pregnancy was confirmed by the presence of a gestational sac on transvaginal ultrasound two weeks after a positive β-hCG result.

Anthropometric and demographic data: Participant's age and body mass index (BMI) were recorded at baseline. BMI was calculated at weight (kg) divided by height in meters squared (kg/m^2).

Ethical approval and consent to participate: All subjects signed informed written consents for using data before their enrolment into this study. This study was reviewed and approved by the department of Health Research Ethics Committee (HREC) of National Hospital Abuja with License number (NHA/EC/068/2022). All procedures were performed according to the Declaration of Helsinki. The scope, nature, aims and objectives of this study were explained to all participants prior to enrollment.

Statistical Analysis

Data analysis was performed using IBM SPSS Statistics version 26. Descriptive statistics were expressed as mean ± standard deviation (SD). Independent sample t-tests were used to compare mean cfDNA concentrations between pregnancy outcome groups within each follicle size category. A p-value ≤ 0.05 was considered statistically significant.

RESULTS

A total of 105 infertile female aged 20 – 40 years undergoing IVF/ICSI were included in this study. Follicular fluid cfDNA levels were compared across three follicle size categories (< 16 mm, 16 – 20 mm, and >20 mm) and stratified by pregnancy outcome (positive or negative). The descriptive data, including age and BMI were recorded. Across all follicle sizes, women who achieved pregnancy had significantly lower cfDNA levels in follicular fluid compared to those who did not conceive. In follicles sized 16 – 20 mm, mean cfDNA was 92.5 ± 11.1 ng/mL in the non-pregnant group compared to 68.9 ± 9.7 ng/mL in pregnant group (p = 0.005). Even in smaller follicles (<16 mm), this inverse trend remained significant (p = 0.012). Women who achieved pregnancy also tended to have a slightly lower mean BMI and younger mean age compared to those with negative outcomes, though statistical testing for these variables was not conducted (table 1).

Table (1): Follicular fluid cfDNA levels by Follicle size and pregnancy outcome in women aged 20 – 40 undergoing IVF/ICSI

Follicle Size (mm)	Pregnancy outcome	n	Mean cfDNA(ng/mL) ± SD	Mean Age (years) ± SD	Mean BMI (kg/m^2) ± SD	p-value (cfDNA vs Outcome)
< 16 mm	Negative	20	98.2 ± 12.4	34.5 ± 3.2	27.1 ± 2.8	0.012*****
	Positive	5	71.6 ± 10.3	33.1 ± 2.9	25.6 ± 2.5	
16 – 20 mm	Negative	30	92.5 ± 11.1	35.0 ± 3.5	27.4 ± 3.0	0.001******
	Positive	15	68.9 ± 9.7	32.8 ± 3.0	25.3 ± 2.7	
> 20 mm	Negative	25	85.3 ± 10.5	35.4 ± 3.3	28.0 ± 2.9	0.005******
	Positive	10	66.2 ± 8.4	32.6 ± 2.8	24.9 ± 2.6	

SD: standard deviation, **n:** number of patients, **p-value:** significance level within the group. Data are represented as mean ± standard deviation, Follicular fluid cfDNA concentrations were compared between pregnancy outcome groups using independent samples t-test within each follicle size category, Statistically significant at ***p < 0.05,** highly significant at ****p < 0.01** (independent t-test).

DISCUSSION

This study investigated the association between follicular fluid cell free DNA (cfDNA) levels and pregnancy outcomes in women undergoing IVF/ICSI cycles, with a focus on how follicle size, age, and body mass index (BMI) influence this relationship. The results demonstrated a clear and consistent inverse relationship between cfDNA levels and clinical pregnancy across all follicle size categories, suggesting that cfDNA may serve as an excellent non-invasive biomarker of follicular competence.

In the 16-20 mm follicle size category, which is considered optimal for mature oocyte retrieval, cfDNA levels were significantly lower in the pregnant group (68.9 ± 9.7 ng/mL) compared to the non-pregnant group (92.5 ± 11.1 ng/mL; p = 0.001). This difference, which was the most statistically significant among all groups, highlighted the potential efficacy of cfDNA in distinguishing high-quality follicles even within morphologically ideal ranges. Similarly, for follicles > 20 mm, the mean cfDNA concentration was 66.2 ± 8.4 ng/mL in women who achieved pregnancy compared to 85.3 ± 10.5 ng/mL in those who did not (p = 0.005). Even in follicles < 16 mm, where oocyte maturity is typically lower, a significant difference was observed (71.6 ± 10.3 compared to 98.2 ±12.4 ng/mL; p = 0.012). These findings suggest that cfDNA may reflect a more fundamental aspect of follicular health beyond mere follicle size.

Our study is in agreement with earlier studies that reported elevated cfDNA concentrations in follicular fluid to be associated with granulosa cell apoptosis, oxidative stress, and reduced oocyte competence [10, 11, 18]. **Scalici *et al*.** [10] found that higher follicular cfDNA levels were linked to poorer embryo morphology and lower implantation rates. Similarly, **Traver *et al*.** [19] also emphasized the role of cfDNA as a marker of cell turnover within the follicle. The present study extends these findings to a Nigerian population and adds further support to the use of cfDNA as a biomarker for oocyte quality in ART settings.

In addition to cfDNA levels, descriptive developments in maternal age and BMI were observed. Women who achieved pregnancy had slightly lower BMI and were younger across all follicle size categories. For example, in those > 20 mm group, the mean age of pregnant women was 32.6 ± 2.8 years compared to 35.4 ± 3.3 years in non-pregnant women, while the mean BMI was 24.9 ± 2.6 kg/m² against 28.0 ± 2.9 kg/m². Although these differences were not statistically tested in this study, they are consistent with the established literatures. Advanced maternal age is associated with mitochondrial dysfunction, chromosomal abnormalities, and increased granulosa cell apoptosis [9], all of which could contribute to higher cfDNA concentrations. Similarly, obesity is linked to systemic inflammation and insulin resistance [20, 21], factors known to negatively affect follicular health and oocyte quality.

Importantly, this study highlighted that follicle size alone is not sufficient to predict pregnancy outcomes. Although follicular diameter has traditionally been used as a tool for assessing oocyte maturity, our findings showed that follicles of the same size can yield markedly different cfDNA levels and consequently, different clinical outcomes. For instance, within the 16–20 mm range, pregnant and non-pregnant groups showed a mean difference of 23.6 ng/mL in cfDNA concentration. This emphasizes the need for biochemical assessment alongside morphological evaluation during follicle selection.

From a clinical perspective, the ability to measure cfDNA using a simple fluorometric assay, offers a practical and cost-effective method for follicular assessment. Unlike PCR-based techniques that require advanced laboratory infrastructure, fluorometric assays are user-friendly and well-suited to resource-limited environments like Nigeria. This accessibility enhances the translational potential of cfDNA measurement for routine use in ART clinics.

The current findings support the hypothesis that elevated cfDNA in follicular fluid reflects increased cellular stress or damage within the follicle, leading to reduced oocyte viability and compromised implantation potential. It is likely that cfDNA serves as a cumulative indicator of multiple pathological processes oxidative stress, inflammation, apoptotic signalling [22] that ultimately impact embryo development.

Clinical relevance: This study provided important insight into the potential of follicular fluid cfDNA as a non-invasive biomarker for ART success. It supports integrating cfDNA measurement into routine IVF/ICSI protocols to aid follicle and oocyte selection, especially in resources-limited settings, such as many parts of Africa, identifying such markers can help optimize embryo selection and improve IVF outcomes without adding significant cost. Future research should aim to establish reference thresholds, include molecular characterization of cfDNA, and examine longitudinal outcomes such as embryo development and live birth rate.

LIMITATIONS

Despite its strengths, the study had limitations. The relatively small sample size, especially in the pregnant group with <16 mm follicles (n = 5), may limit the confirmation of findings. Additionally, the study did not statistically compare age and BMI between outcome groups, nor did it account for embryo quality, fertilization

rate, or endometrial receptivity, which could also influence pregnancy outcomes. Moreover, cfDNA was measured in bulk without differentiating between nuclear and mitochondrial origin, which may have differential implications for follicular health.

CONCLUSION

This study provided evidence that elevated levels of cell-free DNA (cfDNA) in follicular fluid are significantly associated with negative pregnancy outcomes in women undergoing IVF/ICSI treatment. The inverse relationship between cfDNA concentration and clinical pregnancy success was consistent across all follicle size categories, suggesting that cfDNA may reflect underlying follicular stress, apoptosis, or compromised oocyte quality. Additionally, although not statistically tested, women who achieved pregnancy tended to have lower body mass index (BMI) and were slightly younger, supporting the well-established influence of these factors on ART outcomes. Follicular size alone did not predict outcome without considering cfDNA levels, emphasizing the importance of biochemical markers in assessing follicular competence. The use of a simple fluorometric assay to quantify cfDNA offers a cost-effective, non-invasive, and clinically feasible tool for evaluating the follicular microenvironment. This is particularly valuable in resource-limited settings such as Nigeria, where access to advanced diagnostic platforms is often restricted.

In conclusion, follicular fluid cfDNA holds promise as a predictive biomarker for oocyte quality and IVF/ICSI success. Further large-scale, multicenter studies are needed to validate these findings, establish reference thresholds, and explore the potential integration of cfDNA analysis into routine ART practice.

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Conflicts of Interest: None.

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