



Research Article

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# Cell Free Fetal DNA As Biomarker for Pregnancy Loss Disorders

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## Abstract

Cell free fetal DNA (cff DNA) is apoptotic fetal DNA that is shed from placenta and flows in maternal blood. This can be used as an indicative biomarker to rule out pregnancy associated complications. The objective of this study is to investigate the correlation of the concentration of cff DNA with intrauterine deaths. The RHD gene (Rh blood group, D antigen) was used as fetal DNA marker for the confirmation of fetal DNA and GAPDH gene (Glyceraldehyde-3-phosphate dehydrogenase) was used as internal control. The control group comprises of 54 healthy singleton pregnant women without any history of pregnancy associated complications or fetal chromosomal abnormalities. The control group was confirmed by follow up for the delivery of normal baby.

Case group consisted of pregnant women who were showing abnormal gynecological findings/history of previous miscarriages or chromosomal abnormalities. Intrauterine death (IUDs) was confirmed after following up the pregnancy. T-test was applied to find out the correlation between control and case groups. We have observed significantly higher quantities of cell free fetal DNA in case group as compared to control group (P value <0.0001, r<sup>2</sup>=0.4266). The case group was further subdivided into five groups depending upon the reason of intrauterine death of fetus. A significant correlation has been observed among all the five case subgroups as compared to control group (P value <0.0001). Our study concludes there is a significant increase in the concentration of cell free fetal DNA of IUD cases before onset of the conditions.

## Keywords

Cell free fetal DNA, Apoptotic fetal DNA, Intrauterine death, Oli gohydraminos, Multiple trisomes, Miscarriages

## Abbreviations

IUDs: Intrauterine Death; cff DNA: Cell free fetal DNA; RHD: RH blood group, D antigen; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; NIPT: Non-Invasive Prenatal Testing; TOP: Termination of Pregnancy; CHD Congenital Heart Disease; mt DNA: mitochondrial DNA; FGR: Fetal Growth Restriction

## Introduction

The prenatal determination of sex chromosomes was the opening of a new era to investigate the disorders in developing fetus [1,2]. Fuches and his colleagues in 1960 determined gender to rule out sex linked recessive diseases; hemophilia from amniotic fluid [3]. Advancements in the field of prenatal diagnosis starting from intrauterine detection of genetic defects [4] and first prenatal diagnosis of Down's Syndrome [5] to preimplantation genetic

diagnosis [6] a broad spectrum of prenatal genetic tests have facilitated the parents about the wellbeing of their unborn child.

Around 3% to 5% of pregnancies lead to still birth or the infants with malformation due to chromosomal abnormalities. Aneuploidy is one of the major types of chromosomal abnormality that results the cells with extra or missing chromosomes. During the normal process of meiosis, cellular division produces haploid sperm and

eggs, containing one copy of chromosomes from each parent which fertilize to make a diploid zygote. Data suggested that errors during spindle fiber formation due to the less elasticity of aged maternal cells results in the fetus with aneuploidies [7,8].

According to American College of Obstetricians and Gynecologists maternal age alone should not be taken as a threshold to offer prenatal screening rather it should be available to all women. The criteria for the selection of prenatal diagnosis to the parents having increased risk of aneuploidies can be determined by advanced maternal age (35 years or above), previous deliveries with aneuploidies, still births, spontaneous abortions and chromosomal aneuploidies/translocations/inversions in either of the parent [9].

Nowadays, a wide range of prenatal tests are available for pregnant women to assure the well-being of the unborn child which fall into two groups: the non-invasive and the invasive method. Non-invasive prenatal testing utilizes blood of pregnant women to detect cell free fetal DNA (cff DNA) to screen fetal abnormalities. This method was recognized by Lo et al in 1997 [10]. Invasive prenatal testing includes amniocentesis, chorionic villi sampling and fetal blood sampling. These tests are performed under sonographic control by specialized obstetricians who provide the specimen for genetic analysis. Importantly, all invasive techniques carry a significant procedure-related risk for an abortion. Generally, invasive procedures are arranged upon securing high risk of Non-Invasive Prenatal Testing (NIPT) of pregnant woman [10].

Due to the risk of fetal abnormalities [9] and chances of miscarriages during invasive prenatal sampling, the use of cell free fetal DNA is considered a more attractive alternative of prenatal diagnosis. Cff DNA is apoptotic fetal DNA that shed from villous trophoblast during the development of fetus [11]. In addition to the role of cff DNA in detection of chromosomal abnormalities and single gene disorders, studies have shown the association of significant increase in the level of cff DNA in maternal plasma

during pathological conditions for example scleroderma, [12] preeclampsia, [13,14] trisomy 21 [15] intrauterine growth restriction [16,17] and preterm labor [18]. Hence cffDNA can be used as an alternative screening marker for the detection of pregnancy loss disorders. The objective of this study is to investigate correlation of the concentration of cff DNA with intrauterine deaths in Pakistani female population.

## Material & Methods

### Ethical Statement

The study was approved by the ethical review committee of Institute of Biomedical & Genetic Engineering (IBGE) Islamabad. It was conducted according to the Declaration of Helsinki. The informed patient consent was taken before the all the research activities and analysis.

### Inclusion Criteria

Samples were collected from General Hospitals of Islamabad from January 2020 to December 2021 after the 10<sup>th</sup> week of gestation. The control group comprises of 54 healthy singleton pregnant women without any history of pregnancy associated complications or fetal chromosomal abnormalities who delivered healthy babies at full term (37 weeks of gestation or above). Confirmation of control group was done after the delivery of normal baby. Case group (n=18) consisted of pregnant women who have shown abnormal gynecological findings/history of previous miscarriages or previous birth with chromosomal abnormalities. These pregnancies were followed up for the clinical outcome so intrauterine deaths (IUDs) or miscarriages were confirmed after following up the pregnancies. Some IUDs were due to unknown causes, others happened as a result of pregnancy associated disorder; oligohydramnios or due to multiple trisomies (causes are summarized in table 1).

**Table 1:** Distribution and clinical characteristics of case sub-groups.

Group No	Complications	Number (n)	Average gestational age at time of sampling (weeks)	Average maternal age at time of sampling (years)	Average gestational age at IUD (weeks)	Concentration of cff DNA (ng/ml)
1	Intrauterine deaths (IUDs) due to unknown causes	n=7	19.85	27.71	30.3	3128.57
2	IUDs and pregnancy with oligohydramnios	n=3	19.33	27.33	29.3	6366.66
3	IUDs with abnormal sonographic parameters	n=4	26.25	33.25	32.5	6950
4	IUDs with multiple trisomies (trisomies of chromosome 21, 13, 18, X, Y)	n=2	19.5	34	29	5050
5	Miscarriages (abortion before 20 week of gestation)	n=2	13	28	29	7000

**Abbreviations:** Intrauterine death (IUDs), Cell free fetal DNA (cff DNA)

### Exclusion Criterion

- Pregnancy loss due to Injury or infection
- Induced abortions for normal fetus (except in case of intrauterine death)
- Fetal loss due to Diabetes or hypertension.

### Sample Processing

5ml of peripheral blood was obtained in EDTA vacutainer. Plasma was extracted from maternal blood by centrifugation of blood at 2500RPM for 10 min at 4°C. Re-centrifugation of the plasma was done at 16000 RPM for 10 min at 4°C. The plasma extraction was performed on the same day as phlebotomy. Plasmas were kept at -70°C for storage. 1ml plasma was used for cell free fetal DNA extraction through slight modification of manufacturer protocol for blood and body fluids (QIAamp DNA blood mini-Kit, Qiagen, Cat No. 51106. Extracted cff DNA was diluted in 100 µl of buffer AE (Qiagen) and kept at -70°C for storage. cffDNA was quantified by Nanodrop 2000c (Thermo Scientific).

The RHD gene (Rh blood group, D antigen) was used as fetal DNA marker for the confirmation of fetal DNA (RHD gene amplification in cell free fetal DNA extracted from negative blood group female bearing Rh factor positive fetus is the confirmation of the presence of fetal DNA) and GAPDH gene (Glyceraldehyde-3-Phosphate Dehydrogenase) was used as internal control (this in-house gene will amplify both fetal & maternal DNA following the protocol of Devyser RHD (8-A060) using Slan -96P real time PCR System. For the confirmation of trisomies (trisomy 18, 21 and 13)

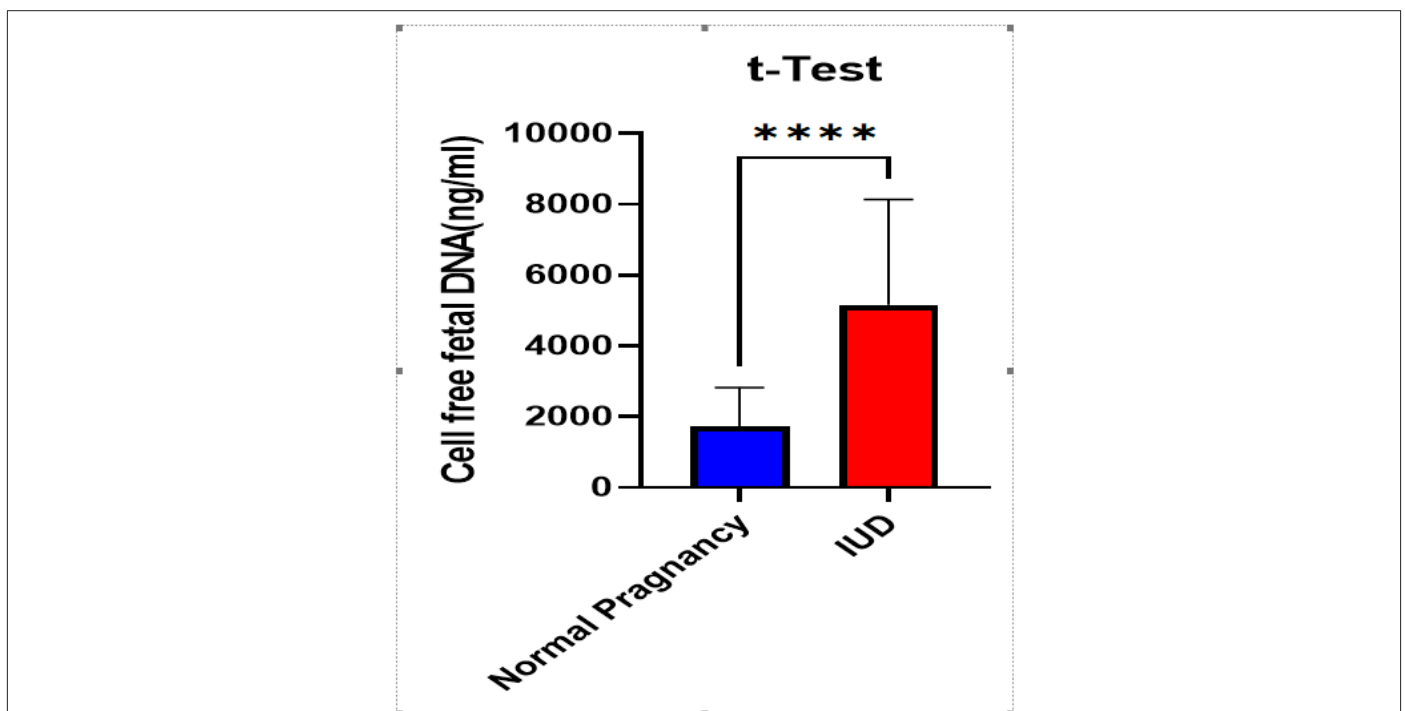
Rapid aneuploidies Detection (Devyser Compact v3, 8-A017.3-25) was done following manufacturer instructions.

### Statistical Analysis

The quantity of cell free fetal DNA of case and control group were analyzed using T-test. Statistical software GraphPad Prism version 9.3.1 was used for statistical analysis of data. The data was expressed in comparison of concentration of cell free fetal DNA in case and control groups. Concentration of cell free fetal DNA among case subgroups with control group and comparison of gestational ages and maternal ages between case and control group.

### Result and Discussion

Samples were collected from General Hospitals of Islamabad from January 2020 to December 2021 after the 10<sup>th</sup> week of gestation. The control group comprises of 54 healthy singleton pregnant women without any history of pregnancy associated complications or fetal chromosomal abnormalities who delivered healthy babies at full term (37 weeks of gestation or above). Confirmation of control group was done after the delivery of normal baby. Case group (n=18) consisted of pregnant women who have shown abnormal gynecological findings/history of previous miscarriages or previous birth with chromosomal abnormalities. These pregnancies were followed up for the clinical outcome so intrauterine deaths (IUDs) or miscarriages were confirmed after following up the pregnancies. Multiple trisomies were confirmed by QF-PCR from abortus fetus. Then our study correlated for the concentration of cell free fetal DNA in case and control group.



**Figure 1:** Significant increase in the concentration of cff DNA of complicated pregnancies as compared to the normal pregnancy.

A significant correlation ( $P$  value  $<0.0001$ ,  $r^2=0.4266$ ) has been found for the concentration of cell free fetal DNA in case group vs control group. (Figure 1). Moreover, a significant correlation has been observed among all the five case subgroups (Figure 2) as

compared to control group ( $P$  value  $<0.0001$ ). However, there was no significant correlation between gestational ages of case and control groups ( $P=0.4160$ ,  $r^2=0.009342$ ) (Figure 3) and maternal ages of both the groups;  $p= 0.4492$ ,  $r^2= 0.008326$  (Figure 4).

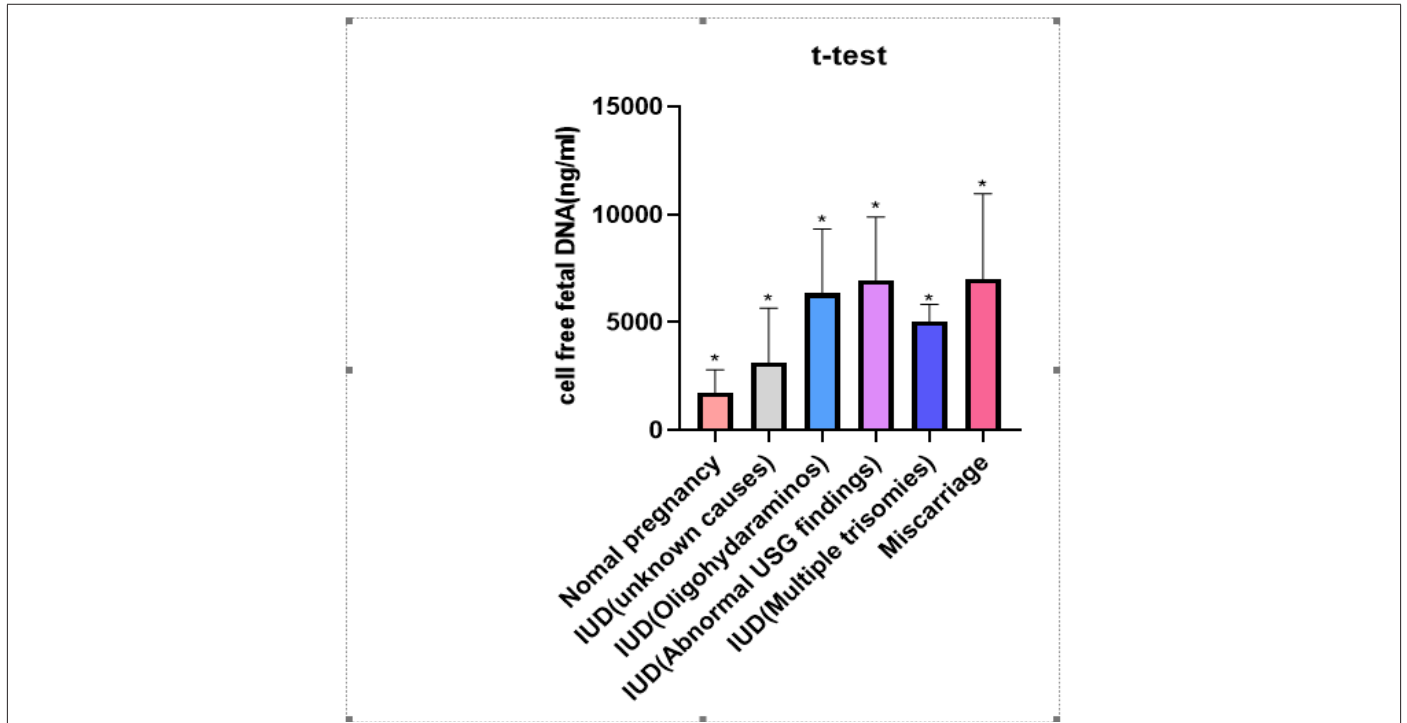


Figure 2: Median cff DNA concentrations of all study groups.

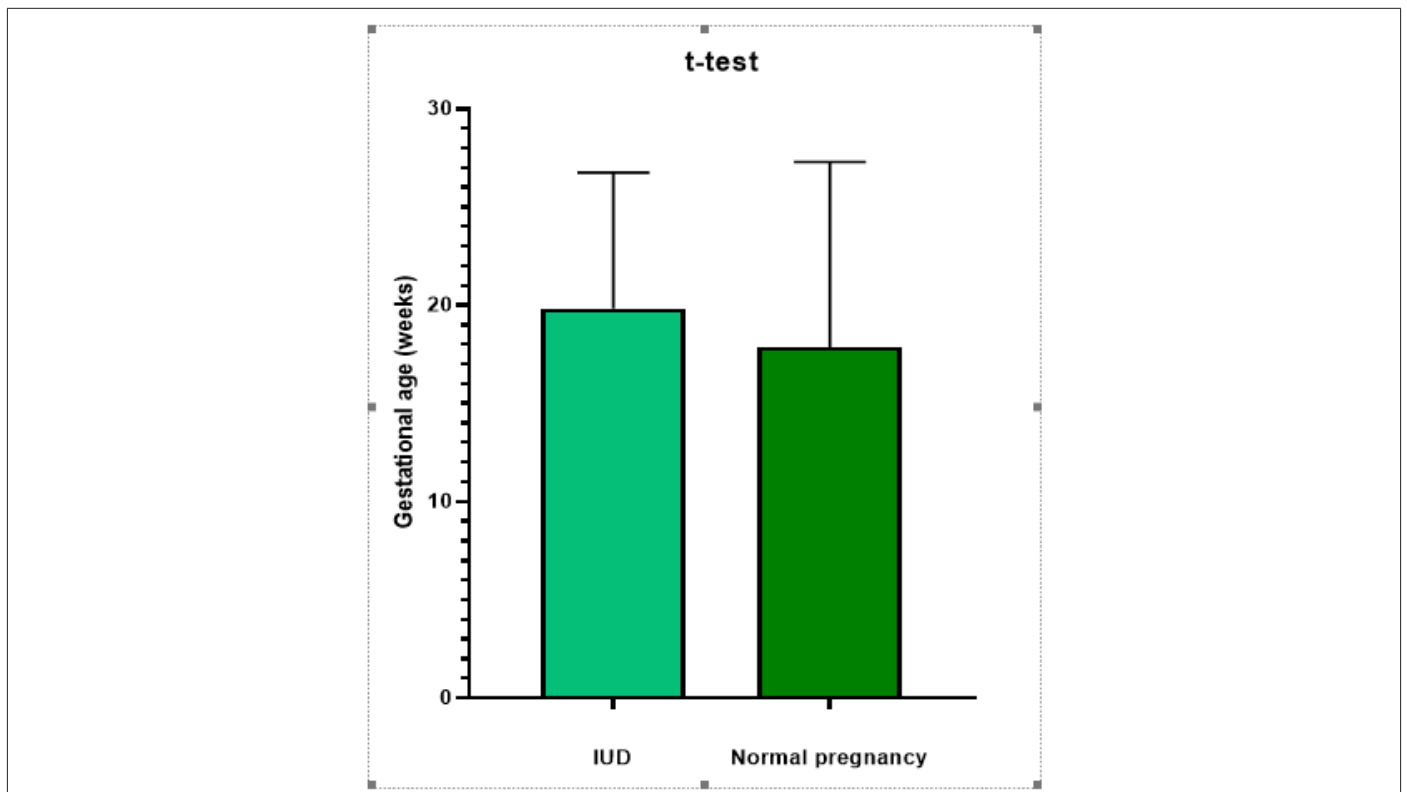
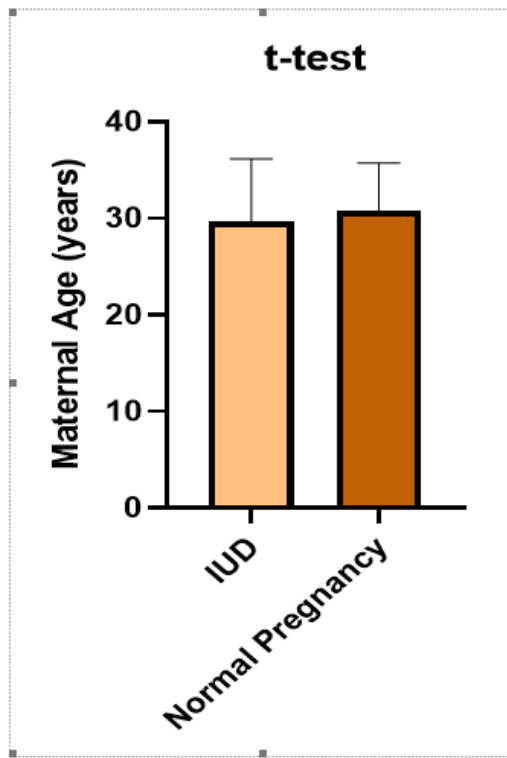


Figure 3: Median gestational ages (weeks) of normal and IUD groups at the time of sampling.



**Figure 4:** Median maternal ages (years) of normal and IUD group at the time of sampling.

Concentrations of cell free fetal DNA were significantly high even at early gestational stages in all the pregnancies which ended up with fetal deaths due to unknown causes, IUDs, miscarriages, multiple trisomies or oli gohydramionis. Distribution, clinical characteristics and concentration of cff DNA of case subgroups is described in Table 1. Clinical characteristics, pregnancy outcome and concentration of cff DNA of case and control group have been shown in table 2. The average gestational age and maternal age of

the control group is 17.471 weeks and 30.660 years respectively. The average gestational age and maternal age of case group is 20.3 weeks and 29.6 years respectively (Table 2). The average concentration of cell free fetal DNA of control group is 1740.56 ng/ml (Table 2) whereas the average concentration of cell free fetal DNA of case group is 5161.1ng/ml (Table 2) which is many folds higher as compared to control group. Sample size is 61 to reach confidence interval of 95%.

**Table 2:** Clinical characteristics & concentration of cff DNA of studied groups (average)

Groups	Average gestational age at time of sampling (weeks)	Total no. of patients	Average maternal Age at time of sampling (years)	Concentration of cff DNA (ng/ml)	Pregnancy outcome
Control group	17.47	54	30.66	1740.56	Normal baby birth at full term
Case Group	20.38	18	29.61	5161.11	Intrauterine death

**Abbreviations:** Cell free fetal DNA (cff DNA)

This is a primarily study on Pakistani population based on the evaluation of pregnancy out come by initial measurement of concentration of cell free fetal DNA. Recently transgenic Green fluorescent protein (GFP) mice were used to scrutinize that the continual increase in concentration of cell free fetal DNA till labor has not been linked maternal inflammatory response [19]

Elevation in total amount of cell free fetal DNA in maternal blood was previously reported significantly high in trisomy 21 and preterm labor using Y specific markers for the confirmation of cell free fetal DNA [18-22]. We have used RHD gene amplification in cell free fetal DNA extracted from negative blood group female baring Rh factor positive fetus for the confirmation of the presence of fetal

DNA as fetal marker and concluded that the elevated concentration of cffDNA can be used as an indicative biomarker to rule out any pregnancy associated complication later in pregnancy.

This is to be noted that cell free fetal DNA concentration has increased many folds before any indication of pregnancy loss or chromosomal abnormalities in case group as compared to control group. The confirmation about the clinical outcome of pregnancies of case group and control group was done before applying statistical analysis. This is worth mentioning that the gestational age at the time of sampling is far before the onset of pregnancy loss (pregnancy losses and their reason were later confirmed when we followed up our cases). Thus, we conclude that concentration of circulating fetal DNA has increased many folds in pathological conditions as compared to normal pregnancies the possible cause of increasing concentration of cell free fetal DNA in pathological problems is due to increase in apoptosis of placental cells.

The research implication of our study is to determine the threshold of increasing concentration of cffDNA in pregnancy loss disorders. This can be an easy approach to detect pregnancy loss disorders like IUDs miscarriages and aneuploidies. This preliminary data has the potential to be used in clinical applications if carried out on a large number of samples. This is a cost effective and attractive approach to identify pregnancy loss conditions. No doubt that prenatal diagnosis helps the parents and obstetricians for timely decision of termination, but it is not limited to Termination of Pregnancy (TOP) rather it has many other potential benefits. In higher risk cases, where a couple is having children with chromosomal abnormalities; normal result can reduce the obvious parent anxiety which is very important to relieve stressed out pregnant woman [21]. Knowledge of anomalies allows certain preinatal clinical arrangements. For example, there is a significant association between congenital heart disease (CHD) and Down's syndrome [21]. Similarly, there are chances of delivery with diaphragmatic hernia in aneuploidies [22]. The appropriate operative interventions would be lifesaving in such cases. Awareness about delivery with chromosomal abnormality augments the parents to prepare both emotionally and financially and facilitates the parents to develop a management plan for raising a child with special needs. Prenatal testing through cell free fetal DNA has opened a new era of non-invasive diagnosis of human health condition before birth [23]. Over ages, our medical history exceeded far for diagnosis more than treatment. However, the ultimate goal is to treat all medical conditions prenatally, that is not possible if we will not be able to see the whole depiction of disorder inside the womb.

Cell free fetal DNA has opened a novel approach to predict worse developmental pregnancy outcomes. Studies have reported elevated levels of cell free fetal DNA in maternal plasma [24,30] and in maternal serum cell free mitochondrial DNA (mt DNA) [25] in preeclampsia. Conversely there have been low levels of maternal

serum cell free mitochondrial DNA in early gestational 11 to 12+6 weeks in preeclampsia [26]. Therefore it is a need to develop consensus upon predicting diagnostic/prognostic value [27]. A plasma RNA quantification panel has been developed for detection of Fetal Growth Restriction (FGR) and preeclampsia which requires investigations for larger patient cohorts [28]. In line with our study Borna et al reported significant association between concentrations of cell free fetal DNA & pregnancy associated complications of gestational diabetes, chronic hypertension, still births, fetal trisomies, preeclampsia preterm delivery and intrauterine growth restriction [29].

## Conclusion

Elevated concentration of cffDNA can be used as a biomarker to rule out any pregnancy associated complication later in pregnancy. It is to be noted that after following up of the entire pregnant woman included in our study, we correlated the elevated concentration of cell free fetal DNA in case group. This could be a cost effective and rapid screening for detection of pregnancy associated complications. Our data has shown increased concentration of fetal DNA extracted from maternal plasma in all suspected subjects before the any clinical indication of fetal demise [30]. The limitation of our study is confined number of patients which are taken from tertiary care hospitals of a confined area moreover inclusion of only willing patients is also a limiting factor. However, based on this preliminary data potential clinical applications can be developed if carried out on a large number of samples. As we included only RHD gene as fetal marker more fetal markers, for example DNA methylation markers for the confirmation of fetal origin of cell free fetal DNA [23] can be included to generalize the study. More fetal markers may imply accuracy in the utility of early detection of pregnancy related complications [31].

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## Author's Contributions

**Author 1:** Idea/hypothesis, scientific work, data collection, statistical analysis and paper writing.

**Author 2 & 3:** Patients/sample submission and gynecological investigations according to the criteria of study.

## Conflict of Interest

The authors declare no conflict of interest.

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