ORIGINAL RESEARCH

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Effect of Levothyroxine Sodium Intake on the Fetal Fraction in Non-Invasive Prenatal Testing: A Cross-Sectional Study

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ABSTRACT Objective: Non-invasive prenatal testing (NIPT) and fetal fraction (FF) are shown to be affected by various factors like maternal characteristics or medications. Recently medications are now undergoing evaluation as influencing factors. Still no data are presented on the relation between levothyroxine, NIPT and FF results. The study's aim is to assess the effect of levothyroxine sodium on the FF. **Material and Methods:** This retrospective case control study was conducted with medical records of pregnant women who underwent NIPT between 2016-2020 at our institution. Women with multiple gestation, body-mass index ≥25, abnormal fetal karyotype, pregnancy with assisted reproductive techniques, those without FF report and non-euthyroid patients were excluded. The pregnant euthyroid women included in the study were divided into two groups: using and not using levothyroxine. Maternal characteristics, FF of the NIPT, TSH, FT4 values and levothyroxine dosing were noted and compared between the two groups. **Results:** Data were collected from 51 pregnant women using levothyroxine and 102 pregnant women who did not. There was no difference in demographic characteristics, also no significant difference was shown in TSH and FT4 between the groups (p=0.180, p=0.920). The mean FF level in pregnant women using levothyroxine is lower, but the situation did not reach statistical significance (p=0.070). **Conclusion:** The knowledge of relation between FF and medications is largely based on very limited data. This is the first report of assessing levothyroxine effect on FF percentage in NIPT. Levothyroxine sodium was not confirmed to be associated with any significant change in the FF.

Keywords: Non-invasive prenatal testing; prenatal diagnosis; thyroxine

Non-invasive prenatal testing (NIPT) uses cell-free fetal DNA (cfDNA) that is shown in the maternal circulation, and presumed to originate from the trophoblasts. It was first identified by Lo et al., and used to indicate the Y chromosome to diagnose fetal sex.[1] The source of fetal cfDNA in the maternal circulation is thought to be mainly apoptosis of placental cells (syncytiotrophoblast) and secondarily fetal cell traffic across the placenta. However, maternal hematopoietic cells are the source of most maternal cfDNA.[2]

Circulating cfDNA was observed to be highly fragmented. The fetal fraction (FF) is the percentage of all cfDNA in maternal blood, which originated from the fetal-placental unit. Fetal-placental cfDNA can be isolated in maternal blood from 5 weeks of gestation.[3] However, FF is in fact very low before 10 weeks. The concentration of fetal cfDNA increases slightly between 10 and 20 weeks of gestational age and then increases until term.[4] Sufficient amount of cfDNA -approximately 3 to 4%- must be existent for the correct test results.

FF is a trend topic recently. Still it should be carefully interpreted for NIPT results. FF percentage is shown to be affected by various factors such as ges-

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tational age, sample collection, maternal weight, fetal karyotype, medications, conception with assisted reproduction, and multiple gestation.[5-7]

Maternal thyroid hormones have a strong influence on pregnancy, particularly on the placenta, and they are involved in the proliferation, survival functions of trophoblastic cells.[8] Also in vitro results confirm that both thyroid hormone deficiency or excess may compromise trophoblastic cell function.[9] Thyroid hypo-function is observed to affect fetoplacental unit growth, distorting the decidualization, vascularization, and development of the placenta, increasing apoptosis, and reducing the proliferation of trophoblasts.[9] Accordingly, it is suggested that FF percentage may be altered in the pregnant women with levothyroxine intake. This study assesses the effect of levothyroxine sodium on the FF of a NIPT.

MATERIAL AND METHODS

A retrospective case control study was conducted on pregnant women who underwent NIPT between 2016 and 2020 in the institution which is designated as NIPT testing, as almost 3,000 patients tested annually. The exposed group included the females who were receiving oral levothyroxine sodium before the pregnancy and at the time of the testing. The control group was defined as the pregnant women who were not receiving any medication. Women with multiple gestation, body mass index (BMI)≥25, abnormal fetal karyotype, pregnancy with assisted reproductive techniques, those without FF report and non-euthyroid pregnant were excluded. Gestational age was determined from the measurement of the crown-rump length (CRL) of the fetus, which was performed just before NIPT and thyroid stimulating hormone (TSH), FT4 sampling. If a woman had more than one pregnancy during the research period, only the first one was included in the study. Maternal characteristics (age, gestational age, BMI, smoking status, gravida, parity), FF as reported (%), and levothyroxine sodium dosing were noted from the NIPT registry form. All parameters like ultrasonography, NIPT, TSH and FT4 results were recruited from the hospital's computer based data. Parameters were compared between the groups.

In the perinatology clinic, NIPT indications of the patients were determined as the following: advanced maternal age (≥35 years), combined trisomy 21 risk between 1/300 and 1/1,000 or sole biochemical risk more than 1/1,000 with normal fetal nuchal translucency (NT) in the first trimester screening, one fetal soft marker on ultrasonography for trisomy 21.

Patients' venous blood samples were collected in streck tubes and studied in the MiSeq NGS (Illumina, U.S.) platform and NextSeq (Illumina, U.S.). After plasma isolation and storing are achieved, the workflow was performed according to the recommendations of the producer (Clarigo™, CE-IVD Marked, Multiplicom, Belgium). Cloud based "Clarigo Reporter, the Initial Version (Multiplicom, Belgium)" software was used for data analysis. The bioinformatic analysis was based on the correlation of samples which are studied under the same conditions. Having FF 4% or over and at least 2 M reads per sample is required. The cases with a FF 4% or over and Z-score above 3.5 and the trisomy evidence is 0.5 or over are automatically called positive for the related trisomy and the cases with Z-score lower 3.5 and the trisomy evidence -2 or lower are called negative. Low FF results are obtained and manual studies are needed on those. NIPT procedure and associated data analysis set up used were as previously reported by Koc et al.[10] Euthyroid defines pregnant women with normal thyroid functions. First and second trimester-specific intervals were defined as; TSH: 0.005-3.65 and 0.01-3.63 mIU/L, FT4: 0.72-1.79 and 0.71-1.26 ng/dL in Turkish pregnant women.[11] TSH, free T4 levels were measured with chemiluminescent method by using an Immulite 2000 otoanalyzer (Immulite XPi, Siemens, Germany).

The local institutional review board approved this study (date: January 25, 2021, no: 2021/01-06). This study was performed consistent with the Declaration of Helsinki ethical principles. Shapiro-Wilk test was used for normality tests, box plot and histogram were used as graphical methods. Mann-Whitney U test for ordinal or continuous variables was performed. Variables were summarized using mean, standard deviation, median and percentiles. Spearman's correlation coefficient was used to assess the correlation between the variables. A p value of

p<0.05 was considered as statistically significant. Statistical analyses were conducted using the SPSS (SPSS Inc, Chicago IL).

RESULTS

Of 10,275 performed tests; duplicate data, pregnant women with multiple gestation, BMI≥25, pregnancy with assisted reproductive techniques, abnormal fetal karyotype and no report of FF and non-euthyroid patients were excluded. From 1,039 patients left, 51 of them were left using levothyroxine and 900 without when missing data were excluded. Randomized sampling was performed using SPSS in the control group. Finally, 51 pregnant women with levothyroxine sodium intake and 102 pregnant women without were analyzed. Characteristics of the pregnant women, FF and TSH are presented in Table 1.

No significant difference was shown in the maternal age, gestational age, BMI, gravida and parity between the groups (p=0.060, p=0.953, p=0.771, p=0.587, p=0.672 respectively). One percent (n=1) of the pregnant women in the control group and 2% (n=1) of the pregnant women in the medication group were smokers (p=1.000). In the control group, 100% and in the case group 82% (n=42) of the pregnant were euthyroid.

No significant difference was shown in FF% between the groups (7.6% vs 8.3% p=0.070). Additionally, no significant difference was shown in TSH and FT4 levels between the patients (p=0.180, p=0.920) (Table 1). The mean levothyroxine dose was 65.19±39.07 mcg (minimum-maximum: 25-175). No correlation was found between levothyroxine dose and FF (rho=-0.175, p=0.219). Also, no correlation was reported between TSH and the FF (rho=0.050, p=0.538) in pregnant women with levothyroxine intake.

DISCUSSION

NIPT depending on FF is a widely used aneuploidy screening test in pregnancy. Several factors influence the FF. In this research, it was suggested that levothyroxine use could affect FF%, however no significant difference was observed between the patients with and without levothyroxine use. Furthermore, no correlation was recognized between FF% and levothyroxine dose. This is the first report assessing levothyroxine sodium effect on FF percentage in NIPT.

FF is defined as the percentage of total maternal plasma cfDNA. Accordingly, maternal plasma cfDNA contains both maternal and fetal origins of cfDNA. However, what we call the fetal DNA is fundamentally placental.[12] The typical FF threshold for an adequate result is between 2% and 4%.[13] While higher FF means greater detection rates of aneuploid and euploid pregnancies, lower FF can be interpreted as decreased detection or no result. Besides the high cost, higher sequencing depths can be used to compensate for very low FFs.[14]

Studies have shown that gestational changes in patients with hypothyroidism result in disturbed placental development, with reduced proliferation and increased apoptosis of trophoblastic cells through

TABLE 1: Maternal characteristics, FF and TSH of the pregnant women with and without an intake of levothyroxine.

	Pregnant using levothyroxine sodium (n=51)		Pregnant not using levothyroxine sodium (n=102)		
	X±SD (minimum-maximum)	Median (25p-75p)	X±SD (minimum-maximum)	Median (25p-75p)	P*
Maternal age (years)	35,37±6,73 (16-47)	36 (33-40)	34,15±5,21 (20-43)	36 (31-38)	0,060
Gestational age (week)	14,8±2,89 (11-22)	14 (13-15)	14,28±2,04 (10-22)	14 (13-15)	0,953
BMI (kg/m^2)	22,25±1,39 (20-24)	22 (21-24)	22,17±1,44 (19-24)	22 (21-23,25)	0,771
Gravida	1,66±0,84 (1-4)	1 (1-2)	1,80±1,00 (1-4)	1 (1-3)	0,587
Parity	0,62±0,77 (0-2)	0 (0-1)	0,71±0,87 (0-3)	0 (0-1)	0,672
FF (%)	7,67±4,91 (2,9-29,7)	6,1 (5-9,1)	8,37±3,93 (3,5-22)	7,95 (5,27-10,42)	0,070
TSH (mIU/L)	2,14±1,10 (0,06-3,64)	2,41 (1,34-3,25)	1,95±0,84 (0,17-3,47)	1,91 (1,34-2,62)	0,180
FT4 (ng/dL)	0,87±0,20 (0,66-1,78)	0,83 (0,75-0,94)	0,86±0,47 (0,71-1,25)	0,84 (0,73-0,96)	0,920

*Mann-Whitney U test; FF: Fetal fraction; TSH: Thyroid stimulating hormone; SD: Standard deviation; BMI: Body mass index.

modifications in the endocrine, immune, and angiogenic profiles at the maternal-fetal interface.[9,15] While hyperthyroidism can alter placental morphogenesis and increase the proliferative activity, inflammatory environment of hypothyroid individuals affects the process of trophoblastic actions.[8,9] In the study assessing rats treated with L-thyroxine, the authors observed that an improved anti-inflammatory course may result in an increase in the FF.[9]

Documented maternal and fetal influences on FF could be grouped as positive and negative correlation and no effect. While increased gestational age, CRL, and fetal trisomy 21 are positively associated, negatively correlated factors can be listed as maternal weight, maternal BMI, maternal blood volume, multiple pregnancy, fetal mosaicism, fetal trisomy 18, trisomy 13, digynic triploid pregnancy and preexisting hypertension, ethnic origin.[16] Also, no relevance was observed between FF and maternal age, fetal sex, NT measurement, and preexisting diabetes mellitus.[16] There is still considerable ambiguity between the FF% ranges, that can lead to exploration trimester, BMI, ethnic, or any other factor-specific FF percentages.

Rare maternal medications have been studied. Low-molecular-weight heparin (LMWH) is the first drug reported to have an adverse effect on NIPT.[17] They reported an unusually high proportion of small DNA fragments while underlying mechanism is unknown. These findings are consistent with a recent case report observing a low FF while using LMWH and hypothesized reduction trophoblast apoptosis.[18] Nevertheless, others have shown acetyl salycillic acid or heparin did not affect FF.[19] The same study stated metformin might be correlated with lower FF.[19] Although metformin was shown to reduce the secretions leading to proliferation and metabolism, medication was not found directly associated with the FF. Another repeated failed NIPT in a woman with an autoimmune disease case demonstrated the patient taking acetyl salycillic acid, prednisolone, and LMWH.[20] Still, low FF in the patient could be due to the disease itself rather than medications. Intravenous immunoglobulin was also observed not to be associated with FF.[21]

This study's findings showed that even with a levothyroxine intake, TSH rates were not higher than those of the other group; nevertheless, they were mostly in the reference ranges. The lack of statistical significance of the study is presumably secondary to small sample size and other undocumented factors that could affect trophoblastic actions leading dissolve any expected effects. It is hypothesized that levothyroxine use could affect FF%; however no significant difference was observed between the patients with and without levothyroxine use. Furthermore, no correlation was recognized between FF% and levothyroxine dose. Thus, clinicians should not raise any concern about the timing of the test or any altered results in patients with levothyroxine sodium use. Yet considering limited data on the specific subject, our results will provide new insight for health professionals.

CONCLUSION

The knowledge of relation between FF and medications is largely based on very limited data. This is the first report of assessing levothyroxine sodium effect on FF percentage in NIPT. Levothyroxine sodium was not confirmed to be associated with any significant change in the FF.

Source of Finance

During this study, no financial or spiritual support was received neither from any pharmaceutical company that has a direct connection with the research subject, nor from a company that provides or produces medical instruments and materials which may negatively affect the evaluation process of this study.

Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Design and Consept: *Alperi İleri, Suna Yıldırım Karaca;* ***Data Collection:*** *Alper İleri, Suna Yıldırım Karaca, Hande İleri, Hakan Gölbaşı;* ***Analysis and Interpretation:*** *Alper İleri, Hande İleri;* ***Literature Review:*** *Hande İleri;* ***Writing Manuscript:*** *Alper İleri, Suna Yıldırım Karaca, Hande İleri, Hakan Gölbaşı, Alkım Gülşah Şahingöz Yıldırım, Yaşar Bekir Kutbay, Altuğ Koç, Mehmet Özeren;* ***Critical Review:*** *Altuğ Koç, Mehmet Özeren.*

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