The Affect of Different Immunoassay Systems and Preanalytical Variables on Second Trimester Aneuploidy Screening Test Results

İkinci Trimester Anaploidi Tarama Test Sonuçlarına, Farklı İmmünoassay Sistemlerin ve Preanalitik Değişkenlerin Etkisi

Özlem ÖZTÜRK,[a]
Mustafa ÖZTÜRK,[b]
Funda GÜCEL,[c]
Muhittin A. SERDAR,[d]
Taner ÖZGÜRTAŞ,[a]
Mustafa ULUBAY,[e]
Zafer AKSU,[a]
Serkan TAPAN[a]

Clinics of
[a]Biochemistry,
[e]Gynecology and Obstetrics,
Gülhane Training and Research Hospital,
[b]Clinic of Obstetrics and Gynecology,
Etimesgut Military Hospital,
[c]Clinic of Biochemistry,
Etlik Zübeyde Hanım Gynecology
Training and Research Hospital,
Ankara
[d]Department of Biochemistry,
Acıbadem University Faculty of Medicine,
İstanbul

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Yazışma Adresi/*Correspondence:*
Özlem ÖZTÜRK
Gülhane Training and Research Hospital,
Clinic of Biochemistry, Ankara,
TÜRKİYE/TURKEY
oozturk@gata.edu.tr

ABSTRACT Objective: Triple test is a screening test used in prenatal diagnosis of the disease that chromosomal defects such as Trisomy 18, 21 (Down syndrome) and neural tube defects (NTD). The objective of this study was to investigate the effects of sample storage conditions and different instruments on triple test individual risk results. **Material and Methods:** Serum samples were divided into four aliquots to measure alpha fetoprotein (AFP), beta human chorionic gonadotropin (β-hCG) and unconjugated Estriol (uE3). The one group of samples were measured on the same day, the other samples were then kept in the refrigerator (+4°C) and measured at Day 2 and at Day 7, in the deep freezer (-20°C) at Day 7 respectively. Each sample was individually measured in Beckman DXI 800 (Instrument 1) and Siemens IMMULITE 2000 (Instrument 2) Instruments, and MoM values and patient's individual risks were calculated for AFP, β-hCG and uE3 tests using PRA (Prenatal Risk Calculation, Benetech Software, Toronto) and PRISCA 4.0 (Prenatal Risk Calculation, TYPOLOG Software/GmBH, Hamburg, Germany) programs respectively. **Results:** The difference between AFP MoM values was observed for the 1st and 2nd day of the 1st instrument ($p < 0.05$). In comparison of day 1st and 7th day of measurement results differences were observed for AFP and β-hCG at +4°C and AFP and uE3 MoM values at -20°C. ($p<0.05$). The difference between AFP MoM values was observed for the 1st and 2nd day of the 2nd instrument ($p<0.05$). When 1st and 7th day measurement results were compared, it was seen that there was a difference in uE3 and β-hCG MoM values in both the +4°C and -20°C pending samples ($p<0.05$). There was a significant difference ($p<0.05$) in MoM values of two Instruments, obtained from β-HCG, uE3 and AFP tests. Despite a significant difference in analytical variation, no significant differences were found between 2 instruments after evaluating patient's individual risks ($p=0.58$, $p=0.59$, $p=0.33$, $p=0.65$). **Conclusion:** Waiting time for samples is a preanalytical variable that influences the individual risk results from triple test. For Down Syndrome, it is considered that different analytical performances have no effects on individual risk for patients whose individual risk is not near the limit value.

Key Words: Chorionic gonadotropin, beta subunit, human; estriol 3-sulfate; afp protein, human; analytic sample preparation methods

ÖZET Amaç: Triple test; Trizomi 18, 21 (Down Sendromu) gibi kromozomal bozuklukların ve nöral tüp defekti (NTD) gibi hastalıkların prenatal tanısında kullanılan bir tarama testidir. Bu çalışmanın amacı örnek saklama koşullarının ve farklı cihazların triple test, bireysel risk sonuçlarına etkisini incelemektir. **Gereç Yöntemler:** Serum örnekleri alfa fetoprotein (AFP), beta human koryonik gonadotropin (β-hCG) ve ankonjuge estriol (uE3) testleri çalışılmak üzere 4 kısma ayrıldı. Alikotlanan örnekler sırası ile aynı gün (1. gün), buzdolabında (+4°C) bekletilerek 2. gün, buzdolabında (+4°C) bekletilerek 7. gün ve buzdolabının derin dondurucu bölümünde (-20°C) bekletilerek 7. gün çalışıldı. Her örnek Beckman DXI 800 (cihaz 1) ve Siemens IMMULITE 2000 (cihaz 2) cihazlarında ayrı ayrı çalışıldı ve sırası ile PRA (Prenatal Risk Hesaplama, Benetech Software, Toronto) ve PRISCA 4.0 (Prenatal Risk Hesaplama,TYPOLOG Software/GmBH, Hamburg, Germany) bilgisayar programları kullanılarak AFP, β-hCG ve uE3 testleri için MoM değerleri ve hastaların bireysel riskleri hesaplandı. **Bulgular:** Cihaz 1 için 1. ve 2. gün ölçüm sonuçları karşılaştırıldında AFP MoM değerleri arasında fark olduğu görüldü ($p<0,05$). 1. gün 7. gün ölçüm sonuçları karşılaştırıldında ise +4°C'de bekleyen örneklerde AFP ve β-hCG, -20°C'de bekleyenelerde ise AFP ve uE3 MoM değerlerinde fark olduğu görüldü ($p<0,05$). Cihaz 2 için 1. ve 2. gün ölçüm sonuçları karşılaştırıldında AFP nin MoM değerleri arasında fark olduğu görüldü ($p<0,05$). 1. gün 7. gün ölçüm sonuçları karşılaştırıldığında ise hem +4°C hem de ve -20°C'de bekleyen örneklerde uE3 ve β-hCG MoM değerlerinde fark olduğu anlaşıldı ($p<0,05$). İki cihaz karşılaştırıldığında ise β- HCG, uE3 ve AFP testleri MoM değerleri arasında anlamlı bir farklılık ($p<0,05$) olduğu saptandı. Cihazlar arasındaki analitik varyasyona rağmen hastaların bireysel riskleri değerlendirdiğinde 2 cihaz arasında fark olmadığı tespit edildi ($p=0,58$, $p=0,59$, $p=0,33$, $p=0,65$). **Sonuç:** Örnek bekleme süresi Triple test bireysel risk sonuçlarını etkileyen preanalitik bir değişkendir. Down Sendromu bireysel risk sınır değerine yakın olmayan hastalar için farklı analitik performansların bireysel risk üzerine etkisi olmadığı değerlendirildi.

Anahtar Kelimeler: Koryonik gonadotropin, beta altünitesi, insan; östriol 3-sülfat; afp protein, insan; analitik örnek hazırlama yöntemleri

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Triple test is a screening test that is used for prenatal diagnosis of chromosomal abnormalities, e.g., trisomy 18, 21 (Down Syndrome), and diseases, e.g., neural tube defect (NTD), and currently performed on a routine basis by many laboratories.

Triple test, a non-invasive test, indicates the individual risk for a pregnant woman. In this test, alpha fetoprotein (AFP), human chorionic gonadotropin (hCG) and unconjugated estriol (uE3) parameters are measured in the serum of pregnant woman in certain gestational weeks (weeks 16-18), and values of these parameters are standardized in multiples of median (MoM) based on gestational week using special computer programs. Parameters, such as ultrasonographic measurement results, age and weight and race of pregnant, if she smokes and has diabetes mellitus, are included in the same program, and individual risks are separately calculated for Trisomy 18, Trisomy 21 and NTD of pregnant women.

An AFP value higher than 2.5 MoM' is considered a high risk for NTD; 1/270 is considered a individual risk limit value for Down Syndrome and it is 1/100 for Trisomy 18.[1]

A sample collected from a pregnant woman for triple test is sometimes not possible to deliver to the laboratory on the same day. There is no agreement on how to maintain stabilization of samples during such period and how sample delay time of analysis, a pre-analytical variable, affects the results of individual risk results.

This test is also measured at different laboratories using different analytical methods, and individual risks are measured by different computer programs. Inconsistence may occur in individual risk results delivered by different laboratories for pregnant woman because biological variations are added to these analytical variations. Such inconsistence results in anxiety in pregnant woman, and even may lead pregnant women to have invasive procedures (e.g., amniocentesis, cordocentesis).

Our objective in this study was to investigate the effects of different analytical performances, delay time of analysis, a pre-analytical variable, and conditions on triple test.

MATERIAL AND METHODS:

This study included 12 singleton pregnancy who had between 16+1 and 18+5 gestational weeks and mean age was 27,4 (21-32) years old. They presented at Etlik Zübeyde Hanım Gynecological Diseases Training and Research Hospital, Medical Biochemistry Department to have triple test. All of the pregnants were informed about study. Peripheral venous blood (5 ml) was obtained from each pregnant woman and drawn into two blood collection plastic tubes (red closure, BD USA) containing. Blood samples were taken by same nurse. Two blood samples were taken for each patient. All the serum samples were separated by centrifugation for 10 min at 1,000 × g. One of the serum samples stayed in the Etlik Zübeyde Hanım Gynecological Diseases Training and Research Hospital Medical Biochemistry Department. The other tube was send to Gülhane Training and Research Hospital Medical Biochemistry Department (10 minutes walk distance from Etlik Zübeyde Hanım Gynecological Diseases Training and Research Hospital). After then, serums were divided into four parts. Samples in the first part were studied on the same day (Day 1). Samples in the second part were kept in the refrigerator (+4 °C) and studied after 24 hours (Day 2). Samples in the third part were kept in the refrigerator (+4 °C) and measured after 1 week (Day 7). Samples in the fourth part were kept in the deep freezer (-20 °C) and measured after 1 week (Day 7). Samples were studied only one time. In each sample, AFP, β-hCG and uE3 tests were separately performed by chemiluminescence enzyme immunoassay test procedure using Beckman DXI 800 (Instrument 1) and Siemens IMMULITE 2000 Instruments (Instrument 2).

Laboratories where we conducted our study process quality control materials at 2 levels on daily basis. Instrument 1 uses quality control materials from Randox (United Kingdom). Level 1 internal quality control CV values for β-HCG, uE3 and AFP tests are 7.7%, 9% and 6.4% respectively. Level 2

internal quality control CV values are 4.8% 6.2% and 4.7% respectively. Instrument 2 uses quality control materials from Bio-rad (USA). Level 1 internal quality control CV values for β-HCG, uE3 and AFP tests are 9.0%, 8.6% and 3.4% respectively, and Level 2 internal quality control CV values are 7.5%, 8.0% and 3.6%.

Performance criteria of the triple test; Instrument 1 β-HCG, uE3 and AFP tests limit of detection 0,5 mIU/mL, 0.1 ng/mL 0.5 ng/mL, intra assay CV values are between 1.8-3.6%, 1.7-6.1%, 1.6-4.8%, total cv values are between 3.1-6.6%, 3.4-10.7%, 2.6-4.8%, recovery is between 101-111%, 88-91%, 103-105% range of linearity 1350 mIU/mL, 6.9 ng/mL, 3000 ng/mL respectively.

Performance criteria of the triple test; Instrument 2 β-HCG, uE3 and AFP tests limit of detection 0.4 mIU/mL, 0.1 ng/mL 0.2 IU/mL, intra assay CV values are between 2.5-6.6%, 3.7-7.9%, 2.1-6.3%, total cv values are between 4.5-7.5%, 5.7-12.3%, 4.5-12%, recovery are between 102-112%, 91-113%, 92-103% range of linearity 3000 mIU/mL, 30 ng/mL, 300 IU/mL respectively.

The laboratory that uses Instrument 2 is a member of external quality control program (RIQAS), and bias values for β-HCG, uE3 and AFP tests are 6.8, 7.3 and 8.4 respectively. The laboratory that uses Instrument 2 is not member of external quality control program.

PRA (Prenatal Risk Calculation, Benetech Software, Toronto) and PRISCA 4.0 (Prenatal Risk Calculation, TYPOLOG Software/GmBH, Hamburg, Germany) were used respectively to calculate individual risks. Determination of gestational week was based on ultrasonographic biparietal diameter (BPD) measurements performed on the date of collection of serum sample. AFP, β-hCG and uE3 measurement values were divided by hormone median value of normal gestational population with the same gestational week to calculate the MoM values. These MoM values, age of the mother and other data (weight, smoking habit, presence of diabetes mellitus, twin pregnancy) were statistically analyzed to calculate individual risks for pregnant women.

Based on these test results, individual risk threshold value (For both of the prenatal screening test programe) was considered 1/250 for Down Syndrome and 1/300 for Trisomy 18.

Approval of Gülhane Training and Research Hospital Ethics Committee was obtained for the study.

SPSS 22.0 and Medcalc packet programs was used for statistical analyses. Wilcoxon test was used to investigate differences between MoM values of biochemical tests and differences in individual risks, and those with $p<0.05$ were considered significant. Bland-Altman graph was used to comparison of two instruments.

RESULTS

The MoM variances of β-HCG, uE3 and AFP tests for both of the Instruments were shown (Figures 1-3). Plot of β-HCG, uE3 and AFP tests MoM differences (Day1, Day 2, Day 7 (+4) and Day 7 (-20)) between Instrument 1 and Instrument 2 were shown (Figures 4-6).

Statistical analyses revealed that generally significant differences ($p<0.05$) were present in β-HCG, uE3 and AFP tests between 2 Instruments, however no significant differences were found between 2 Instruments when evaluated patient's individual risks for Down syndrome ($p=0.58$, $p=0.59$, $p=0.33$, $p=0.65$) (Table 1).

According to results of β-hCG and uE3 measurements performed by Instrument 1, there were no significant differences in MoM values of Day 1 and Day 2 ($p=0.95$, $p=0.09$ respectively). However, a significant difference was observed in the result of AFP measurement ($p<0.05$). Measurement results from samples that were kept for 7 days were compared to results of Day 1, and differences ($p<0.05$) were present in β-hCG and AFP MoM values of samples that were kept at +4°C but no differences were present in uE3 MoM values ($p=0.38$). There were no differences in β-hCG MoM values of samples that were kept at -20°C ($p=0.30$) but significant differences were found in AFP and uE3 MoM values ($p<0.05$).

TABLE 1: Variances in individual risk ratios.

		HCG (kU/L) median MoM (25%-75%)	p	uE3 (ng/ml) median MoM (25%-75%)	p	AFP (ng/ml) median MoM (25%-75%)	p	Down syndrome mean risk ratio (min-max)	p
Day 1st	Instrument 1	0.92 (0.66-1.43)	p<0.05	0.96 (0.86-1.13)	p<0.05	0.75 (0.59-0.93)	p=0.20	1:2430 <1:1000-1:844	p=0.58
	Instrument 2	0.85 (0.65-1.30)		1.15 (1.08-1.33)		0.77 (0.59-0.96)		1:2654 1:9520-1:693	
Day 2nd	Instrument 1	0.92 (0.71-1.41)	p<0.05	0.91 (0.82-1.03)	p<0.05	0.98* (0.78-1.19)	p<0.05	1:3016* <1:1000-1:790	p=0.59
	Instrument 2	0.77 (0.64-1.11)		1.28 (1.05-1.45)		0.81* (0.65-1.02)		1:2953* <1:1000-1:734	
Day 7th (+4)	Instrument 1	1.03* (0.77-1.79)	p<0.05	0.99 (0.86-1.14)	p=0.61	1.16* (0.89-1.43)	p<0.05	1:3043* <1:1000-1:535	p=0.33
	Instrument 2	0.70* (0.48-0.91)		0.93* (0.86-1.16)		0.74* (0.66-1.02)		1:4288* <1:1000-1:1845	
Day 7th (-20)	Instrument 1	0.77 (0.59-1.38)	p<0.05	0.83* (0.73-0.97)	p<0.05	0.96* (0.70-1.13)	p<0.05	1:3203* <1:1000-1:730	p=0.65
	Instrument 2	0.62* (0.47-0.82)		0.95* (0.81-1.06)		0.76 (0.58-0.94)		1:3951* <1:1000-1:1697	

Instrument 1: BeckmanDXI 800; Instrument 2: Siemens IMMULITE 2000.

* Significant changes between days of instruments (instrument 1; 1st, 2nd, 7th day +4°C and 7th -20°C, instrument 2; 1st, 2nd, 7th day +4°C and 7th -20°C) are shown.

hCG: Human chorionic gonadotropin; uE3: Uncongugated estriol; AFP: Alpha fetoprotein; MoM: Multiples of median.

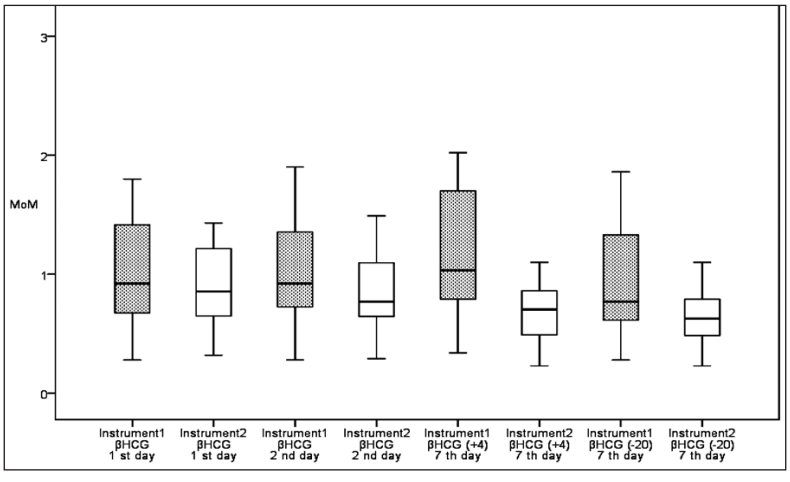

FIGURE 1: The MoM variances of β-HCG test for both of the Instruments (1st, 2nd, 7th day +4°C and 7th -20°C).

MoM: Multiples of median; β-hCG: Beta human chorionic gonadotropin.

According to results of β-hCG and uE3 measurements performed by Instrument 2, there were no significant differences in MoM values of Day 1 and Day 2 (p=0.32 p=0.27 respectively). However, a significant difference was present in the result of AFP measurement (p<0.05). Measurement results from samples that were kept for 7 days were compared to results of Day 1, and there were differences in β-hCG and uE3 MoM values of samples that were kept at +4°C and -20°C (p<0.05), but no differences were found in AFP MoM values (p=0.07, p=0.16 respectively).

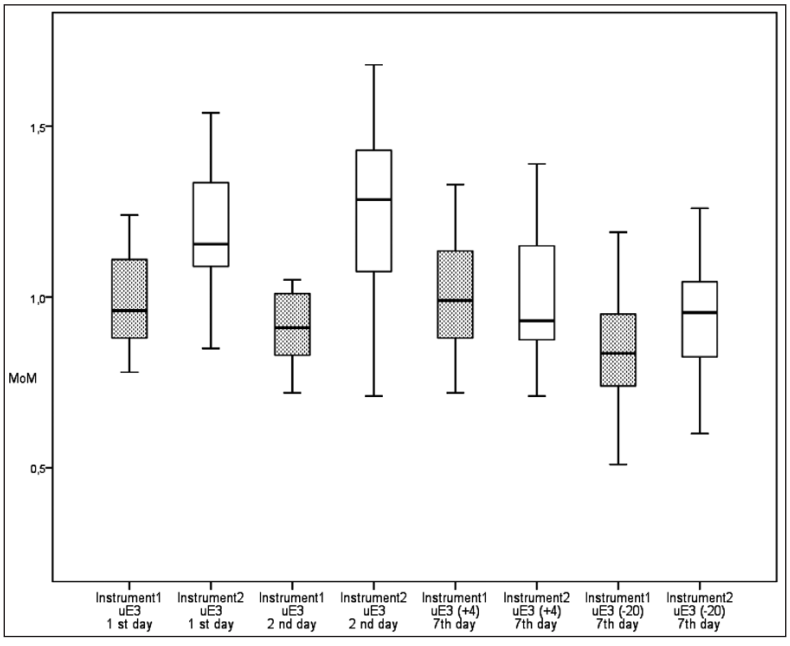

FIGURE 2: The MoM variances of uE3 test for both of the Instruments (1st, 2nd, 7th day +4°C and 7th -20°C).
MoM: Multiples of median; uE3: Uncongugated estriol.

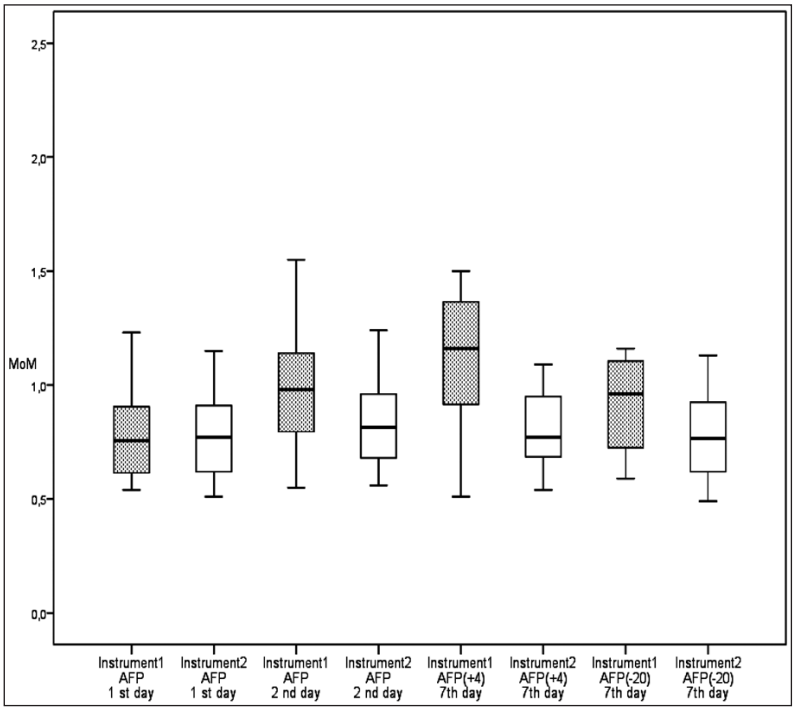

FIGURE 3: The MoM variances of AFP test for both of the Instruments (1st, 2nd, 7th day +4°C and 7th -20°C).
MoM: Multiples of median; AFP: Alpha fetoprotein.

DISCUSSION

Triple test, a prenatal screen test, is widely used to identify fetal chromosomal abnormalities and fetuses at risk for neural tube defects. As with any biochemical test, accuracy of triple test results depends on timely and properly collection of samples, proper transfer to laboratory, and regular maintenance and calibration of Instruments used for testing.

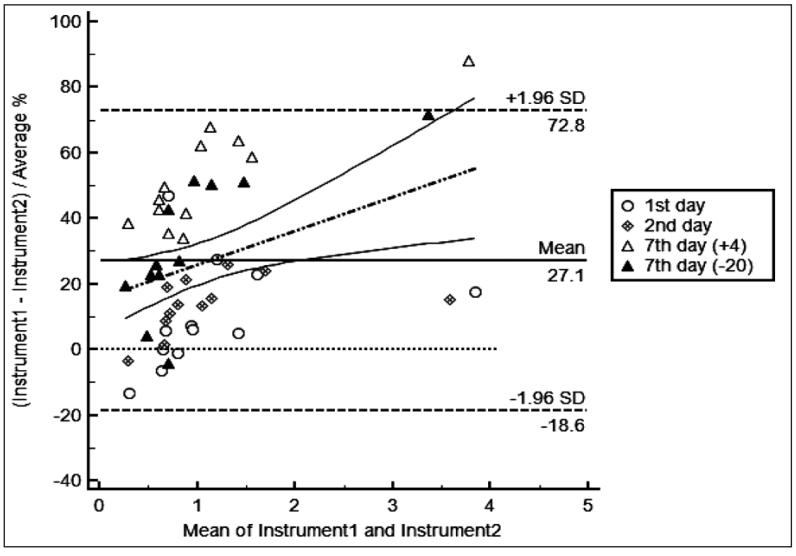

FIGURE 4: Bland-Altman plot for β-HCG test. The bias between the Instrument 1 and Instrument 2 for all samples.
β-hCG: Beta human chorionic gonadotropin.

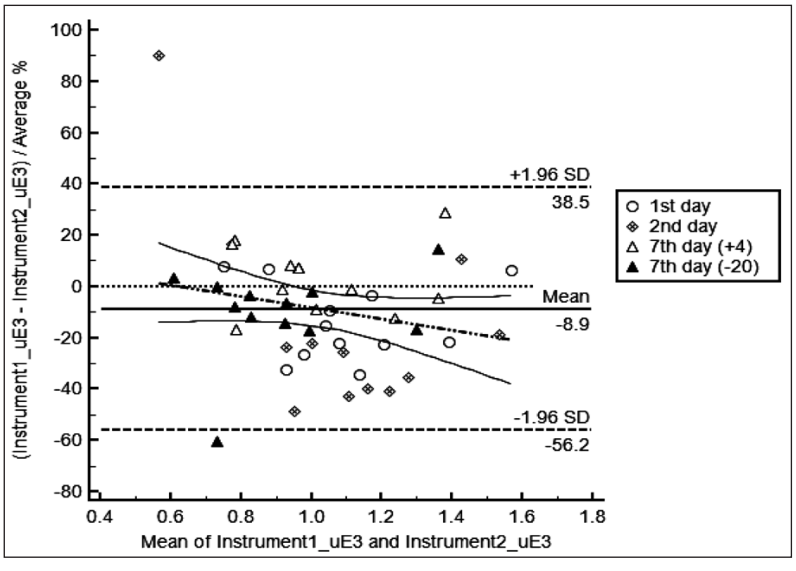

FIGURE 5: Bland-Altman plot for uE3 test. The bias between the Instrument 1 and Instrument 2 for all samples.
uE3: Uncongugated estriol.

Delivery time of patient's samples to laboratories is a pre-analytical variable. There is no agreement on stabilization of serum samples for triple test. Kit prospectuses indicate that serum samples maintain their stabilization at 2-8°C for 3 days and at -20°C after 3 days whereas several laboratories (Mayo Medical Laboratories) report that they maintain their stability at 2-8°C for 7 days and at -20°C for 90 days. Based on a large scale of studies, some authors recommend not keeping the samples for more than 6 days with minimum delivery time to laboratories.[2]

Despite using different kits, β-hCG and uE3 tests remained stable for the first 24 hours in our study. On contrary to some studies, AFP test was unable to maintain its stability.[3] For β-hCG test, it is known that stabilization problems occur in samples kept at +4°C and particularly at -20°C with high concentrations.[2,4]

In our study, stabilization problems were observed in both uE3 and β-hCG tests for samples kept at +4°C and -20°C for 1 week. In literature, it has been emphasized that the amount of uE3 may

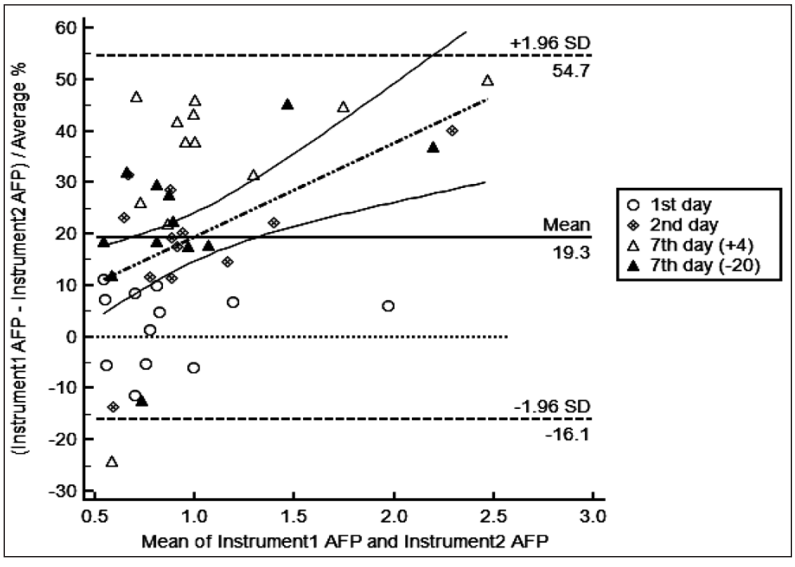

FIGURE 6: Bland-Altman plot for AFP test. The bias between the Instrument 1 and Instrument 2 for all samples.
AFP: Alpha fetoprotein.

change depending on the hydrolysis of conjugate estriol.[5] It has been shown that β-hCG may be elevated by 10% in serum due to seconder dissociation and degradation of intact hCG.[2,4,6] Stabilization problem for these tests appeared not to affect patient's individual risks for Trisomy 18. However, individual risk results for Down syndrome varied significantly ($p<0.05$) between days. The significant difference between Day 1 and Day 2 was attributed to variance in AFP test results. As a result, keeping samples in the refrigerator at +4°C even for 1 day appeared to have effects on individual risk results for Down syndrome in our study.

To what extent does it influence the individual risk for a pregnant woman to perform prenatal tests by different laboratories using different analytical systems? There is no agreement on the answer to this question which is constantly asked by clinicians. According to several authors, the effect of analytical variations of β-HCG, AFP and E3 tests on individual risk appears minor; however, some studies report that individual risks are sensitive to minor differences in analytical performances.[7,8] It has been shown that there may be a difference between β-hCG measurements in different analytical systems, especially as different standards are used or the measurement of nicked hCG and other hCG variants in serum affects the measurement.[9]

In addition, Serdar et al. reported that analytical variations have a great impact on second trimester risk estimation procedures and some articles have reported borderline cases as being problematic.[8,10,11] A survey report of external quality control samples for 9 clinical laboratories in Korea showed variable results for the borderline cases that necessitated standardization.[12]

Indeed, it is suggested that the variation in individual risks would become greater when different software programs used to calculate risks are included in different analytical performances.[8]

In our study, although % CV values of tests performed by two laboratories were within acceptable limits, there were differences in 2 analytical systems for β-hCG and uE3 tests at Day 1 ($p<0.05$) whereas no differences were present for AFP test ($p<0.20$).

For samples that were studied at Day 2 and samples that were kept at -20°C for 7 days and studied, differences were found in analytical systems for each of 3 tests ($p<0.05$). For samples that were kept at +4°C for 7 days, no differences were present in 2 analytical systems for only uE3 test ($p<0.61$).

However, analytical differences in two systems did not affect the individual risk results calculated for Down syndrome and trisomy 18. Sev-

eral studies reported that individual risk for a pregnant woman carrying a fetus with Down, syndrome could vary from 1/502 to 1/80 depending on analytical differences in laboratories, and argued that it would significantly affect the clinical diagnosis.[13] In our study, variances in individual risk ratios (Table 1) were not statistically significant and did not affect the clinical diagnosis because the patient population consisted of individuals who were at risk lower than individual risk limit values of 1/250 for Down syndrome and 1/100 for Trisomy 18.

Studies performed so far either assessed stabilization of samples or compared the methods.[3,4,14] This study both assessed stabilization of samples and evaluated variations due to using different Instruments.

Limitations of our study: we were unable to compare individual risks for NTD because two laboratories used different programs to calculate the risks. Also, the selected study population did not include patients whose individual risk was positive or near the limit value for Down syndrome, therefore variations in individual risks for patients in this population were unable to examine.

As a result, our study can be considered pre-study providing information on pre-analytical and analytical assessment of triple test. It would be appropriate to perform similar studies that use more samples and engage more than two laboratories. We also consider that presence of patients whose individual risk ratio is positive or near clinical limit value in the study group would further present the importance of pre-analytical and analytical variables.

"The authors declare that there are no conflicts of interest and no financial support".

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