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Fine-tuning ofroutine combined first-trimester screening: The ratio of serum-free-beta-human chorionic gonadotropin (fβ-hCG) to pregnancy-associated plasma protein-A (PAPP-A) could improve performance of Down syndrome screening program, a retrospective cohort study in Iran

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Abstract.
OBJECTIVES: To evaluate the performance of the current national screening policy for Down syndrome (DS) in Iran and suggest a more efficient protocol with a wealth of a large series of first-trimester screening (FTS) data obtained from Nilou medical laboratory. To fulfill this aim, detection rate (DR), positive screening rate (PSR), false negative rate (FNR) and odds of being affected given a positive results (OAPR) were calculated at different cutoff risk. In the latest update of DS screening program in Iran, there is no place for intermediate group to be further investigated. Next, we proposed a novel parameter namely the ratio of fβ-hCG multiple of the median (MoM) value to PAPP-A MoM value to delicately categorize FTS results in a way that reduce FNR without imposing unnecessary anxious and extra money on most families.
METHODS: The present investigation was conducted retrospectively on 197,210 pregnancies undergoing FTS for aneuploidies in Nilou medical laboratory, Tehran, Iran, from March 2015 to February 2016.
RESULTS: Intermediate risk group is important as 23 out of 45 FN fellin the range 1:250 to 1:1100. By applying the proposed index, the ratio of fβ-hCG MoM to PAPP-A MoM and subsequent decision about NIPT, 8 out of 23 FN cases in intermediate group could be detected.
CONCLUSION: Compared with the current policy, our novel proposed approach had better performance and could be applied by the Iran National Health Service to improve the screening program guideline.

Keywords: Down syndrome, fβ-hCG, pregnancy-associated plasma protein-A, PAPP-A

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1. Introduction

Down syndrome (DS) is in the spotlight of any prenatal screening program. To identify an affected fetus at first trimester the results of maternal free β-human chorionic gonadotropin (fβ-hCG) and pregnancy associated plasma protein A (PAPP-A) as biochemical markers combine with nuchal translucency (NT) ultrasound scan at 10 to 13 weeks of gestation to produce the final risk [1]. National screening policy for DS in Iran as depicted in Fig. 1 has set the cutoff risk 1:250 to categorize test result into high or low risk groups. Accordingly, intermediate risk group with risk value 1:251 to 1:1100 in previous protocol has no place for further workup in the current national practice. Of note, screening program is not a diagnostic test and despite the strict rules applied in the laboratory testing, the inevitable errors might arise in pre-, intra- and post-analytical steps which keep the screening result assurance near but not equal to 100% [3]. This raises concerns about risk figures around the cut off value reasoning that all combined effects of acceptable errors could turn the final risk of, say, 1:245 to 1:255 or vise versa. This is especially worrisome for a result categorized as screen negative. Furthermore, the program should not candidate high number of pregnancies as false positive which means imposing extra cost and anxious. Therefore, striking the balance, here determining cutoff risk and introducing policies for categorizing pregnancies as true as possible is of utmost importance particularly in a nationwide scale program.

In this study our large sample of 197,210 FTS data from pregnancies referred to Nilou medical lab opened up an opportunity to evaluate the efficiency of antenatal screening for DS in Iran and also draw out a novel index, namely the ratio of fβ-hCG multiple of the median (MoM) value to PAPP-A MoM value and related cutoff risk to delicately categorize FTS results in a way that reduce false negative rate (FNR) without imposing unnecessary anxious and extra money on most families. The proposed approach could be applied by the Iran National Health Service to improve the screening program guideline.

2. Methods

The present investigation was conducted retrospectively on 197,210 pregnancies undergoing FTS for aneuploidies in Nilou medical laboratory, Tehran, Iran, from March 2015 to February 2016. Nilou medical laboratory is a large private laboratory with $\sim$ 200,000 referrals in a year from all over Iran for prenatal screening. There are approximately, 50,00 births per annum In Iran (https://www.sabteahval.ir/en). Thus, significant portions ($\sim$ 13.3%) of prenatalscreened results in Iran are administrated in our laboratory, allowing inclusive statistical analysis to be driven. Serum PAPP-A and fβ-hCG was measured by Electrochemiluminescence Immunoassay (Elecsys) by Cobas 6000 (Roche Diagnostics, Basel, Switzerland) with analytical sensitivities of fβ-hCG and PAPP-A 0.2 ng/mL and 5 mU/L, respectively. Individual fβ-hCG and PAPP-A measurements were transformed to MoM values using latest obtained medians of 8000 corresponding data in our laboratory. cFTS risk results were calculated using the LMS Alpha program, version 8 (http://www.lmsalpha.com/). The suggested protocol of Iran national health services and cutoff risk 1:250 was applied for trisomy 21 in cFTS to assign risk results as high, intermediate and low risk (in previous protocol intermediate risks received commentsfor further practice e.g. noninvasive prenatal test or NIPT). MoMs were corrected for maternal weight, diabetic status, smoking, and ethnicity. The outcome of all pregnancies was recorded by following-up until the delivery. cFTS program integrates the results of maternal serum PAPP-A and fβ-hCG and fetal nuchal translucency (NT) ultrasound scan obtained by experts certified by the Fetal Medicine Foundation during gestational weeks $11 + 0$ to $13 + 6$. For high risk pregnancies chorionic villus sampling (CVS) or amniocentesis for fetal karyotyping were offered. Cell free DNA testing was applied for those with intermediate risk (1:251_1:1100). FPR, FNR, DR, and OAPR were determined. False negative results were confirmed by karyotyping of infants. In the presence of positive screening results, amniocentesis confirmed the positive results.

3. Results

3.1. Demographic profile of FTS screened population

Data on cases of FTS performed on a total of 197,210 pregnancies referred to Nilou medical laboratory were collected. There were a total of 304 DS fetuses in the screened population (259 confirmed true positive (TP) and 45 confirmed FN fetuses). An individual risk of DS was calculated for each screened pregnancy. The DS risk was considered as positive if the risk value is 1:250 or greater. A risk value of 1:250

Figure 1 Screening test strategy for trisomy 21 currently in use in Iran

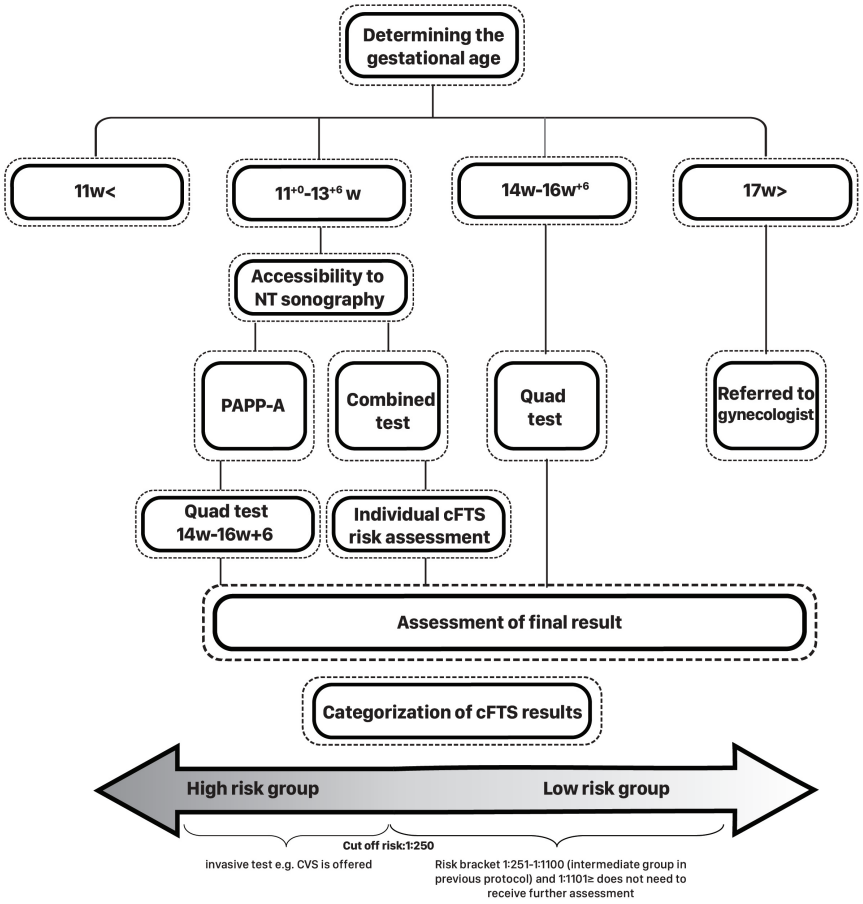

Fig. 1. Screening test strategy for trisomy 21 currently in use in Iran.

belongs to the risk to a 35-year-old woman at week 12 of gestation. Age distribution of studied pregnancies has been tabulated in Table 1. The mean maternal age was 28.7 years. More than 17% of participants were above 35 years old. Demographic profile of FTS screened population including distribution of fetal NT thickness in millimeters, maternal PAPP-A, free β-hCG expressed as MoM and status of other factors in pregnancies are shown in Table 2.

Demographic characteristics of 45 FN cases for DS including maternal age, gestational age, combined risk figures of trisomy 13, 18 and 2, NT in millimeters, β-hCG and PAPP-A expressed as MoM, the novel introduced index fβ-hCG MoM/PAPPA MoM and measurement of sonographic findings at first trimester are set out in Table 3.

To evaluate the performance of current FTS program of DS in Iran, cumulative (Table 4) and non-cumulative

Table 1
Maternal age distribution of 197210 combined first trimester screening data

Age distribution	Number of cases	Percentage
< 20	10882	5.6
21–25	26623	13.5
26–29	61906	31.3
30–34	63091	31.9
35–40	29384	14.9
> 40	5324	2.8
Total	197210	100.0

(Table 5) values for FPR, DR and OAPR were calculated for different risk cutoffs from 1:10 to $<$ 1:1100. At proposed Iranian health service cutoff risk (1:250), the cumulative FPR, DR and OAPR are 3.3, 85.2% and 1:25.1 respectively.

For better evaluation of DS screening program, the distribution of DS cases and screening parameters val-

Table 2
Maternal demographic characteristics, ultrasound measurements and biochemical results categorized by cutoff risks for Down syndrome ($n = 197{,}210$)

	High risk group ($\geqslant$ 1:250)	Intermediate risk group (1:251–1:1100)	Low risk group ($\leqslant$ 1:1101)	p value
$N = 197{,}210$	$N = 6362$	$N = 19298$	$N = 171550$	–
NT (MoM)	1.61 ± 0.67	1.18 ± 0.25	0.92 ± 0.20	0.000^{a}
Mean $\pm$ SD				0.000^{b}
PAPPA (MoM)	0.85 ± 0.34	0.89 ± 0.46	0.98 ± 0.42	0.057^{a}
Mean $\pm$ SD				0.046^{b}
fβ HCG (MoM)	1.40 ± 0.65	1.35 ± 0.72	1.35 ± 0.72	0.000^{a}
Mean $\pm$ SD				0.000^{b}
Diabetic	106/6362	180/19298	497/171550	0.023^{a}
	(1.67%)	(0.93%)	(0.29%)	0.000^{b}
Smoker	35/6362	108/19298	1952/171550	0.957^{a}
	(0.56%)	(0.56%)	(1.14%)	0.000^{b}
Twin pregnancies	707/6362	467/19298	4258/171550	0.000^{a}
	(11.11%)	(2.42%)	(2.48%)	0.000^{b}
IVF	71/6362	503/19298	2164/171550	0.016^{a}
	(1.11%)	(2.61%)	(1.26%)	0.053^{b}

NT, nuchal translucency; MoM, multiples of the median; PAPP-A, pregnancy-associated plasma protein-A; fβ-hCG, free-beta-human chorionic gonadotropin; IVF, in-vitro fertilization.; ^{a}: p value obtained through comparison between low risk and intermediate risk groups; ^{b}: p value obtained through comparison between low risk and high risk groups.

ues including FPR, DR and OAPRs were calculated and categorized in specific cutoff risk intervals $>$ 1:50, 1:51–1:250, 1:251–1:1100 and $<$ 1:1100 depicted in Table 5. The significant portion of DS cases (59.9%) were born from pregnancies with FTS risk $>$ 1:50 (OAPR = 1:9.7). At the risk interval 1:51–1:250, although DR can be 25% increased, invasive test should be offered to 61 screen positive mothers in order to detect one DS fetus.

In the intermediate risk group (risk 1:250_1:1100), 505 mothers should be offered further evaluation (NIPT or even invasive tests) to detect only one case of DS. On this wise, in DS screening program, at cutoff risk of 1:250 and greater, screening parameters values are not evenly distributed and highly profitable.

To optimize OAPR values and improve DR, a novel parameter namely the ratio of fβ-hCG MoM to PAPP-A MoM was applied for all cFTS results. Majority of DS cases had the ratio greater than 3. However, Cutoffs of 2.5, 3 and 3.5 were applied and the corresponding DR and OAPR values were calculated (data is presented for cutoff 3). Cutoff risk 3 showed the best performance (Table 6). Considering the ratio of fβ-hCG MoM/PAPP-A MoM, OAPR was reduced to 1:51.6 for the interval 1:50_1:250 and to 1:234.5 in 1:251_1:1100 intermediate group.

To check the competency of the NT thickness in 95th and 99th percentiles expressed as both millimeters and MoM in differentiating 45 screen negative cases, they were categorized according to the corresponding CRL range (Table 7). Only one pregnancy with NT = 2.45 mm in 99$^{\text{th}}$ percentile (CRL: 74.1_84.0) could be spotted as screen positive.

4. Discussion

This study provides the first comprehensive assessment of screening program for DS in Iran with a wealth of 197,210 FTS results inquired in Nilou medical laboratory database from March 2015 to February 2016. To evaluate the efficacy of DS screening protocol, DR, FPR and OAPR were calculated. In the latest update of DS screening program in Iran, there is no place for intermediate group to be further investigated or at least received public fund services. If the latest protocol had been employed, 23 DS with intermediate risk might not be diagnosed. Although, the reason is simply to avoid imposing unnecessary work-up and anxiety on most families with intermediate risk result (OAPR: 1:505.6), from the view point of laboratory it is worrisome.

By a wealth of roughly one million prenatal screening tests carried out in our laboratory in Iran, here we propose a novel index, the ratio of fβ-hCG MoM to PAPP-A MoM to better categorize FTS results particularly for those fall in the intermediate group. This ratio was 3 and greater for majority of DS pregnancies, hence the cutoff point 3 was chosen for assessment. Table 8 tabulated comparison of three conditions by considering intermediate group and suggested novel index. Cuckle has previously discussed the advantage of using ratio of particular parameters in certain conditions

Table 3
Demographic characteristics of the 45 false-negative cases in details of women referred to Nilou medical lab for first trimester prenatal screening

	Maternal age	GA	Combined risk figure T21	Combined risk figure T18	Combined risk figure T13	NT	MoM fβ-hCG	MoMP APP-A	fβ-hCG moM/PAPPA moM	Sonographic findings	Results[a]
1	33	11 + 5	443	< 30000	< 30000	0.96	2.63	0.27	9.74	Normal	DS
2	30	12 + 4	3329	< 30000	< 30000	1.10	2.48	0.67	3.70	Normal	Mosaeism DS
3	35	12 + 3	788	< 30000	< 30000	1.40	1.24	0.54	2.30	Normal	DS
4	21	12 + 6	351	< 30000	< 30000	2.40	1.46	0.76	1.92	Normal	DS
5	32	11 + 3	265	< 30000	< 30000	1.60	1.13	0.56	2.02	Normal	DS
6	32	11 + 2	506	< 30000	< 30000	1.60	1.62	0.72	2.25	NB = present	DS
7	23	12 + 5	2136	< 30000	< 30000	1.38	2.85	0.42	6.79	Normal	DS
8	35	12 + 1	2136	< 30000	< 30000	1.30	1.32	0.54	2.44	Normal	DS
9	37	12 + 3	355	< 30000	< 30000	1.61	4.06	0.79	5.14	Normal	DS
10	31	12 + 3	839	< 30000	< 30000	2.00	2.89	1.19	2.43	Normal	DS
11	28	12 + 4	3992	< 30000	< 30000	1.10	2.45	0.93	2.63	NB = present	DS
12	33	12 + 4	2592	< 30000	< 30000	0.90	2.25	0.68	3.31	Normal	DS
13	31	13 + 3	480	< 30000	< 30000	1.90	2.76	0.57	4.84	Normal	DS
14	31	13 + 1	265	< 30000	< 30000	1.85	7.48	0.86	8.70	Normal	DS
15	30	12 + 5	1671	< 30000	< 30000	1.20	5.11	1.08	4.73	Normal	DS
16	30	12 + 3	806	< 30000	< 30000	1.20	3.45	0.67	5.15	NB = present	DS
17	26.9	11 + 3	1154	< 30000	< 30000	1.26	0.61	0.26	2.35	NB = present	DS
18	29.4	12 + 2	3631	< 30000	< 30000	2.10	1.23	0.37	3.32	NB = present	47XY + 21
19	37	13 + 5	354	< 30000	< 30000	2.20	1.62	0.44	3.68	NB = present	DS
20	38.4	13 + 0	3112	< 30000	< 30000	2.10	0.51	1.40	0.36	Normal	DS
21	37.9	13 + 5	525	< 30000	< 30000	1.80	2.44	1.06	2.30	NB = present	DS
22	40.9	13 + 3	631	< 30000	< 30000	1.60	0.82	1.10	0.75	Normal	47XY + 21
23	31.5	13 + 3	2918	< 30000	< 30000	1.60	1.48	0.77	1.92	Normal	DS
24	28.2	13 + 5	24811	< 30000	< 30000	1.50	0.68	0.95	0.72	Normal	DS
25	19.4	12 + 2	12991	< 30000	< 30000	1.10	1.86	0.86	2.16	Normal	DS
26	31.2	12 + 2	1212	< 30000	< 30000	1.71	1.50	0.58	2.59	NB = present	DS
27	23.9	12 + 2	735	< 30000	< 30000	1.56	1.00	0.60	1.67	NB = present	DS
28	40	12 + 0	734	11625	< 30000	1.80	0.78	1.17	0.67	Normal	47XY + 21
29	25.4	12 + 5	300	< 30000	< 30000	1.33	1.64	0.98	1.67	NB = present	DS
30	33.5	13 + 5	4579	< 30000	< 30000	1.40	1.19	0.68	1.75	Normal	DS
31	29.1	13 + 0	585	< 30000	< 30000	2.20	1.58	0.38	4.16	NB = present	DS
32	20.5	12 + 5	282	< 30000	< 30000	1.63	2.60	0.87	2.99	Normal	DS
33	21.6	12 + 5	704	< 30000	< 30000	2.45	1.07	0.45	2.38	NB = present	DS
34	39.6	12 + 4	2204	< 30000	< 30000	1.43	1.37	1.40	0.98	Normal	DS
35	38.3	13 + 0	1144	< 30000	< 30000	1.00	1.14	1.80	0.63	Normal	DS
36	39.4	12 + 6	386	< 30000	< 30000	1.50	1.37	1.02	1.34	NB = absent	DS
37	20.9	12 + 5	1669	< 30000	< 30000	0.99	2.10	0.59	3.56	Normal	DS
38	37.5	2 + 12	1820	< 30000	< 30000	1.36	1.30	0.78	1.67	Normal	DS
39	39.9	12 + 5	1497	< 30000	< 30000	1.59	0.88	1.13	0.78	Normal	DS
40	27.6	0 + 12	6124	< 30000	< 30000	1.00	1.67	0.51	3.27	Normal	DS
41	30.5	12 + 2	1169	< 30000	< 30000	1.80	1.34	0.85	1.58	NB = hypoplastic	47XX + 21
42	28.4	12 + 2	521	< 30000	< 30000	1.20	1.63	0.44	3.70	NB = present	DS
43	36.4	11 + 5	439	< 30000	< 30000	1.10	2.07	0.76	2.72	NB = present	47XX + 21
44	37.2	13 + 0	621/924	394/723	21225/ < 30000	1.9 & 1.7	0.88	0.62	1.42	NB = present	47XY + 21
45	34.3	12 + 0	2100	< 30000	< 30000	1.40	0.95	0.43	2.21	NB = present	47XY + 21

GA: gestational age; NT: nuchal translucency; MoM, multiples of the median; PAPP-A, pregnancy-associated plasma protein-A; fβ-hCG, free-beta-human chorionic gonadotropin; [a]Karyotypes for cytogenetically confirmed results are shown.

to differentiate affected pregnancies from unaffected ones in a more effective manner [6,7]. Kagan et al. showed that PAPP-A MoM and free β-hCG MoM in affected pregnancies were 0.5 and 2 respectively. Using these values, the ratio of fβ-hCG to PAPPA would be 4 [8]. Cowans et al. reported fβ-hCG MoM of 2 and PAPP-A MoM of 0.549 for 722 DS pregnancies respectively which yield the ratio of 3.6 [9]. In the current investigation, in risk range of 1:251_1:1100, 8 out of 23 DS fetus can be detected if this parameter is applied. DR would increased by 2.65% with negligible increase in FPR (FPR = 0.95%). the ratio of fβ-hCG MoM to PAPP-A MoM could capture at least 8 affected cases.

Table 4
Performance of current first trimester screening program in Iran for detection of trisomy 21 at different selected risk cut-offs (n = 197,210)

Cut-off risk	Cumulative number of cases	FPR (%)	No of TP	No of TN	No of DR (percent)	OAPR	Prevalance
> 1:10	552	0.28	118	186	38.8	1:4.7	
> 1:50	1771	0.89	182	122	59.9	1:9.7	
> 1:100	3379	1.70	211	93	69.4	1:16	
> 1:150	4138	2.09	230	74	75.6	1:18	
> 1:250[a]	6508	3.30	259	45	85.2	1:25.1	
> 1:300	7479	3.80	263	41	86.5	1:28.4	
> 1:350	8187	4.20	265	39	87.2	1:30.9	
> 1:400	8801	4.50	269	35	88.5	1:32.7	
> 1:450	9776	4.90	272	32	89.9	1:35.9	
> 1:500	10574	5.40	274	30	90.1	1:38.6	
> 1:1000	15969	8.10	281	23	92.4	1:56.8	
> 1:1100	18138	9.20	282	22	92.8	1:64.3	
< 1:1100	179072	–	21	0	100	–	
Sum	197210	–	304	0	100	–	1:649

FP: false positive, TP: true positive, TN: true negative, DR: detection rate, OAPR: odds of being affected given the positive results; [a] Risk cut off used to identify screen positive women according to the current suggested Iranian national health services.

Table 5
Diagnostic rate and other screening parameters at particular cut-off interval (OAPR: 61.5)

Cut-off risk intervals	No. of cases	FPR (%)	Total of Down syndrome cases	Increase in DR	OAPR
> 1:50	1771	0.89	182	59.9	01:09.7
1:50–1:250	4737	2.39	77	25.3	1:61.5
1:251–1:1100	11630	4.80	23	7.6	1:505.6
< 1:1100	179072	–	22	–	–
Sum	197210		304		

FPR: false positive rate, DR: detection rate, OAPR: odds of being affected given the positive results.

Table 6
Evaluated ratio of fβ-hCG MoM/PAPR-A MoM ratio3.0 at different selected risk cut-offs (n = 197,210)

Cut-off risk intervals	No. of cases	No o f cases with ratio = 3.0	FPR (%)	TP/Total of DS	DR total	OAPR
> 1:50	1771	–	–	–	182/304 (59.9)	–
1:50–1:250	4737	1341	0.68	26/73	208/304 (68.4)	01:51.6
1:250–1:1100	11630	1879	0.95	8/23	216/304 (71.05)	04:54.9
< 1:1100	179072	8693	4.40	7/22	227/304 (74.7)	21:41.8
Sum	197210	14758	7.50			

FPR: false positive rate; TP: true positive; DR: detection rate; OAPR: odds of being affected given the positive results.

Table 7
NT thickness of 45 screen negative cases for DS expressed as millimeters and MoM in different percentiles (50^{th}, 95^{th} and 99^{th}) according to the corresponding CRL range

CRL (mm)	GA	No of cases	Observed NT[a] (mm)	50^{th} or median NT[b] (mm)	95^{th} NT[b] (mm)	99^{th} NT[b] (mm)	95^{th} NT[b] (MoM)	99^{th} NT[b] (MoM)
45.0–54.0	11 W + 1D–12 W + 0D	8	0.9–1.8	1.31	2.1	2.7	1.71	2.32
54.1–64.0	12 W + 1D–12 W + 5D	23	0.9–2.45	1.5	2.3	2.8	1.67	2.09
64.1–74.0	12 W + 6D–13 W + 3D	9	1.0–2.2	1.7	2.6	3.0	1.65	2.00
74.1–84.0	13 W + 4D–14 W + 2D	5	1.4–2.1	1.8	2.8	3.2	1.64	1.92

CRL: crown-rump length, GA: Gestational age; DS: down syndrome, NT: nuchal translucency; [a] Data of 45 screen negative cases for DS expressed as minimum and maximum values observed in each category. [b] The reference value of NT thickness (50^{th}, 95th, and 99th percentiles) were extracted from 197210 FTS results belongs to the defined categorized CRL brackets.

According to the aforementioned findings, we suggest the following decision pathway which could improve the first trimester prenatal screening program for DS.

1. Pregnancies with cFTS risk result of higher than 1:50 be categorized as high risk group and offered a diagnostic tests e.g. CVS. Conventional karyotyping or array CGH could be used as

Table 8

Comparison of screening parameters (detection rate, false positive rate and odds of being affected given a positive results) for three conditions: 1. intermediate group is not included, 2. Intermediate group is included. 3. Intermediate group is included and suggestion of NIPT for those with with fβ-hCG MoM to PAPP-A MoM $\geqslant 3$

FTS (n)	DS prevalence in our study	1. Intermediate group not included			2. Intermediate included			3. Intermediate group is included and NIPT is suggested for those with with fβ-hCG MoM to PAPP-A MoM $\geqslant 3$		
		DR	FPR	OAPR	DR	FPR	OAPR	DR	FPR	OAPR
197210	1:649[a]	85.2%	3.3%	1:25	92.8%	9.2%	1:64.3	87.8	4.2	1:31.4

DR: detection rate, FPR: false positive rate, OAPR: odds of being affected given the positive results. [a] DS prevalence is 1:700 in the United States [4]. Two contributing factors to this slight increase are: 1. High proportions of mothers above 35 year old (17.7%) hence inescapable higher prior risk and 2. Recheck of screen positive results sent by other laboratories. Along with the current alarming trends to later childbearing and association with pregnancy complications [5], we highly recommend the Iran health services to make plans for good management of pregnancies by providing public education, revising insurance coverage and assessing socio-economic aspects of this tendency.

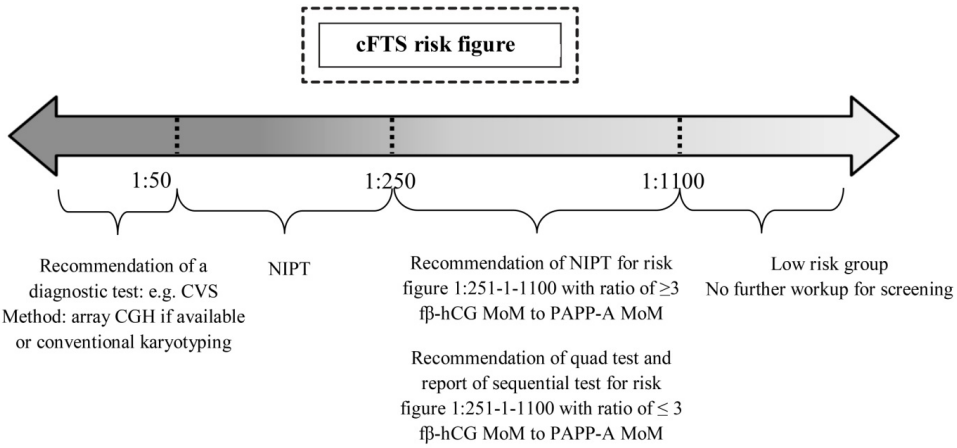

Fig. 2. Our suggested protocol for Down syndrome screening in Iran. A cutoff risk $\geqslant$ 1:50 for invasive testing and recommendation of non-invasive test as NIPT for risk figure between 1:51–1:250 and also 1:251–1:1100 with the ratio of fβ-hCG MoM to PAPP-A MoM $= 3$ could improve the efficacy of DS screening program in Iran. We highly suggest Iran health service to reassess the latest program by including the intermediate group 1:251–1:1100 for further evaluation.

method of investigation. Vogel et al. highlighted the importance of chromosomal microarray in detection of abnormal results for pregnancies at increased risk [10]. They demonstrated that common aneuploidies and copy number variations (CNVs) are distributed unevenly at different cut-off points as significant portion of aberration at cFTS risk $>$ 1 in 50 are common aneuploidies (detected via NIPT or conventional karyotyping) while pathogenic CNVs (detected via array CGH) were more prevalent in the group with a risk between 1 in 100 and 1 in 300. Hence, for pregnancies with risk results $\leqslant$ 1:300, the presence of any structural anomalies, increased NT or intrauterine growth restriction (IUGR) or a positive family history of mental retardation other than common chromosomal anomalies should prompt considering array CGH if available.

2. Pregnancies with increased NT $\geqslant$ 3 mm or NT $\geqslant 99^{\text{th}}$ percentiles be offered diagnostic test e.g. CVS. NT scan is a measurement of fluid-filled subcutaneous space found behind the fetal neck in the first-trimester of pregnancy during 11.3–13.6 weeks. For the reasons that increased fetal NT irrespective of final risk values raises concern about DS or other congenital anomalies and it has DR of $\sim$ 60% for DS by itself, we assessed its competency in discrimination of affected pregnancies in FN group. Table 7 indicates by inclusion of 95^{th} and 99^{th} NT, only one DS fetus with NT $=$ 2.45 mm in 99^{th}, would have been detected.
3. Pregnancies with cFTS risk result in the range of 1:51_1:250 should be offered NIPT. As the study of Vogel et al. revealed [10], due to the higher prevalence of pathogenic CNVs in risk values between 1 in 100 and 1 in 300, women should be notified of this risk to make decision between NIPT and invasive testing.

4. Pregnancies with cFTS risk result 1:251_1:1100 and the ratio of fβ-hCG to PAPPA $\geqslant 3$ should be offered NIPT.
5. Pregnancies with cFTS risk result 1:251_1:1100 and the ratio of fβ-hCG to PAPPA < 3 should be offered quad marker and report of sequential test
6. Pregnancies with cFTS risk result $\leqslant$ 1:1101 should be classified as low risk group and assessment of alpha-Fetoprotein test (AFP) should be offered at 16–18 weeks of gestation.

Figure 2 summarizes our suggested protocol for DS screening in Iran. Government, laboratories and parents' priority and expectancy are different for a screening protocol such as antenatal screening. Government prefers financing a protocol with minimum money burden. Laboratory concern is reporting less FN cases even at the expense of imposing extra money or anxious and parents' desire is taking more affordable and less stressful and risky route. Here, we proposed a protocol to be implemented which is assumed to be satisfactory for all three modalities.

In conclusion, our study accentuates the importance of large sample size as it accommodates getting a superb view of the whole picture. The classical way of combining biochemical and sonographic findings with a specific risk cutoff to finally report a risk figure is effective. Nonetheless, the big data provides researchers with the opportunity to fine tune the guideline in a way that is more efficient and informative. As Linquist et al. by a large sample size of 110712 FTS, emphasize on importance of considering the serum marker PAPP-A and fβ-hCG independently to decide about NIPT after cFTS [12], our data propose the ratio of fβ-hCG MoM to PAPP-A MoM to be considered for intermediate risk group to decide about NIPT. The risk cut off 1:250 which is currently applied to categorize cFTS results into high and low risk group in Iran, is better to plunge into 1:50 with the offer of NIPT for risk figures 1:51_1:250 and also 1:251_1:1100 with ratio fβ-hCG MoM to PAPP-A MoM $\geqslant 3$.

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