Preliminary Studies on Triple Marker Test to Screen Pregnant Women for Various Birth Defects

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Summary

Triple test (maternal serum alfa-feto protein AFP, human chorionic gonadotropin hCG and unconjugated estriol uE3) is being frequently carried out on pregnant women in order to pick up 'high risk' cases associated with chromosomal aneuploidies and/or open foetal defects. These biochemical markers show gradual change in circulating levels across gestation and abnormal levels are found associated with foetal abnormalities. The high risk cases picked up by the triple test are then subjected to specific tests to confirm the defect. In the present cross-sectional study we have established median levels of all the three markers from 12 to 22 weeks of gestation in Indian pregnant women and show that the levels of AFP & hCG are markedly different in them as compared to other ethnic groups especially in later gestational weeks, whereas uE3 remains elevated throughout. The present study further reveals the significance of using Indian norms for identifying high risk cases as some Down syndrome cases may go undetected when Western norms are used since only 1.7% cases are 'screen positive' as compared to 3.6% cases when norms generated in the present study are used to calculate the risk. On retrospective analysis, 4 cases with affected pregnancy outcome were 'screen positive' only when present study norms were applied to calculate the risk.

Introduction

A baby with birth defects imposes a great degree of socio-economic burden and psychological stress on the family. Antenatal screening of the pregnant women to possibly identify a group who are at an increased risk of carrying a fetus with certain congenital anomalies, can be given an option of specific diagnostic tests. Once an anomaly incompatible with life is diagnosed, termination of pregnancy can be advised to the couple. On the other hand, if the anomaly is compatible with life, optimal treatment in neonatal period can be planned in an efficient manner and may help in preparing the parents psychologically. Obstetric decision making for caesarean section is also influenced by the presence of anomalies.

Down syndrome is one of the most commonly found congenital abnormality associated with mental retardation, developmental defects and certain congenital malformations. In our country alone, about 21,580 Down syndrome babies are born every year (Kaur and Verma, 1995). Maternal age has long been reported to be associated with birth of Down syndrome child. The chances of an elderly woman giving birth to a Down syndrome baby are high, being about 1 in 380 if she is 35 years and above (Harper, 1988); Haddow et al 1994, Kaur and Verma, 1995; Rai, 1995; Saller and Canick 1996 a,b). However, such elderly group comprises only 5% of the entire pregnant population and accounts for the birth of about 20% of Down syndrome babies. More than 90% of all children and 70-80% of children with Down syndrome are born to women younger than 35 years of

age (Haddow et al 1994, Pauker and Pauker 1994). Therefore it becomes necessary to adopt methods to screen these young age group pregnant women for possible birth defects in their foetuses.

Triple test is a simple and well established prenatal screening test which significantly enhances the sensitivity of screening for various chromosomal aneuploidies such as Down syndrome as well as open foetal defects like neural tube defect (Harper, 1988; Haddow et al 1994; Kaur and Verma 1995; Rai 1995; Saller and Canick 1996a,b). In the triple test, maternal serum is analysed for circulating levels of alpha feto protein (AFP), unconjugated estriol (uE3) and human Chorionic Gonadotropin is a glycoprotein produced by trophoblastic cells of placenta (Braustein et al 1976) and estriol is one of the three major naturally occurring estrogens, produced by the feto-placental unit (Buster 1983). The levels of all the three markers are constantly changing throughout the period of gestation.

In a triple test protocol, when screening is done (usually in the second trimester), the levels of AFP, hCG and uE3 if found abnormal make the pregnancy "high risk" which can then be investigated further by detailed malformation scan, invasive procedures for chromosomal analysis and/or identification of Acetyl Cholinesterase isoenzyme in amniotic fluid which is a useful diagnostic marker for neural tube defects (NTD).

Several published reports indicate that median values for AFP, hCG and uE3 at most gestational ages differ in various ethnic groups viz. White, Black, Hispanic and Oriental women (Cuckle et al, 1987; Benn et al 1997; Wald & Cuckle 1977; Cuckle and Wald 1990; Wald and Cuckle 1989; Shaprio et al 1975). Shaprio et al (1975) reported that mean AFP in 24 women of Asian origin was only 57% of that in Caucasian women. Cuckle et al (1987) supported these findings and reported that AFP level in Asians (originating from Indian subcontinent) was 94% of the value in non-Asians ($P<0.02$). They further proposed that reduced AFP values in Asian women were due to inadequate production of AFP by foetal cells as median amniotic fluid AFP levels were also low in them. Contrary to these reports, Benn et al (1997) reported relatively higher median values of all the 3 markers in patients grouped as 'other' (oriental and Indian), particularly in later gestational ages. Crandall et al (1983) also reported a sudden increase of maternal serum AFP values in the Oriental group at 22 weeks of gestation. The observations have however, been based on relatively few patients.

Recently in a retrospective study, Gilbert et al (1996) reported an increase in false positive rates (from 3.4 to 12.3%), when Western norms (derived from white women in UK) were applied on the Indian Asian (Pakistani, Bangladeshi, Indians, African Indians) women settled there. Moreover, two babies with Down syndrome (born to Indian Asian women) went undetected in their study. The authors therefore, stressed on a need for a separate set of norms for ethnic minorities with proven efficiency, as they form a significant proportion of the obstetric population in UK.

It was therefore felt essential, to generate median values of AFP, hCG and uE3 in Indian Pregnant women at various weeks of gestation, as a prerequisite to use triple marker test as an indicator of in-utero abnormalities. To date such data are not available. This data will be useful not only for better antenatal care in our subcontinent, but will also aid the prenatal care of obstetric population, from India, settled abroad. Therefore in the present study we have estimated median values for AFP, intact hCG & uE3 from 12 to 22 weeks of gestation (confirmed by ultrasound) in Indian pregnant women (attending out patients department at Nowrosjee Wadia Maternity Hospital, Mumbai). In addition, we report our preliminary experience on the discrepancies that arose when Western norms were used to screen Indian pregnant women especially during retrospective analysis of five cases where women gave birth to an affected fetus.

Material and methods

Sample collection: Pregnant women (at least 50 per week of gestation from 12 to 22 weeks) attending the our patients department at Nowrosjee Wadia Maternity Hospital, Mumbai, participated in the study. Blood samples were collected, serum separated and stored at -70C in aliquots. Maternal weight, height, caste, date of last menstrual period (LMP), previous obstetric history and the diabetes status were recorded. Gestational age was determined by ultrasound measurement of biparietal diameter. Ultrasound (USG) dates were used for gestational age correlation when the discrepancy between the USG and LMP dates was greater than 10 days, else gestational age was based on the time since the first day of the last menstrual period (LMP). Twin pregnancies and pregnancies of mothers having history of diabetes were excluded from the analysis.

Estimation of AFP, hCG and uE3: Commercial kits for all the 3 markers were obtained from Diagnostic Systems Laboratories, Webster, Texas, USA. AFP and intact hCG were analysed by immunoradiometric assay (IRMA). uE3 was analysed by radioimmunoassay (RIA). All the samples were run in duplicates. Inter and intra assay variation was monitored and was 3.6% and 4.9%

respectively. Known controls of high and low values provided with the kit were always run in the assays.

Data analysis: Data analysis was done using SAS (Statistical Analysis Software, North Carolina) package. The median values obtained in the present study, at various weeks of gestation, were checked for mortality and were compared with the published median values in White, Black and Hispanic women (Benn et al 1997). In order to study whether our median values give a better indication of 'at risk' compared to western median values, we analysed and calculated individual multiple of median (MOM) values of 175 randomly selected women, using both present study and Western norms for White women (Benn et al 1997). Multiple of median (MOM) is calculated by dividing the patient specific value by the specific median values for that gestational age in normal women. Theoretically the value should be 1.0 MOM in unaffected cases and is either more or less in 'high risk' case. Cut off values used to identify high risk were 0.8 MOM for AFP and uE3, 2.0 & 0.8 MOM for hCG – for chromosomal defects and a value greater than 2.0 MOM of AFP for neural tube defects.

Results

Table I summarises the raw and weight adjusted median values for AFP, hCG and uE3 from 12-22 weeks of gestation in Indian pregnant women. Very high or very low median values were not excluded from the data base. Weight adjustments were done according to the method published earlier (Benn et al 1997). Mean, standard errors and absolute ranges are also mentioned. Figure 1 is a graphic representation of the median values for all the 3 markers in Whites, Blacks and Hispanic women (data of Ben et al, 1997) and the Indian women (present study data). AFP values in Indian women appeared to be higher in the later gestational ages (20-22 weeks). The values showed an abrupt increase from 60.4 to 102.8ng/ml (19-20 weeks) and rose further to 148.3ng/ml at 22 weeks. hCG values also appear to be higher in Indian women as compared to other ethnic groups in late gestational ages (18-22 weeks). In the White, Black and Hispanic women hCG values show a gradual fall from 15 to 21 weeks whereas in Indian women hCG levels are more or less steady from 16-19 followed by a slight increase from 23.3 to 30.1m IU/ml (19 to 21 weeks) and then a fall back to 21.3 mIU/ml at 22 weeks. uE3 values appeared to be higher in Indian women at all the weeks of gestation as compared to the other ethnic groups.

Analysis and identification of 'high risk' cases using both present study and Western median values, on 175 randomly selected cases, revealed discrepancies in picking up of 'high risk' women as shown in table II. Using all 3 markers, present study norms identified 3.6% as 'screen positive' whereas Western norms identified only 1.7%. For neural tube defects, the screen positive rates were 7.6% and 10% using present study and Western norms respectively (Table II).

The comparison becomes more interesting in 4 cases associated with adverse pregnancy outcome depicted in table III. When Western norms were used for calculating patient specific MOM values, uE3 was not indicative of any defect in any of the 4 cases (since uE3 value was always more than 1.0 MOM). On the other hand, uE3 MOMs were less than 1 in all cases using Indian norms, correlating well with the pregnancy outcome.

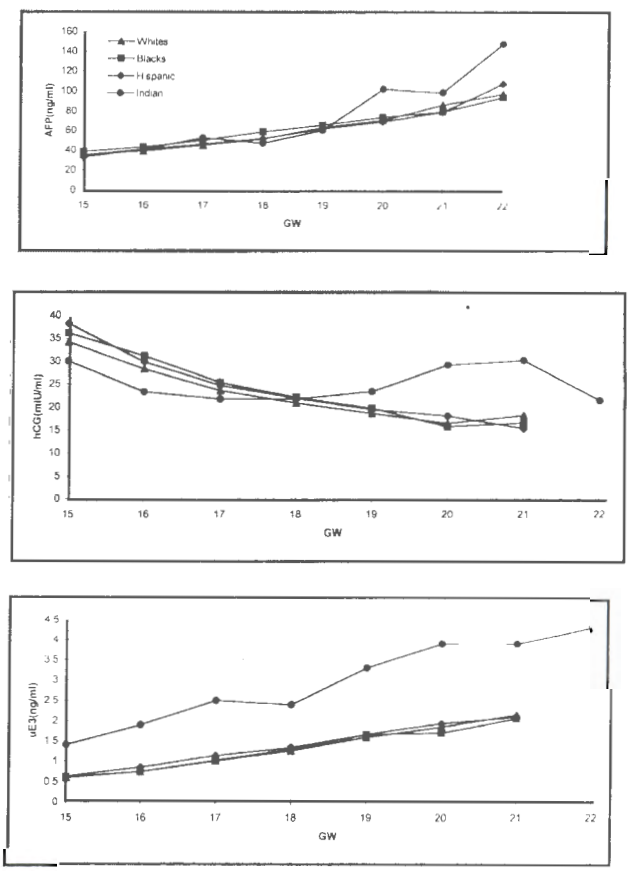

Figure 1: Graphical representation of weight adjusted median values in White, Black and Hispanic women (Benn's data, 1997) and in Indian women (present study data) from 15 to 22 weeks of gestation. Written permission of Dr Peter A Benn has been taken for using his data in the present paper.

Table I: Raw and weight adjusted median values per week of gestation for AFP, hCG and uE3. Very high and very low median values were not excluded from the data base while analyzing the median values. Mean and standard error (SE) are also provided, after excluding the values 10th and 90th percentile below and above the median values.

AFP GW	N	Median unad.	Median adj.	Mean ± SE	Range
12	50	23	16.1	21.6 ± 2.3	10.0 – 40
13	51	27.5	23.1	30.4 ± 2.3	12.0 – 54
14	50	30	27	32.1 ± 1.8	17.0 – 52
15	55	42.5	34.4	44.3 ± 2.9	25.0 – 78
16	50	43	41	47.5 ± 3.5	22.0 – 92
17	51	53	52.7	53.2 ± 3.1	29.0 – 80
18	51	53	47.2	58.2 ± 4.8	28 – 110
19	55	72	60.4	71.2 ± 4.1	37 – 120
20	50	109	102.8	113 ± 7	54 – 180
21	51	117.5	99.2	118.9 ± 7.8	30 – 160
22	55	175	148.3	161.4 ± 11	50 – 240
hCG GW	**N**	**Median unadj.**	**Median adj.**	**Mean ± SE**	**Range**
12	50	19.5	24.8	50.7 ± 17.5	12.0 – 110
13	51	50	44.2	64.5 ± 8	12.0 – 120
14	50	32.5	25.6	32.5 ± 3.1	12.5 – 57
15	55	32.5	29.6	37.5 ± 4.8	14.5 – 82.5
16	50	32.5	23.2	30.8 ± 3.2	15.5 – 61.3
17	51	23.5	21.7	26.5 ± 3.4	10.0 – 60
18	51	26.3	21.7	30.6 ± 4.6	11.0 – 72.5
19	55	30	23.3	37.3 ± 5.5	8.75 – 100
20	50	40	29.1	36.7 ± 5.5	8.25 – 85
21	51	38.8	30.1	42 ± 6.5	19 – 82.5
22	55	23.5	21.3	25.3 ± 3.5	10.25 – 55
uE3 GW	**N**	**Median unadj.**	**Median adj.**	**Mean ± SE**	**Range**
12	50	0.7	0.6	0.7 ± 0.1	0.26 – 1.2
13	51	0.7	0.8	0.9 ± 0.1	0.12 – 2.4
14	50	1.3	1.2	1.2 ± 0.1	0.15 – 2.5
15	55	1.7	1.4	1.7 ± 0.2	0.5 – 4.5
16	50	2.1	1.9	2.1 ± 0.2	1.1 – 3.0
17	51	2.5	2.5	2.6 ± 0.2	1.6 – 4.1
18	51	2.6	2.4	2.8 ± 0.3	1.5 – 3.8
19	55	3.5	3.3	3.5 ± 0.3	0.3 – 4.8
20	50	4	3.9	4 ± 0.3	2.0 – 6.0
21	51	4.2	3.9	4.3 ± 0.3	3.2 – 6.3
22	55	4.7	4.3	4.5 ± 0.3	2.5 – 6.4

GW: Gestational week, No: Number of patients; unadj: unadjusted, adj: adusted by weight (lbs)

Discussion

The present study, involving more than 500 Indian pregnant women reveals that the circulating levels of all the three markers viz. AFP, hCG and uE3 are different from other ethnic groups especially in late gestational weeks (Fig 1). The values for AFP and hCG levels appear to rise during the later gestational week a trend not observed in White, Black or Hispanic women. Both hCG and AFP levels increased during late gestational weeks whereas uE3 remained elevated throughout in the present study. A similar abrupt rise in maternal serum AFP was reported by Crandall et al (1983) in his Oriental group, where the AFP levels rose

Table II: Identification of 'screen positive' cases by analysis of 175 cases using both Western and present study norms (reduced AFP, uE3 and increased hCG indicates Down syndrome; all three markers reduced indicates high risk for either Trisomy 13, 18 or sex chromosome aneuploidy; raised AFP alone is indicative of open neural tube defect).

	According to Western Norms		According to present study norms	
AFP↓ hCG↑	13 cases	(7.5%)	7 cases	(4.2%)
AFP↓ hCG↑ uE3↓	3 cases	(1.7%)	6 cases	(3.6%)
AFP↓ hCG↓	8 cases	(4.7%)	7 cases	(4.2%)
AFP↓	20 cases	(10%)	15 cases	(7.6%)

Table 3: Retrospective analysis of four cases where the baby was born affected. MOM calculations has been done using both Western and present study median values. (reduced AFP, uE3 and increased hCG indicates Down syndrome; all three markers reduced indicates high risk for either Trisomy 13, 18 or sex chromosome aneuploidy; raised AFP alone is indicative of open neural tube defect).

Case no	MOM (Western norms) AFP	hCG	uE3	MOM (Present study norms) AFP	hCG	uE3	Pregnancy outcome
1.	0.3	0.4	1.5	0.2	0.2	0.7	Trisomy 13
2.	0.6	0.4	1.0	0.4	0.2	0.5	Trisomy 18
3.	0.7	54.9	1.6	0.6	33.3	0.7	Trisomy 21
4.	0.8	0.7	4.2	0.5	0.6	0.9	Congenital malformations

from 69 to 128.5 ug/l (21 to 22 weeks). Benn et al (1997) also noted a similar increase in AFP levels in the group labeled as "other" (but the values were based on fewer observations). Incidentally, differences in hCG values between Asian and Caucasian pregnant women are known to exist resulting in high screen positive rate in Asian group (Ford et al 1998). Therefore, one needs to be very careful in using Western norms to screen Indian women for 'high risk' for fetal defects.

The discrepancies noted in the median values of Indian women, in the present study, may also explain the possibility that the 2 babies, born to women of Asian Indian origin, reported by Gilbert et al (1996), which were missed by screening for Down syndrome could be because of the differences in the median values of AFP in Indian and Western populations. Such possibility emphasizes the importance of using set of norms specific for Indian population and further indicates that median values for Triple Markers should be specifically applied to a given population, keeping in mind their origin and ethnicity.

At present, the reason for the differences observed between Indian and other women is not clearly understood. As the number of observations per weeks, in the present study, were less and as the very high and very low values were not eliminated from the data base, we wanted to validate our results, to ensure that we were looking at a normal population and that differences from the Western population are not due to less number of samples per week of gestation. Furthermore, if the differences observed in our study as compared to the data published by Benn et al (1997) were to be attributed to less number of samples per week of gestation the differences should exist even at earlier gestational age. As shown in Figure 1, in earlier gestational weeks, the trend remains similar for all the three markers with a distinct difference in both AFP and hCG specifically from 20-22 weeks. Statistical analysis of the present study data showed normal distribution, the differences appear to be biological and warrant further investigations.

Ethnic influences (cultural and religious beliefs, uncertainty of age) have been felt to be responsible for the discrepancy in AFP value between various ethnic classes (Gilbert et al 1996). Moreover, menstrual cycle date error results in many false results. The sensitivity and specificity of serum screening is reported to be greatly improved if ultrasound dates are used (Wald et al 1992 Gardosi and Mongelli 1993). In the present study, record of both LMP and gestational age determination by ultrasound examination was maintained in every case. More than 35% of cases showed discrepancy in the gestational age determination by the two methods. Therefore, for the purpose of the analysis we based gestational age confirmed by ultrasound reports.

Besides biological factors and assay conditions, triple marker median values are known to be affected by maternal weight and race. Maternal weight and AFP concentrations in serum are inversely related (Crandall

et al, 1983). The median weight for our study population was 45kg which is less than that reported for white (68.4kg) and black (73.7kg) population by Benn et al (1997). However, adjusting the AFP values with maternal weight and ultrasound examination of gestational age does not abolish the differences in the AFP values noted in the present study (Fig 1).

With growing awareness, increasing number of pregnant women are being subjected to the triple test especially in the metropolitan cities of our country. Currently, reports are being given in 'ranges' which overlap considerably between various gestational weeks, making it difficult to interpret the results. MOM (multiple of medians) appears to be a better method of reporting. MOMs are independent of gestational age and have several advantages in reporting over standard deviations & ranges such as mathematical robustness and a more precise estimate of the detection rate (Wald et al 1977).

It should be realized that prenatal screening is a screening and not a diagnostic test for fetal aneuploidies. It is associated with a lot of false negative and false positive rates. This should be kept in mind prior to subjecting a woman to the test. The couple should be appropriately counseled prior to the test as it can result in extreme parental anxiety (Smith 1995). It is usually impossible to give accurate prognostic advice in 'screen positive' cases and this uncertainty increases the level of anxiety. Analysis of fetal chromosome by karyotyping is the diagnostic test but is not easily affordable and available to a pregnant woman in our country. Fluorescent in situ hybridization procedure is a new test and can detect aneuploidies within 2-3 days, using specific chromosome probes (D'Alton et al 1997). But its use is not yet widely employed in our country. Nonetheless, triple marker screening coupled with FISH would help to reduce maternal anxiety period and would hopefully be acceptable to both obstetrician and parents.

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