TRIPLE TEST ROLE IN IDENTIFYING CHROMOSOMAL DISORDERS IN THE SECOND TRIMESTER OF PREGNANCY

ANDREEA LITEANU[1,2*], ALINA ZLĂVOG[2], VLAD ARTENIE[1]

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Abstract: The triple test plays a very important role in identifying chromosomal disorders, in the prenatal screening of the second pregnancy trimester. The scope of our research resides in investigating the level of human chorionic gonadotropin, alpha-fetoprotein and unconjugated estriol (markers that make-up the triple test), in the serum sampled and analysed from a group of 135 pregnant women. The observation of the above mentioned markers is made in order to identify the pregnancies that present a higher risk for the appearance of chromosomal disorders. We also, decided to associate the values gathered for human chorionic gonadotropin, alpha-fetoprotein and unconjugated estriol, with the maternal age. The interpretation of the data was made using the PRISCA 4.0 software, considering by default the gestational age, smoking, *in vitro* fertilization, diabetic status, medical history of the mother. We must say that the patients were pregnant in the second trimester, period specific for triple test survey and are not the same patients included in the double test survey.

Following the conducted biochemical analyses normal values were obtained, values that fit the ranges specified in the specific literature, but also values that were outside the normal ranges, indentifying in this way pregnancies with high risk for 21 and 18 trisomy.

INTRODUCTION

Finding some serum biochemical markers that can be determined in the second trimester of pregnancy and that are useful in the estimation of the risk for chromosomal aberrations of the fetus led to the development of the survey named triple test (Bogart et al., 1987; Cuckle et al., 1994).

The triple test includes determination of the serum markers: alpha-fetoprotein, human chorionic gonadotropin and unconjugated estriol.

Alpha-fetoprotein (AFP) is a plasma fetal protein dominant during the pregnancy. AFP is a glycoprotein with a unique polypeptidic chain, with a molecular weight of 65000-70000 daltons. From the whole molecule, the protein component represents 96%, while the carbohydrate one represents 4%. In the human embryo AFP synthesis takes place in the yolk sac, liver and gastro-intestinal tract. From the synthesis places, AFP in secreted in the fetal plasma, where in can be identified beginning with the 6^{th} week of pregnancy.

AFP concentration during the pregnancy grows progressively. AFP levels are higher in the pregnancies associated with open neural tube defects and in average lower in the presence of the Down syndrome and 18 trisomy (Cuckle, 2000 ; Heyl, 1990).

Human chorionic gonadotropin (HCG) is a complex sialoglycoprotein. This glycoprotein-hormone is produced during pregnancy. Initially, HCG is secreted by the trophoblastic cells in the blastocyst development, afterwards by the scitiotrophoblastic cells of the placenta. Its serum levels present the next aspect: rises from the moment of the fecundated ovule implantation, peaks in the 8-12 weeks interval, decreases gradually till in the 18-20 weeks interval, after which it stays in a plateau till delivery. HCG concentration is higher in the pregnancies associated with Down syndrome and lower in the presence of 18 trisomy (Laborator Synevo, 2006 ; Veduta et al, 2007).

Unconjugated estriol constitutes an important biochemical marker, in a prenatal screening for Down syndrome. Unconjugated estriol is produced by the feto-placentary unit; its level in the maternal serum rises progressively during pregnancy and is typically low in pregnancies associated with Down syndrome and 18 trisomy (Laborator Synevo, 2006).

The scope of the present study resides in investigating the levels of the markers that constitute the triple test (AFP, HCG, UE_3), in order to indentify pregnancies with high risk for development of chromosomal disorders.

MATERIALS AND METHODS

Researches were conducted on biological probes sampled from 135 women, pregnant in the second trimester (15-22 weeks), period optimal for the triple test investigation. Ultrasound and sampling of the biological probes were conducted on the same day (the pregnancy age being the same both during the ultrasound and during the biochemical

investigations). All of the biological probes of all the patients, with a pregnancy age of 15 to 22 weeks, were accepted, regardless of the fertilisation type (natural or *in vitro*), type of pregnancy (monofetal or twins), or of different fetal disorders found with the ultrasound. The probes that were unsuited for the determination of the biochemical markers necessary for the triple test were rejected, namely hemolysed and lipemic. Tests were conducted on the automate analyser Immulite 1000, of the medical analyses laboratory S.C. MEDICALTEST S.R.L. Bacău, Iaşi branch and interpreted through the means of the PRISCA 4.0 software, software known for his utility in the calculation of the multiple of median (MoM) corrected for the variable factors such as: gestational age, weight of the mother, race, smoking or not, diabetic status, monofetal or twin pregnancy, procedures for the *in vitro* fertilization. Once the MoM is calculated and corrected, the similarity ration is calculated for each of these values and the combination of all of the similarity rations with the risk presented by the maternal age (a priority risk) leads to the final risk (Muller et al., 1999).

Results of the biochemical tests are expressed in IU/mL for AFP, in MIU/mL for HCG and in ng/mL for unconjugated estriol. Statistical interpretation of the gathered values was made through the Student test (Văleanu, Hîncu,1990), revealing the values obtained from pregnant women from the second age group (26-29 years), third age group (30-35 years), respectively the fourth group (>35 years), dependent of the values obtained from the first age group.

RESULTS AND DISCUSSIONS

The 135 pregnant women included in the present study were divided in four groups depending of the maternal age: a first age group comprised the pregnant women with the ages between 21-25 years (n=18), a second age group comprised of pregnant women with ages between 26-29 years (n=53), a third age group comprised of pregnant women with ages between 30-35 years (n=50) and a fourth age group comprised of pregnant women with ages over 35 years (n=14).

In table 1 the results obtained after determination of the values for the three markers, AFG, HCG and UE_3, in the pregnant women serum are presented, the women being separated in the four age groups.

For AFP the median value calculated for the pregnant women from the first age group is 38.900 IU/mL, for those in the second age group is 34.881 IU/mL, for those in the third age group is 30.726 IU/mL, and for those in the fourth age group is 26.823 IU/mL (table 1).

A study conducted on pregnant women in the second pregnancy trimester, depending of the gestational age, presented values in the interval 10-300 IU/mL and different median values depending the gestational age as follows: at a number of 605 pregnant women, with a gestational age of 16 weeks, a median value of 28.5 IU/mL was calculated; at a number of 569 pregnant women, with a gestational age of 17 weeks, a median value of 32.6 IU/mL was calculated; at a number of 431 pregnant women, with a gestational age of 18 weeks, a median value of 37.3 IU/ml was calculated. These probes were all processed on Imulite 1000 the same type of analyser used in our present study (Siemens Medical Solution Diagnostics, Haddow *et al*, 1992).

Table 1. Median values calculated for AFP, HCG and UE_3 in pregnant women from the four age groups**.**

	AFP (IU/mL)		**HCG(MIU/mL)**		**UE_3 (ng/mL)**	
n=18 (21-25 years)	Median (M)	38.900	Median (M)	26541.667	Median (M)	3.352
	Standard Error (Es)	0.76461	Standard Error (Es)	940.05238	Standard Error (Es))	0.08352
	t	-	t	-	t	-
	p	-	p	-	p	-
n=53 (26-29 years)	Median (M)	34.881	Median (M)	28798.320	Median (M)	2.961
	Standard Error (Es)	0.23738	Standard Error (Es)	452.37045	Standard Error (Es)	0.03086
	t_1	5.01975	t_1	2.163	t_1	4.39261
	p_1	<0.001	p_1	0.05>p>0.01	p_1	<0.001

n=50 (30-35 years)	Median (M)	30.726	Median (M)	28505.890	Median (M)	2.534
	Standard Error (Es)	0.22850	Standard Error (Es)	349.51350	Standard Error (Es)	0.02907
	t_2	10.24281	t_2	1.95849	t_2	9.25408
	t_3	12.61092	t_3	0.51156	t_3	10.078
	p_2	<0.001	p_2	>0.05	p_2	<0.001
	p_3	<0.001	p_3	>0.05	p_3	<0.001
n=14 (>35 years)	Median (M)	26.823	Median (M)	32864.571	Median (M)	1.827
	Standard Error (Es)	0.76818	Standard Error (Es)	1601.97258	Standard Error (Es)	0.353
	t_4	9.33841	t_4	3.40413	t_4	12.10415
	t_5	7.59005	t_5	2.44275	t_5	11.42419
	t_6	2.42990	t_6	2.65829	t_6	7.15953
	p_4	<0.001	p_4	<0.001	p_4	p<0.001
	p_5	<0.001	p_5	0.05>p>0.01	p_5	p<0.001
	p_6	0.05>p>0.01	p_6	0.05>p>0.01	p_6	p<0.001
N=135						

The median values that we calculated for the pregnant women in the four age groups are close to those from the speciality literature, with little differences that can be explained through the fact that we did not analysed the pregnant women strictly after the same gestational age (for example: only for 16 weeks pregnant women, separated from 17 weeks pregnant women), but rather for the second trimester pregnancies depending on the maternal age. Considering the first group of pregnant women as our control group, for the patients in the second, third and fourth age groups the results are very significant, as follows: for the patients in the second age group $p<0.001$ (89.6%), for those in the third age group $p<0.001$ (78.96%) and as expected for those in the fourth age group the results were very significant (68.95%), as you can observe from the table 1 and figure 1.

In the case of the second marker analysed, from the component of the triple test and namely HCG, the following results were obtained: for pregnant women in the first age group a median value of 26541.667 MIU/mL was calculated, for those in the second age group a median value of 28798.32075 MIU/mL was calculated, for those in the third age group a median value of 28505.890 MIU/mL was calculated and for those in the fourth age group a median value of 32864.571 MIU/mL was calculated (table 1).

The reference values found in the speciality literature are comprised between 6140 and 103000 MIU/mL. The studies conducted by Siemens on a group of 593 pregnant women, of which 72 were in the second pregnancy trimester, reported on the 72 pregnancies a median value of 40989, specifying that each lab must establish its own reference values, their values serving only as guide (Siemens Medical Solution Diagnostics ; Haddow *et al,* 1992).

The data we gathered fit in the reference interval of 6140-103000 MIU/mL and are close to the literature median values. The differences between the median values calculated by us and those related by Siemens are explained through the fact that the interval we obtained for the pregnant women in each age group is much smaller then the one in the speciality literature, but the total number of analysed pregnant women is much bigger. In the pregnancies associated with the Down syndrome, the HCG levels are ≥ 1.97 MOM, and in those associated with 18 trisomy have a MoM value for HCG ≤ 0.55.

The MoM calculation for each marker consists in dividing the obtained value to the median correspondent to the gestational age. MoM correction is made by comparison of the obtained values for the specific patient with the general median value calculated for a population of pregnant women, these having normal pregnancies. In the present case, the highest values

were obtained for the pregnant women form the fourth age group (>35 years), recording a 123.82% increase from the results obtained for the pregnant women from the first age group, control group (21-25 years), the modifications being significant 0.005>p>0.001(table 1 and figure 2). The results obtained for the pregnant women from the second and third age groups were slightly significant (0.05>p>0.001) and respectively unsignificant from a statistical point of view, p>0.05 (108.5%, respectively 107.4%).

For the third analysed marker, from the component of the triple test and namely free or unconjugated estriol(UE_3) the following median values were obtained: for the pregnant women from the first age group a median value of 3.352 ng/mL was calculated, for the pregnant women in the second age group a median value of 2.961 ng/mL was calculated, for the pregnant women in the third age group a median value of 2.534 ng/mL was calculated and for the pregnant women in the fourth age group a median value of 1.827 ng/mL was calculated (table 1).

The reference interval found in the speciality literature, for the second trimester of pregnancy in 0.46-7.41 ng/mL, according to Siemens studies, encountered median values being different for each laboratory. Siemens studies on 268 pregnant women, but in the third pregnancy trimester (after 23 weeks of pregnancy), revealed a reference interval comprised between 2.9 and >30 ng/mL with a median value from 6.5 to 23 (Siemens Medical Solution Diagnostics ; Haddow *et al,* 1992).

The data we obtained fit in the reference interval of 0.49 – 7.41 ng/mL and are closed to those found in the speciality literature. The differences between the median values we obtained and those related by Siemens are explained through the fact that the interval we calculated for pregnant women from each age group is much smaller than the one reported in the literature and mainly through the fact that Siemens conducted the survey on greater gestational ages, more than 23 weeks of pregnancy, where it is normal for the unconjugated estriol values to be higher.

As we mentioned earlier, unconjugated estriol is typically low in high risk of trisomy associated pregnancies. In this sense, we observed that the lowest values were obtained in pregnant women from the last age group (>35 years), recording a fall of 54.50% as to the results obtained for the pregnant women from the first age group, control group (21-25 years), modifications being significant p<0.001 (table 1 and figure 3). The results obtained for the pregnant women from the third and second age group are also significant from the statistical point of view (p<0.001), observing a diminishing of the values regarding the maternal age and namely: for the pregnant women from the second age group (26-29 years) a drop of 88.33% as to the first age group was recorded, and for the third age group (30-35 years) a 75.59% drop was recorded.

The final result of the triple test analysis, that represents the risk (high or low) for a trisomy appearance, is expressed regarding the MoM. The degree of risk for each pregnant woman is based on combining the obtained result with the maternal age by a complex mathematic algorithm using a software such as PRISCA 4.0, which is the one we used in our study.

The tests are interpreted as being with high or low risk, depending of the cut-off vale set for each trisomy. In the case of the 21 trisomy the cut-off value is 1/250, and in the case of the 18 trisomy the cut-off value is 1/100.

After the determination of the immunological markers, the obtained values were statistically worked by using PRISCA software edition 4.0 of DIAGNOSTIC PRODUCTS CORPORATION, USA. The PRISCA software 4.0 is an application that provides a statistical value to the risk for the Down syndrome (21 trisomy) and for the Edwards syndrome (18

trisomy), in the first and second pregnancy trimester and for the neural tubes defects in the second pregnancy trimester. The risk calculated through PRISCA, for a pregnant woman, is not a test for confirmation for chromosomal abnormalities, but has the scope, in the *in vitro* diagnosis, to be used as an additional support, in her decision to undertake or not the diagnosis procedures.

The biochemical risk for Down syndrome at birth is calculated based on corrected MoM for each of the three markers and maternal age at birth. The risk for the 18 trisomy at birth is calculated based on the corrected MoM for each of the three markers and maternal age. (Muller et al.,1999). PRISCA 4.0 compares the result obtained with the median specific for the gestational age in order to express the result as MoM, for each of the parameters: AFP, HCG, UE_3, during the second trimester of pregnancy.

Speciality literature offers data both on the triple test as an analysis in its self, but also on each marker individually. Hence Akalin et al. (2007), researching the biochemical screening in the second trimester of pregnancy, on 700 pregnant women, excluding the ones with twin pregnancies, using Immulite One analyser, after AFP analysis, gained a median equal with 32.5 and values comprised in the 14.5-95 IU/mL interval. Analysing HCG he gained a value equal to 20961 and values comprised in the 2260-60775 MIU/mL interval. For UE_3 he gained a value equal to 2.70 and values comprised in the 0.77-9.10 ng/mL interval. Cumulating the obtained data, he concluded that significant differences were given by the median value of alpha-fetoprotein ($p<0.001$) in the pregnancy period of 16-19 weeks. Following the analysis of these values we can see that they are similar if not even close to those obtained in our study (table 1).In the studies conducted by Johnson et al. (1984), the necessity of the maternal weight and race, in the utilisation of MoM, is related. Also, Reynolds et al. (2006), unveils the significant differences ($p<0.001$) between corrected MoM values depending on the maternal weight. Wald et al (2006) showed in its studies, the importance of the MoM calculus for the values obtained, resulting in a better screening and thus reducing the appearance rate of false positive results.

Analysing these information we can say that our results are in concordance with the ones in the speciality literature, which show that the patients with ages between 18 and 35 years, regarding the gestational age, smoking, *in vitro* fertilisation, diabetic status, medical history of the pregnant woman, have a lower rate of appearance of high risk pregnancy as do the ones with a maternal age of over 35 years.

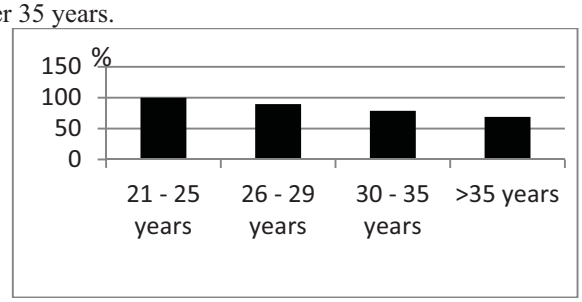

Figure 1. Relative AFP values, obtained for pregnant women from the last age group (>35 years), compared to the first, second and third age group

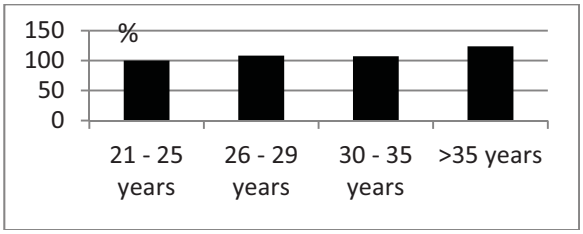

Figure 2. Graphic comparative representation of the relative values (%) for HCG, obtained after the analysis of the pregnant women serum from the four age groups

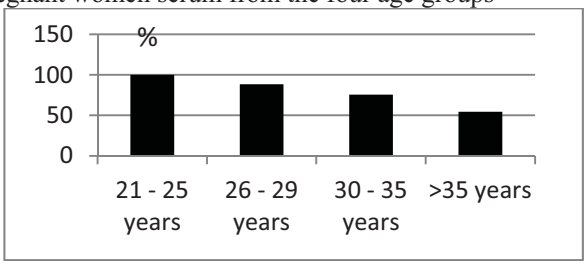

Figure 3. Representation of the relative values of unconjugated estriol obtained in patients from the four age groups

As we said earlier on the total of 135 pregnant women was divided in four age groups. The first group is taken as a control group in the statistical analysis of all of the age groups in this study, because no pathological values were recorded, which means that the pregnant women from the 21-25 years age group do not have risk pregnancies for chromosomal disorders and neural tubes defects.

General analysis of the results on groups shows us the following: the second age group presents a single case of a pregnancy with high risk, from the third age group, 30-35 years, formed from 50 patients, three of those gained a pathologic result, and the rest obtained normal results specific for low risk pregnancies. As the maternal ages progresses, so does the number of cases with risk pregnancies. Regarding the precedent age group, this third group has to more risk pregnancies, the total number of analysed cases being similar in the two groups.

Analysing the last age group where we expect to get more risk pregnancies, as the things evolved, we observe the following: from a total of 14 pregnant women, 4 recorded pathologic results, meaning they have pregnancies with high risk for the appearance of chromosomal disorders. Reported to the number of total pregnant women analysed, these situation is the most critical. To reveal the importance of maternal age in prenatal screening, in figure 4, we represented the percentage for the results obtained after conducting the triple test on patients in the fourth age group, the group with the most pathological results recorded.

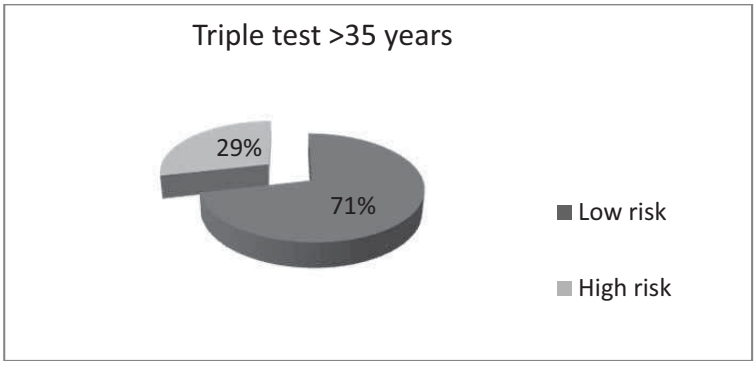

Figure 4. The percentage value of the results obtained after conducting the triple test on patients from the >35 years age group

CONCLUSIONS

The obtained results from the investigated pregnant women, on age groups, for AFP are in correlation with the data from the speciality literature, which demonstrates the importance of the maternal age in the prenatal screening. Also, according to the speciality literature, HCG is the most stable marker, being secreted by the placenta and correlated with the AFP values, it gives it a special importance in the gathering of the results. The association of these markers with UE_3, in the triple test, reveals a clear image in the final result in the prenatal screening with a low rate of false positive results, pointing out a good correlation between the biochemical markers and ultrasound.

The obtained values after testing the biochemical markers, in combination with the ultrasound data, maternal age and medical history of the mother, represents a feasible prenatal screening, a fact that is found both in our study but also in those in the speciality literature. The method of interpreting the triple test through the PRISCA software is exact and until now does not need adjusting. Because of the fact that in the first age group (21-25 years) for any of the pregnant women we got no pathological results specific for pregnancies with high risk of appearance of chromosomal disorders and the number of pregnant women with high risk pregnancies is higher to the patients in the fourth age group (>35 years), we can conclude that the obtained results depend largely on the maternal age.

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[1] "Alexandru Ioan Cuza" University of Iaşi, Faculty of Biology, B-dul Carol I, Nr.20A, 700506, IAŞI, ROMANIA
[2*] "Medicaltest" Clinical Laboratory, IAŞI, ROMANIA
* andreeaberbece@yahoo.com