THE IMPORTANCE OF THE DOUBLE TEST IN IDENTIFICATION OF HIGH RISK PREGANANCIES FOR CHROMOSOMAL DISEASES DEVELOPMENT

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Abstract: The double test plays an important role for the identification of chromosomal diseases and for the prenatal screening in the first pregnancy semester. The current work is focused on the investigation of free beta-human chorionic gonadotropin (free β-HCG) and pregnancy- associated plasma protein-A (PAPP-A) levels (markers part of the double-test) from the blood serum of 132 pregnant women in order to identify the high risk pregnancy for chromosomal diseases development. Also the levels of beta-human chorionic gonadotropin (free β-HCG) and pregnancy-associated plasma protein-A (PAPP-A) were investigated with respect to the maternal age. The interpretation of the results was achieved using the PRISCA v. 4.0 software, considering also the gestational age, smoking, *in vitro* fertilization, diabetic status and the medical history of the pregnant women. All investigated patients were in in the first semester of pregnancy, the specific period for tacking the double test. The biochemical investigations showed that most of the investigated patients presented normal values, within the interval reported in the literature and only a few cases were identified as being with high risk for developing trisomy for the chromosome 21 or 18.

INTRODUCTION

The identification of some suitable serum markers that can be used for assessing the risk of chromosomal aberrations in the first semester of pregnancy lead to development of the double-test (Agarwal, 2003 ; Kagan et al., 2008).

This test combines the two serum markers: the levels of free beta-human chorionic gonadotropin (free β-HCG) and pregnancy-associated plasma protein-A (PAPP-A)

The pregnancy-associated plasma protein-A is a glycoprotein derived from the placenta. During the pregnancy is produced in large amounts by the trophoblast and liberated in the mother’s circulatory system. The serum levels of this protein increase with the gestational age, especially in the last part of the pregnancy. Recent studies showed that the decrease of PAPP-A levels during the pregnancy is associated with chromosomal abnormalities of the fetus (Laborator Synevo, 2006 ; Veduta, Vladareanu, 2007).

The free beta-human chorionic gonadotropin is considered to be an even more relevant marker in the first semester of pregnancy. In pregnancies associated with the Down syndrome, the levels of free beta-HCG are >2 MoM. In the presence of trisomy 18, the levels of free beta-HCG are considerable lowered.

The purpose of this study is the investigation of the markers comprising the double-test (PAPP-A and free beta-HCG) in order to identify pregnancies with high risk for the development of chromosomal maladies.

MATERIALS AND METHODS

All investigations were performed on serum samples from 132 women in the first semester of pregnancy (10-13 weeks), the optimum period for performing the double test. Very important and also required is the fact that both the Ultrasound investigations and the serum sample were performed in the same day. In this manner, the gestational age is the same for both the Ultrasound investigations and the double-test, eliminating errors which might affect the precision of the obtained result. If the date of the Ultrasound is different compared to the blood sampling it must be specifically stated and the errors affecting the result cannot be eliminated. Biological samples were accepted from all the patients with an gestational age between 10 and 13 weeks, no matter of the fertilization method used (natural or *in-vitro*), pregnancy type (mono-fetal or twin), or various abnormalities, dis-functions and diseases identified using Ultrasound. Nevertheless the samples which were not suitable for performing the double test (hemolytic or lipemic) were rejectd. All determinations were performed using the automated analyzer Immulite 1000, belonging to the S.C. MEDICALTEST SRL BACĂU, IAŞI branch and interpreted using the PRISCA v 4.0 software. The PRISCA 4.0 program is able to calculate the corrected multiple of median tacking into account various factors such as: gestational age, mother’s bodyweight, smoking or non-smoking, the diabetic status, the pregnancy type, procedures used for fertilization. Once the corrected MoM was obtained, the similarity ratio is calculated for each of these values. By combining each of these ratios with the risk correlated with the maternal age (an major risk) the final result is obtained (Muller et al., 1999 ; Kevin, 2005).

The results of the biochemical investigations are expressed as mIU/mL for PAPP-A and as ng/mL for free β-HCG. The statistical significance was assayed using the Student test (Văleanu, Hîncu,1990) by grouping the data by mother's age and comparing each group with an random control consisting of the first age group.

RESULTS AND DISCUSSIONS

The 132 pregnant women selected were divided in four group according with their age: 16 in the first age group, consisting of women between 21-25 years old, a second group of 51 women between 26-29 years, 46 women in the third group of age between 30 – 35 years and 19 in the fourth group older then >35 years.

In table 1 are presented the results obtained by measuring the leveled of the two investigated markers PAPP-A and free beta-HCG in the serum of the investigated women from each of the four age groups.

For PAPP-A, the mean value for the first age group was of 2.166 mIU/mL, for the second group was of 2.048 mIU/mL, for the third group the recorded mean value was of 1.942 mIU/mL, and for the fourth group was of 1.568 mIU/mL (table 1).

A previous study on 283 voluntary patients has recorded values situated in the interval of 0.3 şi 10 mIU/mL, with an mean of 3.64 on Immulite 1000 and 3.70 on Immulite 2000 (Siemens Medical Solution Diagnostics ; Wald *et al.,* 1996).

The recorded mean values fort the pregnant women belonging to the four categories are lower, but nevertheless are more close to each other, probably due to an more strict interval on which the results were recorded. Our study was performed only on women in the first semester of pregnancy, making the recorded values interval to be more strict, more specific. Taking into account that in the first age-group no pathological results were encountered, we considered it as the control group. Compared to this group, the results recorded for the second age-group showed no statistically significant differences $p>0.05$(94.5%). Nevertheless, the results recorded for the third age group showed some slight significant differences $0.05>p>0.01$(89.65%), while the forth age-group as expected the differences are highly significant $p<0.001$(72.39%) (table 1, Figure 1).

For the second investigated marker, part of the double test – the free beta-HCG, the recorded levels were as follows: for the pregnant women from the first age-group the mean value was 48.756 ng/mL, for those from the second age-group the mean was 59.53 ng/mL, for the third group 45.689 ng/mL and for the fourth group 59.792 ng/mL(table 1).

The reference values recorded in the literature are between 2 - 200 ng/mL. The studies performed by Siemens using 20 volunteers reported the following values: 60.2 ng/mL from serum, 59.6 ng/mL from heparinized blood, 59.8 ng/mL from blood with EDTA. The levels for the free beta-HCG recorded on 116 pregnant women were between 0.75 şi 129 ng/mL with an average of 27.0 ng/mL on Immulite 1000 and 28.1 ng/mL on Immulite 2000. (Siemens Medical Solution Diagnostics ; Wald *et al.,* 1996).

The data recorded by us are inside the reference interval of 2 – 200 ng/mL and are close to the levels reported in the literature. The differences between values reported in this study and those reported by Siemens could be explained by the fact the interval for ech group of pregnant women in the current study is much smaller.

As stated above, in the pregnancies associated with Down syndrome the levels of free beta-HCG are below 2 MoM (multiple of median). The calculation of MoM for each marker consists of dividing the value with the median of the gestational age. The correction of the MoM is performed by comparing the obtained value for the specific patient with a mean value from a

population of normal pregnant women. In this case the highest values were recorded at women from the last group of age (>35 years), with an increase of over 122.63% compared with the results obtained for the control group (first age-group, 21-25 years), all recorded differences being significant $p<0.001$(table 1 and figure 2). The differences recorded for the pregnant women from the second and third age-group were statistically not significant ($p>0.05$).

The final result of the double test analysis is the risk (low or high) of trisomy development and it is expressed as a function of MoM. The risk level for each subject is based on the combination of the obtained result with the maternal age using an complex mathematical algorithm using software programs such as PRISCA 4.0 (used in our study).

The tests are interpreted as low or high risk based on a cut-off value fixed for each type of trisomy. In the case of 21 trisomy the cut-off is 1/250, and in the case of 18 trisomy the cut-off value is 1/100.

After the determination of the immunological markers, the obtained values were statistically interpreted using the PRISCA v. 4.0 software, DIAGNOSTIC PRODUCTS CORPORATION, SUA. The program is a software application which calculates the statistical value for the risk of development the Down syndrome (trisomy 21) and for the Edwards syndrome (trisomy 18), in the first and the second semester of pregnancy, as well as for neural tube defects in the second semester of pregnancy. The risk calculated using PRISCA software for a given woman is not a confirmation test, but has the role of an additional support in deciding whether a more profound diagnostic procedure is requited or not.

The biochemical risk for developing Down syndrome at birth is calculated on the basis of corrected MoM for each of the two markers and maternal age at birth. PRISCA 4.0 compares the obtained result with the median specific for the age of the pregnancy in order to formulate the final result as MoM, for each of the parameters PAPP-A and free beta-HCG, during the first semester of pregnancy.

Some results from the literature are addressing the double test as a test on its own, and not applied specifically for each markers. In this manner Bellver et al. (2005), analyzing the biochemical screening in the first semester of pregnancy, no matter the fertilization system used conclude that significant differences were due to maternal age, obtaining an $p<0.001$ for patients over 33 years old. Also, the determinations performed by Dr. Elisabeta Kovacs at the 1-st Obstetric and Gynecology Clinic of Emergency District Hospital, Cluj-Napoca, between 2003 – 2005, indicated statistical significant results when the Immulite 1000 automated analyzer was used to perform the double test (Kovacs, 2009). The results obtained by Kovacs, 2009 are in good concordance with the data obtained in our set of determinations, being the differences obtained being statistically significant for the women in the last age-group ($p<0.01$, table 1).

A similar conclusion was reported by the work performed by Adriana Stana (Stana Adriana, Popescu Gabriela**,** 2010), which emphasis that the best method is the simultaneous use of the combined screening and nuchal translucency, the combined risk for trisomy 18 being calculated using the PRISCA software.

All this data are in very good accordance with our observations that patients of age between 18 and 35 years old have a more diminished rate of risk pregnancy compared with those of over 35 years old.

Table 1: Mean values of PAPP-A and free beta-HCG levels determined at pregnant women grouped by age

PAPP-A(mIU/mL)			**free beta-HCG** (ng/mL)		
n=16 (21-25 years)	Mean (M)	2.166	**n=16 (21-25 years)**	Mean (M)	48.756
	Standard error (ES)	0.08489		Standard error (ES)	1.35542
	t	-		t	-
	p	-		p	-
n=51 (26-29 years)	Mean (M)	2.048	**n=51 (26-29 years)**	Mean (M)	59.53
	Standard error (ES)	0.03547		Standard error (ES)	1.89847
	t_1	1.290		t_1	0.68397
	p_1	>0.05		p_1	>0.05
n=46 (30-35 years)	Mean (M)	1.942	**n=46 (30-35 years)**	Mean (M)	45.689
	Standard error (ES)	0.031		Standard error (ES)	0.81503
	t_2	2.479		t_2	1.93929
	t_3	2.236		t_3	0.71231
	p_2	0.05>p>0.01		p_2	>0.05
	p_3	0.05>p>0.01		p_3	>0.05
n=19 (>35 years)	Mean (M)	1.568	**n=19 (>35 years)**	Mean (M)	59,792
	Standard error (ES)	0.099		Standard error (ES)	2.52648
	t_4	4.590		t_4	3,84913
	t_5	4.564		t_5	3,99692
	t_6	3.609		t_6	5,31248
	p_4	<0.001		p_4	<0.001
	p_5	<0.001		p_5	<0.001
	p_6	<0.001		p_6	<0.001
N=132					

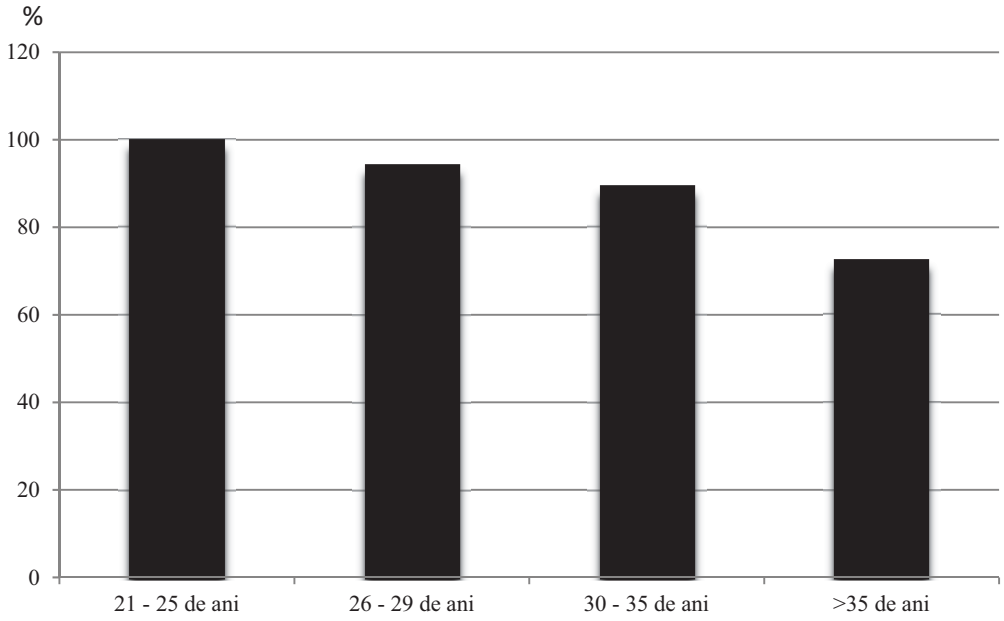

Figure 1. Relative values (%) of PAPP-A levels measured at pregnant women over 35 years old compared with the first, second and third age-group.

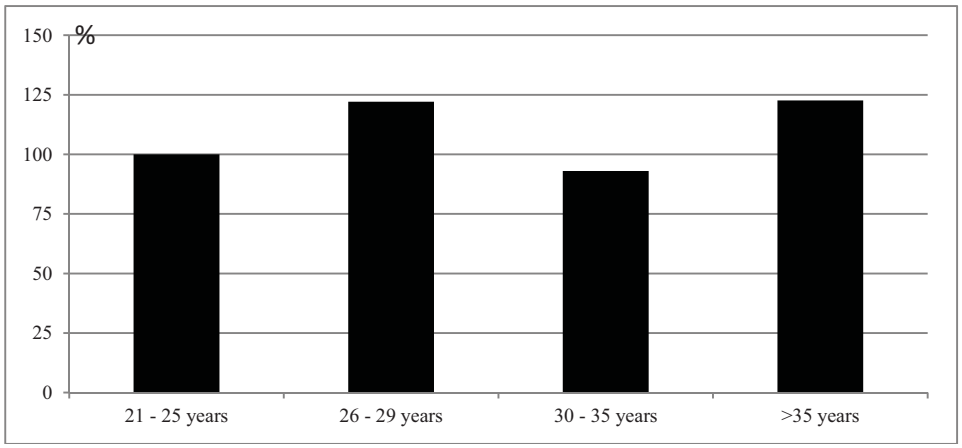

Figure 2: Graphical representations of the relative values for free. beta-HCG levels in serum from pregnant women grouped by age.

As stated earlier, the 132 pregnant women were group according to their age, forming 4 groups. The first group was selected as control group, as no pathological values were detected and the women aged between 21 – 25 do not present an high risk for development of chromosomal maladies.

Out of a total of 51 patients aged between 25 – 29 years, two were detected as presenting a high risk for development of trisomy 21.

Out of a total of 46 patients of aged between 30-35 years, 6 were detected as presenting high risk pregnancies.

The patients from the last group, from a total of 19, 9 were detected as presenting high risk pregnancies and the other 10 (53%) were with low risk. 7 out of the 9 these high risk patients were for trisomy 21, while the other two (10%) for both trisomy 21 and trisomy 18. These last two are more special cases as one patient is 42 years old smoker and the other is 38 years old.

In order to emphasis the importance of the maternal age in the prenatal screening, in figure 3 we present the percentage results of the double test performed on the patients from the forth age group, the one with most pathological cases.

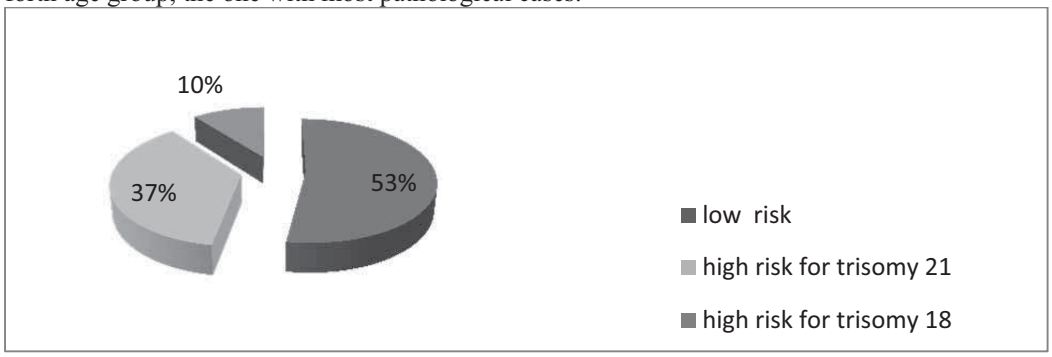

Figura 3: Procentage of the results of the double test performed on women from the fourth age group

CONCLUSIONS

The results obtained in this study, grouped by the age of the pregnant women for PAPP-A are well correlated with those reported in the literature, highlighting the importance, of maternal age in the prenatal screening. Also, according the literature, free beta-HCG levels is a very important marker which, together with PAPP-A levels, makes the double test and assures a very low rate of false positive hits, offering a very good correlation between the biochemical investigations and the ultrasound investigations.

The values obtained by evaluating the biochemical markers, combined with the ultrasound data, maternal age, mothers medical history, is a feasible prenatal screening, fact proved by both our study and the literature. The method of interpretation of the double test results using the PRISCA software is precise and does not require any manual adjusments. Due to the fact that in the first age group (21 – 25 years) no pathological results were recorded and the number of high risk pregnancies is increased as the patient is older, we can conclude that the result depend most on the maternal age.

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