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ORIGINAL ARTICLE

The Effect of Inhibin A on Prenatal Screening Results for Down Syndrome in the High Risk Czech Pregnant Women

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SUMMARY

Background: **Currently, prenatal testing is based on an ultrasound examination, the testing of certain biochemical markers and, most recently, also on the analysis of fragments from the extracellular DNA of the fetus in the mother´s blood.**
The aim of this work was to verify whether inhibin A testing during pregnancy can help influence the risk distribution of Down syndrome screening results in high risk population and thus possibly reduce the number of unnecessarily invasive procedures, or for better stratification of risks when deciding on non-invasive DNA testing.
Methods: **The concentrations of inhibin A were measured using a chemiluminescent immunoassay in two groups of screening tests. The first group (triple test) included a total of 277 pregnant women; the second group (integrated test) included 91 pregnant women. Risk assessments of screenings were performed using Alpha software, LMS.**
Results: **The resulting risk for pregnant women without the determination of inhibin A was higher or equal to 1:300 (triple test) and 1:150 (integrated test). Inhibin A was then measured in the monitored groups and the risk was recalculated. In the first group (triple test) the risk was lower than 1:300 in 152 pregnant women and in the other group (the integrated test) in 47 pregnant women. At the end of the study, all results were compared with the outcome of the pregnancy.**
Conclusions: **The results obtained show that the inclusion of inhibin A in screening protocols reduces the number of positive results in high risk population screened without inhibin A.**
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INTRODUCTION

Inhibins are glycoproteins that belong to the transforming growth factor beta family (TGF-β) [1], which counts more than 60 proteins and also includes activins that are structurally similar but differ in terms of function. AMH (Anti-Müllerian hormone) also belongs to this family of proteins. Inhibin was first described in 1923 [2] and is characterized as a gender hormone, whose most important function is to regulate the production of the follicle-stimulating hormone (FSH) in adenohypophysis cells

[3,4]. Inhibins in women are produced in the ovarian granulosa cells, during pregnancy inhibin A is produced by the yellow body and the placenta. Inhibins play an important role in oocyte maturation and regulating folliculogenesis. While inhibin B is primarily produced by small antral follicles, inhibin A is the hormone of mature forms of oocytes and pregnancy. Its action reduces the production of FSH. However, it is evident that the function of inhibin includes a much wider range of effects, which not only apply to the reproductive system, but as one of the main reproductive hormones, it is also involved in the paracrine regulation of folliculogenesis and steroidogenesis, and it affects the biochemical processes in other organs [5,6].

Structurally, inhibins are made up of dimeric glycoproteins which are present in the form of the α subunit and subunits β_A and β_B Subunit α is the same for both inhibin molecules and the specificity is given by the presence of subunit β_A or subunit β_B. Subunit α has a molecular weight of about 20 kDa and is bound by a disulfide bond to one of the two possible β subunits (molecular weight 13 kDa). The β subunit is present in two modifications - β_A and β_B. Therefore, inhibin is present in two isoforms. If only subunit β creates the dimer, the activin molecule is created, which can exist in 3 isoforms. A total of at least 9 biologically active forms of inhibin have been found [7-9]. These forms differ in their molecular weight, which is affected by the amino acid sequence extension on subunits and thus a greater glycosylation at the C-terminal end of the alpha subunit [10, 11].

Inhibin is encoded by 3 genes, which are responsible for the production of the three peptides mentioned; peptide α, peptide β_A and β_B. The alpha subunit of inhibin in humans is located on chromosome 2 (2q33-q36), β_A and β_B subunits are located on chromosome 7 (7p15-p13) and 2 (2cen-q13), respectively [12,13]. Only heterodimeric subunits $\alpha\beta_A$ and $\alpha\beta_B$, which are connected by a covalent disulfide bridge, create the active forms of inhibin A and B. Both forms of inhibin not only suppress FSH secretion in the pituitary gland but also have a local modular effect on gonadal steroidogenesis [14,15]. Mature inhibins are quickly removed from circulation, their biological half-time is approximately 3 - 6 minutes for inhibin A [16,17] and about 3 minutes for inhibin B [16]. In an experiment with radioactive labeled inhibin A, it was discovered that it accumulates in the spleen, adrenal gland, bone marrow, and ovaries [18].

The source of inhibin A in pregnancy is a yellow body and later the placenta. Inhibin A (and also activin) have a paracrine and autocrine function in the human placenta and locally affect placental hormone production, cellular immunity, cell growth and differentiation of the placenta and embryo. Inhibin A also enters into maternal circulation and can have an endocrine effect during pregnancy. Serum level changes in these proteins reflect changes in placental synthesis and secretion [4]. Inhibin A is one of the main proteins in circulation during pregnancy [19,20]. Its serum level decreases approximately from the 8th to the 16th week of gestation. The level remains lower during the second trimester, but then increases up to five times during the third trimester and reaches its peak during the 36th week of gestation [6]. The majority of inhibin, which is detectable in the serum, is most likely of placental origin [19,21]. The function of inhibin A during pregnancy has yet to be clarified but it is very likely that it has autocrine and paracrine effects on the placenta and the corpus luteum [22]. Placental cytotrophoblasts and syncytiotrophoblasts secrete inhibin A which inhibits the placental secretion of hCG and progesterone. Certain biochemical substances, which are produced by the placenta during pregnancy, might be used as screening markers for genetic diseases e.g., Down syndrome. It has been discovered that the increase in the level of inhibin A is somewhat associated with the presence of Down syndrome. This can be used in combination with other biochemical markers which are produced by the fetoplacental unit [15].

Inhibins and activins are both members of the TGF-β family and they both have the binding membrane protein ActR II (activin type II receptor) in common, which has a high affinity for activin A and B [23]. It also binds inhibin but with a lower affinity, and it acts as an activin antagonist (its binding blocks the formation of the receptor complex). Degradation of inhibin occurs, similarly to other peptide hormones, by the process of receptor-mediated endocytosis [24].

Screening for Down syndrome and other aneuploidies during pregnancy

During pregnancy, a woman's body goes through many changes which affect the production of biochemical markers. Some of these markers are specific for the activity of the fetoplacental unit, and it has been discovered that their production may be affected by the presence of certain genetic diseases of the fetus [25]. The most common genetic defect is Down syndrome which is caused by the trisomy of chromosome 21. With regards to this, in common practice, pregnancy is monitored by using free β HCG and PAPP-A during the first trimester of pregnancy [26]. In the second trimester of pregnancy the levels of total HCG, alpha-1-fetoprotein (AFP), and unconjugated estriol (uE3) are set [26]. Some screening protocols also include the measurement of inhibin A in the second trimester of pregnancy [27]. Unfortunately, inhibin A is practically never used for these purposes in the Czech Republic. Besides the testing of biochemical markers for the purpose of prenatal screening, measurements of specific ultrasound measurements are also used. The most common ultrasound parameter, which is a key part of screening in the first trimester, is the measurement of nuchal translucency (NT), which is evaluated together with the values of free β HCG and PAPP-A [28]. This first-trimester screening is referred to as a combined test and can be performed in different variations [29]. These can differ in the number of ultrasound markers which are entered into the calculations for the

risk results. If a screening is performed in the second trimester and is based solely on the measurement of biochemical markers, it is called a triple test or quadruple test, based on the number of markers used. In the first case, this means testing for HCG, AFP, and uE3. In the second case, inhibin A is added to the testing along with the previous three biochemical markers. The purpose of screenings is to have sensitivity as high as possible while keeping false positives as low as possible. For this reason, it is convenient to integrate the results from the screenings performed in the first trimester with the results obtained in the second trimester. This type of screening is called an integrated test [30].

The overall screening result is routinely presented by numerical risk which indicates that the fetus defect being searched for is present with certain probability. To calculate risk, several kinds of statistical models, which are a part of the computer programs, are used. These models are derived from population studies [31,32]. In these studies, it was discovered that each of the markers used for the detection of a certain disease (in this case Down syndrome) has a particular statistical value. It is also clear that the effectiveness of such screenings is related to the number of screening markers used whether it be biochemical or ultrasound. The result of the screening determines the likelihood of the presence of the disease, but the final answer, whether the disease is actually present or not, can only be given after running a diagnostic test. In regards to prenatal diagnostics, it is a genetic examination of the fetal cells after performing an invasive procedure, either by chorionic villus sampling (CVS) or amniotic fluid collection (AMC). It is evident that the more effective the screening is, the less Down syndrome fetuses are not revealed and the less unnecessary invasive procedures need to be done. At the end of 2011, a new screening test was introduced into clinical practice for the first time and unlike previously performed (conventional) screening tests it was based directly on the analysis of the genetic status of the fetus [33]. This type of test is referred to as NIPT (Non-Invasive Prenatal Testing) or as a prenatal DNA test. The principle of this screening test is based on the analysis of small fragments of extracellular DNA of the fetus, which are found in the mother's bloodstream. These small DNA fragments are formed during the apoptosis of the trophoblast cells and then flow into the bloodstream of the mother. Compared to routine tests (combined and integrated screening tests), NIPT has the highest sensitivity and the lowest false positivity. However, its disadvantage is a larger technological and economic demand and smaller availability. Currently, there are protocols designed to combine conventional screening with NIPT (Reflex DNA testing) [34]. These models are designed to both improve the parameters of conventional screening but, also keep screening economically accessible.

In the Czech Republic, inhibin A is not routinely used as a biochemical parameter in prenatal screenings for Down syndrome. Our goal was to determine how inhibin A could contribute to the improved precision of the screening in two groups of pregnant women who were first screened without inhibin A. The paper describes the proportion of women who were at high risk in the triple test and remained at high risk after adding inhibin A to the screening protocol and similarly the proportion of women who were at high risk in the integrated test and remained high risk with inhibin A as well.

MATERIALS AND METHODS

Groups of pregnant women tested and the calculation of risk

A comparison of Down syndrome screening results was performed in two groups of pregnant women. The first group included women who underwent second trimester screening in the form of a triple test and their screening results were positive – the risk of the presence of the disease in the fetus was higher than or equal to 1:300. A total of 277 pregnant women were tested in this group. The second group of women underwent an integrated test. This group included 91 pregnant women who had positive screening results. We used 1:150 for the positivity cutoff for the integrated test. From the original 373 pregnant women tested, 5 had to be excluded. The reason was that their screening results were positive, but not positive in regards to Down syndrome. The final number in both groups of pregnant women was 368. To determine the risk of Down syndrome we used the software Alpha, which calculates risk in both the first and the second trimester of pregnancy [35]. In the group of pregnant women who underwent a triple test, the biochemical parameters of AFP, total HCG, and uE3 are routinely used for risk calculation. In addition, it is necessary to know the age of the women, accurate gestational age determined by ultrasound, the date of blood sample collection, and the weight of the pregnant women. Two other biochemical parameters - PAPP A and free β HCG - were assessed when defining the risk in the first trimester of pregnancy in the group of pregnant women, who later underwent the complete integrated test. Furthermore, ultrasound measurement of nuchal translucency is performed and added to the calculation of the final risk [36]. Informed consent was obtained from all participants enrolled to the study.

Inhibin A testing and the revised risk calculation for the presence of Down syndrome

For inhibin A testing, we used a Beckman Coulter Access system (Beckman Coulter, Brea, CA, USA) [37]. The Access Inhibin A assay is based on a sequential two step immunoenzymatic ("sandwich") chemiluminescent assay of dimeric inhibin A in human serum and plasma as an aid in the diagnosis and monitoring of various hormonal reproductive disorders. The concentration of inhibin A determined by this kit can be used in combination with HCG, AFP, and unconjugated estriol concentrations to assess the risk of Down syndrome (tri-

somy 21). This approach was verified on the multivariate analysis [32,33,38].
After measuring inhibin A, the risk of occurrence of the disease was recalculated for all results and both sets were statistically compared. In a percentage of the pregnant women, invasive diagnostics were also performed, but in terms of statistical evaluation it was in a small number of cases.
For the purpose of prenatal testing, it is necessary to have the medians specified for inhibin A values for the individual weeks of pregnancy in which the measurements are performed. We used the software Alpha (Version 8, Logical Medical Systems, London, UK) to calculate the risk, which included median values for this methodology.

Statistical analysis
Statistical analyses were performed by using the statistical software package SAS Version 9.3 (SAS Institute, NC, USA). The data was analyzed for normal distribution. The data is presented as median and IQR (interquartile range). We used the Kolmogorov-Smirnov test to decide whether two random samples have the same statistical distribution based on the screening test with and without inhibin A. All tests were two-sided and significance was defined as a p-value of 0.05. The Wilcoxon paired test was used to compare TPR and FPR for the triple test and integrated test.

RESULTS

Of the 368 patients enrolled in the study, 277 (75.3%) were tested using a screening test which is referred to as the triple test (group A) and 91 pregnant women (24.7%) were tested using a screening test which is referred to as the integrated test (group B). For both groups the initial risk of the given screening protocol had solely positive results. In the triple test (group A) the cutoff 1:300 was used and in the case of the integrated test (group B), the cutoff 1:150 was used. The median age of pregnant women in group A was 33.9 years (IQR - 30.2; 36.6); (IQR - Interquartile range). The median of inhibin A was 121.4 pg/mL; (IQR - 88.9, 174.7), median weight was 67 kg (IQR - 60; 76), median gestational age was 113 days (IQR - 109; 116), the median of this risk without inhibin A was 0.01 (IQR - 0.006; 0.025). This means a 1/100 risk for women tested by triple test. After using the results of inhibin A and recalculating the risk, the median of risk was set to 0.003 (IQR - 0.001, 0.008). In group B, the median age was 35.6 years (IQR - 32.9; 37.8), level of inhibin A was 148.2 pg/mL (IQR - 96.2, 208.5), weight was 68 kg (IQR - 32.9; 37.8), gestational age was 112 days (IQR - 110; 115), and the median of this risk without inhibin A was 0.02 (IQR - 0.01; 0.04). This means a risk of 2/100 in the case of patients who had completed the integrated test. After measuring inhibin A in these patients and recalculating the risk, the median of risk was set to 0.006 (IQR - 0.002; 0.022). The results and basic demographic data are summarized in Table 1.
Statistically significant differences were detected in the age of pregnant women (p-value = 0.002) tested by triple test or integrated test and also in the level of inhibin A (p-value = 0.016). There were no significant differences in the weight and gestational age of pregnant women (p-value = 0.866 and p-value = 0.077, respectively).
We recalculated the results of revised risk after adding inhibin A to the screening tests (triple test and integrated test) and then divided them into two categories, which we labeled as „true positive risk“ and „false positive risk“. We chose these labels to compare the risks calculated in the original screening protocols without inhibin A and revised risk in protocols with inhibin A. These labels are in no way related to the false positivity of the screening protocols.

Change in risk after recalculation with inhibin A in the triple test
We discovered that after adding inhibin A and calculating the new risk for the triple test, 125 women (45.1%) remained in the high-risk group and had a risk higher than or equal to 1/300. We labeled this group as „true positive risk - triple (TPRT)“. In the case that the risk was less than 1/300 after adding inhibin A, we labeled this group as „false positive risk - triple (FPRT)“. This group included 152 pregnant women (54.9%) (Figure 1).
The risk median in the group TPRT was 0.09 (IQR - 0.005; 0.025), the median in group FPRT after adding inhibin A and calculating the new risk was 0.0014 (IQR - 0.0007; 0.025), p-value was less than 0.0001 (Figure 2)

Change in risk after the recalculation with inhibin A in integrated tests
We used the same approach in the case of the integrated test where the original risk limit for splitting the results into „positive“ and „negative“ groups was set at 1/150. After the addition of inhibin A, “true positive risk - integrated (TPRI)” was detected in 44 (48.4%) cases. Consequently, the risk after recalculation was still higher than or equal to 1/150. The median of risk for TPRI was 0.024 (IQR - 0.0125; 0.0697). In terms of risk results, which were placed into the group „false positive risk - integrated (FPRI)” after recalculation, the median of risk was 0.0024 (IQR - 0.0013; 0.0036) and this group included 47 (51.6%) pregnant women. (Figure 3 & Figure 4)

DISCUSSION

The aim of this study was to verify whether inhibin A, as the additional biochemical parameter tested during the second trimester of pregnancy, could influence and change the risk distribution of the results of prenatal

Table 1. Basic demographic data of groups tested.

Parameter	Triple test (group A)	Integrated test (group B)	p-value
n	277	91	
Age (years)	33.86 (30.15; 36.56)	35.62 (32.85; 37.76)	0.002
Weight (kg)	67 (60; 76)	68 (61; 78)	0.866
Gestational age (days)	113 (109; 116)	112 (110; 115)	0.077
Risk of D.S. without inhibin A	0.018 (0.006; 0.025)	0.011 (0.011; 0.040)	
Inhibin A (pg/mL)	121.41 (88.88; 174.71)	148.21 (96.21; 208.5)	0.016
Total risk with inhibin A	0.003 (0.001; 0.008)	0.006 (0.002; 0.022)	

Table 2. Verification of MoM in the inhibin A groups measured.

Group of measured samples	Number of samples in the group	MoM
1	42	0.96
2	25	1.05
3	17	1.00
4	60	1.02
5	117	0.92
6	112	1.01
Total	373	0.96

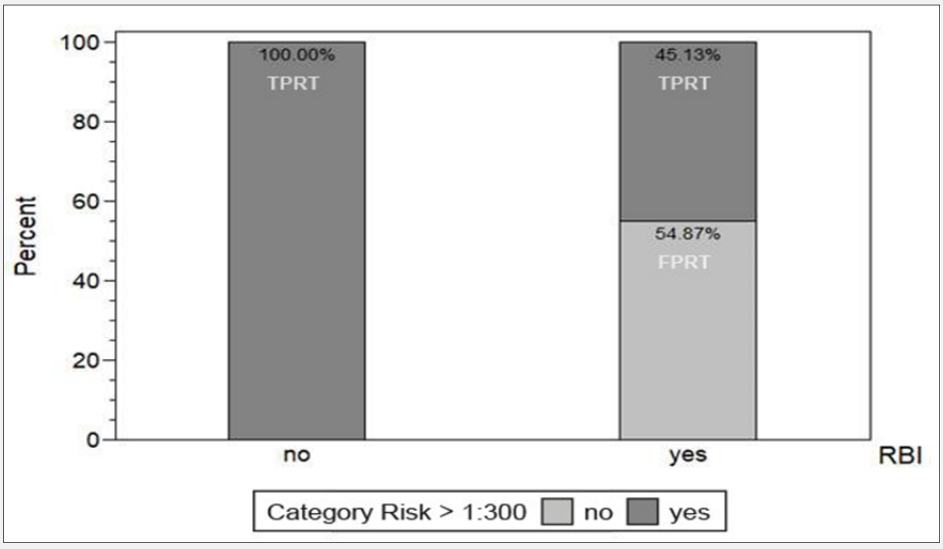

Figure 1. Stacked bar chart of the triple test.

The left column shows all observations with a risk greater than or equal to 1/300 (valid for a protocol without inhibin A). The right column indicates the revised risk with inhibin A and the ratio of patients whose risk is higher than 1/300 (TPRT) to the patients whose risk after recalculation with inhibit A is already less than the cut off 1/300 (FPRT).

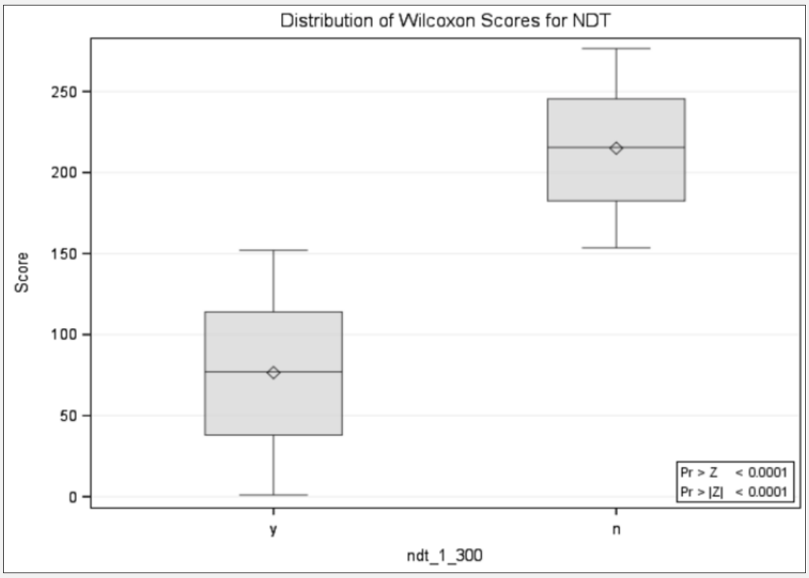

Figure 2. The boxplot of scores (the order of sorted values) of the Wilcoxon two-sample test.

Ndt_1_300 indicates the risk with inhibin A in the triple test. The graph is categorized by the variable of false positive risk where y = risk was less than the set level of 1/300 (FPRT), n = risk was higher than the set level of 1/300 (TPRT). It is noticeable from the graph that the lower the order of the given value is, the lower the risk is. The average value of FPRT order was 76.5 and for TPRT the average value was 215. Splitting risk into two groups (FPRT vs. TPRT) after recalculation with inhibin A, clearly shows that this parameter has a statistically significant effect on risk assessment.

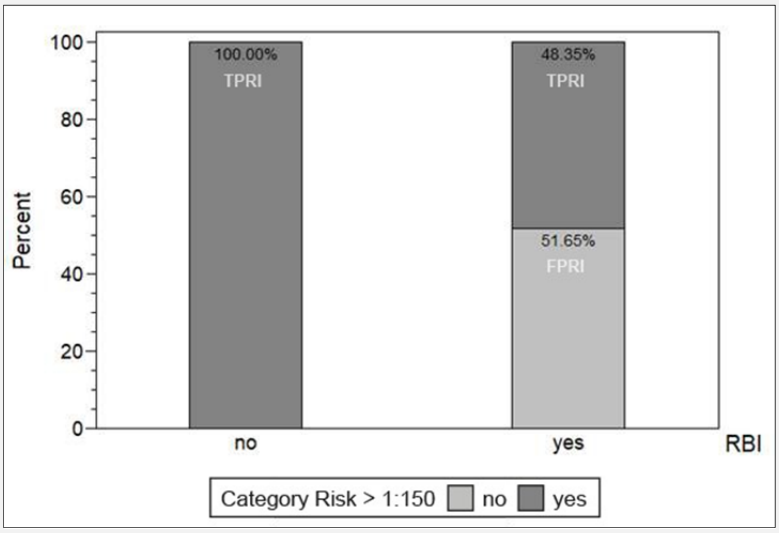

Figure 3. Stacked bar chart of the integrated test risk.

The left column shows all observations with a risk higher than or equal to 1/150 (valid for protocols without inhibin A). The right column indicates the revised risk with inhibin A and the ratio of patients whose risk is higher than 1/150 (TPRI) to the patients whose risk after recalculation with inhibin A is already less than the cutoff 1/150 (FPRI).

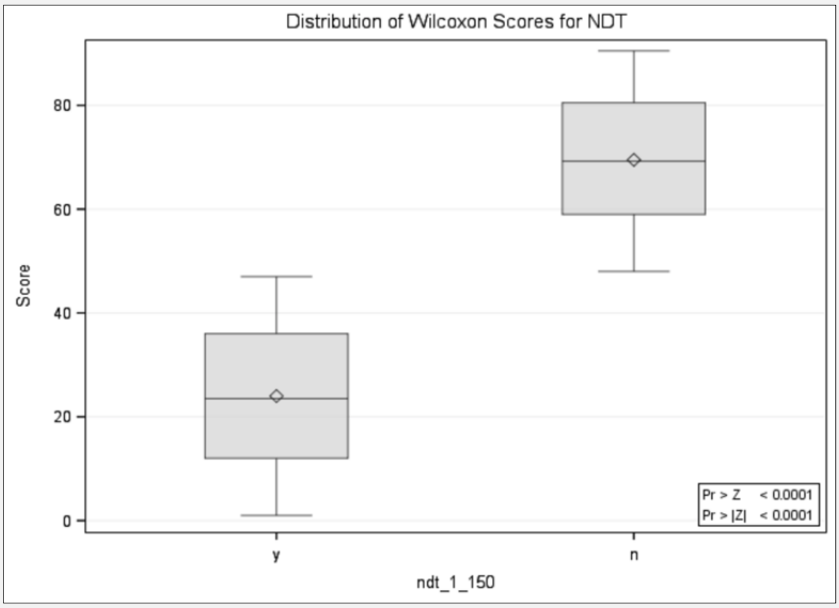

Figure 4. The boxplot of scores (the order of sorted values) of the Wilcoxon two-sample test.

Ndt_1_150 indicates the risk with inhibin A in the integrated test. The graph is categorized by the variable of false positive risk where y = risk was less than the set level of 1/150 (FPRI), n = risk was higher than the set level of 1/150 (TPRI). Also, from the graph similar to the graph of the triple test, it is clear that the lower the order of the given value, the lower the risk. The average value sequence of FPRI was 24 and TPRI was 69.5. In this case, after adding inhibin A to the screening protocol a significant risk distribution occurred.

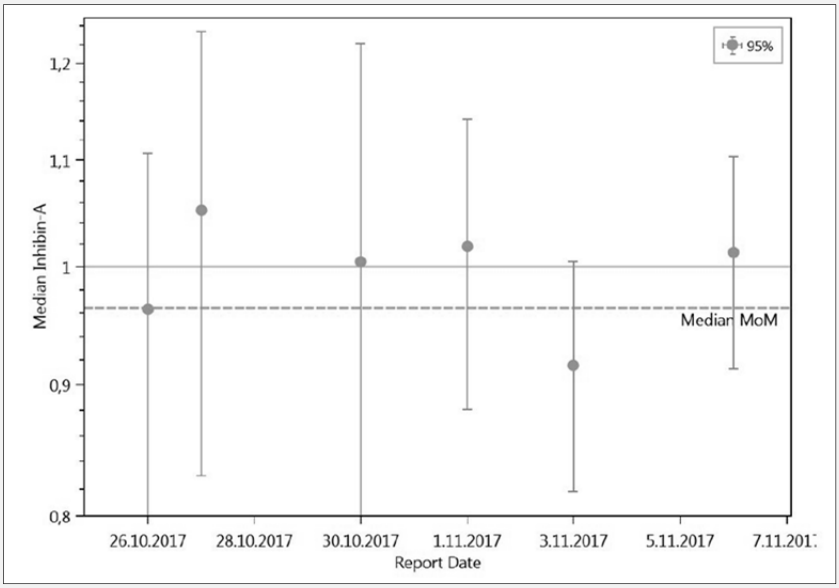

Figure 5. Analysis of MoM in the tested groups for inhibin A using Alpha software - output from the statistical module of the software.

screening for Down syndrome in a high risk group of pregnant women. The presented paper does not provide evidence that adding inhibin A reduces the number of positive results and thus serves as a significant screening marker.

To confirm this evidence, evaluation of the proportion of women who were low risk using the two tests without inhibin A and who became high risk after the addition of inhibin A, need to be performed. The role of inhibin A as marker was previously published [31,39]. Assessing the effectiveness of the screening protocol is done by comparing sensitivity, false positivity, and finally by expressing the positive predictive value of the screening test.

We performed our sample measurements in 6 runs and always used Alpha software to verify the median value of the selected group. The program allowed us to evaluate the quality of biochemical measurements performed after calculating the risk for the presence of Down syndrome and in regards to this, all measurements met the required criteria and were within the 95% interval of reliability. The example of the evaluation is listed in Figure 5.

The multiples of median (MoM) for each group tested are shown in Table 2. The total MoM of all measurements made was 0.96. Since the inhibin A values obtained met the qualitative assumptions for inclusion in prenatal screening, the inhibin A values measured were used to calculate the revised risk of Down syndrome as is described in the Results section.

Screenings should reveal fetuses affected by the disease in as many pregnancies as possible, but on the other hand unnecessary other tests should not be carried out on the basis of a false positive result. A very high percentage of false positive results are an undesirable phenomenon when performing any type of screening. Our small study was designed to verify which part of positive results from the prenatal screenings for Down syndrome would change to negative after adding inhibin A. The results confirmed that by adding inhibin A to both the triple and integrated tests, the number of positive results were significantly reduced and thus also the number of unnecessary invasive procedures and related genetic analyses. Due to the size of the tested groups and organization of the study, the intention was not to prove how sensitivity and false positivity of the studied screening protocols change. For this purpose, several thousand pregnancies would have to be examined, including cases with proven fetal damage and the entire study would have to contain screening results of the entire population, not only the ones with positive results. All cases of affected fetuses would have to be identified with the help of prenatal diagnostics or by monitoring the genetic status of the children after delivery. Invasive diagnostics were performed and were based on the results of screenings without inhibin A on a some of the pregnant women in our study. In some of the pregnancies we were able to obtain information about the baby's health after birth. Unfortunately, this was only in a few cases as described below.

Invasive diagnostic testing

When screening results come back positive, pregnant women are offered to undergo invasive diagnostic testing. Invasive diagnostic testing was performed on 56 women from a group of patients who underwent the triple test. After adding inhibin A to the risk calculation and splitting the women into two groups, with a risk higher than 1:300 (TPRT) and less than 1:300 (FPRT), there were 28 women in both groups who underwent invasive diagnostic testing. In the TPRT group, two pregnancies with Down syndrome were confirmed, the triple test and quadruple test (triple test with inhibin A) accurately revealed pregnancies with an affected fetus. In the second group FPRT which initially had a positive risk, the risk changed to negative after adding inhibin A and the invasive diagnostic test detected an affected fetus in one pregnancy. In this case, risk recalculation with inhibin A provided an incorrect negative result which was significantly influenced by a high level of AFP.

Invasive diagnostic tests were performed on 39 women from a group of patients which underwent the integrated test. After adding inhibin A to the risk calculation and splitting the women into two groups, with a risk higher than 1:150 (TPRI) and less than 1:150 (FPRI), invasive procedures were performed on 21 pregnant women from the first group and 18 women from the second group. In the group TPRI, two pregnancies with Down syndrome were confirmed. In this case, the integrated test and the integrated test with inhibin A correctly revealed pregnancies with an affected fetus. In the second group FPRI which initially had a positive risk, the risk changed to negative by adding inhibin A and the invasive diagnostic test detected an affected fetus in one pregnancy. In this case, risk recalculation with inhibin A provided an incorrect negative result which was evidently affected by a clearly negative ultrasound examination.

CONCLUSION

The presented study has proven that the use of inhibin A, as an additional biochemical parameter, has a significant effect on the distribution of calculated risks in determining the presence of Down syndrome in the triple and integrated tests. Reducing the number of false positive results affects the quality of prenatal testing and this can reduce the number of unnecessary invasive procedures. If a conventional screening test was used in combination with non-invasive DNA testing, better stratification of risk for the presence of genetic disorders could contribute to a more effective use of NIPT. However, it is clear that for the overall objective assessment of inhibin A efficiency in screening protocols, a much larger general population of pregnant women would have to be tested. Information about the presence

or absence of the genetic disease in the fetuses of examined pregnancies also needs to be evaluated, either during the pregnancy or after the birth.

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Declaration of Interest:
The authors state that there are no conflicts of interest regarding the publication of this article.

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