AMNIOTIC FLUID SPECIFIC BIOCHEMICAL PARAMETERS FOR PRENATAL DIAGNOSIS OF MUCOPOLYSACCHARIDOSIS IN NON-IMMUNE HYDROPS FETALIS

By

Iman M. El-Shabrawy., Fagr B. El-Shahat, Amina Ahmed Bauomy., and Hamed M. Yossif*

From

Medical Biochemistry and Obestatrics & Gynacology Departments Faculty of Medicin, Mansoura University*

ABSTRACT

52 amniotic fluid (AF) of women at (18-22 weeks) of pregnancy (10-15ml each were sampled because of abnormal ultrasound findings mainly non-immune hydrops fetalis (N.I.H.F). They were analysed by a procedure involving AF supernatant analysis for total glycosaminoglycans (Total GAG) by formation of complexes with Quinacrine reagent and the pregnancies at increased risk of mucopolysaccharidosis (MPs) i.e with increased total GAG (over 100 mg/l) (12 cases) were enzymatically assayed for a group of enzymes : N-acetyl-B-glucose aminidase (NAGA), β-glucuronidase (B-GLA), β-galactosidase (B-GALS) and Aryl sulfatase (ASA) by measuring the fluorescence of the corresponding 4-methylumbelliferyl- glycoside to each one. Eight cases of different types af MPs were diagnosed. The reported procedures allowed detection of 4 cases of type VII MPs, 2 cases of type VI MPs, 1 case of type III B MPs and 1 case of type IVB MPs. 4 cases of unclassified MPs were found. Because of the poor prognosis of MPs fetuses so the need for a reliable chemical method for estimation of total GAG in AF is the goal of our study. We aimed to know if that Quinacrine method is a potentially valuable analytical tool in the prenatal diagnosis of MPs as a primary step, which must be followed by enzymatic activities assay of expected defective enzymes in AF to detect the type of MPs. This study showed that Quinacrine method is the best chemical method for total GAG-

AF determination with subsequent enzymatic assay for MPs subtyping However, enzymatic assay for subtyping of MPs is an area of research which must have more attention and study efforts and this study is a trial to be one of these efforts.

INTRODUCTION

Mucopolysaccharidosis are genetic diseases resulting from the deficiency of one of the enzymes involved in the catabolism of glycosaminoglycans leading to their accumulation in lysosomes (Tokiedak et al., 1998). Ten enzyme deficiencies in six different clinical types of MPs are known (Table I) (Piraud et al., 1993).

The aetiological mechanisms leading to non-immune fetal hydrops are complex and their impact are variable at different stages of gestation (Jauniaux, 1997).

MPs is suspected before delivary by fetal ultrasound examination which shows scalp oedema, cardiomegaly, hepatomegaly and/or ascites. In addition clinical symptoms of MPs do not appear immediately after birth. Different types of MPs represent a remarkable percentage of non-immunehydrops fetalis after exclusion of other causes. Chemical examination and enzymatic assay of AF obtained by an amniocentesis could be performed to confirm or rulout this hypothesis. So, prenatal diagnosis is more specific (Piraud et al., 1996).

Contribution of fetal urine to the composition of amniotic fluid varies with gestational age; At 12 weeks of gestation, the fetal kidney begins to be functioning. Affected fetuses excrete storage substrates in urine, thus methods used for screening these disorders in urine can be used in AF supernatant allowing the prenatal diagnosis of MPs after 16 weeks of amenorrhea (Piraud et al., 1996).

The precise prenatal diagnosis of MPs relies on enzymatic diagnosis which are often tedious and expensive and must therefore be preceded by the study of AF total GAGs by a simple rapid, sensitive and specific chemical method (Piraud et al. 193)

Thus, the first step in the diagnosis of MPs relies on total GAG determination in AF. Old qualitative tests as spot test and precipitation test as well as quantitative tests reported high incidence of falsy (Berry, 1987). In addition electrophoretic separation of

GAG not only is time consuming but, it also doesn't allow subtypes of MPs to be distinguished, some abnormal bands (KS) can not be visualized as it migrates with other compounds (C.S) Also the expected electrophoretic pattern can not be obtained in some cases as the electrophoretic pattern may be variable (Piraud et al., 1993).

The method presented in this study is based on the formation of complexes with Quinacrine reagent which is simple, fairly, specific for GAG and it added the advantage that it also measures KS, which could not be detected by earlier tests (Mitra and Blau, 1978).

As screening of MPs has prenatal described presentations more than postnatal analysis so, the aim of this work is to predict the base of a schadule for prenatal diagnosis of MPs by a primaly simple, rapid, sensitive, specific and unexpensive chemical method which can be followed by enzymatic diagnosis for subtyping in positive cases.

MATERIAL AND METHODS

In this study 52 AF samples were obtained by transabdominal amniocentesis according to abnormal ultrasound findings (scalp oedema, cardiomegaly, hepatomegaly and/or ascitis) at 18-22 weeks of pregnancy of females aged between 23-34 years. Out of them 12 cases of suspected MPs were detected due to increased total GAG. A sample of 10-15 ml AF was obtained from each woman enclosed in the study from obestatrics and Gynacology Department of Mansoura University Hospital. Another 10 AF samples from women at (18-22 weeks) of pregnancy with matched age referred for amniocentesis for Fetal examination tests were used as controls. Freshly obtained AF vas centrifuged at 2000 rpm for 10 min and the clear supernatant was separated and stored at -20°C until used.

The selected samples according to increased AF level of total GAG were classified as MPs risk group, in addition to control group so this study included 2 groups:

I-Control group: included 10 women with AF of normal total GAG level.

2-MPS risk group: included 12 women with AF increased total GAG level.

Samples were analysed for:

-Total GAG: according to the method of Mitra and Blau, (1978).

The principle, of this method is based on the formation of complexes with quinacrine reagent (Scott, 1973). The quinacrine reagent chemicals were supplied by sigma chemical Co, London and used at conc. of 2.5 g/L at PH 4.4. The AF samples do not have to be deproteinized. A reagent blank of distilled water, carried through the entire procedure and chondriotin sulfate standard were used. Absorbance was measured spectrophotometrically at 450 nm.

Thel total GAG concentration of each AF sample was calculated from the absorbance of test sample A (T), of the blank A (B) and of the standard A (s) according to the following relationship .

$$\text{Total GAG} = \frac{A\,(T) - A\,(B)}{A\,((S) - A((B)} \times 100 \text{ mg/ L}$$

AF samples with total GAG around the control range (7-62mg/l) (40 cases) were not included in enzymatic assay as the presence of MPs is not suspected as well as to save the chemicals for cases with higher ranges of total GAG and fairly suspecte d MPs. These cases were directed to Obestatric and Gynacology Department of Mansoura University Hospital to be under clinical follow up to study causes of abnormal ultrasound findings, which was previously detected, other than MPs.

AF samples with increased GAG concentration (MPs risk group) were enzymatically assayed according to the method of Cascola et al., (1979). The assayed enzymes and the condition of each enzyme assay are given in table 2).

Substrates were supplied by koch light Ltd. In all enzyme assays, diluted AF samples were incubated at 37°C with 50 µl of substrates in 0.2 M buffer for the appropriate length of time after which 2.0, ml of 0.2 M potassium carbonate were added to stop the reaction and to develop the fluorogen. Blanks were used in this study to control for non-enzymatic hydrolysis of substrate which was found to be negligable. Enzymatic activities were determined by using the corresponding 4-methyl-umbelliferylglycoside. The fluorescence of released 4- methyl-umbelliferone was measured by fluorometer at 365 nm excitation and 445 nm emission.

The result was compared with fluorescence from standard 4methylumbelliferone. All enzyme activities are reported as n moles of substrate transformed /ml of AF under the conditions stated in Table (2).

Statistical analysis was done by SPSS computer package using students t test to compare between the groups and P value <0.05 was considered significant.

RESULTS

The results of this study showed that there is a very highly significant increase of total GAG levels in AF samples of (18-22 weeks) pregnancy, in MPS risk group when compared to its level in control group as shown in table (3) and Fig. (1).

AF samples which revealed total GAG around the control range (7-62 mg/L) but have abnormal ultrasound findings (40 cases) were throughly clinically examined for other causes of congenital abnormalities or other metabolic diseases at Obestatrics and Gynacology Department

Some cases (8 cases) were undiagnosed and the other cases were having variable degrees of congenital and metabolic diseases. 18 cases undergone abortion, 10 cases undergone preterm delivary and the offsprings were died within a week after birth. 2 cases had stillbirth after full term, 6 cases undergone medical termination of pregnancy and 3 cases had normal labours with ill off springs and one healthy offspring.

The mean values of total GAG were variable in each group. However, we noticed that moderate degrees of total GAG (41.7-61.9 mg/l) more obvious in (undiagnosed, abortion, preterm delivary and still births groups) as shown in table (4) and Fig. (2) .

Also this study showed that there is a considerable difference of enzymatic activities in AF samples of (18-22 weeks) of pregnancy of control group when compared to MPs risk group.

According to the type of MPs, it was found that the deficiency of enzymatic activity is obvious in one of the assayed enzymes. There were 4 cases deficient in B-GALA (type VII), 2 cases deficient in ASA (Type VI), I case deficient in NAGA (Type III B), and I case deficient in B-GALS (type

IVB). However there were 4 cases of unclassified MPS (suspected by increased levels of AF total GAG) as hown in table (5) and Fig. (2)

Table (I) : Classification of mucopolysaccharidoses .

MPs	Type	E nzymatic defect	Storage products
I	H	α-L-Iduronidase	Dermatan sulfate (DS) and
	S		Heparan sulfate (HS)
II	Hunter	Iduronate sulfatase	SD+HS
III	A	Heparan N-sulfatase	HS
	B	α-N-acetyl glucosaminidase	
	C	Acetyl-CoA α glucosamine N-acetyl transferase	
	D	N-Acetyl glucosamine-6-sulfatase	
IV	A	N-Acetyl galactosamine-6-sulfatase	Keratan sulfate (KS)
	B	β-galactosidase	KS+ galactosides
VI	Maroteaux	Aryl sulfatase	DS
	lamy		
VII	Sly	β-Glucuronidase	DS, HS, chondroitin sulfate
			(CS) or CS only

Quated and modified from Piraud et al., (1993)

Table (2) : Conditions of enzyme assay .

Enzyme	Concentration (mM)	Substrate	Buffer	PH	AF dilution	Incubation time (min)
1) N-Acetyl glucos-aminidase	2.0	4-Mu-2 acetamide-2-deoxy-B-D glucopyranoside	Acetate	4.5	1:10	30
2) -Glucuronidase	5.0	4-MU-B-D glucuronide trihydrate	Acetate	4.0	1:5	30
3) - Galactosidase	1.0	4-MU-B-D galactopyranoside	Acetate	4.0	1:5	60
4) Arylsulfatase	10.0	4-MU-Sulfate potassium salt	Acetate	5.8	1:5	30

Table (3) : Total GAG levels (mg/L) of (18-22 weeks) of pregnancy AF samples in control and MPs risk groups . .

		Total GAG
Control group (n = 10)	Range	6.7-41.6
	Mean	18.8
	SEM	1.94
MPS risk group (n = 12)	Range	102.3-171.2
	Mean	161.7
	SEM	15.9
Student's t		8.09
P		<0.001

P<0.001 is considered very highly significant.

Table (4) : Total GAG levels (mg/L) of (18-22 weeks) of pregnancy AF samples in different groups of prognosis of pregnancy in relation to number of undiagnosed cases .

Group	No of cases	Total GAG	No of undiagnosed cases
Abortion	18	Range 29.3-57.9 Mean 41.9 SEM 2.8	2
Preterm delivary with death of off springs	10	Range 34.2-61.9 Mean 39.5 SEM 3.2	2
still birth after full term	2	Range 41.7-89.4 Mean 45.5 SEM 3.3	1
Medical termination of pregnancy	6	Range 13.5-24.9 Mean 18.9 SEM 1.96	1
Normal labour with ill off springs	3	Range 9.1-23.6 Mean 16.5 SEM 1.8	1
Normal labour with Healthy off springs	1	9.2	1

Table (5) : Enzymatic activities levels (nmol/ml) of (18-22 weeks) of pregnancy AF samples in MPs risk and control groups .

Case no	N-Acetyl glucosaminidase (NAGA)	β-D.Glucuronidase (β-GLA)	β-Glucosaminidase (β-GALS)	Aryl sulfatase (ASA)	Diagnosis
1	6.1	10.48	0.82	0.02*	MPS VI
2	6.8	0.43*	0.78	0.32	MPS VII
3	7.1	18.9	0.85	0.33	Unclassified MPS
4	6.7	39	0.99	0.29	Unclassified MPS
5	7.2	0.03*	0.73	1.01	MPS VII
6	6.6	20.2	0.02*	0.89	MPS IVB
7	5.9	14.3	0.55	0.3	Unclassified MPS
8	6.3	0.35*	0.66	0.35	MPS VII
9	0.93*	16.3	0.44	0.83	MPS III B
10	7.0	0.02*	0.32	1.2	MPS VII
11	5.9	32	0.51	0.05*	MPS VI
12	6.4	12.8	0.77	0.42	Unclassified MPS
Controls	Range 5.9-7.2	5.2-120	0.53-1.3	0.29-1.2	
n=10	Mean 6.99	21	0.91	0.83	
	SEM 0.73	3.1	0.19	0.04	

* = deficient enzyme (DE) mean DE = 0.20 mean DE = 0.035

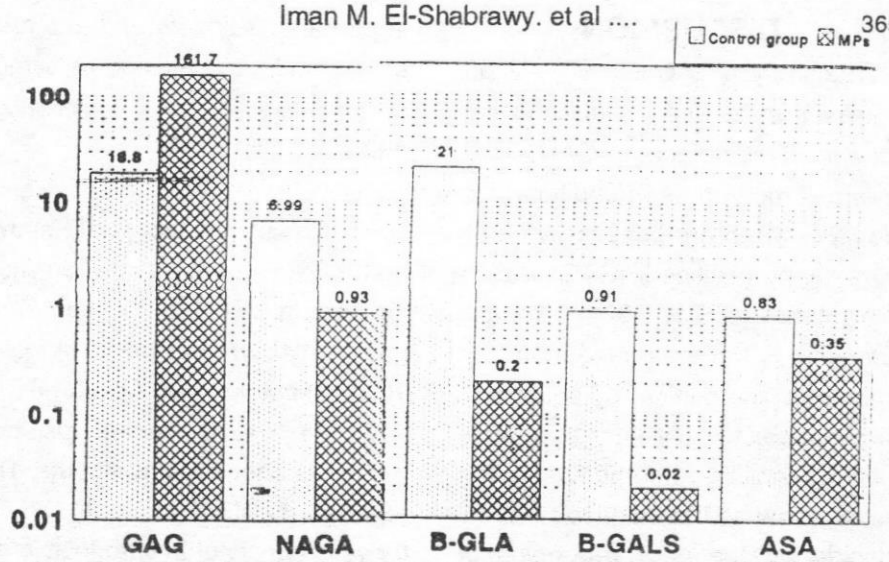

Fig. (1) : Total GAG (mg/L) and mean enzymatic activities (nmol/ml) in AF of (18-22 weeks) pregnancy samples in control group and different types of MPs.

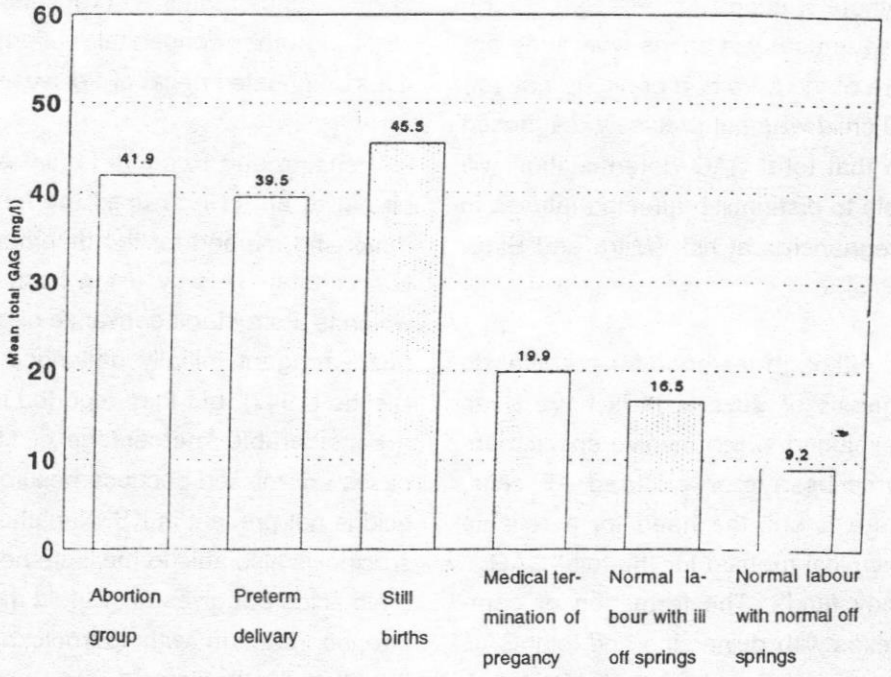

Fig. (2) : Total GAG mean level (mg/l) of (18-22 weeks) of pregnancy AF samples in different groups of prognosis of pregnancy .

DISCUSSION

The study of GAG is of interest because a number of pathological conditions involves defects in GAG metabolism. Among these conditions are the MPS which are genetically determined abnormalities in GAG catabolism (Mitra and Blau 1978). Prenatal diagnosis is the most significant approach as the clinical symptoms of MPS appear usually after a variable delay after birth. In some cases, the results allow a MPs diagnosis and by considering the poor prognosis of these MPs fetuses in utero so, it is a strong indication for medical abortion without waiting (Piraud et al., 1996). This is useful in cases where the nature of the MPs of a previously affected child was not precisely diagnosed so that total GAG determination will help to distinguish affected fetuses in pregnancies at risk (Mitra and Blau, 1978).

Although methods for prenatal diagnosis of several MPs have been developed which involve specific enzyme assays on cultured AF cells, there is still the need for a reliable chemical method for the total GAG in body fluids. The formation of complexes with quinacrine and total GAG is a specific method for GAG including keratan sulphate and it is also a simple and rapid method as samples do not have to be deproteinizied (Mitra and Blau, 1978).

The resuts of this study showed that there is a very highly significant increase in AF of (18-22 weeks) pregnancy total GAG in MPs risk group. This result agree with Mitra and Blau, 1978 who tried Quinacrine method in routine use for several months. They reported that they found it a straight forward consistent reproducible and reliable method. They also found that total GAG were not raised above the normal concentration in pregnancies involving other congenital malformations or inherited metabolic diseases .

Fensom and Bengom (1994) and piraud et al., (1996) used the hexuronic acid method for the determination of total GAG by using Harmine which is a structural derivative of carbazol reagent initially described by Dische (1947), but they reported that a considerable percentage of MPs cases are missed because hexuronic acid is not present in KS. Naphthoresorcinol is also able to measure hexuronic acids but gives an 18-fold more intense reaction with iduronic acid than the glucuronic acid. Thus naph-

thoresorcinol is more specific for DS which accounts for the highest content of iduronic acid among the GAGs (piraud et al., 1993). So the Quinacrine medod in our openion can be considered the chemical method of choice.

In this study AF samples which revealed total GAG around the control range 7-62 mglL but have abnormal ultrasound findings (40 cases) were followed up clinically and their prognosis were variable i.e. 8 cases were undiagnosed, cases ended by abortion, preterm delivary with death of off springs within a week after birth, cases had still births after full term, cases undergone medical termination of pregnancy, cases had normal labour wiith ill off springs and a healthy off spring. This can be explained by the detection of other congenital malformation and other metabolic disease (Piraud et al., 1993).

However, it was noticed that some of the worst prognosis had moderate degrees of total GAG (41.7-61.9 mg/L) (undiagnosed, abortion, preterm delivary and still births groups). As regard that total GAG do not increase above the normal concentration in pregnancies with other congenital malformation or inherted metabolic diseases (Mitra and Blau, 1978). This can be explained by the possibility of presence of MPs but total GAG is moderate because of contribution of fetal urine in AF did not reach its maximum as, it differs with gestational age (Piraud et al., I 996), or the defective enzyme is not very low, or may the decrease in the defective enzyme is masked by the increase of another one.

In fact, the moderate degrees of total GAG increase were better to be assayed by enzymatic subtyping, but because of the shortage of chemicals, we prefered to save it to the most suspected samples and we hope to complete this study by another work focused on moderate degrees of total GAG AF increase as soon as we can.

In this study the activities of 4 different enzymes which are defective in different types of MPs were assayed. To our knowledge, studies on these enzymes are very scanty and it may be the first report of them with the excepsion of B-GLA. Our results concerning B-GLA (type VII MPS) showed a considerable decrease in 4 cases (No 2, 5, 8, 10), these results agree with Van Eyndhoven et al.,

(1998) and Nelson, (1997) who stated that chorionic villus sampling was performed in the 11 th week of pregnancy and B-GLA deficiency indicated that the fetus was affected and after termination of pregnancy in the 12th week, signs of early hydrops fetalis were observed. As well as Molyneux et al., (1997) and Van Dorpe et al., (1996) who confirmed these results by β-GLA assay of cultured fibroblasts which showed markedly deficient β-GLA activity in a more advanced stage of pregnancy after fetal death. Kagie et al., (1992) demonstrated AF deficiency of β-GLA at 25 week of gestation and in the fibroblasts of the fetus which were cultured after fetal death. In addition the results of Piraud et al. (1996) who examined β-GLA in the AF of 20-34 weeks of gestation by electrophoresis which also agree with our results. By electrophoresis Piraud et al., Found increased CS Fraction and abnormal DS and CS bands in the cases with deficient β-GLA.

As regard NAGA (type III B MPs), our results showed a considerable decrease in one case (No. 9) we could not compare it with other results as we found only one reference characterized NAGA electrophoretically from placental extracts from uncomplicated term gestations (Huddleston et al., 1971) and they got two bands (A&B) .

β-GALS (type IV B MPs) was found decreased in one case (No. 6) and ASA (type VI MPs) was found decreased in 2 cases (No 1 & No 11) .

Four cases (No 3,4,7,12) were not classified, this may be explained by the fact that there are six different clinical types of MPs are known with ten enzyme deficiences (Piraud et al., 1993). So, these cases may have defects in enzymes out of the scope of this study.

In conclusion, the prenatal diagnosis of the MPs rests on the prenatal screening for total GAG. This study showed that Quinacrine method is the best chemical method. As soon as increased total GAG is detected, typing of MPs should be done by assaying enzyme activities.

Assaying of enzymatic activities should have much attention in MPs, as this part is poorly studied and needs more effort so that pregnancies at risk could be controlled.

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الملخص العربى

مؤشرات بيوكيميائية نوعية للسائل الأمنيوس لتشخيص مرض أختزان السكريات المتعدده قبل الولادة فى حالات أستسقاء الرأس الغير مناعى للأجنة .

لقد تم أخذ ٥٢ عينة سائل أمنيوس من سيدات فى الأسبوع ١٨ إلى ٢٢ من الحمل (١٠-١٥ مل للعينه) نظراً لوجود ظواهر غير طبيعية عند الفحص بالموجات فوق الصوتية وخاصة الحالات المشتبه فى تشخيصها كأستسقاء الرأس الغير مناعى وقد تم تحليل الطافيه من العينة لتحديد الكمية الكلية للجلوكوز أمينو جلايكانز باستخدام قياس المركبات المتكونة مع كاشف الكويناكرين وكذلك تم قياس نشاط الأنزيمات فى المجموعة التى وجد بها إرتفاع فى مستوى الجلوكوز أمينو جلايكانز الكلى (أكثر من ١٠٠ مل/لتر) وهى المجموعة المعرضة لخطر مرض أختزان السكريات المتعددة (١٢ حالة) وقد تم قياس نشاط مجموعة إنزيمات وهى: ن-أستيل - بيتا جلوكزا ميندار وبيتا - جلوكروناياز وبيتا جالاكتوسايداز والاريلسلفاتيز باستخدام القياس الوميضى. وقد تم تشخيص ٨ حالات من الأنواع المختلفة لمرض أختزان السكريات المتعددة وهم ٤ حالات من النوع السادس وحالتان من النوع الخامس وحالة من النوع الثالث - ب - وحالة من النوع الرابع -ب- وبالأخذ فى الأعتبار بخطورة أصابة الجنين بهذا المرض وضرورة إكتشافه قبل الولادة لذا فقد أصبح إيجاد طريقة كيميائية موثوق بها لتحديد مستوى الجلوكوز أمينو جليكانز الكلى أمر ملحاً. وقد تم إقتراح طريقة الكويناكرين فى هذه الدراسة كأداة تحليلية قيمة وقادرة على التشخيص قبل الولادة كخطوة مبدئية والتى يجب أن يتبعها قياس نشاط الأنزيمات المختلفة فى السائل الأمنيوس لتحديد نوع المرض. ولقد وجد أن هناك نقص فى البحث العلمى فيما يتعلق بهذا المرض وأكتشافه قبل الولادة ولذا يجب أن يحظى بمزيد من الأهتمام والجهود البحثية وهذه الدراسة تعتبر محاولة فى زيادة هذه الجهود .