

Diagnostic utility of chitotriosidase versus total acid phosphatase activity, ferritin and globulin in screening for Gaucher disease

ABSTRACT

Aims: Gaucher disease "GD" is one of the most common glycolipid storage disorders. Several circulating molecules thought to be secreted by Gaucher cells have been studied as biological markers of disease. We investigate and compare the validity of using plasma chitotriosidase "ChT", total acid phosphatase activity, ferritin, and globulin in predicting pediatric patients with GD.

Methodology: A cross sectional case control study, carried out on 49 pediatric patients (29 GD patients and 20 patients suspected to have GD), and in addition to 20 healthy age and sex matched controls. Complete blood counts and liver functions and plasma protein electrophoresis were routinely done for all the studied groups. ELISA assays of plasma ChT and ferritin, colorimetric assay of plasma total acid phosphatase activity and mutation analysis using commercially available strip assay kit after PCR target gene amplification were done.

Results: Significantly higher plasma ChT and total acid phosphatase in GD patients versus the suspected GD patients (P-value <0.05), both markers have the highest AUC, sensitivity and specificity.

Conclusions: Plasma ChT and total acid phosphatase activity, both together are more reliable than ferritin and globulin in screening for GD.

Keywords: Chitotriosidase; total acid phosphatase activity; ferritin; globulin; Gaucher disease screening.

1. Introduction

Gaucher disease "GD" is one of the most common glycolipid storage disorders, caused by a genetic deficiency of lysosomal β - glucocerebrosidase, encoded by GBA gene, leading to accumulation of the substrate glucocerebroside in the cells of the macrophage- monocyte system [1].Glucocerebroside engorged cells, termed Gaucher cells, infiltrate various organs [2], leading to multisystem organomegaly, pancytopenia and bone complication[3]. Molecular genetic analysis is the standard for confirmation of the diagnosis of GD, and offers certain advantages over glucocerebrisdase activity assays, including superior DNA stability, and accurate carrier status determination, and potential to establish certain genotype/phenotype correlations[4].

Several circulating molecules thought to be secreted by Gaucher cells have been studied as biological markers of disease burden[5]. Among these molecules, chitotriosidase, acid phosphatase and ferritin [1-6].Chitotriosidase enzyme (ChT; EC 3.2.1.14) is a human chitinase member of family 18-glycosyl-hydrolases that catalyzes the hydrolysis of natural chitin, which is remarkably homologous to chitinases from plants, bacteria, fungi, nematodes and insects[7], secreted by active tissue macrophages[8]. The clinical utility of plasma ferritin and total acid phosphatase activity as GD biomarkers is still debated [9,10]. The infiltrated reticuloendothelial organ such as Gaucher spleen, may contribute to the production of monoclonal immunoglobulin [11], with overall increase in the plasma globulin levels.

Research on various biochemical markers of Gaucher disease will provide insight into the pathophysiology of the disease and allow the clinicians to pick up GD

cases early. A collection of plasma biomarkers in the form of plasma ChT, total acid phosphatase activity, ferritin and globulin were studied to test which of them, either single or in combination with another, will be the best predictor/s and can be used as primary screening marker/s for GD before making mutation analysis.

2. Materials and methods

2.1. Study population

The present study is a cross sectional case control study, was carried out on 49 pediatric patients divided into two groups; Group A (29 GD patients) (mean age 6.20 years $\pm$ 5.88 SD) (16 males, 13 females), Group B(20 pediatric patients have the inclusion criteria of Gaucher disease) (mean age 6.04 years $\pm$ 4.99SD) (9 males, 11 females), in addition to 20 apparently healthy children, age and sex matched selected as control group (group C) (mean age 8.07 $\pm$ 5.50SD) (17 males, 13 females). They were recruited from the pediatric outpatient clinics and inpatients Pediatric departments of Assiut and Qena University hospitals, which are of the major tertiary referral pediatric hospitals in Upper Egypt according to the inclusion criteria and after approval of the university hospital ethical committee in accordance with the Declaration of Helsinki. Prior to initiation of the study; every subject and his/her parents were informed about the aim of the study and gave a written consent. The study was carried out during the period from January 2015 to May 2016.

Inclusion criteria [12]; clinically: unexplained isolated splenomegaly or hepatosplenomegaly with skeletal abnormalities (bone pain or spontaneous fractures) associated with or without delayed physical and /or mental development and/ or neurologic abnormalities. Laboratory: anemia and/or thrombocytopenia to be confirmed by the mutation analysis.

2.2. *Laboratory measurements*

Complete blood counts "CBC", albumin, globulin, alkaline phosphatase "ALP", bilirubin (total and direct), liver enzymes (ALT and AST) were taken already from the file of the patients. Complete blood counts "CBC" were measured using (Cell Dyn 1800-Abbott diagnostics, Germany). Liver function tests including albumin, globulin, A/G ratio, alkaline phosphatase "ALP", bilirubin (total & direct) and liver enzymes (ALT & AST) were estimated using [Cobas C311 (Roche diagnostics, Germany)].

Five cc of venous blood was drawn from the included children and divided into two tubes: 3 cc on EDTA tubes, centrifuged at 3500 rpm for 15 min and the separated plasma from each tube was stored into aliquots using 1 ml cryotubes at- 20° C until biochemical analysis (for ferritin, total acid phosphatase activity and ChT assays in all studied groups and plasma protein electrophoresis in group A only). 2 cc on another EDTA tubes, stored as whole blood at- 20° C till the time of mutation analysis using strip assay method.

Using commercially available enzyme-linked immune-sorbent assay (ELISA) assay kits(using ELISA multiskan EX microplatephotomter, thermo scientific, STAT FAX-2100, USA) according to manufacturer protocol for measurements of plasma ChT (supplied by Elabscience Biotechnology Co.,Beijing, Catalog No: E-EL-H5620) and plasma Ferritin (supplied by BIOCHECK, INC, 323 Vintage Park Dr., Foster City, CA 94404- Catalog Number: BC-1025).

Using commercially available colorimetric assay kit (using T60 UV visible spectrophotometer. PG INSTRUMENTS LIMITED, Alma park wibtoft, Leicester shreshire, England. LE17SBE. Serial No. 20-1650-01-0010) according to manufacturer protocol for measurement of plasma total acid phosphatase activity (supplied by Bio-Diagnostic Co. Cairo, Egypt, Catalog No: AC 10 10).

Using commercially available strip assay kit for mutation analysis supplied by Vienna Lab Diagnostics GmbH, GaudenzdorferGuertel 43-45, A-1120 Vienna, Austria: The procedure included three steps: (1) DNA isolation, (2) polymerase chain reaction "PCR" amplification using biotinylated primers (using VeriFlex™ 96-Well Thermal Cycler, Applied Biosystems, USA), (3) hybridization of amplification products to a test strip containing allele-specific oligonucleotide probes immobilized as an array of parallel lines. Bound biotinylated sequences were detected using streptavidin-alkaline phosphatase and color substrates.

Plasma protein electrophoresis (Fig.1) was performed using Sebia capillary electrophoresis, Sebia, Inc. according to manufacturer's guidelines. Plasma proteins were separated into 6 components; albumin, alpha1, 2, beta1, 2 and gamma globulin. The separation was carried out on silica capillaries according to their electrophoresis mobility and electroosmotic flow at high voltage in alkaline media.

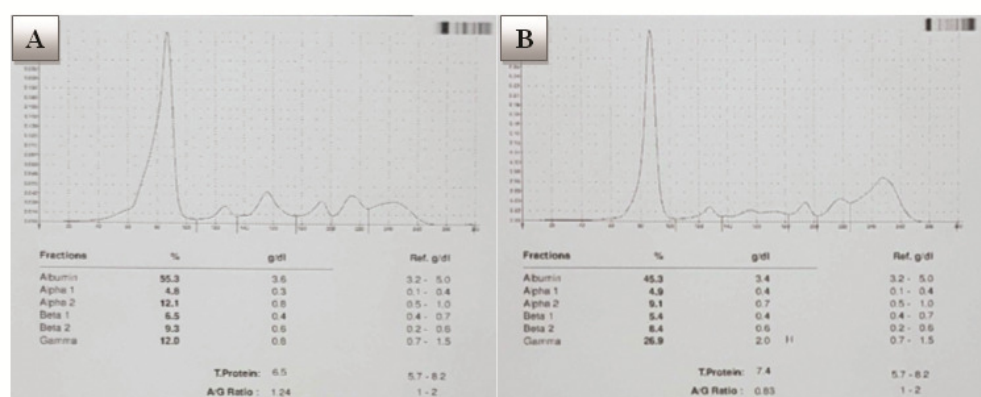


FIGURE 1: Plasma protein electrophoresis; A) Normal. B) Polyclonal hypergammaglobulinemia with inverted A/G ratio.

2.3. Statistical analysis

Analysis of data was done by IBM computer using SPSS (statistical program for social science version 12) as follows: Description of quantitative variables as mean $\pm$ SD .Description of qualitative variables as number and percentage. Unpaired t-test was used to compare quantitative variables, in parametric data.P value >0.05 insignificant, $P<0.05$ significant, $P<0.001$ highly significant.

3. Results

Regarding the frequency distribution of group B cases according to their final diagnoses; mutation analysis for GD was positive in three cases out of 20 (15%), unclassified metabolic disorders were present in 6 cases (30%), leukemia was the diagnosis in 6 cases (30 %), glycogen storage disease type –I in two cases (10%), three cases died before completing the investigatory battery (15%).

Regarding the clinical data of the included GD patients, 24 (75%) have non-neuropathic type 1 GD and 8 (25%) have type 3 GD. Their genotypes were; homozygous for the mutation L444P (30.8%), homozygous for the mutation N370S (15.4%), heterozygous for the mutations N370S and rec Ncil (15.4%), heterozygous for IVS2 +1 and rec Ncil(11.5%), heterozygous for IVS2 +1 and L444P (7.7%), heterozygous for L444P and second alleles were not detected was occurred in 7.7%, while, in 11.5% mutation was present but it was not possible to identify any of the mutant alleles.

TABLE 1: Comparison between the mean hemoglobin levels , platelets counts, plasma, ferritin, chitotriosidase, total acid phosphatase, globulin and A/G ratio) between group A , B and C.

Variables (Mean±SD)	Group A (n= 29)	Group B (n= 20)	Group C (n= 20)	P- value ¹	P- value ²	P- value ³
Hb level (g/dl)	9.46±2.41	9.41±1.93	12.92±0.85	0.849	0.000 *	0.000 *
Platelet count (x10 ³ mm ⁻³)	148.31±102.56	154.38±123.44	264.20±61.40	0.870	0.001 *	0.007 *
Plasma ferritin (ng/ml)	476.69±336.84	703.83±307.65	69.89±59.93	0.127	0.000 *	0.000 *
Plasma chitotriosidas e (pg/ml)	2134.27±965.4	1186.08±439.5	624.55±375.2	0.01*	0.000 *	0.000 *
Plasma total acid phosphatase activity (U/L)	26.99±12.58	17.73±5.37	11.59±4.17	0.01*	0.000 *	0.001 *
A/G ratio	1.32 ± 0.29	1.48 ± 0.36	1.55 ± 0.19	0.384	0.021 *	0.236
Globulin (g/dl)	2.90 ± 0.84	2.73 ± 0.69	2.53 ± 0.33	0.549	0.108	0.418

P₁: A versus B - P₂: A versus C - P₃: B versus C.

P value >0.05 insignificant, * P<0.05 significant, **P<0.001 highly significant.

Comparison between group A , B and C as regard hemoglobin levels, platelets count, plasma chitotriosidase, total acid phosphatase, ferritin, globulin and A/G ratio (Table 1), showing significantly higher plasma chitotriosidase and total acid phosphatase in group A versus B (P-value <0.05).

Plasma protein electrophoresis showed hypergammaglobulinemia in 76% of group A and in 10% of group B. Mutation analysis was positive in 3 cases only of group B (15%) (Table 2). Images showing hepatosplenomegally presented in GD. (Fig.2)

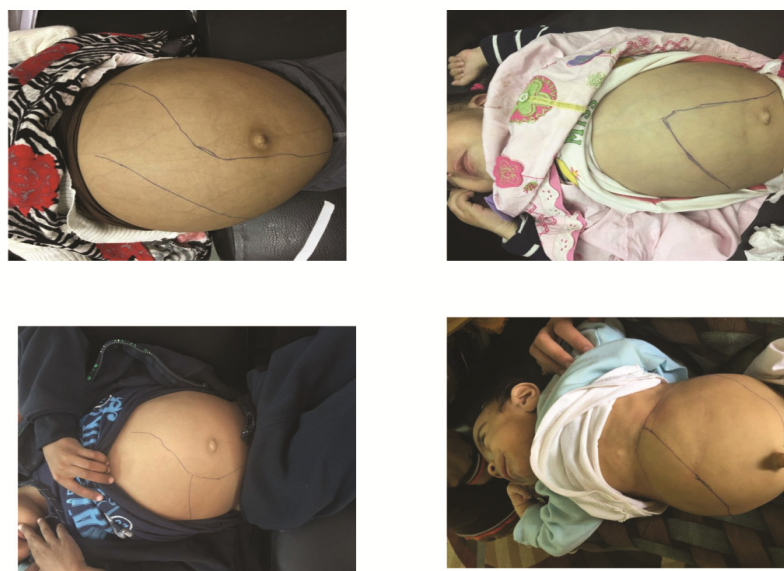


FIGURE 2: Images for some included GD patients showing abdominal distension with hepatosplenomegally

TABLE 2: Plasma protein electrophoresis and mutation analysis results of the included patients.

Variables	Group A (n= 29)	Group B (n= 20)
Hypergammaglobulinemia No. (%)	22 (76)	2 (10)
Positive mutation analysis No. (%)	29 (100)	3 (15)

Regarding the diagnostic utility of ChT, total acid phosphatase, ferritin, globulin and A/G ratio in diagnosing GD (group A and group B) , plasma chitotriosidase

showed the highest area under the curve (AUC= 0.897) followed by total acid phosphatase (AUC= 0.879) , then ferritin (AUC= 0.762) followed by A/G ratio (AUC= 0.689) and Globulin showed the lowest AUC (= 0.631)(Table 3 and Fig.3).

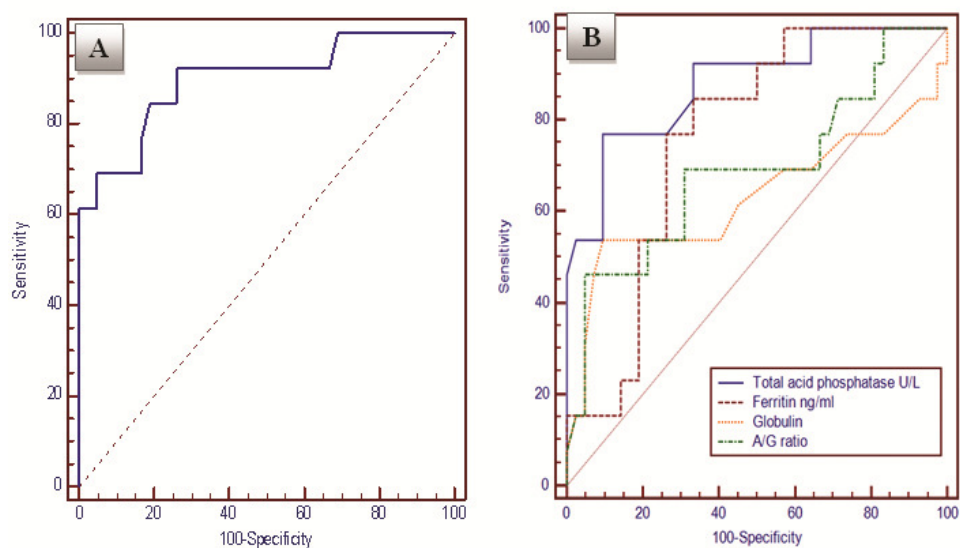


FIGURE 3: Combined ROC curve analysis of plasma chitotriosidase(A); Total acid phosphatase, ferritin, globulin and A/G ratio (B).

TABLE3. Performance characteristics of chitotriosidase, total acid phosphatase, ferritin, globulin and A/G ratio in diagnosing GD.

Variables	Chitotriosidase (pg/ml)	Total acid phosphatase (U/L)	Ferritin (ng/ml)	Globulin (g/dl)	A/G ratio
Best cut off	> 943	> 20	> 141	> 3	≤ 1.18
AUC	0.897	0.879	0.762	0.631	0.689
Sensitivity	92.31	76.92	84.62	53.85	46.15
Specificity	73.81	90.48	66.67	90.48	95.24
PPV	52.2	71.4	44.0	63.6	75.0
NPV	96.9	92.7	93.3	86.4	85.1
Accuracy	78.18	87.3	70.9	81.8	83.6

4. Discussion

.ChT is dramatically induced in disease states where tissue-resident macrophages are chronically activated. It is released by Gaucher storage cells and is increased in the plasma of Gaucher patients [12]. However, the limitation of ChT as a marker of GD is that, 3-5 % of individuals in the general population have no activity due to the presence of a null allele in the ChTgene[8].The increased serum acid phosphatase activity in patients with GD arises by spillage of the enzyme from the glucocerebroside-rich storage cells of the spleen and other tissues[13].

In the present study, the laboratory inclusion criteria among group B were demonstrated by comparing hemoglobin levels and platelets counts in group B

versus group C which revealed significant anemia and lower platelet counts in group B.

The findings of the present study revealed that plasma ChT has the highest area under curve, high plasma ChT and ferritin have the highest sensitivity, while, high total acid phosphatase activity, and globulin and low A/G ratio have the highest specificity in diagnosing GD. All these biomarkers are better negative than positive in predicting GD. Comparison between the plasma levels of such biomarkers among group A versus group B showed significant higher plasma ChT and total acid phosphatase activity in group A than in group B, indicating that plasma ChT and total acid phosphatase activity are the best screening markers could be used for predicting GD patients. Hassan et al. [14] confirmed in their study that plasma ChT, total acid phosphatase activity, ferritin and globulin were collectively helpful biomarkers in evaluation and follow up the effect of enzyme replacement therapy in GD patients. The findings of the present study proved that plasma ChT, total acid phosphatase activity were the most of the studied biomarkers that could be reliable in screening for GD.

The mechanism explaining the relationship between GD and immunoglobulin abnormalities is far from understood [15]. Plasma protein electrophoresis in the present study revealed polyclonal hypergammaglobulinemia among GD patients, although monoclonal or biclonalgammopathies are more specific to Gaucher disease as polyclonal gammopathies may have multiple etiologies, but not assessed in the present study. In agreement with these findings a study done by Arıkan-Ayyıldız et

al.[16] on pediatric patients with GD found that hyperimmunoglobulinemia was present in 77% at diagnosis.

Further studies comparing the emerging glycosylsphingosine or lyso-Gb1 biomarkers versus the studied biomarkers in screening for Gaucher disease on a larger scale, are recommended.

5. Conclusions

To conclude, the present study proves that plasma ChT combined with total acid phosphatase activity are the most helpful primary screening biomarkers for GD and we call for dried blood spot chitotriosidase and total acid phosphatase activity assays , if possible, to be included in the newborn screening program in Egypt.

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