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Diagnostic Utility of Plasma Chitotriosidase, Total Acid Phosphatase Activity, Ferritin and Globulin Levels in Screening for Gaucher Disease

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MHH and AAS carried out the whole biochemical and genetic assays, wrote the first draft of the manuscript, managed the literature searches and designed the required statistical analysis. Authors AEAA and THS were responsible for study concept and design. Author AEAA was responsible for patients' selection and clinical data collections from the included patients. All authors shared in the interpretations of results, read and approved the final manuscript.

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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 GD in pediatric patients.

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Methodology: A cross sectional hospital based study, carried out on 43 pediatric patients (23 GD patients and 20 patients with suspected storage disorder). Complete blood counts, globulin assays, calculated A/G ratio, β-glucocerebrosidase activity assays, plasma protein electrophoresis, ELISA assays of plasma ChT and ferritin, colorimetric assay of plasma total acid phosphatase activity and *GBA* gene mutation analysis, were done for the included patients.
Results: Significantly higher plasma ChT levels and increased total acid phosphatase activity were observed in GD patients versus those suspected to have storage disorders (P-value <0.05). High plasma ChT and ferritin have higher sensitivity, while high plasma total acid phosphatase activity, and globulin and low A/G ratio have higher specificity in diagnosing GD.
Conclusions: Plasma ChT and total acid phosphatase activity, both together are more valid 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 glucocerebrosidase 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 GD [5]. Among these molecules are 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]. Ferritin is often elevated in GD due to chronic inflammation and impaired iron mobilization, which confined to Gaucher cells and not occur in other organs [11]. Gaucher cells produces high amounts of acid phosphatase, in addition to the increased osteoclastic activity and bone resorption with increased production of acid phosphatase from osteocalsts, especially in type-I GD [12]. The infiltrated reticuloendothelial organ such as Gaucher spleen, may contribute to the production of monoclonal immunoglobulin [13], 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, will be the best predictor/s and can be used as primary screening marker/s for GD before performing the mutation analysis.

2. MATERIALS AND METHODS

2.1 Study Population

A cross sectional hospital based study, carried out on 43 pediatric patients divided into two groups; Group A (23 GD patients) (mean age 6.20 years ±5.88 SD) (13 males, 10 females), Group B (20 pediatric patients with suspected storage disorder) (mean age 6.04 years ±4.99SD) (9 males, 11 females). They were selected from the attendant pediatric patients at Assiut and Qena University hospitals, Egypt, according to the selection criteria and after approval of the university hospital ethical committee, in accordance with the declaration of Helsinki. Before starting the study; every included child and his/her parents were informed about the study's aim and an assigned consent in a written form was obtained. The total duration of the study was eighteen months from January 1st 2015 up to May 31st 2016.

Selection criteria [11,14]; clinically: isolated splenomegaly or hepatosplenomegaly of unknown aetiology with bone pain or spontaneous fractures, associated with or without physical and /or mental developmental delay and/ or neurologic abnormalities in the form of spacticity, opisthotonus, ataxia, hyperreflexia or hypertonia . Laboratory: low hemoglobin and/or low platelet count to be confirmed by the low leucocytic β-glucocerebrosidase activity and presence of *GBA* gene mutation.

2.2 Laboratory Measurements

Complete blood count "CBC", globulin assays, calculated A/G ratio and β-glucocerebrosidase activity assays were done for the included patients. Complete blood counts "CBC" were measured using (Cell Dyn 1800-Abbott diagnostics, Germany). Globulin levels and calculated A/G ratio were estimated using [Cobas C311 (Roche diagnostics, Germany)]. β-glucocerebrosidase activity was measured according to Daniels and Glew [15] at National Research Centre, Cairo, Egypt and expressed as µmol/g protein/hour.

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℃ until biochemical analysis (ferritin, total acid phosphatase activity and ChT assays, in addition to plasma protein electrophoresis). 2 cc on another EDTA tubes, stored as whole blood at-20℃ till the time of *GBA* gene mutation analysis using strip assay method.

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 enzyme-linked immune-sorbent assay (ELISA) assay kits by ELISA multiskan EX microplatephotomter, thermo scientific (STAT FAX-2100, USA) according to manufacturer protocol.

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

GBA gene mutation analysis was performed using a strip assay kit (Vienna Lab Diagnostics GmbH, Gaudenzdorfer Guertel 43-45, A-1120 Vienna, Austria). The assay based on polymerase chain reaction (PCR), reverse-hybridization, and detection using streptavidin-alkaline phosphatase and color substrates. Briefly, DNA was extracted; PCR was carried out on VeriFlex™ 96-Well Thermal Cycler, Applied Biosystems, USA, using the provided biotinylated primers according to manufacturer's instruction. PCR products were subjected to hybridization with allele-specific oligonucleotide probes immobilized to the test strip. Results were assessed visually according to the enclosed collector sheet, the positive control square should be positive for assay results to be accepted, as shown below. The assay covered 8 common GBA mutations: 84GG, IVS2+1, N370S, V394L, D409H, L444P, R463C, R496H, with the mutant alleles placed at positions 1-8 and their corresponding wildtype alleles placed at position 10 to 17. For a given mutation, the presence of only colored mutant position is called homozygous mutant, the presence of only colored wild type position is called normal, when both were detected the genotype is called heterozygous, compound genotype could be assigned as well. The assay also included 2 recombinant alleles derived from crossover between the GBA functional gene and pseudogene in the region of exons 9-10 (rec NciI, for recombination between D409H and L444 and rec TL for recombination at V394L and L444P) placed at positions 9 and 18 respectively. The wild type rec reporter probe reads positive for GBA alleles without crossover while positive mutant rec reporter probe, rec TL and rec NciI can be distinguished by the presence or absence of positive staining for mutant D409H [16].

Plasma protein electrophoresis (Fig. 1) was done using Sebia capillary electrophoresis, Sebia, Inc. according to manufacturer's protocol. Plasma proteins separation was performed on silica capillaries according to elctroosmotic flow and their electrophoretic mobility in an alkaline media at high voltage.

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 ±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; *GBA* mutation analysis was positive in three cases out of 20 (15%) confirming the presence of GD, 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%). The diagnosis of the included GD patients was confirmed by the presence of significantly decreased β-glucocerebrosidase activity combined with positive *GBA* mutations (Table 1, Fig. 2).

Regarding the clinical data of the included 26 GD patients (group A and 3 from group B), 20 (77%) has non- neuronopathic type 1 GD and 6 (23%) 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 NciI (11.5%), heterozygous for IVS2 +1 and L444P (7.7%), heterozygous for L444P and second allele was not detected in 7.7%, while, in 11.5% mutation was present but it was not possible to identify any of the mutant alleles (Fig. 2).

Comparison between group A and B as regard hemoglobin levels, platelets count, β-glucocerebrosidase activity, plasma

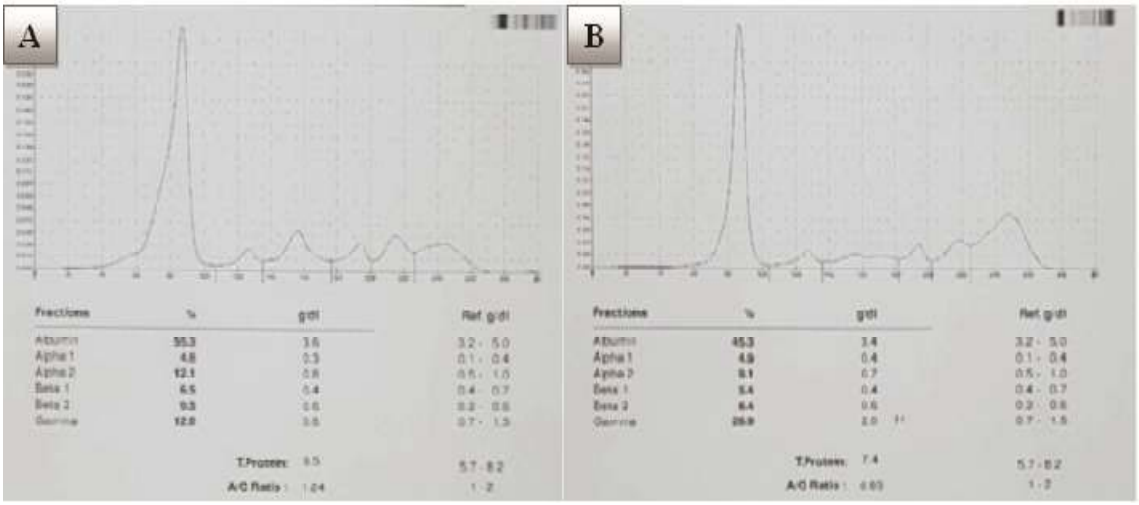

Fig. 1. Plasma protein electrophoresis; A) Normal. B) Polyclonal hypergammagl obulinemia with inverted A/G ratio

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

Variables(Mean±SD)	Group A(n= 23)	Group B(n= 20)	P-value
Hb level (g/dl)	9.46±2.41	9.41±1.93	0.849
Platelet count (x10^3 mm^{-3})	148.31±102.56	154.38±123.44	0.870
β-glucocerebrosidase activity (µmol/g prot./h)	0.28 ±0.2	2.53±1.4	0.000**
Plasma chitotriosidase (pg/ml)	1750.36±705.13	1186.08±439.58	0.01*
Plasma total acid phosphatase activity (U/L)	47.34±10.39	17.73±5.37	0.01*
Plasma ferritin (ng/ml)	703.83±307.65	673.5±240.84	0.127
Globulin (g/dl)	2.90 ± 0.84	2.73 ± 0.69	0.549
A/G ratio	1.32 ± 0.29	1.48 ± 0.36	0.384

** P<0.05 significant; ** P<0.001 highly significant*

chitotriosidase, total acid phosphatase, ferritin, globulin and A/G ratio (Table 1), showing significantly higher plasma chitotriosidase and total acid phosphatase was observed in GD patients versus those with suspected storage disorders (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 for some included GD patients were captured, showing abdominal distension due to hepatosplenomegally (Fig. 3).

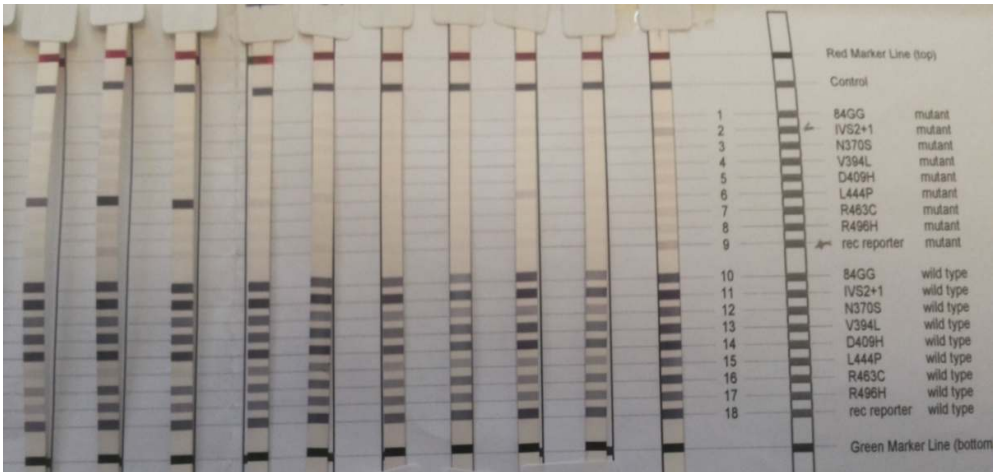

Fig. 2. Results of *GBA* mutation analysis of some included GD patients, using strip assays

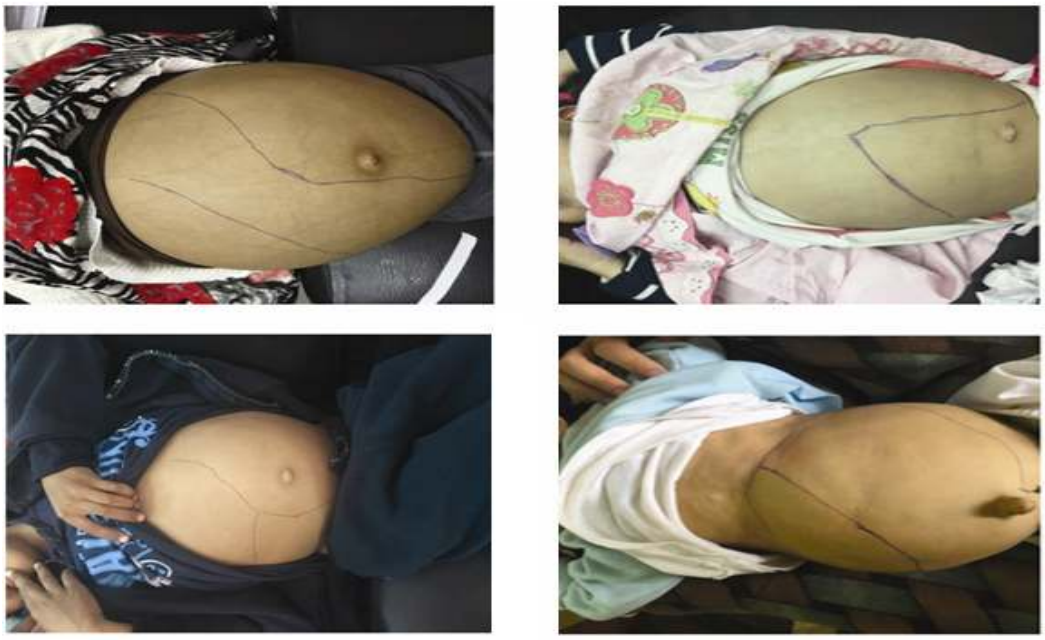

Fig. 3. Images for some included GD patients showing abdominal distension due to hepatosplenomegally

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

Variables	Group A (n= 23)	Group B (n= 20)
Hypergammaglobulinemia No. (%)	17 (74)	2 (10)
Positive mutation analysis No. (%)	23 (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. 4).

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 [14]. 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 *ChT* gene [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, liver and other tissues [17]. As lysosomal enzymes including lysosomal acid phosphatase, can leak from damaged cells under conditions that leave the lysosomal membrane intact which occur in glucocerebroside-rich, glucocerebrosidase deficient storage cells of GD [18].

In the present study, the primary laboratory inclusion criteria among group B was demonstrated by comparing hemoglobin levels and platelets counts in group B versus group A which revealed non-significant differences regarding lower hemoglobin and platelet counts between the two groups.

Table 3. 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

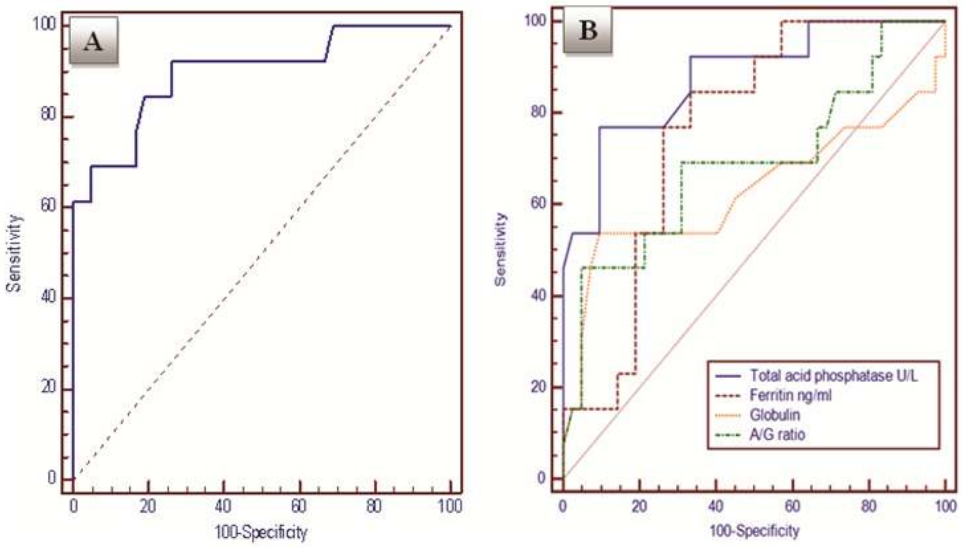

Fig. 4. Combined ROC curve analysis of plasma chitotriosidase (A); total acid phosphatase, ferritin, globulin and A/G ratio (B)

The findings of the present study revealed that high plasma ChT and ferritin have higher sensitivity, while high plasma total acid phosphatase activity, and globulin and low A/G ratio have higher specificity in diagnosing GD. Collectively, high plasma ChT and high plasma total acid phosphatase activity have the highest area under curve (AUC). 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. [19] confirmed in their study that plasma ChT, total acid phosphatase activity, ferritin and globulin were collectively helpful prognostic biomarkers in 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 valid 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 [20]. Plasma protein electrophoresis in the present study revealed polyclonal hypergammaglobulinemia among GD patients, although monoclonal or biclonal gammopathies 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. [21] on pediatric patients with GD found that hyperimmunoglobulinemia was present in 77% at diagnosis. Explanations on the pathogenesis of immunoglobulin disorders in GD have mainly focused on the accumulated glucocerebroside as the principle agent for chronic stimulation of macrophages surrounding Gaucher cells. Cytokines of macrophagic origin could in turn stimulate B-cells leading to a polyclonal gammopathy in GD [20].

Further studies comparing the emerging glycosylsphingosine or lysosomal glucosyl ceramide (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 assay combined with total acid phosphatase activity measurement are the most helpful primary screening biomarkers for GD and we call for dried blood spot chitotriosidase assay, and total acid phosphatase activity measurement if possible, to be included in the newborn screening program in Egypt.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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