Biochemical diagnosis of Wolman disease among patients with suspected lysosomal storage diseases

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Introduction
Lysosomal acid lipase (LAL) deficiency is an autosomal recessive disease, causing two different disorders, Wolman disease (WD) and cholesteryl ester storage disease. WD is the severest type of LAL deficiency. It causes accumulation of lipids in body organs and calcium deposits in the adrenal glands. The phenotypes in infants include hepatosplenomegaly, failure to thrive, jaundice, vomiting, diarrhea, developmental delay, and anemia. It is life-threatening in early childhood; however, the late form appears after puberty and in some cases at an older age, leading to liver dysfunction. A recent method was established to measure the activity of LAL in a dried blood spot, using Lalistat 2 as an inhibitor.

Aim
The aim was to diagnose WD among a group of high-risk patients with organomegaly and establishing the dried blood spot technique at the Biochemical Genetics Laboratory, Center of Excellence for Human Genetics, National Research Centre.

Patients and methods
A total of 83 participants were recruited, comprising 30 controls and 53 patients with hepatosplenomegaly. Four different enzyme activities were measured (Chitotriosidase, B-glucosidase, sphingomyelinase, and LAL).

Results
Overall, 53 patients were diagnosed, comprising 17 patients with Gaucher disease, six patients with Niemann–Pick (NP) disease type A/B, one patient with WD, and 29 patients were suspected to be NP type C or other lysosomal diseases for further investigations.

Conclusion
Plasma chitotriosidase, peripheral leukocytic β-glucosidase, and acid sphingomyelinase enzyme activities should be assessed in all patients with clinical suspicion of WD, to exclude Gaucher disease and NP disease, as they have overlapping signs and symptoms in early childhood. Screening for acid lipase enzyme activity in a dried blood spot test is an accurate specific technique for the diagnosis of WD.

Keywords:
Dried blood spot, Gaucher disease, Lalistat 2, lysosomal acid lipase, Niemann-Pick disease, Wolman disease

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Introduction

Lysosomal acid lipase (LAL) is an essential lysosomal enzyme, hydrolyzing cholesteryl esters and triglycerides and generating free cholesterol and free fatty acids (Boldrini *et al.*, 2004). LAL deficiency disorder is an autosomal recessive disorder (Hoffman *et al.*, 1993), having two phenotypes: Wolman disease (WD), which is the severest form, and cholesteryl ester storage disease (CESD), which is the benign form. CESD is the late onset of the disease; it could be diagnosed from childhood to adolescence (Wolman, 1994). Both are listed under the same code (OMIM # 278000).

WD was first described by Moshe Wolman in 1956. He published a case report with severe malnutrition, adrenal calcifications, hepatosplenomegaly with liver xanthomata, who died at 3 months of age (Abramov *et al.*, 1956). WD is characterized by impaired metabolism of lipids (Young and Patrick, 1970). It is caused by mutations in the *LIPA* gene, producing a defective LAL enzyme (Hoffman *et al.*, 1993). Incidence of WD is 1: 500 000 (Meikle *et al.*, 1999). Bone marrow transplantation achieved long-term successful progress in the patient with WD, where the activity of LAL turned to normal levels, diarrhea stopped, and the levels of cholesterols and triglycerides turned to normal ranges (Krivit *et al.*, 2000). Enzyme replacement therapy has recently been developed using recombinant enzymes produced by egg whites of a transgenic Gallus (Burton *et al.*, 2015). Early

diagnosis of WD with the presence of ERT (Enzyme Replacement Therapy) will be life saving for these patients.

WD shares with Gaucher disease (GD) and Niemann–Pick (NP) types A/B many signs and symptoms, such as hepatosplenomegaly, anemia, jaundice, vomiting, and diarrhea. All three diseases are autosomal recessive, caused by a deficiency of lysosomal enzyme owing to a mutation in the responsible gene.

GD is caused by a deficiency of β-glucocerebrosidase enzyme, which is responsible for breaking down and recycling glucocerebroside (Grabowski *et al.*, 2004).

NP type A/B is mainly caused by deficiency of enzymes responsible for breaking down sphingomyelin and cholesterol. However, in type C/D, the problem is in the cholesterol transport inside the cell. NP type C is the most common type, whereas D is a variant of C.

This study aimed:
(1) To biochemically diagnose WD among a group of high-risk patients with organomegaly and failure to thrive
(2) To establish the dried blood spot technique using Lalistat 2 as the method of choice for the screening and diagnosis of WD.

Patients and methods

This study included 83 children, comprising 30 controls and 53 patients with hepatosplenomegaly, anemia, and failure to thrive. They were recruited from cases referred to the Biochemical Genetics Department, National Research Centre, Cairo, Egypt. Patients' ages ranged from 2 months to 2 years. The control group included 17 males and 13 females, whereas the patients' group included 35 males and 18 females.

A written informed consent was obtained from parents of all participants in the study. An ethical approval was obtained from the Medical Research Ethics Committees at the National Research Centre and the Faculty of Science, Cairo University.

All cases were subjected to the following:
(1) Patients were clinically diagnosed, and the needed laboratory tests were specified by the referring physicians
(2) Measurement of the activity of chitotriosidase, β-glucosidase, and sphingomyelinase enzymes was done to exclude GD and Niemann–Pick diseases (NPD)
(3) Measurement of the activity of LAL enzyme was done to diagnose WD.

Biochemical studies

Plasma chitotriosidase activity assay

This assay was described by Hollak *et al.* (1994). Chitotriosidase cleaves 4-methylumbelliferyl-β-N-N, N"-triacetylchitotriose at acidic pH to liberate fluorogenic 4-methylumbelliferone molecules. The reaction is stopped by an alkaline buffer, and fluorescence is measured.

Peripheral leukocytic β-glucosidase assay

This enzyme is deficient in GD. The assay is based on the method described by Daniels *et al.* (1981).

Peripheral leukocytic acid sphingomyelinase assay

This enzyme is deficient in NP type A/B. The assay with fluorescent substrate is based on the method described by van Diggelen *et al.* (2012).

Dried blood spot method measuring lysosomal acid lipase assay

The assay with fluorescent substrate is based on the method described by Hamilton *et al.* (2012).

First, the activity is measured in the absence of Lalistat 2, to measure the total lipases activity in the elution of the samples, and then in the presence of Lalistat 2, the inhibitor, which is very specific for LAL. The difference between the two concentrations is the real LAL activity.

Results

Table 1 shows the results of age, sex and consanguinity in the two study groups.

Biochemical findings

(1) Plasma chitotriosidase activity was measured for all 83 cases.
   (a) 30 controls had normal plasma chitotriosidase activity (Table 2)
   (b) 53 patients had high plasma chitotriosidase activity
(2) Peripheral leukocytic β-glucosidase activity was measured for 83 participants
   (a) 30 normal controls gave normal activity for the enzyme
   (b) 53 patients were divided into the following:

(i) 17 patients had a very low activity of the β-glucosidase enzyme
(ii) 35 patients had a normal activity of the β-glucosidase enzyme (Table 3)

(3) Peripheral leukocytic acid sphingomyelinase activity was measured for the 83 patients
  (a) 30 normal controls gave normal activity for the enzyme
  (b) 53 patients were divided into the following:
    (i) 47 patients had normal activity, excluding NP type A/B disease
    (ii) Only six patients were diagnosed as NPD patients with a very low activity of the enzyme (Table 4)

(4) LAL activity was measured for the 83 participants.
  (a) 30 controls showed normal activity for the LAL enzyme
  (b) 53 patients showed the following:
    (i) 52 showed normal range of the enzyme activity
    (ii) Only one case showed no activity of the enzyme (Table 5).

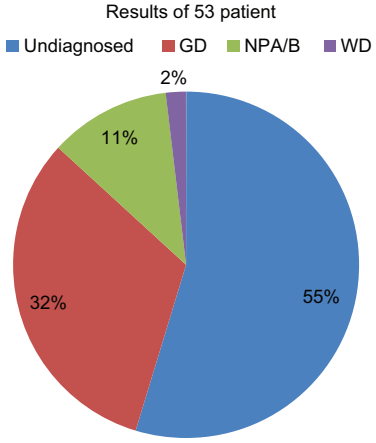
Table 6 and Fig. 1 shows the percentage and final diagnosis of all patients in the study.

Patient with Wolman disease

Clinical manifestations

A 6-month-old male, offspring of consanguineous parents, presented with hepatosplenomegaly, jaundice, anemia, diarrhea, vomiting, and failure to thrive. Computed tomography showed adrenal calcifications. He had high levels of liver enzymes, bilirubin, cholesterol, triglycerides, and low-density lipoprotein. However, he had low levels of high-density lipoprotein and albumin.

Figure 1



Percentage of each type of disease among the patient group.

Table 1 The results of age, sex, and consanguinity in control and patient groups

Parameters	Age (months)	Sex [*n* (%)]		Consanguinity [*n* (%)]	
		Males	Females	Positive	Negative
30 controls	2-24	17 (56.5)	13 (33.5)	15 (50)	15 (50)
53 patients	2-24	35 (66)	18 (34)	44 (83)	9 (17)

Table 2 Plasma chitotriosidase mean activity among the two study groups

Groups	Number of cases	Mean of chitotriosidase activity (normal range, 4-80 μmol/l/h)
Normal controls	30	23
Patients with Gaucher disease	17	3500
Patients with NPD	6	392
Patients with LSD	29	342
Patient with Wolman	1	394

LSD, lysosomal storage disorder; NPD, Niemann-Pick disease.

Table 3 Peripheral β-glucosidase enzyme activity among the two study groups

Groups	Number of cases	Mean of β-glucosidase activity (normal range, 1-5 μmol/g.prot/h)
Normal controls	30	2.5±0.7309
Patients with Gaucher disease	17	0.5±0.501
Patients with NPD	6	2.7±0.8501
Patients with LSD	29	3.4±0.8501
Patient with Wolman disease	1	1.9

LSD, lysosomal storage disorder; NPD, Niemann-Pick disease.

Table 4 Peripheral leukocytic acid sphingomyelinase activity among the two study groups

Groups	Number	Sphingomyelinase mean enzyme activity (μmol/g/h)
Normal controls	30	20.73±9.051
Patients with Gaucher disease	17	23±4.652
Patients with NPD	6	2±8.652
Patients with LSD	29	21.73±9.051
Patient with Wolman disease	1	15

LSD, lysosomal storage disorder; NPD, Niemann-Pick disease.

Table 5 Lysosomal acid lipase enzyme activity among the two study groups

Groups	Number	Mean activity of LAL enzyme (nmol/punch/h)
Normal controls	30	1.5±0.5
Patients with Gaucher disease	17	1.3±0.5
Patients with NPD	6	1.3±0.5
Patients with LSD	29	1.6±0.5
Patient with Wolman	1	0

LAL, lysosomal acid lipase; LSD, lysosomal storage disorder; NPD, Niemann-Pick disease.

Table 6 The final diagnosis of all patients in the study

83 patients and controls	Number
Normal controls	30
Patients with Gaucher disease	17
Patients with Niemann-Pick disease	6
Patient with Wolman disease	1
Undiagnosed	29

Biochemical genetic tests revealed the following:

Level of chitotriosidase was 394 μmol/l/h
Level of β-glucosidase was 1.9 μmol/g.prot/h
Level of acid sphingomyelinase was 15 nmol/g. prot/h
Level of LAL was 0 nmol/punch/h.

High chitotriosidase level is a biomarker of many lysosomal diseases. Normal activity of the β-glucosidase excluded GD, as well as normal acid sphingomyelinase activity excluded NP type A/B disease. LAL was measured, and a complete absence of LAL activity was confirmed. The test was repeated twice and gave the same results.

The patient was tested for other biomarkers with corporation of Hopitaux de Lyon, GHE-Centre de Biologieet Pathologie Est, Biochimie et Biologie moleculaire, and all the results confirmed WD.

Plasmatic lysosphingomyelin = 0.4 nmol/l (normal range, <0.7). Plasmatic lysosphingomyelin isoform '509'=21 nmol/l (normal range, <3). Plasmatic cholestane-3b, 5a, 6b-triol = 4580 nmol/l (normal range, <90). 7-ketocholesterol = 6700 nmol/l (normal range, <30).

Discussion

LAL deficiency is an autosomal recessive disease, caused by a mutation in the gene responsible for the production of LAL enzyme. Mutation in *LIPA* gene causes disruption in the production of LAL enzyme, resulting in huge accumulation of cholesterols and triglycerides inside the liver and the spleen. LAL deficiency has two different phenotypes: WD and CESD. WD is the early onset type, with severe signs and symptoms, and causes death within the first few months of life.

The aim of this study was to diagnose WD among a group of clinically suspected patients with hepatosplenomegaly and failure to thrive. Three storage diseases with similar signs and symptoms were investigated: GD, NP, both are classified as sphingolipids, but WD is classified as one of the lipid storage disorders. All three diseases are autosomal recessive sharing the same signs and symptoms with slight difference. Patients had anemia, hepatosplenomegaly, and failure to thrive. The severity of the symptoms varies according to the diagnosis. WD is the severest of them all, leading to death before the first year of life, similar to Gaucher type 2.

GD is the commonest of the lysosomal genetic disorder caused by deficiency of β-glucosidase enzyme, responsible for breaking down the glucocerebrosides inside the lysosomes. The age of GD reported in this study covers only the infantile spectrum. GD has an incidence of 1: 20 000 to 1: 200 000 (Martins *et al.*, 2009). For instance, Ragab *et al.* (2000) have reported an age range from 1 month to 12 years. El-Morsy *et al.* (2011), reported an age range from 1 to 17 years. Most of these studies agreed that infantile and juvenile forms are the commonest presentations of GD in Egypt, whereas the adult form is less common (Fateen and Abdallah, 2019). In this study, we covered only the infantile form of GD with severe signs and symptoms; still patients with Gaucher constituted 32% of the study group. This agrees with all previous studies, which stated that GD is the commonest lysosomal disorder.

NPD is also a rare inherited disorder, in which large quantities of sphingolipids accumulate in the spleen, liver, lungs, bone marrow, and brain. It is subdivided into A, B, C, D, E, and F. NP types A/B are diagnosed in this study by measuring the sphingomyelinase activity. We distinguished between NP type A and NP type B by two factors. The residual enzyme activity is slightly higher in NP type B than in NP type A. The second difference is that NP type A has visceral and neurological signs and symptoms, whereas NP type B has visceral signs only. NP type C is caused by sequestration of unesterified cholesterol in late endosomal/lysosomal compartment caused by unique abnormalities in intracellular trafficking of endocytosed cholesterol (Yu, 2013). NP type D is supposed to be a variant for C; however, E and F are less common types, which appear in adulthood. NP types A/B are known as type 1 but NP type C is type 2 (NPD type C – National Organization for Rare Disorders, n.d.).

It is obvious, however, that NPD has a wider age range of presentation than GD. This is consistent with the fact that NPD has a wide clinical spectrum that ranges from fatal neonatal disease to chronic neurodegenerative disease (Vanier, 2010). In this study, we covered only the early severe infantile form of the disease with overlapping signs and symptoms with WD. The diagnosed NP type A/B cases constituted 11.3% of the study group, and the undiagnosed cases constituted 54.7% with a question mark for further diagnosis. The high chitotriosidase enzyme activity points to other lysosomal disorder for further confirmatory tests. NP type C is one of the most probably suspected diseases for these patients, sharing many signs and symptoms with the undiagnosed group of patients.

A clear difference in the male to female ratio was found among the patients' group, which was 1.9: 1 for male: female, indicating that there is a male predominancy. Despite the fact that three diseases

are autosomal recessive disorders affecting both sexes equally, this reflects a deep routed tradition in Arab and Eastern countries, who give more medical care to males than females, especially in rural areas, where the consanguinity rate and metabolic disorders are high (Fateen and Abdallah, 2019).

Positive consanguinity among the patients' group was 83%, whereas in the control group, it was 50%. The social habits and traditions of consanguineous marriage are deep routed in Egypt, mostly over 33% (Temtamy and Aglan, 2012). High consanguinity is reported in all storage diseases, and positive consanguinity was at about 81% during 25 years of diagnosing genetic disorders in Egypt (Fateen and Abdallah, 2019). It is the main cause of many genetic disorders in our population (Aboulnasr and Fateen, 2019).

Normal plasma chitotriosidase activity excluded GD, NP types A and B (NPD A/B) and C, as well as WD in the included control group. High activity of the chitotriosidase enzyme is a biomarker for the presence of a lysosomal storage disorder. Gaucher patients showed a very high range of the enzyme activity. This agrees with previous studies on GD in Egypt, where chitotriosidase mean activity was 5185 ± 7461 μmol/l/h) (Fateen and Abdallah, 2019). However, patients with NPD showed also high chitotriosidase activity, but not to the extent of GD (Fateen and Abdallah, 2019). Chitotriosidase activity is elevated in many lysosomal disorders with different ranges. Chitotriosidase activity reflects the body load of the storage material. The patient with WD showed mild elevation of the activity of chitotriosidase. As this is the first study for WD among a high-risk group of Egyptian patients, chitotriosidase can be used as a biomarker for WD; still more specific biomarkers should be considered as well for future studies. Twenty nine patients from the study group showed higher chitotriosidase enzyme activity than the maximum of the normal range, but not to the extent of patients with GD or NPD. These patients need further investigations to exclude other lysosomal disorders, especially NP type C.

The β-glucosidase enzyme activity was measured for all patients and controls. Thirty controls showed normal ranges of the enzyme activity, whereas 17 patients, which constituted 32% of the study group, showed very low residual activity, confirming the diagnosis of GD with very high levels of chitotriosidase activity.

The sphingomyelinase enzyme activity was measured for all patients and controls. Thirty controls showed normal activity for the enzyme. Six patients, which constituted 11% of the study group, showed a very low activity of the enzyme, diagnosed as NP type A/B, with a moderate increase in chitotriosidase activity.

LAL activity was measured for all patients and controls. All showed normal activity for the enzyme except for one patient (1.9% of the study group), who showed zero activity, confirming the diagnosis of WD.

Oxysterols profile is a new sensitive and specific biomarker for the diagnosis of WD. The oxysterol is a derivative of cholesterol obtained by enzyme oxidation. Such compounds play important roles in various biological processes such as cholesterol homeostasis, lipid metabolism (sphingolipids and fatty acids), apoptosis, and autophagy. The mode of action of oxysterols is still poorly understood. Several oxysterols are associated with age-related diseases such as cardiovascular disease, eye disease (cataract and age-related macular degeneration), certain neurodegenerative diseases, and cancers. The activities of oxysterols in these diseases could be owing to their pro-oxidative and pro-inflammatory activities and their ability to act on cellular organelles (mitochondria, peroxisome, and lysosome) that can activate apoptosis and autophagy (Siems *et al.*, 2005). Oxysterols were measured for the patient with WDs and found to be highly increased.

The method of Hamilton *et al.* (2012) used in this study, using a dried blood spot sample to diagnose WD with Lalistat 2 as an inhibitor, proved to be an accurate and efficient method. The diagnosed patient with WD showed all typical signs and symptoms of the disease. All other biomarkers whether chitotriosidase or oxysterols confirmed the diagnosis.

In 2014, another publication used the Hamilton's method and measured the LAL activity in both dried blood spot and fibroblast. They recommended testing LAL activities in cases of suspected lysosomal storage disorders with overlapping phenotype (Gaucher, NP type A/B/C, and others) to avoid missing a diagnosis of LAL deficiency (Civallero *et al.*, 2014). This urged us to use the method in the included high-risk group of patients with severe signs and symptoms in the infantile period.

In 2015, an Egyptian article was published, showing the misdiagnosis of WD in one patient who was thought to have lymphohistiocytosis [familial hemophagocytic lymphohistiocytosis (FHL)]. They recommended that Wolman should be excluded in patients with clinical and laboratory characteristic of FHL (Elsayed *et al.*, 2016). None of the 53 patients included in this study showed any signs of lymphohistiocytosis (FHL). This indicates that WD should be included in other disease groups as well. It also indicates that WD is not uncommon as thought, and more studies should be performed to evaluate the magnitude of the problem, especially with

the presence of ERT for WD, which can save the life of many children.

Adrenal calcification is also a good sign for the suspected diagnosis of WD. It is a rare finding in a few other diseases like Addison disease in which adrenocortical insufficiency owing to the destruction or dysfunction of the entire adrenal cortex takes place. The onset of the disease usually occurs when 90% or more of both adrenal cortices are dysfunctional or destroyed (Ross and Levitt, 2013). TB, myelolipoma, and neuroblastoma are also some of the few causes of adrenal calcifications. The WD-diagnosed patient in this study showed adrenal calcification as well.

The diagnosed patient with WD was a 6-month-old male, to consanguineous parents. The child had hepatosplenomegaly and adrenal calcification. He showed moderate elevation in the chitotriosidase enzyme activity, with normal levels of both β-glucosidase and sphingomyelinase enzyme activities, excluding GD and NPD. He showed zero activity for the LAL enzyme. Oxysterols were measured to this patient as new biomarkers for the disorder and found to be extremely elevated, confirming the diagnosis of WD. No increase in plasmatic lysosphingomyelin excludes NBD A/B; however, the slight increase of lysosphingomyelin isoform '509' is a strong evidence of WD. This patient was diagnosed provisionally as NP type C, before measuring the LAL activity. The increase of plasmatic lysosphingomyelin may refer to NP type C disease, but this pattern of increase points to WD. As plasmatic cholestane-3b, 5a, 6b-triol was highly increased, it confirmed the pattern of WD.

The overlap of the signs and symptoms in the neonatal period showed the importance of exclusion of WD among this group of patients and the importance of the use of oxysterols and lysosphingomyelin isoform as biomarkers to have an accurate and rapid diagnosis of these patients, who mostly die without reaching a final diagnosis.

The method implemented in this study for measurement of LAL proved to be an accurate specific technique for the diagnosis and screening of WD. Biomarkers help in the establishment of the diagnosis and follow-up of WD.

Conclusion

WD is a fatal disease leading to death before the end of 6 months of age. A complete absence of the enzyme LAL due to mutations in *LIPA* gene is the cause of this disease. Adrenal calcification is a major sign differentiating the disease from other fatal disorders with similar signs and symptoms. Oxysterols level is a new biomarker for the screening of WD, as it is highly increased in the patient with WD. Families with a history of WD should be consulted for future pregnancy and available treatment options. WD should be excluded in all patients with severe organomegaly and life-threatening signs and symptoms.

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Conflicts of interest

There are no conflicts of interest.

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