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Research Article

Gaucher disease (GD; glucosylceramidosis) is caused by a deficient activity of the enzyme glucocerebrosidase (GC). Clinical manifestations are highly variable and cannot be predicted accurately on the basis of the properties of mutant GC. Analysis of secondary abnormalities, such as elevated plasma levels of some hydrolases, may help to increase insight into the complicated pathophysiology of the disease and could also provide useful disease markers. The recent availability of enzyme supplementation therapy for GD increases the need for markers as early predictors of the efficacy of treatment. We report the finding of a very marked increase in chitotrisidase activity in plasma of 30 of 32 symptomatic type 1 GD patients studied: the median activity being > 600 times the median value in plasma of healthy volunteers. In three GC-deficient individuals without clinical symptoms, only slight increases were noted. Chitotriosidase activity was absent in plasma of three control subjects and two patients. During enzyme supplementation therapy, chitotriosidase activity declined dramatically. We conclude that plasma chitotriosidase levels can serve as a new diagnostic hallmark of GD and should prove to be useful in assessing whether clinical manifestations of GD are present and for monitoring the efficacy of therapeutic intervention.

Marked Elevation of Plasma Chitotriosidase Activity

A Novel Hallmark of Gaucher Disease

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Abstract

Gaucher disease (GD; glucosylceramidosis) is caused by a deficient activity of the enzyme glucocerebrosidase (GC). Clinical manifestations are highly variable and cannot be predicted accurately on the basis of the properties of mutant GC. Analysis of secondary abnormalities, such as elevated plasma levels of some hydrolases, may help to increase insight into the complicated pathophysiology of the disease and could also provide useful disease markers. The recent availability of enzyme supplementation therapy for GD increases the need for markers as early predictors of the efficacy of treatment. We report the finding of a very marked increase in chitotriosidase activity in plasma of 30 of 32 symptomatic type 1 GD patients studied: the median activity being > 600 times the median value in plasma of healthy volunteers. In three GC-deficient individuals without clinical symptoms, only slight increases were noted. Chitotriosidase activity was absent in plasma of three control subjects and two patients. During enzyme supplementation therapy, chitotriosidase activity declined dramatically. We conclude that plasma chitotriosidase levels can serve as a new diagnostic hallmark of GD and should prove to be useful in assessing whether clinical manifestations of GD are present and for monitoring the efficacy of therapeutic intervention. (*J. Clin. Invest.* 1994. 93:1288–1292.) Key words: Gaucher disease • glucosylceramidosis • chitotriosidase • enzyme therapy • acid phosphatase

Introduction

Gaucher disease (GD)[1] is characterized by accumulation of glucosylceramide (glucocerebroside) in the lysosomes of macrophages. This is due to an inherited deficiency in the activity of glucocerebrosidase (GC), a lysosomal hydrolase (1). Accumulation of lipid-laden macrophages results in hepatosplenomegaly, bone lesions, and, less commonly, in neurological abnormalities. For the most frequent nonneuronopathic phenotype (type 1) no strict correlation has been established between mutant genotypes, residual GC activity, and clinical expression of the disease (1, 2). Even among siblings sharing the same GC genotype a striking difference in clinical symptoms may occur. It has been claimed that one of the most frequent mutant GC genotypes, homozygosity for the N370S mutation, is associated with mild disease (3, 4). However, with this genotype, completely asymptomatic elderly individuals as well as severely affected young patients have been described. The limited correlation between genotype and phenotype suggests a more complicated pathophysiology of the disease and hampers genetic counselling. This has stimulated interest in secondary biochemical abnormalities associated with clinical manifestations of GD. Elevated plasma levels of acid phosphatase 5b (AP) are traditionally considered to be the most prominent secondary biochemical abnormality (1). Moderate increases in plasma levels of β-hexosaminidase, angiotensin converting enzyme, and lysozyme have also been described (1). Recently enzyme supplementation therapy with alglucerase has become available. Since alglucerase is extremely costly and clinical improvement during enzyme supplementation therapy is slow (usually requiring at least 3–6 mo of treatment) (5, 6), the search for secondary biochemical abnormalities as possible early indicators of response to treatment has become an issue of increasing importance.

Here we report on an extreme elevation in chitotriosidase activity in the plasma of type 1 GD patients. The increased plasma levels of this novel enzyme were studied in relation to those of AP and lysozyme. Its possible source was investigated. The clinical significance of these findings with respect to diagnosis, disease severity, and the monitoring of the efficacy of enzyme supplementation therapy is discussed.

Methods

All GD patients studied (16 males and 16 females; 3–72 yr old) were known to us either by contact with the Netherlands Gaucher Society or by referral to the Academic Medical Center. The diagnosis of GD was based on deficient glucocerebrosidase activity in leucocytes and/or urine samples (7, 8). The clinical manifestations of 25 patients (11 males and 14 females; 15–72 yr old) were classified as being mild, moderate, or severe by using the modified severity scoring index (SSI) (9), which is based on an assessment of the extent of liver, spleen, and bone involvement and the severity of pancytopenia. Using the SSI, mild disease was present in 7 patients, moderate disease in 12 patients, and severe disease in 6 patients. No patient had acute manifestations of the disease. During family screening, three other individuals (one male and two females; 71, 78, and 96 yr old) were found to have deficient GC activity, but no clinical expression of GD. The control population con-

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1. *Abbreviations used in this paper:* AP, acid phosphatase 5b; GC, glucocerebrosidase; GD, Gaucher disease; MU, methylumbelliferyl; SSI, severity scoring index.

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sisted of 50 healthy volunteers (30 males and 20 females; 23–56 yr old), all of whom were found to have normal GC activity.

EDTA plasma samples were obtained from freshly drawn blood and immediately stored at −20°C.

Four patients were studied during therapy with intravenous alglucerase (Ceredase®, Genzyme Corp., Cambridge, MA) at a dose of 4 U/kg, three times a week.

Chitotriosidase activity was measured by incubating 5 μl of EDTA plasma with 100 μl of 0.022 mM 4-methylumbelliferyl-β-D-*N,N',N''*-triacetylchitotriose (4 MU-chitotrioside; Sigma Chemical Co., St. Louis, MO) as substrate in citrate/phosphate buffer (0.1/0.2 M), pH 5.2, at 37°C. In GD patients, samples were diluted 50× in demineralized water before incubation. After 15 min the reaction was stopped with 2 ml of 0.3 M glycine/NaOH buffer, pH 10.6. Fluorescent 4-methylumbelliferone was measured with a fluorimeter (Perkin-Elmer Corp., Norwalk, CT) at 445 nm. Chitotriosidase activity in the supernatant of cultured cells was measured by incubating 10 μl of the supernatant with 100 μl of substrate mixture for 30 min. The enzyme activities were linear with time of incubation and amount of enzyme (data not shown).

Lysozyme activity towards cell wall suspension of *Micrococcus lysodeikticus* (Sigma Chemical Co.) was determined as described by Mörsky (10).

Acid phosphatase activity was measured using 4-methylumbelliferyl phosphate as substrate in the presence of 3 M mercaptoethanol as described by Chambers et al. (11).

Immunoprecipitation studies were performed with a rabbit antiserum to chitotriosidase (obtained after immunization of a rabbit with the purified enzyme) or with anti-(lysozyme) antibodies (Zymed Labs., Inc., San Francisco, CA). Chitotriosidase was purified from the soluble fraction of a detergent-free homogenate of type 1 Gaucher spleen by a sequence of chromatographic steps, including chromatofocussing, gel filtration, and isoelectric focussing. Antibodies were coupled to protein A–Sepharose 4B beads, and an excess of the immobilized antibodies was incubated with plasma for 1 h at room temperature. After centrifugation, enzyme activities in the supernatants were measured as described above.

Monocytes were prepared as follows: mononuclear cells were isolated from citrated blood, diluted 1:1 with PBS, by Percoll density gradient centrifugation (1.077 g/cm^3, 18 min, 2,000 rpm). After two washing steps, one in PBS supplemented with 0.38% sodium citrate and one in autologous plasma, cells were resuspended in Percoll (1.063 g/cm^3) and carefully layered on Percoll (1.074 g/cm^3). After centrifugation (18 min at 2,000 rpm), monocytes were collected from a band on top of the gradient. The cells were washed and resuspended in culture medium (RPMI 1640 supplemented with L-glutamine and 10% human AB serum). The monocyte preparations had a purity exceeding 85% as judged by Giemsa staining. Monocytes were cultured in plastic petri dishes at a concentration of 4×10^5 cells/ml in 10 ml of culture medium. During 20 d of culture, 1.8 ml of the culture supernatant was collected daily and replaced by fresh medium. After centrifugation, the supernatant was immediately frozen at −20°C.

Results

Fig. 1 shows the plasma levels of chitotriosidase, lysozyme, and AP in 32 untreated symptomatic GD patients (III), 3 asymptomatic GC-deficient individuals (II), and 50 healthy controls (I). Chitotriosidase activity was strikingly increased in 30 GD patients (median, 12,824; range, 3,122–65,349 nmol/ml per h) as compared with asymptomatic GC-deficient individuals (90, 166, and 195 nmol/ml per h) and healthy controls (median, 20; range, 4–76 nmol/ml per h) (Fig. 1 *A*). In the 30 GD patients chitotriosidase was increased ≥ 100-fold, the median value being > 600 times the median value of the control subjects. However, in two GD patients, chitotriosidase activity was almost absent (2 and 3 nmol/ml per h). Mixing experiments indicated that the plasma samples of these patients contained no inhibitors of the enzyme. In the three asymptomatic GC-deficient individuals the elevation with respect to the median control value was only 9.7, 4.5, and 8.3 times.

Since it is known that the 4 MU-chitotrioside substrate can also be hydrolyzed by lysozyme (12), the contribution of this enzyme to the observed chitotriosidase activity was assessed by the measurement of the activity of lysozyme, using a suspension of cell walls of *M. lysodeikticus* as substrate. Lysozyme activity, measured in 27 of the 32 GD patients, was only slightly increased (median, 4.1; range, 1.3–9.5 U/μl) as compared with the controls (median, 1.7; range, 0.7–2.9 U/μl) and the asymptomatic individuals (1.2, 1.7, and 3.5 U/μl). Interestingly, in both GD patients with low chitotriosidase activity, elevations in lysozyme activity (4.1 and 5.0 U/μl) were similar to those in the other GD patients. Comparison of chitotriosidase and lysozyme activities in the 30 GD patients revealed no correlation ($r = 0.15$), indicating that the observed chitotriosidase activity can not be explained by lysozyme activity. More importantly, chitotriosidase activity in GD plasma samples proved to be almost completely precipitable with anti-(chitotriosidase) antiserum, but not at all with anti-(lysozyme) antibodies. On the other hand, plasma lysozyme could not be immunoprecipitated with anti-(chitotriosidase) antiserum. In the two GD patients with very low chitotriosidase activities, immunoprecipitation with anti-(lysozyme) antibodies showed that the low residual activity present was entirely due to lysozyme. The same phenomenon was found in the three control subjects with relatively low chitotriosidase activities (4, 4, and 6 nmol/ml per h) (data not shown).

AP activity was elevated to a variable extent in plasma of all GD patients (median, 2,590; range, 452–9,785 nmol/ml per h in the 30 patients with high chitotriosidase activity; 1,159 and 4,764 nmol/ml per h in the 2 patients with low chitotriosidase activity), when compared with controls (median, 202; range, 94–342 nmol/ml per h) and asymptomatic individuals (100, 130, and 177 nmol/ml per h). In GD patients, the elevations in AP activities were clearly far less prominent than those in chitotriosidase activities (Fig. 1, *A* and *B*).

In GD patients, the macrophages are a likely cellular source of biochemical abnormalities (1). We therefore studied the production and secretion of chitotriosidase activity during differentiation of cultured monocytes into macrophages. During the first 5 d of culture of purified monocytes, chitotriosidase activity was found to be absent both in the cells and supernatant. After morphological differentiation of monocytes into macrophages, the cells began to produce and secrete increasing amounts of chitotriosidase (Fig. 2). AP was also produced and secreted after 5 d of culture (Fig. 2) (see reference 13).

We did not find a correlation between chitotriosidase levels and the severity of clinical manifestations, as assessed by the use of the SSI (data not shown). However, in asymptomatic GC-deficient individuals only mild elevations were found. Likewise, chitotriosidase levels did not correlate with the mutant GC genotype in the GD patients (data not shown).

Four patients were treated by enzyme supplementation therapy for ≥ 1 yr. After 3–6 mo, clinical improvement was apparent with respect to hematological markers and reduction in organomegaly (14). Fig. 3, *A–D*, shows that chitotriosidase levels rapidly declined during enzyme supplementation therapy, starting already within 1–2 mo.

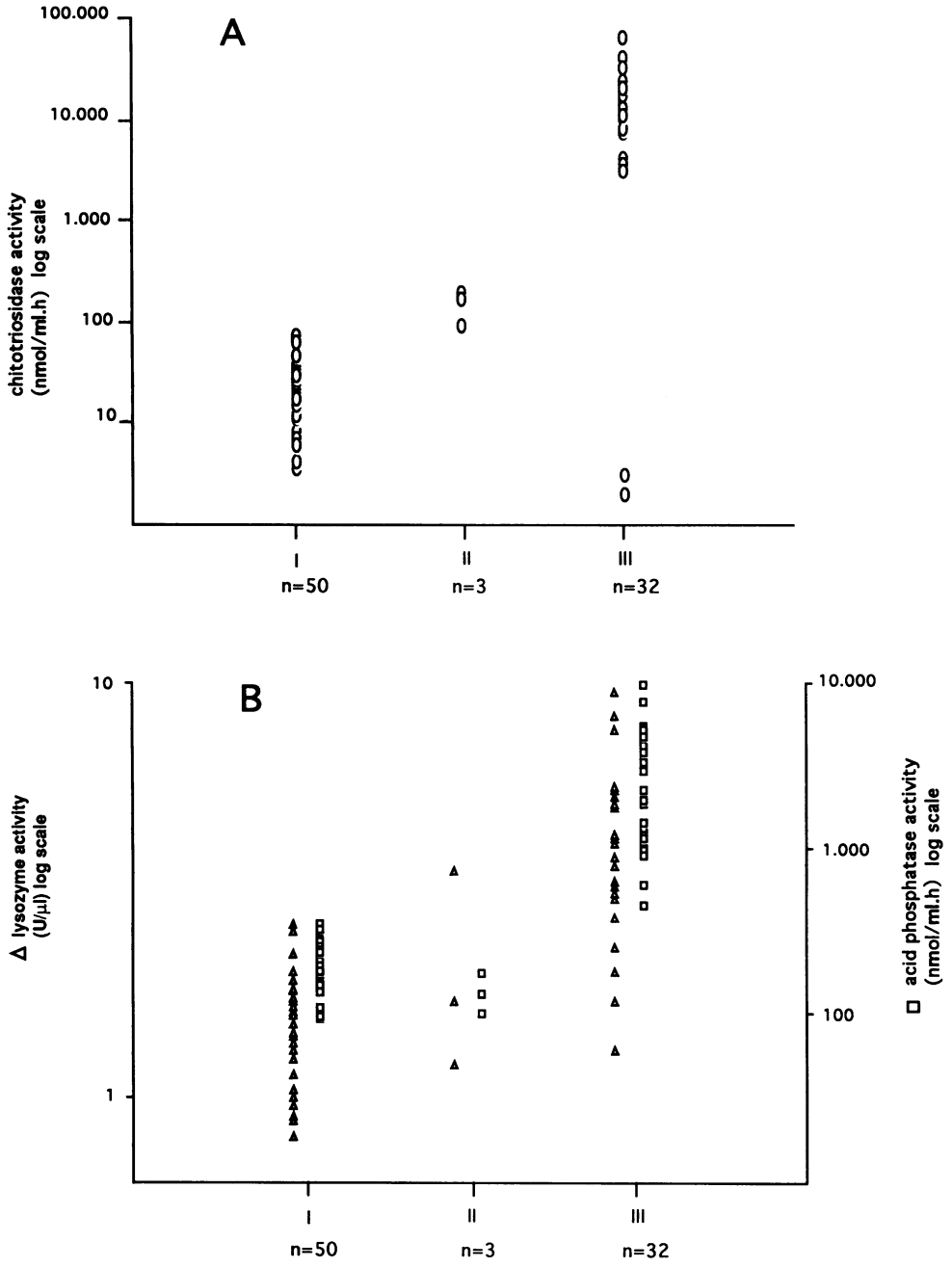

Figure 1. Levels of plasma chitotriosidase (*A*), and lysozyme and AP activity (*B*) in controls (*I*, $n = 50$), asymptomatic GC-deficient individuals (*II*, $n = 3$), and symptomatic GD patients (*III*, $n = 32$).

Discussion

Chitotriosidase activity in plasma samples of untreated type 1 GD patients was found to be strikingly elevated. Moreover, chitotriosidase activity began to decline rapidly after the initiation of enzyme supplementation therapy. Previously, the occurrence of a chitotetraosidase activity in normal human plasma has been reported (15). The chitotriosidase described here resembles the plasma chitotetraosidase with respect to the apparent basic isoelectric point and nonbinding to the lectin concanavalin A. It is therefore possible that chitotriosidase and chitotetraosidase activities are due to a single enzyme. An increase in either chitotriosidase or chitotetraosidase activities in association with pathological conditions has so far not been reported.

The increase in plasma chitotriosidase in GD patients is far more pronounced than the increase in AP activity, which has often been used as an important diagnostic hallmark of the disease (16, 17). Even in asymptomatic GC-deficient individuals a slight elevation in chitotriosidase activity was found, whereas in these individuals AP levels were well within the control range. Because of the simplicity and sensitivity of the assay, determination of chitotriosidase activity can conveniently be used for biochemical confirmation of the diagnosis of GD, in addition to the demonstration of glucocerebrosidase deficiency. Surprisingly, in two of the 32 GD patients studied we repeatedly found an almost complete absence of chitotriosidase activity. In these cases residual enzyme activity was found to be due to lysozyme, which also has catalytic activity towards

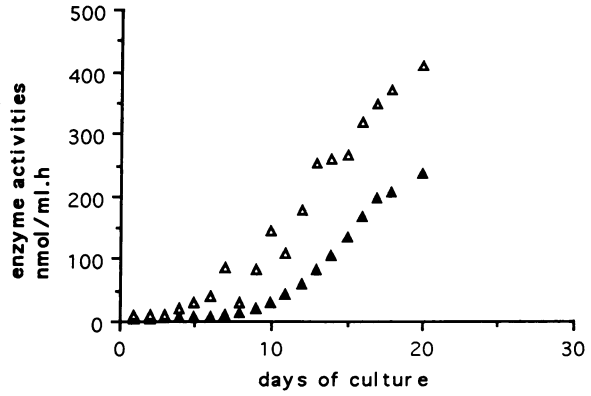

Figure 2. Secretion of chitotriosidase (*filled triangles*) and AP (*open triangles*) in medium of cultured monocytes during differentiation into macrophages. Enzyme activities (means of a duplicate experiment) are expressed in nmol/ml medium per h. Values were corrected for the daily change in medium.

the 4-methylumbelliferyl substrate, although clearly distinct from chitotriosidase. One of the parents of a chitotriosidase-deficient GD patient also lacked enzyme activity, suggesting a familial nature of the deficiency. Absence of chitotriosidase activity was also observed in 3 of 50 controls studied.

The question arises as to what the biological function of chitotriosidase is. Since the two GD patients lacking chitotriosidase activity manifested characteristic clinical symptoms, it is unlikely that chitotriosidase itself contributes to the clinical presentation of GD. On the other hand, the similarity between lysozyme and chitotriosidase with respect to catalytic activity towards the same substrate suggests that chitotriosidase may also have a function in host defense mechanisms, e.g., through cleavage of bacterial cell wall polysaccharide.

Another important question relates to the source of chitotriosidase. GD is characterized by the presence of large numbers of macrophages loaded with glucosylceramide. It therefore seemed likely that these so-called "Gaucher cells" are the main source of the plasma chitotriosidase activity. Our in vitro data support the hypothesis that the enzyme is macrophage derived. However, the rapid decline in chitotriosidase levels after the start of enzyme supplementation therapy, preceding objective clinical improvement, suggests that the production of chitotriosidase is not a simple function of the number of lipid-laden macrophages. This is also suggested by the poor correlation between chitotriosidase levels and SSI, which mainly focusses on the extent of organ involvement in GD. It is more likely that in GD a particular state of activation or differentiation of macrophages or of their precursors leads to the excessive production of chitotriosidase. The rapid decrease in chitotriosidase activity during supplementation with GC may (initially) reflect an alteration in this state rather than a decrease in Gaucher cell mass. The changes in chitotriosidase levels upon enzyme supplementation therapy suggest that the enzyme can be a promising parameter for assessing the clinical response to treatment. More extended studies are needed to relate clinical improvement to decrease in chitotriosidase levels.

Since activated macrophages contribute to the pathophysiology of many diseases, chitotriosidase activity was determined in plasma samples of a number of patients with granulomatous immunological disorders (Wegener's granulomatosis, sarcoidosis) or granulomatous infectious diseases (tuberculosis, leishmaniasis, and leprosy). Elevated enzyme levels were only found for some patients with leishmaniasis and sarcoidosis

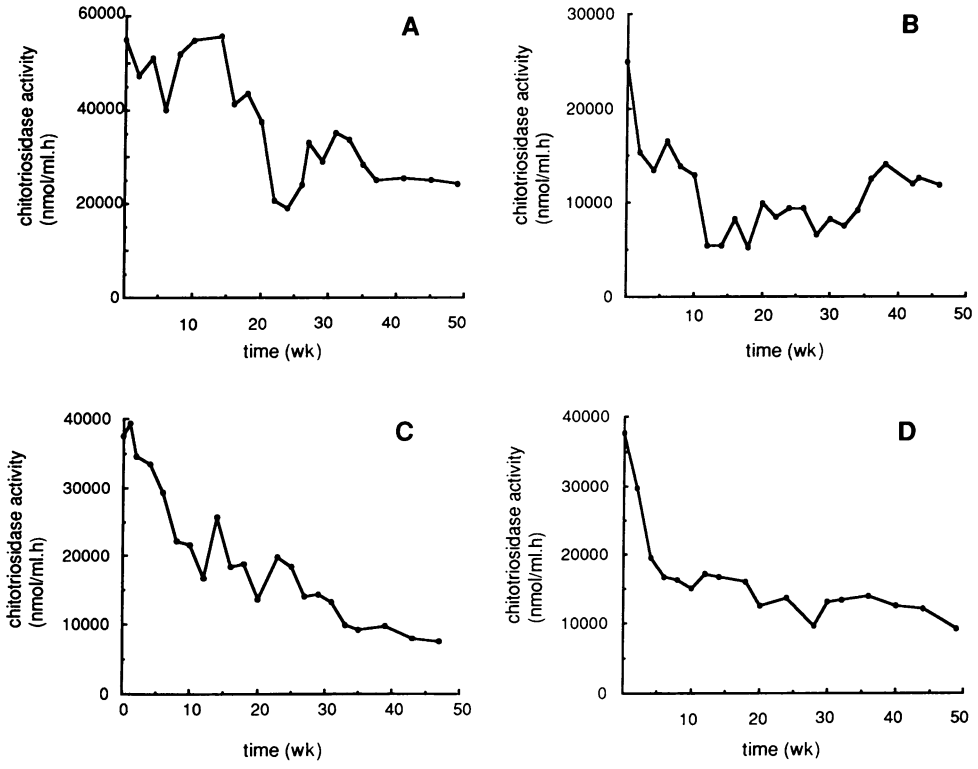

Figure 3. Decrease in plasma chitotriosidase activity in time in four type 1 GD patients (*A–D*) treated with alglucerase (50 U/kg per mo). (control range, 4–80 nmol/ml per h)

(Hollak, C. E. M., and J. M. F. G. Aerts, manuscript in preparation). However, in these cases even the highest chitotriosidase levels did not exceed 1,800 nmol/ml per h, being clearly less than the lowest chitotriosidase activities found for symptomatic GD patients. In plasma samples of patients with acute and chronic myeloid leukemia (diseases that are commonly listed in the differential diagnosis of GD) chitotriosidase activities were within or slightly above the normal range.

It is of importance to note that chitotriosidase levels were found to be only moderately increased in the asymptomatic patients studied and > 100-fold in the mildly affected patients. This difference is unique with respect to secondary markers and suggests that increasing chitotriosidase levels can prove to be a sensitive harbinger of clinical symptoms and consequently may contribute to considerations concerning early therapeutic intervention.

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