

## Original Article

# Individualized screening for chaperone activity in Gaucher disease using multiple patient derived primary cell lines

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**Abstract:** The knowledge of individual response to a therapy, which can be assessed by *in vitro* screening, is essential for the development of therapeutics. Chaperone therapy is based on the ability of small molecules to fold the mutant protein to recover its function. As a novel approach for the treatment of Gaucher disease (GD), ambroxol was recently identified as a chaperone for GD, caused by the pathogenic variants in *GBA* gene, resulting in lysosomal enzyme glucocerebrosidase (GCCase) deficiency. Since ambroxol activity is mutation-dependent, the assessment of the chaperone action requires adaptation of a cell model with genetic format identical to the patient. We compared the chaperone activity of ambroxol using different primary cells derived from GD patients with different *GBA* genotypes. Ambroxol enhanced GCCase activity in cells with wild type *GBA* and in those, compound heterozygous for N370S, but was ineffective in cell lines with complex *GBA* alleles. In cells from patients with neuronopathic GD and L444P/L444P genotype, the response to ambroxol was varied. We conclude that chaperone activity depends on diverse factors in addition to a particular *GBA* genotype. We showed that PBMCs and macrophages are the most relevant cell-based methods to screen the efficacy of ambroxol therapy. For pediatric patients, a non-invasive source of primary cells, urine derived kidney epithelial cells, have a vast potential for drug screening in GD. These findings demonstrate the importance of personalized screening to evaluate efficacy of chaperone therapy, especially in patients with neuronopathic GD.

**Keywords:** Ambroxol, Gaucher disease, chaperone therapy, drug screening, neuronopathic

## Introduction

Personalized medicine started with the knowledge that each individual has a unique genetic background [1]. In some patients, certain drugs do not work as expected; in others, treatment can have toxic effects [2-4]. Therefore, it is desirable to test drug efficacy using *in vitro* cell-based screening assays before prescription of therapy. The key to a successful screening is the adaptation of native cell systems with physiological and genetic format relevant to the patient. Application of stem cell-derived cellular models is expected to improve our ability to speed up the development of novel therapies. Preparation of stem cells from each individual patient however is not only costly but also requires specific expertise. Primary cells can be an ideal source for drug screening for a particular disease, especially for genetic disorders.

Gaucher disease (GD) (OMIM 23080, 231000, 231005), the most common lysosomal storage disorder (LSD), is caused by mutations in *GBA* gene (OMIM 606463), resulting in the deficiency of lysosomal enzyme glucocerebrosidase (GCCase) (EC 3.2.1.45). The clinical Gaucher disease is divided into distinct clinical types whether the Central Nervous System (CNS) is involved. The spectrum for neuronopathic GD ranges from a perinatal lethal form (type 2 GD or acute neuronopathic, GD2) to a slowly progressive neurological disorder (type 3 Gaucher disease, GD3). In non neuronopathic or type 1 Gaucher disease, GD1), there is primary systemic involvement with organomegaly and skeletal disease, or some patients with GD1 occasionally remain asymptomatic.

The *GBA* mutations lead to misfolding of GCCase during the translation process in the endoplasmic reticulum, and altered protein trafficking to

## Chaperone screening in Gaucher disease

**Table 1.** Characteristics of patient-derived primary cells

Patients	Gender	Age	Ethnicity	GBA genotype	GD type	GCase residual activity	Ambroxol treatment	
							Enzyme activity (%)	Protein
1	M	8	Asian	L444P/L444P	GD3	3.5%	No	No
2	M	15	Caucasian	L444P/L444P	GD3	4.2%	211±3.2% 551%*±26%	Increased
3	M	8	Caucasian	L444P/L444P	GD3	3.8%	212±3.2%	ND
4	F	4	Hispanic	L444P/L444P	GD3	5.1%	No*	ND
				D409H/A549P				
5	F	22	Hispanic	L444P/L444P	GD3	7.4%	119±3.5% 216±26#	ND
6	M	21	Hispanic	L444P/L444P	GD3	2.4%	No No#	ND
7	F	12	Hispanic	L444P/L444P	GD3	6.3%	No 158±24#	ND
8	M	50	Ashkenazi	L444P/N370S	GD1	26%	222±14%	ND
9	F	61	Caucasian	L444P/N370S	GD1	6.8%	176±4.4% 304±10%#	ND
<b>Carrier</b>								
1	F	55	Caucasian	WT/N370S		58%	No	ND
2	M	8	Ashkenazi	WT/N370S or L444P		Normal	132±3% UKEC cells	Increased
<b>Control</b>								
1	F	49	Caucasian	WT/WT		Normal	191±5.6% 122±2.5%&	ND
2	F	26	Caucasian	WT/WT		Normal	144±9.8%	ND
3	M	25	Asian	WT/WT		Normal	153±3.3%	ND

No: does not show a statistically significant increase in response to 100 µM Ambroxol. ND: no data. \*Data from fibroblasts and PBMC. #Data from PBMC and macrophages. &Data from fibroblasts and macrophages.

the lysosome [5]. The deficiency of GCase in the lysosome results in chronic accumulation of the substrate glucosylceramide virtually in all cell lineages. However, the involvement of macrophages and their precursors, monocytes result in Gaucher cells, the pathologic hallmark for GD. The current standard of care, enzyme replacement therapy (ERT) administered intravenously successfully treats the systemic manifestation, however is less effective during the advanced stages of disease when the complications ensue. In developing countries, ERT as a treatment modality, is still problematic due to its high cost and limitations with production, distribution, and storage. Another FDA approved therapy for GD is substrate reduction therapy (SRT), and both miglustat and eliglustat act on ceramide synthesis pathway by decreasing the production of the substrate. None of the above therapies are effective for the treatment of neuropathic form of GD. The future of GD therapy may lie in small molecules acting as agents

for enzyme-enhancement therapy (EET). EET is based on the ability of small molecules, or chaperones, to fold the misfolded mutant enzyme. This approach has the potential to cross the CNS and carries the potential to treat the neurological symptoms of GD.

Chaperones show mutation-dependent biochemical chaperoning profiles. It is well studied that different GBA mutations have different effects on protein folding, trafficking, and functional activity [6, 7]. The GCase is extremely prone to bind to site-specific chaperones and propagate enzyme activity in a pH-dependent manner. In 2009, ambroxol was identified as a chaperone for GCase [7, 8]. Ambroxol hydrochloride (C<sub>13</sub>H<sub>19</sub>Br<sub>2</sub>ClN<sub>2</sub>O) is an anti-inflammatory and a Na<sup>+</sup> channel blocker. It was developed for treatment of airway mucus-hypersecretion and hyaline membrane disease in newborns. Ambroxol has unique chaperone characteristics: it works best at both the pH of the endo-

## Chaperone screening in Gaucher disease

plasmic reticulum (ER) and lysosomal environment. At pH 4.3, near lysosomal pH, ambroxol does not inhibit the enzyme but instead becomes an activator of GCase. The 3-D docking model predicts that ambroxol interacts with GCase through hydrogen binding, hydrophobic and  $\pi$ - $\pi$  interactions [7]. Ambroxol interacts with active and non-active sites of the enzyme, by explaining the mixed type of activation/inhibition and pH-dependent activity [7]. However, ambroxol does not have a uniform chaperoning profile for all *GBA* mutations such as L444P [7]. Given that each patient has a unique genetic background, we need a personalized approach to assess the efficacy of chaperones utilizing *in vitro* cell models to achieve therapeutic success for each patient [9, 10].

### Patients and methods

#### Materials

Ambroxol hydrochloride (Abcam, Cambridge, UK), human anti-glucocerebrosidase (*GBA*) antibodies (N-terminal # G4046, C-terminal #G41-71, Sigma-Aldrich, St. Louis, MO, USA), LAMP1 and LC3A/B antibodies (Cell signaling technology, Danvers, MA, USA), NuPAGE SDS running buffer, bolt 8% Bis-Tris Plus gel, Novex ECL chemiluminescent substrate reagents, sample reducing agents, supeScript VIL0 master mix, qubit RNA assay kit, powerUP SYBR green master mix, media 106, low serum growth supplement kit, BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA), sodium taurocholate hydrate, 4-Methylumbelliferyl  $\beta$ -D-glucopyranoside (Sigma-Aldrich, St. Louis, MO, USA), normocin (InvivoGen, San Diego, CA, USA).

#### Patients

The patients' demographics and clinical characteristics are presented in **Table 1**. The diagnosis of GD was confirmed by enzymatic activity and molecular analysis. All patients have given a written informed consent for the collection of samples and analysis of the data. The clinical protocol was approved by the ethics committees and data protection agencies at all participating sites, protocol # 13-CFCT-07, WIBR protocol #20131424.

#### Isolation and growth of primary skin fibroblasts

Tissue samples were obtained from 4 patients (1 female and 3 male, age 3-15 years) (**Table**

**1**). Skin biopsies were placed into 50 ml conical tube and washed in PBS with 1% penicillin/streptomycin solution (ThermoFisher Scientific, Rockford, IL, USA). Skin fibroblasts were cultured as per standard methodology in 24 well plates with complete media 106 (media 106, low serum growth supplement kit and normocin, ATCC, USA). After first passages, fibroblast cells were grown in complete media 106 until confluent stage and sub-cultured at a split ratio 1:4 for young cultures. Cultures were terminated before passage 10 or when the fibroblasts were unable to reach confluence after two weeks.

#### Isolation, purification and growth of urine-derived kidney cells

Fresh 25-50 ml of mid-stream urine samples were collected from a male patient with GD and a healthy control. The samples were processed immediately by transferring to sterile 50 ml conical tubes and then centrifuging at 400 $\times$ g for 10 min. After the supernatant was removed, the pellets were washed three times with PBS containing 1% ampicillin/streptomycin and centrifuged again at 400 $\times$ g for 10 min. Cells were then plated in a 24-well dish with renal epithelial cell basal media supplemented with renal epithelial cell growth kit (ATCC) and normocin (InvivoGen). While most cells from urine failed to attach; kidney epithelial cells attached to plate surfaces. The culture media was changed every 2-3 days until a single or few cells formed colonies. The urine kidney epithelial cells were split using 0.05% trypsin when culture cells reached formation of large colonies. After first passages, kidney epithelial cells were continuously grown in complete renal epithelial cell basal media and sub-cultured at a split ratio of 1:2 for young cultures. Maximum passage number was 6-8 passages, or until kidney epithelial cells were unable to reach confluence and started to undergo apoptosis.

#### Isolation, purification and culture of peripheral blood monocytes (PBMC)

PBMC were purified from blood samples from patients with GD using Lymphoprep™ reagent and SepMate™ tubes (Stemcell Technologies, Vancouver, Canada). Lymphoprep™ was added to the lower compartment of the SepMate tube. Blood was mixed with PBS containing 2% fetal bovine serum (FBS) in a 1:1 ratio, and then layered on top of Lymphoprep™ following manu-

## Chaperone screening in Gaucher disease

facturer's protocol. Samples were centrifuged at 800×g for 20 min at 18°C with the brake off. The upper plasma layer was discarded. The PBMC layer was removed carefully, washed three times with PBS and centrifuged at 300×g for 8 min at room temperature between each wash. Isolated PBMC were incubated in 5% CO<sub>2</sub> in phenol red-free RPMI media with 10% FBS.

### *Differentiation of M2-macrophages from PBMC*

Freshly isolated PBMC had been used for macrophage differentiation. PBMC (10 million in 1 ml) were resuspended in an appropriate amount of monocyte attachment medium (2 ml per well in 6 well plate) then incubated for 2 h in 5% CO<sub>2</sub> cell culture incubator to let monocytes attach to the surface. After aspiration of the media along with floating cells, a complete M2-macrophage generation media was added. M2 differentiation media contained: RPMI 1640 supplemented with 10% FBS, 1% normocin, 2 mM glutamine, 1% Na-pyruvate, 1% non-essential amino acids (NEEA) and 50 ng/ml human recombinant M-CSF (ThermoFisher Scientific, Rockford, IL, USA). After 6 days 100% by volume of fresh complete M2 macrophage differentiation media was added, and 2 days later media was completely replaced with fresh media. On day 10 macrophages were treated with ambroxol at indicated intervals and concentrations.

### *Protein isolation and Western blot analysis*

Whole-cell extracts (WCE) were prepared in radioimmunoprecipitation (RIPA) buffer. Protein concentrations were determined using the BCA protein assay kit (ThermoFisher Scientific, Rockford, IL, USA). 30-40 µg of WCE were separated on mini protein TGX stain free gel (Bio-Rad, Hercules, CA, USA) and electroblotted using the Trans-Blot® Turbo™ midi PVDF transfer packs. The ChemiDoc™ MP imaging system (Bio-Rad, Hercules, CA, USA) was used to visualize and quantitate optical density (IOD) for each band. The IODs of bands of interest were normalized to the loading control, beta-actin, and the normalized value of the controls were set to 1 for comparison between separate experiments.

### *RNA isolation and quantitative real-time-PCR (qPCR)*

RNA was extracted from patient and control PBMCs using the RNeasy mini kit from Qiagen

(Valencia, CA, USA). The superScript®VILO™ cDNA synthesis kit was used to reverse-transcribe RNA using random hexamers primers. Samples were prepared using PowerUp™ SYBR® master mix. Individual samples were run in triplicate. mRNA transcript levels were compared to loading control, GAPDH were measured using StepOnePlus™ Real-time PCR System (ThermoFisher Scientific, Rockford, IL, USA). The primers were purchased from ThermoFisher Scientific (Rockford, IL, USA) ([Supplementary Table 1](#)). Analyses and fold differences were determined using the comparative CT method. Fold change was calculated from the  $\Delta\Delta CT$  values with the formula  $2^{-\Delta\Delta CT}$  relative to mRNA expression in untreated control.

### *Measurement of GCase activity*

The assay to measure GCase enzymatic activity in cells was carried out as described [11, 12], using 4-methylumbelliferyl b-D-glucopyranoside as the artificial substrate. Released 4-methylumbelliferone was measured using a fluorescence plate reader (excitation 360 nm and emission 460 nm). Briefly, for preparation of lysates, the frozen cells were lysed in cold H<sub>2</sub>O. Protein concentrations were determined with BCA assay kit (Pierce, Rockford, IL, USA). The reaction was started by addition of 5 or 10 µg of protein into substrates solution in 0.1 M citrated buffer, pH 5.2, supplemented with sodium taurocholate (0.8% w/v). The reaction was terminated by adding 0.4 ml of 0.2 M glycine sodium hydroxide buffer (pH 10.7).

### *Statistical analysis*

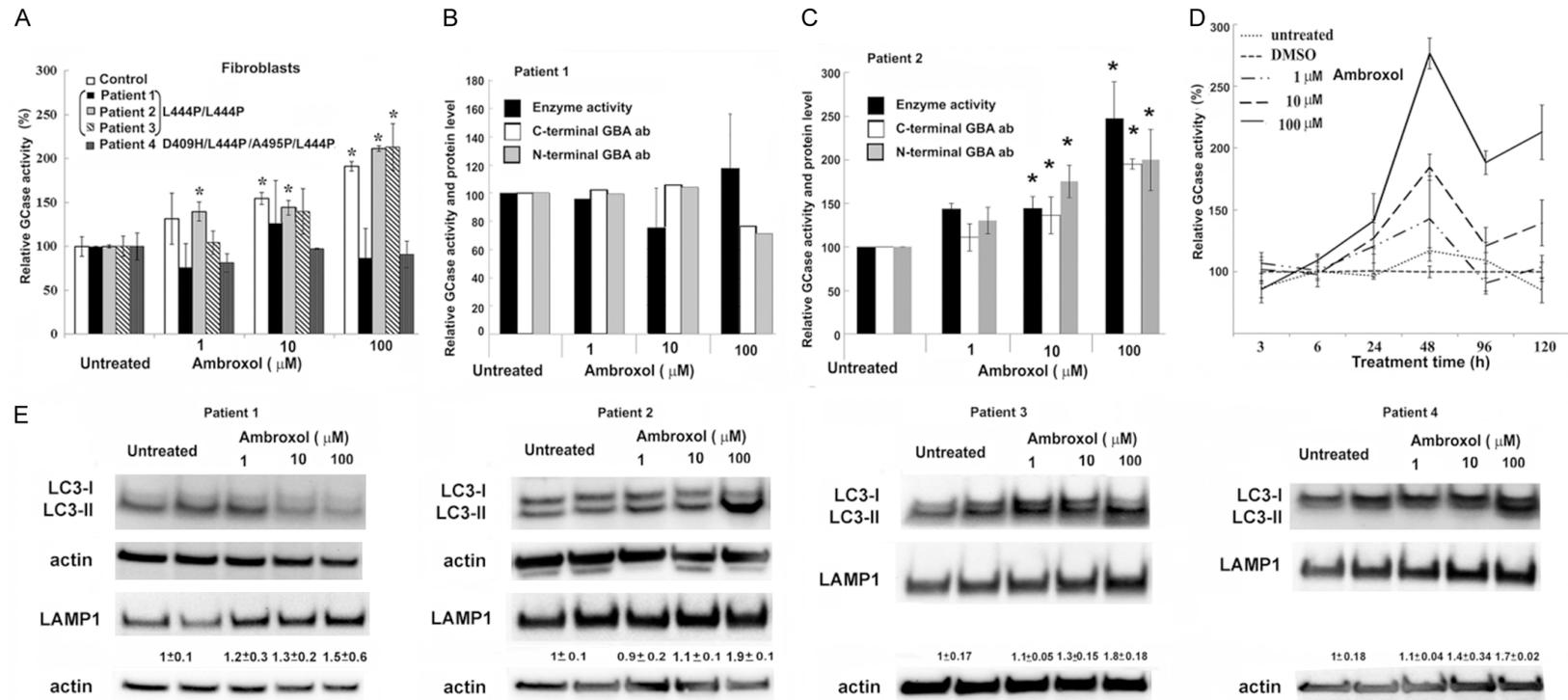
Statistical analyses were performed using Student's *t* test with 2-tailed distribution and 2-sample equal variance or 1-way ANOVA followed by Student-Newman-Keuls using GraphPad Prism (GraphPad, San Diego, CA, USA).

## Results

### *Chaperone activity of ambroxol in patient derived fibroblasts*

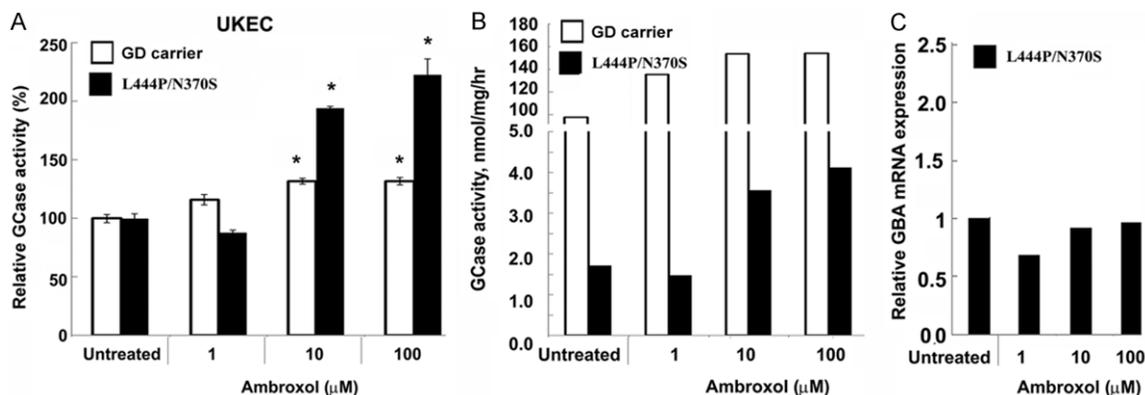
Dermal fibroblasts are an established research model for GD. Fibroblasts appear 7-10 days after the biopsy and get established in 4-8 weeks before screening. The most common GBA mutation leading to GD3 is L444P/L444P. However, it is not clear whether ambroxol is able to fold this mutated enzyme in fibroblasts derived from GD3 patients with L444P/L444P

## Chaperone screening in Gaucher disease



**Figure 1.** Ambroxol chaperone activity in fibroblasts from GD patients with L444P/L444P and D409H/L444P/L444P/A549P genotypes. A. Fibroblasts were cultured for 5 days in presence of increasing concentrations of ambroxol. GCase enzyme activity was estimated as relative levels towards to untreated control. Each bar represents the average  $\pm$  stdev.  $P < 0.05$  vs. untreated control; Student's *t* test. B. Fibroblasts derived from patient 1 show no enhancement of GCase activity or protein level in response to ambroxol treatment. Fibroblasts were treated for 5 days in the presence of ambroxol. Whole cell extracts (30 mg) were prepared and immunoblotted using anti-GBA antibodies. Results represent enzyme activity level (black bar), immunoblotting with C-terminal (white bar) and N-terminal (gray bar) anti-GBA antibodies. Quantification of relative levels of GBA normalized to actin from separate experiments in which control were untreated fibroblasts. Untreated control set as 100%. C. Fibroblasts derived from patient 2 show enhancement of GCase activity in response to ambroxol treatment. Values are averages  $\pm$  SEM.  $*P < 0.05$  vs. untreated control; Student's *t* test. D. Ambroxol induced GCase activity in time- and concentration-dependent manner, as determined by enzyme activity assay. GD3 fibroblasts were derived from patient 2. Each bar represents the average  $\pm$  stdev vs untreated control; Student's *t* test. E. Following ambroxol treatment, representative Western blot showing LC3-I/LC3-II and LAMP1 protein expression level in fibroblasts derived from patient 1, 2, 3 and 4. Quantification of relative level of LAMP1/actin from 3 different experiments.

## Chaperone screening in Gaucher disease



**Figure 2.** Ambroxol increases GCCase activity in urine kidney epithelial cells. (A, B) GCCase activity in UKEC cells derived from a healthy control (white bar) and a GD patient with genotype L444P/N370S (black bar) cultured for 5 days in presence of increasing concentrations of ambroxol. The data are expressed as % GCCase activity compared to respective untreated cells (A) and as GCCase concentration nmol/hr/mg protein (B). Each bar represents the average  $\pm$  sem. \* $P < 0.05$  compared with an untreated group. (C) Q-PCR was performed to determine relative *GBA* mRNA expression levels after 5 days ambroxol treatment.

and with 3-6% of residual GCCase activity. GCCase activity was measured and compared to controls after treating the cells with ambroxol. GCCase activity was increased in control fibroblasts (**Figure 1A** and [Supplementary Figure 1A](#)) suggesting ambroxol's ability to accelerate folding and trafficking of wild type GCCase from endoplasmic reticulum to lysosomes [7, 13]. Consistent with a dose response effect, in two patient cell lines, there was an increase in GCCase activity (**Figure 1A** and [Supplementary Figure 1A](#)), however fibroblasts from patient 1 showed no increase in GCCase activity following ambroxol treatment (**Figure 1A** and [Supplementary Figure 1A](#)). Ambroxol did not lead to a change in enzymatic activity in D409H/L444P fibroblasts (patient 4). Fibroblasts from patient 1 similarly showed no change in protein or mRNA levels (**Figure 1B** and [Supplementary Figure 1B, 1C](#)). In fibroblasts from patient 2, there was an increase in GBA protein, but not mRNA levels (**Figure 1C** and [Supplementary Figure 1D, 1E](#)). These findings demonstrate that not all GCCase mutants respond to ambroxol similarly, and especially the genotype L444P/L444P exhibit varied responses. To determine the optimal time to enhance mutated GCCase, fibroblasts (patient 3) were treated with ambroxol at intervals of 3 h, 6 h, 24 h, 48 h, 4, and 5 days. GCCase activity increased after 24 h, with a maximum peak at 48 h (**Figure 1D**).

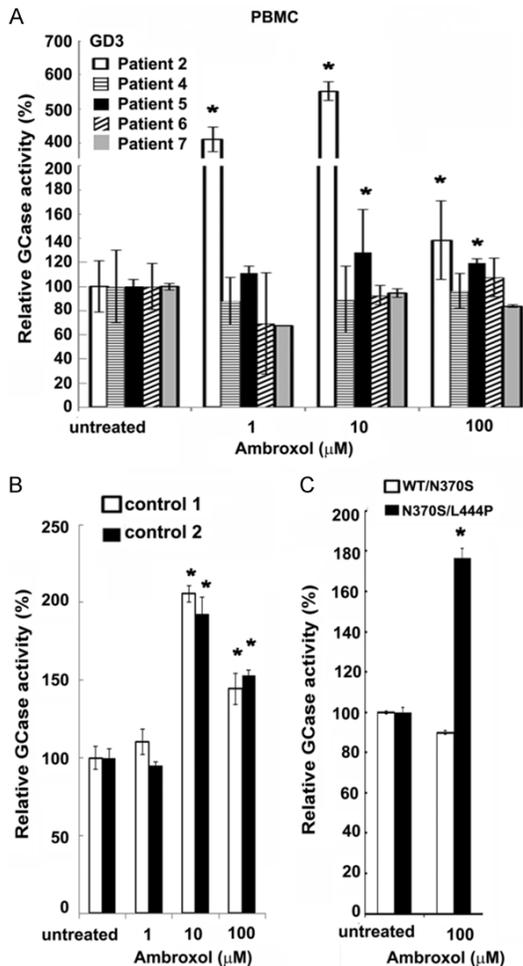
GCCase deficiency is associated with alterations in autophagy-lysosomal function. Recent reports indicate that ambroxol may have dual action:

chaperone activity and restoration of lysosomal dynamics for the proper functioning of GCCase [13-15]. To examine the effects of ambroxol on autophagy-lysosomal pathway, the autophagy marker LC3/II and lysosome marker LAMP1 were used. Western blot analysis demonstrated an increase in the level of LC3-II with ambroxol treatment in all fibroblasts except for patient 1 that also didn't have a change in GCCase activity (**Figure 1E**). For LAMP1, fibroblasts from all patients demonstrated increased levels after ambroxol treatment (**Figure 1E**). In summary, ambroxol enhanced GCCase activity and improved autophagy-lysosomal dynamics in fibroblasts derived from GD 3 patients. Moreover, these results clearly suggest that the prediction of ambroxol chaperone activity cannot be done alone based on the type of GCCase mutation.

### *Monitoring chaperone response in urine kidney epithelial cells (UKEC)*

We investigated whether a non-invasive source of primary cells from patients could serve as a cell model for chaperone screening, the effect of ambroxol on the GCCase activity in urine derived kidney epithelial cells (UKEC) was studied. UKEC derived from an asymptomatic GD carrier and a patient with GD and L444P/N370S mutation were treated with ambroxol. GCCase activity increased in UKEC lines in a concentration-dependent manner, similar to that observed in fibroblasts (**Figure 2**). mRNA analysis was concurrent with that of the fibroblast,

## Chaperone screening in Gaucher disease



**Figure 3.** Ambroxol chaperone activity in PBMCs. A. PBMCs derived from GD patients with L444P/L444P genotype, were cultured for 5 days in the presence of increasing concentrations of ambroxol. The data are expressed as % GCase activity compared to respective untreated cells. Each bar represents the average  $\pm$  stdev. \* $P < 0.05$  Student's t-test compared with an untreated group. B. Ambroxol induced GCase activity in control PBMC as determined by enzyme activity assay. PBMCs derived from healthy donors were cultured for 5 days in the presence of ambroxol. The data are expressed as % GCase activity compared to respective untreated cells. Each bar represents the average  $\pm$  stdev. \* $P < 0.05$  Student's t-test compared with an untreated group. C. PBMCs derived from a Gaucher carrier patient with WT/N370S genotype and a GD patient with N370S/L444P genotype were cultured for 5 days in presence of 100  $\mu$ M of ambroxol. The data are expressed as % GCase activity compared to respective untreated cells  $\pm$  stdev. \* $P < 0.05$  Student's t-test compared with an untreated group.

and showed that ambroxol-treated kidney cells did not have increased mRNA level, (Figure 2C). Thus, UKEC are a suitable model for testing effi-

cacy of enzyme enhancement therapy with pharmacological chaperones.

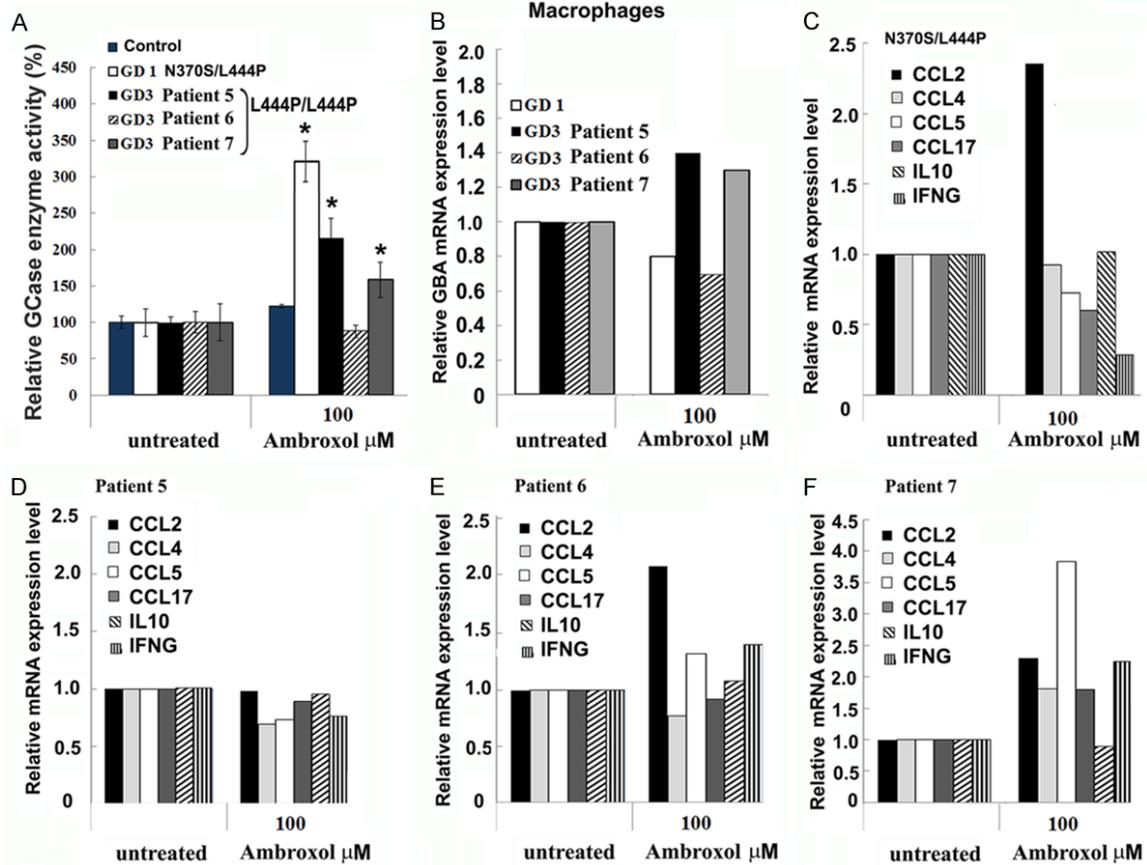
### *An express-screening model for GD peripheral blood mononuclear cells*

An expedited screening for ambroxol chaperone efficiency could be done in 5 days using PBMC. Screening of PBMCs from five patients with either L444P/L444P or D409H/L444P/A495P/L444P genotypes demonstrated that cells from different patients responded differently to ambroxol (Figure 3A and Supplementary Figure 2A). PBMCs from patient 2 with the lowest GCase activity showed the best chaperone response (Figure 3A), consistent with ambroxol induced increase in GCase activity in fibroblasts. D409H/L444P/A495P/L444P PBMCs showed no increase in GCase activity in the presence of ambroxol, also similar to the fibroblasts. In samples 5, 6, and 7 derived from siblings carrying L444P/L444P mutation, only PBMCs from patient 5 showed an increase in GCase activity with ambroxol. (Figure 3A and Supplementary Figure 2A). Ambroxol elevated GCase activity in control PBMCs (Figure 3B and Supplementary Figure 2B). The chaperone efficiency of ambroxol was assessed in a GD carrier (WT/N370S) and the GD patient with N370S/L444P genotype and Parkinsonism, PBMCs were treated with ambroxol. Ambroxol increased enzyme activity in PBMCs from the patient with GD, but not from the Gaucher carrier (Figure 3C). These results supports that individual screening is essential to predict the efficacy of ambroxol. In contrast to the fibroblast data, the highest concentration (100  $\mu$ M) of ambroxol showed inhibition of GCase activity in PBMCs.

### *Patient-derived macrophages as a GD model for chaperone screening*

In GD, macrophages, with distinct subpopulations such as M2, are suggested to be the primary disease effectors [16, 17]. M2 macrophages were differentiated from PBMCs isolated from a control, a GD1 patient with N370S/L444P, and GD3 patients with L444P/L444P genotypes (Figure 4). Macrophages, along with PBMCs, derived from GD1 patient showed ambroxol chaperone activity (Figure 4A). Consistent with the previous data, in patients with the L444P/L444P, the response to ambroxol treatment varied. Macrophages and PBMC derived

## Chaperone screening in Gaucher disease



**Figure 4.** Macrophages as a GD model for screening chaperone activity. (A) Macrophages derived from a healthy donor (control), GD patients with L444P/N370S, and L444P/L444P genotypes, were differentiated from PBMCs and treated for 5 days in the presence of 100 mM ambroxol. The data are expressed as % GCase activity compared to respective untreated cells  $\pm$  stdev. \*P < 0.05 Student's t-test compared with an untreated group. (B) Ambroxol treatment did not change GCase mRNA levels in macrophages. Q-PCR was performed to determine relative GBA mRNA expression levels from macrophages derived from GD patients with L444P/N370S and L444P/L444P genotypes. Macrophages were differentiated from PBMC and treated for 5 days in the presence of 100 mM of Ambroxol. (C-F) Q-RT-PCR was performed to determine mRNA expression levels of CCL2, CCL4, CCL5, CCL17, IL10 and IFNG in macrophages derived from GD1 patient (C), GD3 patients with chaperoning activity to ambroxol (C-E), and GD3 patient not responded to ambroxol (E).

from patient 5 showed increasing GCase activity in the presence of ambroxol (Figure 4A). Surprisingly, ambroxol led to an enhanced GCase activity in macrophages from patient 7, but no effect was observed in PBMCs (gray color bar, Figures 3 and 4). Despite an increase in the GCase activity level, the mRNA expression level did not change with ambroxol (Figure 4B).

Substrate accumulation is suggested to trigger macrophage activation, enhanced expression of immune response genes, and transformation of monocytes into macrophages [16]. For that reason, we studied the effect of ambroxol on expression of cytokines, interferon gamma (IFNG), and interleukin 10 (IL10). An increased CCL2 mRNA level was observed after ambroxol

treatment in GD1 and GD3 macrophages (Figure 4). CCL4 and CCL5 levels did not change, except for an increase in CCL5 levels in macrophages from patient 7 (Figure 4F). A decrease in the expression of IFNG and CCL7 occurred only in N370S/L444P macrophages after ambroxol treatment, but not in L444P/L444P macrophages (Figure 4). Altogether, data demonstrate that ambroxol also changes the expression profile of genes involved in immune pathways in macrophages.

### Discussion

Enzyme enhancement or chaperone therapy is a promising approach for the treatment of GD, especially when the CNS is involved. Recently,

## Chaperone screening in Gaucher disease

an open-label pilot study with ambroxol showed promising results in patients with neuronopathic GD with N188S, G193W, F213I/RecNcil and D409H/IVS10<sup>-1</sup>G>A genotypes [10]. Ambroxol demonstrated good tolerability while enhancing GCase activity and improving neurological manifestations. In this study, we focused on screening cell lines from GD3 patients with L444P/L444P and compared them with the cell lines harboring H409/L444P/A549P/L444P and N370S/L444P mutations as in a given population of patients with the subacute neurological form of Gaucher disease (GD3), the homozygosity for L444P is the most common. However, it is not clear whether ambroxol is able to fold GCase mutant L444P/L444P. In previous studies, fibroblasts with L444P/L444P mutation demonstrated neither increased protein levels nor enzymatic activity upon ambroxol exposure [6, 7]. While ambroxol increased GCase activity, protein and RNA levels in L444P/L444P fibroblasts in another study [13]. As such, we investigated ambroxol chaperoning activity in primary fibroblasts derived from patients with the genotype L444P/L444P, and with 3-6% range of residual GCase activity. Ambroxol showed time and concentration-dependent chaperoning activity in control fibroblast, however not all L444P/L444P fibroblast lines responded positively to ambroxol. This result, together with the contradicting previously published data, suggests that the chaperone activity of ambroxol could be modified by diverse factors and not only by the individual's genetic variants. Therefore, we highlight the importance of individualized screening for choosing chaperone therapy using ambroxol for patients with GD. It is well-known that the ER-folding system is complex and plays a central role on folding of misfolded mutant proteins. GCase as a lysosomal enzyme go through quality control and several protein modifications before shuttle to Golgi apparatus. Following further modification, GCase is trafficking to the lysosomes [18]. Mutant GCase enzymes are recognized as misfolded proteins and undergo through excessive degradation instead of trafficking to lysosome especially the L444P mutant of GCase [19]. Different mutations of GCase demonstrated varying degrees of degradation and demonstrated different level of residual enzyme activity. In GD1 cell lines with N370S/N370S, where residual GCase activity is 20% of normal, ambroxol was shown to sig-

nificantly increase the enzymatic activity and protein levels [6, 7]. Similarly, ambroxol increases GCase activity in fibroblasts harboring N188S/G193W, F213I/RecNcil, and D409S/IVS10<sup>-1</sup>G>A mutations [10]. In contrast, the genotype F213I/L444P having 5% residual GCase activity does not respond to ambroxol [6, 10]. Additionally, ambroxol increases lysosomal integral membrane protein-2 (LIMP-2) which mediates GCase trafficking to lysosomes and activate GCase endogenous activator saposin C [15, 20]. During the process of translation and trafficking of GCase, ambroxol enhances the removal of L444P mutant protein from the endoplasmic reticulum, however this does not always lead to recovery of the enzyme activity [20]. Thus, the wide clinical spectrum observed in GD patients especially with L444P homozygotes cannot be explained only by the contribution of a genetic mutation [21]. The balance between lysosomal enzyme degradation, folding and trafficking is determined by the more than 75 proteins of the ER proteostasis network. As such, components of proteostasis network may play a vital role by re-arrangement ambroxol-mutant GCase forming interaction to other proteins during trafficking and maintaining enzyme activation. For example, inhibition of ERdj3-mediated mutant GCase with enhancing of calnexin-associated folding is able to rescue of lysosomal L444P GCase in fibroblast L444P cell line [22].

Dermal fibroblasts are the most common research model for LSDs, including GD. Initially, cultured fibroblasts were used to measure GCase activity to confirm GD diagnosis [7]. Later, cultured skin fibroblasts became the popular model to study molecular and biochemical aspects of GD, including the original screening of ambroxol as a GCase chaperone [7]. We were able to complete the ambroxol screening in two to three weeks, when fibroblasts demonstrated good proliferative rate with a low passage number. But, ambroxol concentration and time course treatments for screening in fibroblasts were not standardized and varied from one publication to another. The reported range for the optimal concentration of ambroxol, i.e., when ambroxol enhances the activity of the mutant GCase without toxic effects, has a wide range from 1 to 100  $\mu$ M concentration. In fibroblasts, GCase has been reported to have half-life of approximately 60 h, so treatment dura-

## Chaperone screening in Gaucher disease

tion for 5 days often has been used [7, 10, 13]. In this study, ambroxol exhibited time-dependent chaperoning profiles after 24 h treatment, and the maximum enzyme activity was observed at 48 h.

Obtaining tissue material from pediatric patients requires invasive procedure such as biopsy or phlebotomy. Moreover, access to a medical facility is required for this kind of sample collection. We have now established primary UKEC as a cell model for screening assays. Stably-transfected podocytes have been used previously to study molecular and the pharmacogenetics aspect of Fabry disease (FD), where kidney dysfunction is a frequent complication [23, 24]. However, to our knowledge, kidney cells are not yet studied as a GD cell model. Kidney epithelial cells are regularly sloughed off the renal tubule into the urine [25], and can be collected from urine and selectively grown under conditions supporting proliferation of epithelial cells [26]. 25-100 ml of mid-stream urine can be collected and samples may be processed immediately or stored at +4°C for up to 24 h, allowing the transport of samples. UKEC cell colonies become visible 1-2 weeks, after which can be passaged. Cells stop dividing after 5-6 passages, providing a time frame of two to three weeks for ambroxol screening.

GD pathology involves macrophage lineage cells, including monocytes, and dendritic cells, T and B cells [16, 27]. In macrophages, the accumulation of GCase substrate in the lysosomes leads to formation of "Gaucher cells" [28]. Gaucher cells become intensely stimulated and release chemokines and cytokines, some of which are utilized as GD biomarkers. Moreover, Gaucher cells accumulate in various tissues, and are associated with the propagation of tumor associated macrophages [28]. Therefore, PBMCs and macrophages are the most relevant cell type to screen efficacy, toxicity, and inflammatory response in GD. Similar to the fibroblast and UKEC data, ambroxol demonstrated chaperone activity in PBMC and macrophages. However, cells derived from various patients with genotype L444P/L444P, including PBMCs and macrophages derived from three siblings with GD3 responded differently to the ambroxol treatment.

Synonymous single nucleotide polymorphism (SNPs) could affect messenger RNA splicing,

stability, protein structure and protein folding [29-31]. Increasing evidence suggest that in future, SNPs analysis could help to predict individual's response to treatment. Over 4000 SNPs of GBA genes have been reported, including 98 pathogenic SNPs, 34 likely benign, or uncertain significant SNPs, it is possible that variability in response to ambroxol treatment may be related to SNP.

Ambroxol is a drug with anti-inflammatory activity, and has been historically used for the treatment of acute and chronic bronchopulmonary diseases. It is known that patients with L444P/L444P genotype have a high risk for lung involvement [32]. Furthermore, Gaucher macrophages release proinflammatory cytokines such as CCL2, CCL5, IF gamma, IL-6, IL-8, IL-18, IL10 [16]. Elevated cytokine levels are associated with bone and pulmonary pathology of GD. Ambroxol was shown to decrease the secretion of IL-2, IF gamma, IL-1, and TNF-alfa in PBMCs stimulated with phytohemagglutinin or lipopolysaccharide [33]. In the present study, macrophages respond to ambroxol by changing expression profile of genes involved in immune pathways. Ambroxol stimulated the expression of CCL2, a chemokine which regulates migration and infiltration of monocytes/macrophages. Expression analysis of other immune pathway genes suggests, among patients with GD, there are differing responses. Altogether, our results show that macrophages respond to ambroxol by changing expression profile of immune genes, thus chaperone screening should also include immune activation studies, especially in patients with significant bone or pulmonary disease.

### Summary

We evaluated chaperone therapy using ambroxol in different types of primary cells derived from patients with Gaucher disease. Ambroxol demonstrated a positive chaperone effect in primary cells with N370S/L444P genotype; however, in cells derived from patients with L444P/L444P mutation, there was not always a response to ambroxol. Patient-derived primary cells can be used to identify patients who are likely to respond to chaperone treatment. The individualized screening for ambroxol is an example how personalized medicine can improve the efficacy of certain medications while preventing the untoward effects.

# Chaperone screening in Gaucher disease

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## Disclosure of conflict of interest

None.

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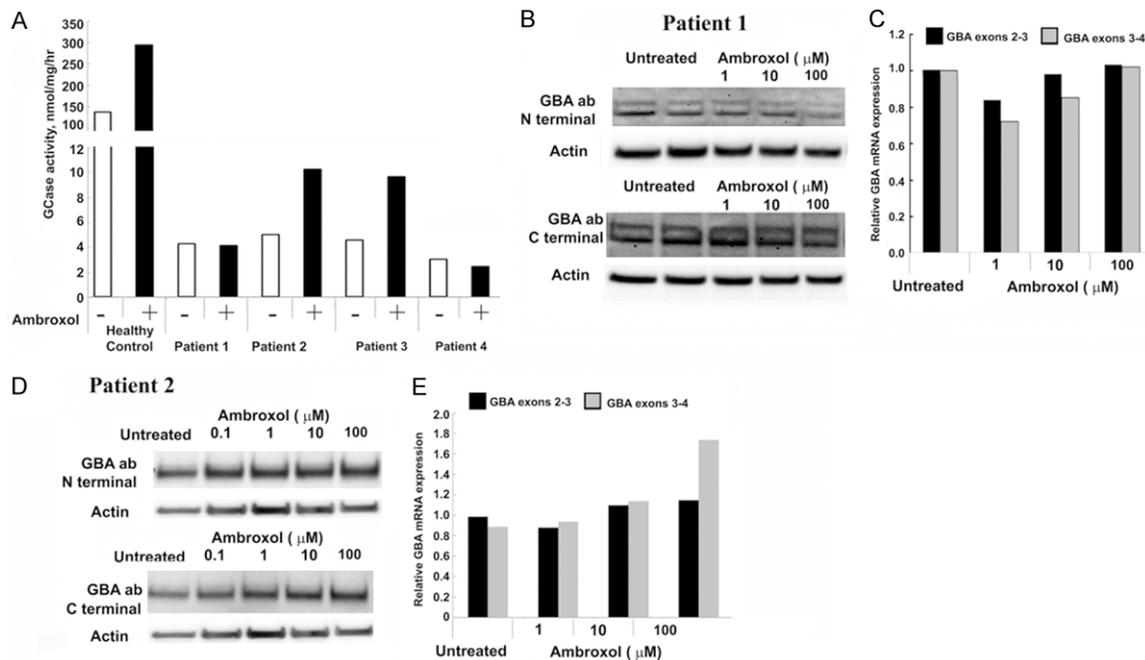
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## Chaperone screening in Gaucher disease

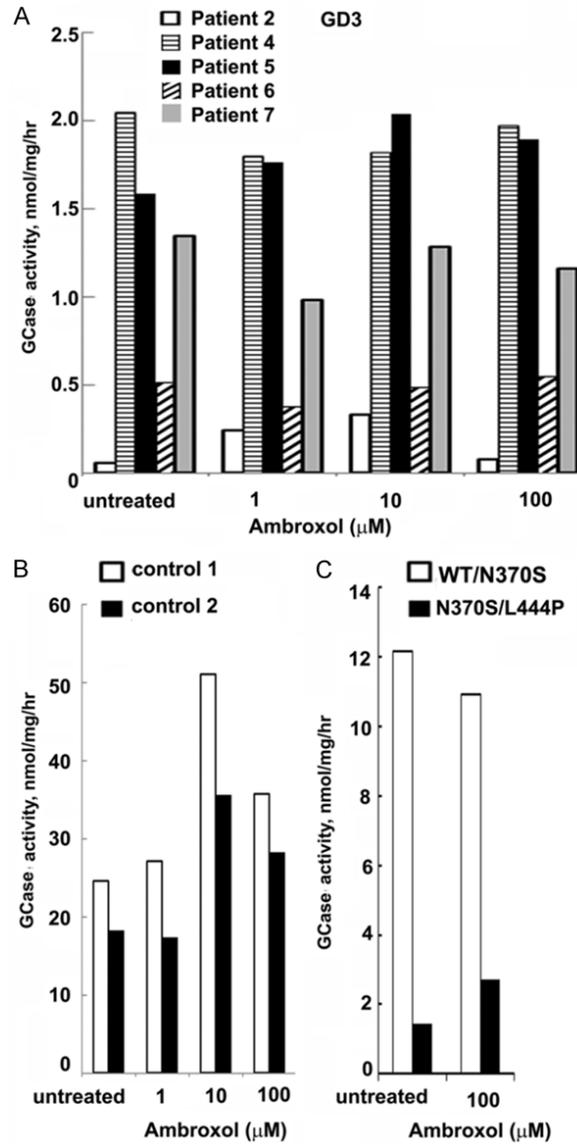
**Supplementary Table 1.** Primers used for RT-PCR

Name	Abbreviation	Sequence (5'-3')
acidic $\beta$ -glucosidase	GBA exon 2-3	5'-gcagccagaacagaagtcc-3' 5'-gcataggttaggtgcggat-3'
	GBA exon 3-4	5'-accacaatgtccgcctactc-3' 5'-cgacgcctgcttcaccacct-3'
Chemokine (C-C motif) ligand 11	CCl2	5'-cagccagatgcaatcaatgcc-3' 5'-tgattcctgaaccactct-3'
Chemokine (C-C motif) ligand 4	CCL4	5'-aagctctgcgtgactgtcctgt-3' 5'-aagcttctcgcgggtgaaga-3'
Chemokine (C-C motif) ligand 5	CCl5	5'-atcctcattgctactgcctc-3' 5'-gccactgggtagaataactcc-3'
Chemokine (C-C motif) ligand 17	CCL17	5'-ccagggatccatgggtttgtactgttc-3' 5'-cctactgtggctcttcttctccctggaa-3'
Interleukin 10	Il-10	5'-gcctaacatgcttcgagatc-3' 5'-ctcatggctttgtagatcc-3'
Interferon gamma	ING	5'-ccaacgcaaagcaatacatga-3' 5'-ccttttgcctccctgttta-3'



**Supplementary Figure 1.** Measurement of ambroxol activity in fibroblasts from patients with GD 3 and healthy controls. A. Following ambroxol treatment, level of GCase enzyme activity was increased in the control sample and fibroblasts cells derived from patient 2 and 3. Fibroblasts were cultured for 5 days in presence of increasing concentrations of ambroxol. Enzyme level was calculated as nmol/mg/hr. B. Fibroblasts derived from patient 1 show no enhancement of GCase protein level in response to ambroxol treatment. Western blot panels represent immunoblotting with C-terminal (white bar) and N-terminal (gray bar) anti-GBA antibodies. C. Q-PCR was performed to determine *GBA* mRNA expression levels from patient 1 using two sets of *GBA* primers covering exons 2-3 (black bar) and exons 3-4 (gray bar), respectively. Level of untreated cells was set to 1. D. Fibroblasts derived from patient 2 show enhancement of GCase level in response to ambroxol treatment. Western blot panels represent immunoblotting with C-terminus (white bar) and N-terminus (gray bar) anti-GBA antibodies. E. *GBA* mRNA levels were not significantly different between treated and untreated groups. Q-PCR was performed to determine *GBA* mRNA levels from patient 2 using two sets of *GBA* primers: covering exons 2-3 (black bar) and exons 3-4 (gray bar), respectively.

## Chaperone screening in Gaucher disease



**Supplementary Figure 2.** Ambroxol chaperone activity in PBMC. A. PBMCs derived from GD3 patients after 5 days treatment with ambroxol. GCCase enzyme activity is measured as nmol/mg/hr. B. PBMCs derived from two healthy donors (white and black panels) were treated for 5 days in the presence of 1, 10 and 100 mM of ambroxol. GCCase enzyme activity is represented as nmol/mg/hr. C. PBMCs derived from a patient with WT/N370S and GD patient with N370S/L444P genotype were treated with 100 mM of ambroxol for 5 days. GCCase enzyme activity is represented as concentration (nmol/mg/hr).