BIOCHEMICAL AND MOLECULAR GENETIC STUDIES ON GAUCHER DISEASE IN PORTUGAL
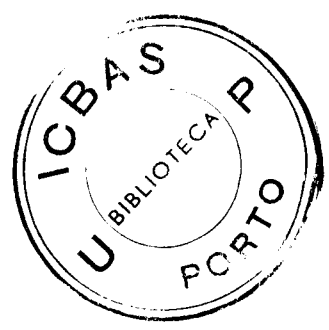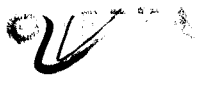
THE N370S GLUCOCEREBROSIDASE GENE MUTATION

Lúcia Maria Wanzeller Guedes de Lacerda

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The N370S glucocerebrosidase gene mutation

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The work reported herein was conducted at the Unidade de Enzimologia, Instituto de Genética Médica Jacinto Magalhães (IGMJM), Porto, Portugal, in fulfillment of the Ph.D. work, under the supervision of Dr. M.C. Sá Miranda (IGMJM) and co-supervision of Prof. Dr. J. Aerts (University of Amsterdam) and Prof. Dr. M.J. Mascaranhas (ICBAS).

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De acordo com o disposto no nº 2 do Artº 8º do Dec. Lei 388/70, esclarece-se serem da nossa responsabilidade a execução das experiências que estiveram na base dos resultados apresentados neste trabalho (excepto quando referido em contrário) assim como a sua interpretação e discussão.

Nesta tese foram utilizados em parte, resultados contidos nos seguintes trabalhos pessoais ou em colaboração, já publicados ou submetidos para publicação.

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SUMMARY

Type 1 Gaucher's disease (GD), an autossomal recessive disorder, is the most common lysosomal storage disease in the Portuguese. The primary metabolic defect consists in the deficient activity of glucocerebrosidase, which leads to the accumulation of the glycosphingolipid glucosylceramide in lysosomes of macrophages. The occurrence of these cells (Gaucher cells) in tissues and organs underlies the most common signs of pancytopenia, organomegaly and skeletal deterioration but a striking variability in clinical expression and course of type 1 GD can be observed. The delineation of the mutations in the glucocerebrosidase locus and the emergence of enzyme supplementation therapy required more complete understanding of the determinants of genotype/phenotype relationships and the modulation of glucocerebrosidase activity. Of the more than 100 disease associated alleles, the N370S has significant frequency in various ethnic and demographic groups, being the most common causal mutation in the Ashkenazi Jews and in the Portuguese.

In this thesis, the frequency of the N370S mutated allele in the Portuguese population was determined by DNA analysis of 2000 individuals. On the basis of the obtained gene frequency (0.0043), an incidence of 1/55000 homozygotes was calculated to existe in the Portuguese population and a significant number of these patients were predicted to remain undiagnosed, most probably due to a milder clinical presentation. To address the question of the origin of this mutation in the Portuguese, the association to an intragenic polymorphic marker was investigated. The occurrence of the N370S allele on a unique haplotype argued against locus heterogeneity between the Ashkenazi Jews and the Portuguese. In order to study Portuguese affected families in which a significant number of mutations remain unidentified, an assay for carrier detection was developed, based in the measurement of glucocerebrosidase activity per amount of cross reacting material.

The study of glucocerebrosidase activity in peripheral blood total leucocytes of genotyped GD patients showed that patients carrying at least one N370S allele presented higher residual activity and that glucocerebrosidase was predominantly extracted in a complex form containing saposin C and phosphatidylserine. Moreover, although plasma glucosylceramide concentration was found to be significantly correlated with score of patients clinical severity, the clinically milder N370S homozygotes presented also significantly less abnormal plasma chitotriosidase activity (a putative marker of the lipid loaded macrophage activation) and tartrate resistant acid phosphatase (TRAP) activity (which probably reflects osteoclastic activity). Importantly,

although this indicates that genotype is implicated in the outcome of a clinical phenotype, the study of the peripheral blood T lymphocyte major subsets showed an association between clinical signs of bone pathology and statistically significant decrease of the $CD8^+$ T lymphocyte subset. This finding suggests thus possible regulatory interaction of T lymphocytes in the pathophysiology of GD.

The results obtained in the study of GD patients receiving enzyme suplementation therapy allow to conclude that the lipid-loaded macrophages were indeed involved in the release of glucosylceramide, chitotriosidase, β-hexosaminidase and TRAP activity and that their plasma levels may constitute quantitative markers for the correction and/or prevention of ongoing formation of the glucosylceramide storage cells.

The advances obtained in the study of the proposed goals may provide useful disease markers and contribute to obtain more insights into the complicated pathophysiology of the disease in order to finally facilitate appropriate interventive therapy to prevent the morbid long-term manifestations of GD.

RÈSUMÈ

La maladie de Gaucher du type 1 présente une transmission autossomique et récessive et est le plus fréquent défaut de l´accumulation lysosomal chez les Portuguais. Le défaut métabolique primaire consiste dans le déficit de l´activitée de la glucocérébrosidase, résultant en une accumulation intralysosomal du glycosphingolipide glucosylcéramide dans les macrophages. La présence de ces cellules (les cellules de Gaucher) dans les tissues et organes est à l´origine des plus fréquents syntomes de la pancytopénie, de la viscéromégalie et de la détérioration osseuse mais une grande variétée de l´expression clinique et de l´évolution de la MD du type 1 peut être observée. L´identification des mutations dans le locus de la glucocérébrosidase et l´aparition de la thérapie de suplémentation enzymatique requèrent une plus profonde compréhension de la relation entre les déterminants du génotype/phénotype et des méchanismes impliqués dans la régulation de l´activitée de la glucocérébrosidase. Des plus de 100 allèles mutés associés à la maladie, le N370S a une fréquence significative dans de nombreux groupes ètniques et démographiques, ètant la pluis frequente mutation dans le Juifs Ashkenazi et le portuguaise.

Dans cette thèse la fréquence de l´allèle muté N370S chez la population portuguaise a été déterminée à partir de l´analyse de l´ADN de 2000 nouveaux nés. Ayant comme base la fréquence du gene obtenue (0.0043), une incidence de 1/55000 homozygotes a été calculée dans le portuguaise et on prévoit um nombre significatif de ces malades sans être diagnostiqués, probablement dû à une présentation clinique pluis légère. Afin d´ étudier l´origine de cette mutation ches le portuguaise, l´association à um marqueur polymorphique intragénique à été étudiée.. La présence de l´allèle N370S sur un unique haplotype contrarie l´hypothèse de l´hétérogénéité des locus entre les Juifs Ashkenazi et les Portuguais. Afin d´étudier les familles portuguaise affectées dans lesquelles un nombre significatif de mutations est encore non identifié, un essai, pour détecter les porteurs, a été dévelopé à partir de la détermination de l´activité enzimatique de la glucocérébrosidase par unitée de matériel immuno reconnu.

L´étude de l´activitée de la glucocérébrosidase dans les leucocytes totaux du sang périphérique des malades génotypés a révélée que les malades portant au moins un allèle N370S aprésentent une activitée résiduelle plus grande et aussi que la glucocérébrosidase est extraie prédominantement sous une forme complexe contenant la saposin C et la phosphatidylsérine. De plus, même si cela la concentration plasmique de la glucosylcéramide a été significativement corrélationée avec le degré de sévérité clinique chez les malades de Gaucher, les homozygotes N370S,

cliniquement moins sévères, aprésentent aussi une activitée plasmique de la chitotriosidase (un marqueur putatif de l´activation du macrophage par l`accumulation lipidic) et une activitée de la phosphatase acide résistante au tartrate (TRAP) (probablement traduisant l´activitée ostéoclastique) moins anormaux. Même si cela semble indiquer que le génotype est associé à un phénotype clinique, l´étude du subgroupe majoritaire des lymphocytes T dans le sang périphérique a permis une association entre des signes cliniques de pathologie osseuse et des défauts statistiquement significatifs dans le subgroupe des lymphocytes T CD8+. Ces résultats sugèrent ainsi une intéraction régulatoire possible des lymphocytes T dans la pathophysiologie de la MG.

Les résultats obtenus dans l´étude des malades de la MG suivant une thérapie de suplémentation enzymatique ont permis conclure que les macrophages chargés par des lipides sont en fait impliqués dans la libération de glucosylcéramide, chitotriosidase, β-hexosaminidase et l´activitée de la TRAP et que leur niveaux de plasma peuvent constituir des marqueurs quantitatifs pour la correction et/ou prèvention de la formation des cellules d´ accumulation.

Les progrès obtenus dans l´étude des objectifs propos peuvent fournir des marqueurs utils pour cet maladie et contibuent dans l´obtention de plus d´informations sur la compliquée pathophysiologie de la maladie afin de finalment faciliter une thérapie d´intervention adéquade.

RESUMO

O tipo 1 da doença de Gaucher é uma doença hereditária de transmissão autossómica recessiva e constitui a patologia de sobrecarga lisosomal mais frequente na população portuguesa. O defeito metabólico primário consiste na deficiente actividade enzimática da glucocerebrosidase com a resultante acumulação de glicolipido glucosilceramida nos macrófagos. Da ocorrência destas células (designadas células Gaucher) nos tecidos e orgãos, resultam os sinais clínicos de pancitopenia, organomegalia e envolvimento ósseo; esta patologia é no entanto caracterizada por uma grande variabilidade de apresentação e evolução clínica. A identificação de mutações do *locus* da glucocerebrosidase bem como o aparecimento da terapia de suplementação enzimática requer um melhor conhecimento dos determinantes da relação genótipo/fenótipo bem como da modulação da actividade da glucocerebrosidase. De mais de 100 alelos associados á doença, a mutação N370S apresenta uma frequência significativa nos vários grupos étnicos e demográficos, e constitui a mutação causal mais comum em Judeus Ashkenazi e nos portugueses.

Neste trabalho, a determinação da frequência do alelo mutado N370S na população portuguesa foi determinada pela análise do DNA de 2000 indivíduos. Com base na frequência genética obtida (0.0043) foi calculada uma incidência de 1/55000 homozigotos na população portuguesa. Este valor permitiu prever a existência de um elevado número de doentes não diagnosticados provavelmente devido a uma apresentação clinica menos severa. No sentido de estudar a origem da mutação N370S nos portugueses efectuou-se a análise da associação genética entre esta mutação e um polimorfismo intragénico. O ocorrência do alelo N370S no contexto de um único haplótipo argumenta contra a hipótese de heterogeneidade do *locus* entre os doentes portugueses e os de ascendência judaica Ashkenazi. O desenvolvimento de um ensaio baseado na determinação da actividade da glucocerebrosidase por quantidade de proteína imuno-reconhecida permitiu estudar as famílias afectadas nas quais um elevado número de alelos mutados permanece por identificar.

O estudo da actividade da glucocerebrosidase nos leucócitos do sangue periférico mostrou que os doentes portadores de pelo menos um alelo mutado N370S apresentavam actividade residual superior á dos restantes e que a glucocerebrosidase era predominantemente extraída na forma complexa contendo saposina C e fosfatidilserina. Apesar de se verificar a existência de correlação significativa entre o nível plasmático da glucosilceramida e o grau de severidade clínica, os homozigóticos N370S, geralmente afectados com menor severidade, foram também caracterizados pela apresentação de uma actividade de quitotriosidase (um putativo marcador da

activação do macrófago com acumulação de glucosilceramida) e da fosfatase acida resistente ao tartarato, TRAP, (a qual provavelmente reflecte a actividade osteoclástica) significativamente menos anormal que os restantes doentes. No entanto, embora estes resultados sejam indicadores da implicação de um genótipo para um determinado fenótipo, o estudo das subpopulações linfocitárias maioritárias do sangue periférico mostrou a existência de uma associação entre a ocorrência de sinais clínicos de patologia óssea e uma diminuição estatisticamente significativa da subpopulação linfócitária $CD8^+$. Este achado sugere a possibilidade de uma interacção reguladora dos linfócitos T na patofísiologia da doença de Gaucher.

Os resultados obtidos no estudo de doentes de Gaucher submetidos a terapia de suplementação enzimática permitiram concluir que células Gaucher estão de facto envolvidas na libertação da glucosylceramida, das enzimas quitotriosidase, TRAP e β-hexosaminidase, podendo o nível plasmático destas constituir marcadores quantitativos da correcção e/ou prevenção da acumulação destas células.

O avanço obtido no estudo dos objectivos propostos poderão providenciar marcadores úteis bem como contribuir para o melhor conhecimento da complexa patofísiologia da doença de forma a finalmente facilitar uma intervenção terapêutica adequada á prevenção das manifestações a longo prazo lesivas da doença de Gaucher.

ABBREVIATIONS

4-MU	4-Methylumbellyferone
ABMT	Allogenic bone marrow transplantation
AD	After Death
ATP	Adenosine triphosphate
BC	Before Christ
BMT	Bone marrow transplantation
bp	Base pair
BSA	Bovine serum albumin
CD-	Cluster of differentiation
cDNA	DNA complementary to RNA
Da	Dalton
DEAE-	Diethylaminoethyl-
DMSO	Dimethylsulphoxide
DNA	Deoxiribonucleic acid
dNTP	Deoxinucleotide triphosphate
dTT	Dithiothreitol
EBA	Enzime binding assay
EDTA	Ethylenediamine tetra-acetic acid
EtBr	Ethidium bromide
FDA	Food and drug administration
Fis	Inbreeding coefficient
Fst	Fixation index
GD	Gaucher disease
GM1	N-acetyl-neuraminosyl-gangliotetraosylceramide
GM2	N-acetyl-neuraminosyl-gangliotriaosylceramide
GM3	N-acetyl-neuraminosyl-lactosylceramide
GM-CSF	Granulocyte macrophage colony stimulating factor
HEPES	N-(2-hydroxyethyl)-piperazine-N-2-ethanesulphonic acid
HLA	Human leucocyte antigenes
Ig	Immunogloblin

IFN-γ	γ-Interferon
IL-	Interleukin-
Kb	Kilo base pair
KDa	Kilo Dalton
K_M	Michaelis constant
M-CSF	Macrophage colony stimulating factor
Mr	apparent molecular mass
NADP	Nicotinamide-adenine dinucleotide phosphate
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PKC	Protein kinase C
PKU	Phenylketonuria
PMSF	Phenylmethylsulphonyl fluoride
PS	Phosphatidylserine
rpm	Rotations per minute
SSI	Severity score index
SAP	Saposin activator protain
SDS	Sodium dodecyl sulphate
Taq	*Thermus acquaticus*
T-	Taurocholate
TLC	Thin layer chromatography
TNF-α	Tumor necrosis factor α
TRAP	Tartrate resistant acid phosphatase
Tris	Tris (hydroxymethyl) aminomethane

Chapter 1
General introduction to Gaucher Disease

Lysosomes contain a variety of acid hydrolases responsible for the degradation of proteins, lipids and carbohydrates that are internalized via endocytosis. A large number of genetically determined defects in lysosomal hydrolases are known, manifested either by the absence of a particular hydrolase or by defects in its activity, resulting in accumulation of undegraded substracts, a progressive increase in size and number of lysosomes, and onset of a lysosomal storage disease with its typical clinical manifestations (reviewed in Neufeld, 1991). At the present time more than 30 different lysosomal storage disorders have been reported, nearly all of which are inherited in an autossomal recessive manner. The incidence at birth of lysosomal disorders varies from 1 in 100,000 to 1 in 250,000. The total group of lysosomal storage disorders has an incidence of approximately 1:5,000 to 1:10,000 (Galjaard and Reuser, 1984; Reuser et al, 1994).

1.1   Biochemical defect

The most prevalent lysosomal storage disease, Gaucher disease (Barranger and Ginns, 1989; Grabowski et al, 1990; Beutler and Grabowski, 1997), is an autosomal recessive disorder of glycosphingolipid metabolism, characterized by accumulation of glucocerebroside in reticuloendothelial cells (Aghion, 1934) due to the deficient activity of glucocerebrosidase (D-glucosyl-N-acylsphingosine glucohydrolase, E.C.3.2.1.45) (Brady et al, 1965; Patrick, 1965). This enzyme cleaves the β-glucosidic linkage of glucosylceramide to produce ceramide and glucose (Brady et al, 1966), the latter being degraded by lysosomal acid ceramidase to sphingosine and fatty acid (Figure 1-1). The fatty acid chain length varies, the most abundant component being stearic acid (C_{18}) in brain, and C_{20} to C_{24} fatty acids in spleen, liver and blood cells (Mansson et al, 1978).
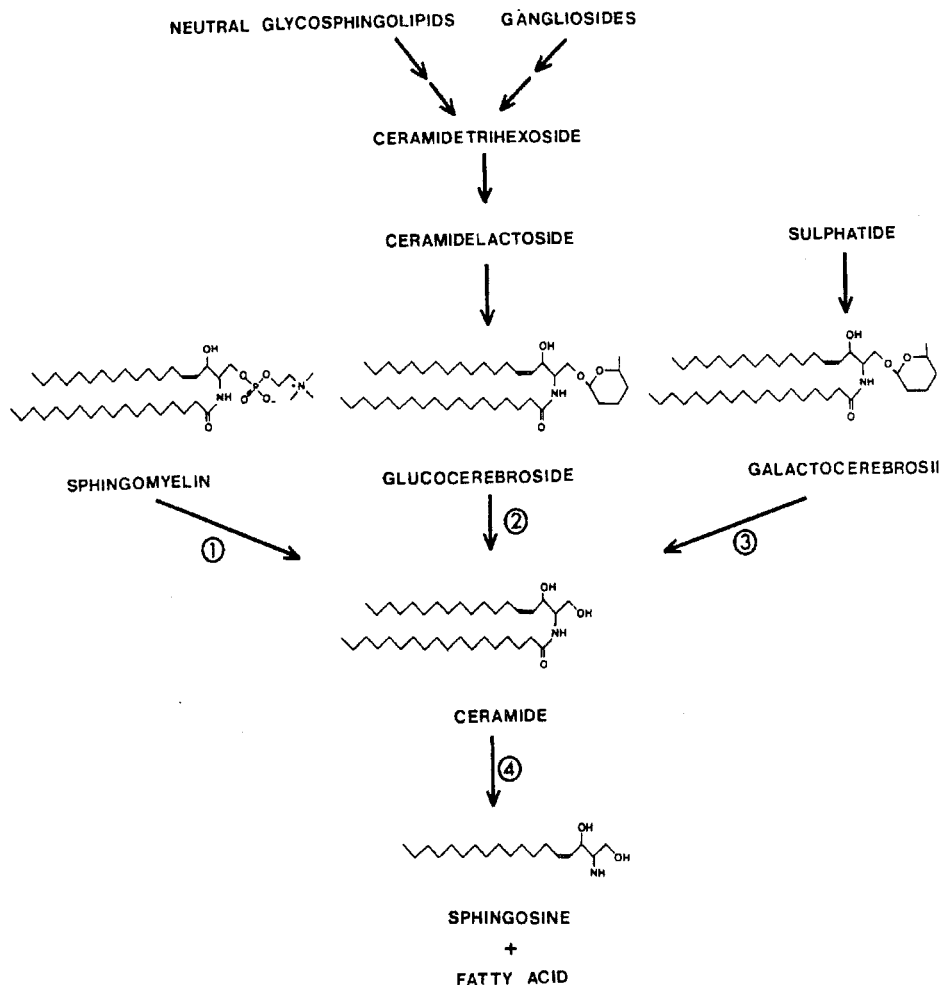
Figure 1-1

Lysosomal degradation of sphingolipids. Enzimes are 1, sphingomyelinase; 2, glucocerebrosidase; 3 galactocerebrosidase.

1.2 Phenotypic presentation and classification by characteristic symptoms

In his medical school thesis in 1882, Philippe C. E. Gaucher described a female patient with an enlarged spleen that he believed was an epithelioma. Descriptions of additional patients with similar presentations began to appear, and the eponym Gaucher disease was ascribed (Gaucher, 1882).

The clinical manifestations of Gaucher disease are heterogeneous. On the basis of the occurrence and age of onset of neurological symptoms three different phenotypic variants of GD are generally distinguished:

-Type 1 (McKusick 230800), is the non-neuronopathic form of GD (the individuals are free of primary neurologic involvement; any neurologic symptoms observed in these

patients are secondary to systemic complications of the illness), and the age of onset of clinical symptoms and the severity can differ to a large extent. Symptomatic patients exhibit variable splenomegaly (4 to 70 times normal), with accompanying haematological abnormalities like anaemia, thrombocytopenia, and ocasionally, leukopenia. The spleen is known for it specialised vascular structure: it contains lymphocytes and macrophages that serve unique filtering, scavenging and immune functions. The enlarged spleen in GD causes cytopenias by haemodilution, sequestration and premature destruction of the blood elements in phagocytes that line its expanded sinusoids and cords. Increased haematopoiesis may initially compensate but continuing enlargement of the spleen and local effects of Gaucher cells in the bone marrow ultimately lead to the syndrome of hypersplenism. Severe abdominal pain due to infarcts, mechanical interference with adjoining organs, growth retardation (in children), or cachexia (in adults) occur; in severely afflicted patients, even high cardiac output failure may occur. Easy bruising, epistaxis, and bleeding from gums are common. Low haemoglobin levels lead to dyspnea, tachypnea, and fatigue. Hepatomegaly (1.5 to 10 times normal) is another common manifestation. Hepatic function may be impaired, resulting in elongation of prothrombin time and partial thromboplastin time. Skeletal involvement is frequent in patients and can cover the spectrum from osteopenia to severe destruction of bone. Erlenmeyer flask-like deformities of the distal femur are characteristic of the disorder but occur in about 30% of patients. Aseptic necrosis of femoral head is the most common destructive bony lesion (reviewed in Beutler and Grabowski, 1995).

Besides the very heterogeneous presentation and variable progression of type 1 GD, a number of patients have been described with moderately enlarged spleens who were otherwise asymptomatic (Brady, 1978), or even symptom-free individuals were identified who presented both mutated alleles of the glucocerebrosidase gene and a markedly deficient enzymatic activity (Zimran et al, 1991; Sibille et al, 1993; Beutler and Grabowski, 1995). Although type 1 is classically devoid of neurological symptomatology, association to Parkinsonian symptoms have been reported in adult Gaucher patients (Baumann et al, 1995).

-Type 2 (McKusick 230900), the acute neuronophatic form of GD, is a rare panethnic disease presenting a very homogeneous clinical phenotype characterized by hepatosplenomegaly, anemia and trombocytopenia noticeable at 3 months of age, and universally present by 6 months with severe neurological dysfunction: trismus, strabismus, and retroflexion of the head. Seizures may occur, and the infant becomes hypotonic and apathetic pre-terminally. Extensive brain-stem involvement leads to progressive bulbar palsies with concomitant aspiration pneumonia and feeding

difficulties. These problems are compounded by extensive pulmonary infiltrative disease. Death usually occurs by about 9 months, but some of these patients live for 2 years and the examination of the brain show gliosis and cell death (reviewed in Beutler and Grabowski, 1995).

A subset of type 2 Gaucher patients clearly have a different and more severe phenotype, namely signs and symptoms presented prenatally or at birth and death frequently ensued within hours to days or at the most 2-3 months. Although skin involvement in GD had previously been considered uncommon and usually limited to pigmentary changes (Goldblatt and Beighton, 1984), this subgroup of type 2 Gaucher patients displays ichthyosis (a collodion baby phenotype) (Lui et al, 1988; Lipson et al, 1991; Sidransky et al, 1992a).

-Type 3 (McKusick 231000), the sub-acute juvenile neuronopathic form of GD is distinguishable of type 2 by a late onset, a heterogeneous presentation and a slowly progressive development of neurological symptoms by adolescence, in addition to the symptoms found in type 1 of the disease. Death usually occurs in childhood or early adolescence from hepatic or pulmonary complications (reviewed in Beutler.and Grabowski, 1995)

Three distinct subtypes of type 3 disease are now recognized: Type 3a describes progressive neurological involvement beginning in adolescence but mild systemic manifestations; type 3b describes severe systemic disease but mild neurological involvement (Brady et al, 1993); type 3c describes corneal clouding, left-sided cardiac valvular calcification, and oculomotor apraxia but only mild visceral and skeletal manifestations (Abrahamov et al, 1995).

1.3   Ocurrence and ethnic prevalence

Like many autosomal recessive disorders, GD shows a prominent ethnic predilection: to Ashkenazi Jews in type 1 and to Norbottnian Swedes in type 3.

Type 1 GD is the most common form, the occurrency is panethnic with an estimated incidence of about 1 in 40,000-200,000 births, and is considered to be prevalent among the Ashkenazi Jews in which the incidence is 1 in 625-1,500 (Galjaard and Reuser, 1984; Reuser et al, 1994; Grabowski, 1995), and the carrier frequency is of 1 in 10-20 (reviewed in Beutler and Grabowski, 1995).

Type 2 GD, panethnic in incidence, is the least common form of GD with an incidence striking fewer than one in 100,000 individuals (Erikson, 1994).

Type 3 GD is also relatively rare, affecting less than one in 50,000-100,000 individuals. It is panethnic, although there is a cluster of the disease in Norrbotten, the northern province of Sweden, and common ancestors have been traced back to the 17th century (Erikson, 1994; and also reviewed in Beutler and Grabowski, 1995).

1.4 Reticulo-endothelial cells lipid accumulation

In lysosomal disorders the substances to be degraded are enclosed within the lysosomes and usually there seems to be no feedback regulation or influence of the substrate concentration on other biochemical pathways. When the residual activity of the degrading enzyme falls below the critical threshold level, substrate influx exceeds the degradation rate and the excess substrate accumulates continuously. Since undegraded substrates cannot (or only very slowly) leave the lysosomal compartment, these organelles are converted into storage granules which steadily increase in size and number. When they reach a large volume they interfere with the cell's normal function, giving rise to a storage disease (Conzelmann and Sandhoff, 1983/84).

Although every cell in Gaucher patients is deficient in glucocerebrosidase the acumulation of glucocerebroside is most pronounced in macrophages of the mononuclear phagocyte system (Parkin and Brunning, 1982). These cells arise from the bone marrow, and they are components of the reticuloendothelial system: (alveolar) macrophages, histiocytes, Kupffer cells and osteoclasts. By this way, numerous enlarged, lipid-laden histiocytes called Gaucher cells are present in the organs of patients with Gaucher disease (Gaucher, 1882). Macrophages are not only increased in size but also in number. These abnormal macrophages accumulate widely, resulting in hepatosplenomegaly, skeletal disease, bone marrow failure, hypermetabolic state and pulmonary infiltration. The glucocerebroside that accumulates in these cells arises from phagocytosis and degradation of blood group glycosphingolipids, globosides and lactosylceramide from respectively senescent erythrocytes and leukocytes (Kattlove et al, 1969), since the main fatty acid of the glucocerebroside that accumulates is docosanoic acid (C-22) which is the major fatty acid in the glycolipids of blood cells (Mansson et al, 1978). Liver hepatocytes are not involved in the turnover of blood cells and appear to avoid accumulation of glucocerebroside by excreting it into the bile (Tokoro et al, 1987). However, it can not be excluded that uptake of lipoproteins, the carrier of plasma glucocerebroside, by macrophages contributes to the high level of storage material in these cells.

In neurons of patients with type 2 GD the glucosylceramide accumulated probably arises from the catabolism of endogenous gangliosides (acidic sphingolipids).

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Gangliosides undergo rapid turnover via synthesis and catabolism during brain development and the formation of neural circuits. This is supported by the finding that in the ceramide composition of the accumulated glucosylceramide isolated from cerebral and cerebellar cortex, stearic acid (C-18) is the predominant fatty acid, (comprising 60-75%), and sphingenine constitutes 66-85% of the long-chain bases, which is similar to the ceramide composition that has been found only in brain gangliosides (Mansson et al, 1978). In type 3 patients the fatty acid composition of the cerebral glucosylceramide shows large differences. Glucosylceramide from cerebellar cortex is partially of extracerebral origin, being the proportion of stearic acid invariably less than 45%; there is a correlation between a large increase of glucosylceramide and a low proportion of stearic acid and high proportions of C-16, C-22 and C-24 fatty acids (Nilsson and Svennerholm, 1982).

Patients with type 1 GD seem to have sufficient residual glucocerebrosidase activity to degrade glucocerebroside arising from ganglioside turnover in the brain during development. However, the much larger load of glucocerebroside originating from leukocytophagy and erythrocytophagy cannot be completely catabolized and progressively accumulates in the peripheral organs (reviewed in Brady et al, 1993).

The age of onset, progression, and severity of the disease should, to a large extent, depend on the rate at which the substrate accumulates. A total deficiency of an enzyme completely blocks the catabolism of its substrate, which therefore accumulates rapidly, giving rise to a severe disease with early onset and rapid progression. In contrast, even a low residual activity of the affected enzyme may still be sufficient to degrade a large proportion of the incoming substrate and will thus retard the storage process considerably, leading to a mild form of the disease with late onset and slow progression. Thus the symptomatology of the disorder should mainly depend on the influx rates of the substrate in the different organs and cell types. A moderate residual activity may still be sufficient to cope with a comparatively slow influx of the respective substrate in one organ or cell type, whereas in another organ or cell type, where the same substrate is synthesised or acquired at a higher rate, the same residual activity may prove to be insufficient (Conzelmann and Sandhoff, 1983/84).

However, in GD there is no simple relationship between the disease manifestations and the extent of Gaucher cell infiltration or tissue glycolipid content. Thus, the macrophage response on glucosylceramide loading seems to be a critical factor in tissue damage but the mechanisms are unknown.

Gaucher cells or Gaucher-like cells can also be present in other disorders, including all leukemias - most often chronic granulocytic leukemia (Kattlove et al, 1969) - Hodgkin`s

disease (Lee et al, 1982), thalassemias (Zaino et al, 1971), congenital dyserythropoietic anemias (van Dorp et al, 1973), malignancy (e.g. multiple myeloma) (Scullin et al, 1979; Papadimitriou et al, 1988), infectious diseases and aquired immune deficiency syndrome (AIDS) (Solis et al, 1986; Esplin, 1992). The presence of Gaucher cells in patients with chronic myelocytic leukemia is due to the production of sphingolipid from granulocytes, which is greatly increased since the total body granulocyte pool and the granulocyte turnover rate are markedly increased (Kattlove et al, 1969).

1.5 Ultrastructure of epiderme and permeability barrier homeostasis

The permeability barrier of mammalian epidermis is mediated by a system of highly organized lamellar bilayers localized to the intercellular spaces of the *stratum corneum* (SC)-protein enriched corneocytes embedded in a lipid-enriched intercellular matrix (reviewed in Elias and Menon, 1991). These bilayer structures are enriched in relatively hidrophobic species, i.e., ceramides, cholesterol, and free fatty acids, and depleted in the phospholipids associated with bilayer formation in other cellular membranes (Gray and Yardley, 1975; Elias et al, 1979; Lampe et al, 1983). These hydrophobic species derive, in part, from a mixture of precursors, including phospholipids and glucosylceramide, contained in the epidermal lamellar body (Menon et al, 1986; Elias et al, 1988), a distinctive secretory organelle that delivers these lipids, along with co-packaged hydrolytic enzymes (Freinkel and Traczyk, 1985; Grayson et al, 1985), to the intercellular spaces at the base of the *stratum corneum*. After secretion, lamellar body contents undergo a series of morphologic transformations leading to the formation of intercellular lamellar unit structures characteristic of the *stratum corneum* of terrestrial mammals (Elias and Menon, 1991; Landmann, 1988). These structural alterations are accompanied by depletion of glucosylceramides and phospholipids, with an increase in ceramides and free fatty acids within the *stratum corneum* interstices (Gray and Yardley, 1975; Elias et al, 1979; Lampe et al, 1983; Nemanic et al, 1983). Epidermal processing of glucosylceramide to ceramide by glucocerebrosidase is required for barrier homeostasis, and this enzymatic step is regulated by barrier requirements (Holleran et al, 1991; Holleran et al, 1994b). Glucocerebrosidase activity accounts for over 80% of epidermal β-glucosidase activity, and increases in the outer layers (*stratum corneum* and *stratum granulosum*) of murine epidermis (Holleran et al, 1992). An absence of epidermal glucocerebrosidase activity in knockout mice (glucocerebrosidase-deficient transgenic mice, discussed below) leads to abnormal barrier function in association with accumulation of glucosylceramides and depletion of

ceramides in the *stratum corneum*. Moreover the failure of conversion of glucosylceramide to ceramide results in partially processed "immature" lamellar body-derived sheets extending throughout the lower and mid to outer SC interstices. This altered membrane structures in the SC interstices of transgenic mice display abnormal functional integrity, while the epidermal lamellar bodies and their secreted contents at the *stratum granulosum/stratum corneum* interface appeared normal (Holleran et al, 1994a). The presence of ichthyosiform skin changes in both transgenic mice and some neonatal type 2 GD patients suggests that blockade of glucosylceramide hydrolysis, with failure to form normal lamellar bilayer unit structures, not only compromises the integrity of the epidermal permeability barrier, but also provokes an alteration of normal desquamation (Lui et al, 1988; Sidransky et al, 1992a).

The altered lamellar bilayer structures may result from either steric constraints imposed by the additional bulk of the glucose residue and/or the increased hydrogen-bonding capacities provided by the hydrophilic hexose residue (Curatolo, 1987; Pascher et al, 1992). Thus, although increased glucose moieties could result in a loss of barrier integrity because of an alteration of lamellar bilayer structures and increased perfusion of water, a concomitant increase in the cohesiveness of corneocytes, due to increased hydrogen bonding, could lead to the abnormal *stratum corneum* retention, i.e., the excess scale seen in Gaucher mice and in neonatal type 2 patients. A correct molar ratio of ceramides, cholesterol, and free fatty acids is important, but the persistence of glucosylceramide, rather than diminished ceramide is more likely to be the main cause of the membrane abnormalities, based on experimental data with topical application of ceramide (Holleran et al, 1994a).

The lack of skin manifestations in most patients with GD may relate to differences in residual enzyme activity, since ichthyotic skin involvement in type 2 Gaucher patients is associated with severe disease and very low residual enzyme levels in the extracutaneous tissues of these patients (Sidransky et al, 1992a).

1.6 Involved genes: active glucocerebrosidase gene, pseudogene and prosaposin gene

1.6.1 Glucocerebrosidase gene and pseudogene

The human glucocerebrosidase gene is located on region q21 of chromosome 1 (Barneveld et al, 1983a; Ginns et al, 1985; Sorge et al, 1985b). Fifteen other genes have been localized to the same band (Collins et al, 1992). The gene comprises 11 exons and 10 introns, contained within 7604 base pairs. The promoter region is located

approximately 150 base pairs upstream to exon 1 (Reiner et al, 1988; Horowitz et al, 1989). Two functional ATG initiator codons have been identified (Sorge et al, 1987). The downstream ATG was translated more efficiently resulting in a 19-amino acid signal polypeptide which contains a hydrophobic core (Reiner et al, 1987). A 95% homologous pseudogene is located in the same locus at least 50-100 kb downstream from the functional glucocerebrosidase gene (Horowitz et al, 1989; Sorge et al, 1990). The pseudogene, contained within 5769 base pairs, shows missense mutations, deletions in some introns and various base substitutions throughout the gene (Horowitz et al, 1989; Reiner et al, 1988). It is assumed that the glucocerebrosidase pseudogene is non-functional in the production of enzyme due to the absence of a long open reading frame (Sorge et al, 1990). Since the murine genome (O'Neill et al, 1989) does not contain the pseudogene the duplication process must have occurred relatively recently in evolution (Horowitz et al, 1989; Sorge et al, 1990).

The novel metaxin gene is localised within the locus, immediately dowstream to the pseudogene in proximity to and transcribed convergently to the glucocerebrosidase gene (see Figure 1-3). Mutations and/or rearrangements altering metaxin expression may potentially have clinical significance (Bornstein et al, 1995).

1.6.2   Mutations in glucocerebrosidase gene

Cloning of the human glucocerebrosidase gene led to the identification more than 100 mutations, including point mutations (missense mutations), an insertional mutation (frame shift mutation), two deletions, a splicing mutations and three combinations of multiple mutations (crossovers or gene conversions) (reviewed in Beutler and Gelbart, 1997) (Figure 1-2).

Only a limited number of these mutations occur frequently. The four most common mutations are those involving amino acid substitutions at position 370 (N370S), position 444 (L444P), as well as the 84GG frame shift insertion and the IVS2+1 splice junction mutation. These four account for at least 88% of the mutations in Ashkenazic Jewish and at least 70% of European populations (Beutler, 1993; Mistry and Cox, 1993; Horowitz and Zimran, 1994a; Grabowski, 1995).

The most prevalent mutation underlying GD is the A->G transition at nucleotide 5841 in exon 9 of the glucocerebrosidase gene, that leads to the production of an enzyme with a substitution of asparagine to serine at position 370 (N370S) (Tsuji et al, 1988). This allele was not found in neurophatic forms of the disease as well as in Asian (Japonese) patients and represented 70-75% of the mutated alleles in Ashkenazi Jewish type 1 Gaucher patients (Beutler et al, 1992b; Horowitz et al, 1993) and 38-50% in non-

Ashkenazi Jewish patients (Levy et al, 1991; Choy et al, 1991; Sidransky et al, 1992b; Walley et al, 1993; Tuteja et al, 1993). In the Ashkenazim population the N370S mutated allele frequency was found to be 0.032 (Zimran et al, 1991; Beutler et al, 1993).
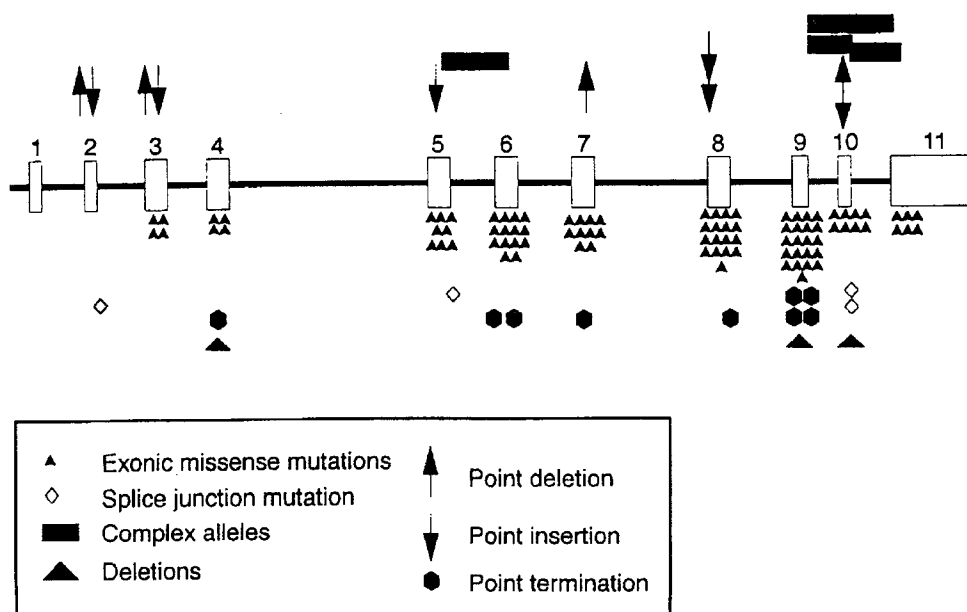
Figure 1-2

Distribution of the mutations that predispose to the Ghacher's disease phenotypes. There are about 108 with a clustrering towards the 3' half of the gene. (Adopted from Grabowski and Horowitz, 1997)

Another relatively frequent mutation, which can be found among the three clinical phenotypes is a T->C transition at nucleotide 6433 in exon 10, leading to an enzyme with a leucine to proline substitution at amino acid 444 (L444P) (Tsuji et al, 1987). This mutation might have been introduced (more than once), either by a point mutation or by recombination with the pseudogene (Zimran et al, 1990). It is the most prevalent mutation in patients with the neuronopathic form of GD, where it accounts for about 50% of the mutated alleles; this mutation is also found in 30% of alleles in type 1 non-Jewish GD patients (only 4% among Ashkenazi Jewish), mostly in a heteroallelic state (Horowitz et al, 1993).

The 84GG mutation is an insertion of an extra G at nucleotide 84 (starting from the first ATG) of the cDNA (Beutler et al, 1991). This is the second most common mutation among Ashkenazi Jewish patients (13%), where it has been described thus far almost exclusively. The frequency of the 84GG allele among Ashkenazi Jews was found to be 0.00217 (Beutler et al, 1993). In the heteroallelic state this mutation is usually associated with type 1 Gaucher disease with severe clinical manifestations (Horowitz

and Zimran, 1994a). Patients homozygous for this "null" mutation have not been found. Homozygosity for 84GG may therefore be lethal in utero or shortly after birth.

The splice junction mutation due to a G to A transition of the first nucleotide of the second intron destroys the 5`donor splice site (He et al, 1992). This splice junction mutation (IVS2+1) accounts for 1-3% of the mutated alleles among Jewish and non-Jewish Gaucher patients (Horowitz et al, 1993). The mutation is also found in patients with both non-neuronopathic and neuropathic GD. Most patients with type 1 GD and the genotype N370S/IVS2+1 develop moderate to severe GD (Horowitz et al, 1993; Horowitz and Zimran, 1994a).

Another relatively frequent mutation (3.5%) appears in all types of GD but only among non-Jewish patients is a C->T transition at nucleotide 6489 in exon 10, leading to an enzyme with an arginine to cystine substitution at amino acid 463 (R463C) (Hong et al, 1990). This mutation is associated with mild to severe clinical manifestations.

In addition to these, the two complex alleles, or "rec" for recombinant, have been referred to as the "pseudogene-like" sequences, in mutated alleles that probably represent rearrangements between the homologous sequences by crossovers or ill-defined rearrangements.

Two related complex alleles with several mutations were reported. The recNcil is composed of three point mutations, namely the 6433 T→C transition (L444P), 6468 G →C transversion (A456P) and 6482 G→C transversion (not associated with any amino acid change). This recombinante allele (it contains exon 10 of the pseudogene) is present in 0.5% and 8% in respectively Jewish and non-Jewish GD patients alleles and is associated with a severe, often neuronopathic phenotype (reviewed in Horowitz and Zimran, 1994a). The recTL complex allele (Zimran and Horowitz, 1994b) is composed of the three point mutations as in recNcil but with a fourth mutation, 5957 G→C transversion (D409H); at least part of exon 9, intron 9 and exon 10 derived from the pseudogene. This recombinant allele has been paradoxically associated with mild clinical manifestation and is shows a similar prevalence (about 2%) in both Jewish and non Jewish mutated alleles.

1.6.3   Polymorphisms in glucocerebrosidase gene

Several neutral (benign) polymorphisms (population allele frequency >1%) in human glucocerebrosidase gene that do not appear to be related to GD have been described (Beutler et al, 1992a). A restriction fragment length polymorphism for the enzyme PvuII, due to a G->A substitution at genomic DNA nucleotide 3931 in intron 6 of the glucocerebrosidase gene, is responsible for the presence (Pv1.1+) or absence (Pv1.1-)

of a 1.1 kb genomic glucocerebrosidase DNA fragment upon PvuII digestion (Sorge et al, 1985a; Zimran et al, 1990). This is the most studied polymorphism in glucocerebrosidase gene and since it is in virtually complete linkage disequilibrium with the 11 other polymorphic sites on the glucocerebrosidase gene (Beutler et al, 1992a), it serves as a surrogate site for all of the other polymorphic sites.

It was shown that the N370S glucocerebrosidase mutation is invariably associated with the Pv1.1- haplotype (Zimran et al, 1990; Beutler et al, 1991). In contrast, mutation L444P was found to be in linkage equilibrium with this PvuII polymorphism (Zimran et al, 1990; Beutler et al, 1991). The incidence of this PvuII polymorphism was reported as being similar in various racial groups and was proposed to be ancient (Sorge et al, 1985a).

1.6.4 Prosaposin gene

The acid exohydrolases that degrade sphingolipids in the lysosomes work in concert with small nonenzymic glycoproteins, called "sphingolipid activator proteins" (SAP`s) for effective interaction with the substrates (reviewed in Sandhoff et al, 1995). Glucocerebrosidase has an activator, designated saposin C. This is a heat-stable small molecular weight protein (80 amino acids) that binds to the enzyme and enhances the *in vivo* hydrolysis of glucocerebroside (Ho and O`Brien, 1971). This protein is encoded by the prosaposin gene which is located on chromosome 10; this gene directs synthesis of a 70 kDa protein, prosaposin, and the posttranslationally protelytic and glycosidic processing generates saposin A, B, C and D (reviewed in O`Brien and Kishimoto, 1991). Saposin A, B and D stimulate the hydrolysis of gluco and galactocerebroside, sulfatides and ceramides respectively (reviewed in Sandhoff et al, 1995). Prosaposin protein, the precursor of the lysosomal saposin proteins, have additional functions as a neurotrophic factor (O´Brien et al, 1994).

Defects in proteins that influence the activity of a lysosomal enzyme as a result of abnormalities of activator proteins (Fürst and Sandhoff, 1992) may also result in a lysosomal storage disease, and a severe form of GD has been reported (Kleinschmidt et al, 1987) as the result of mutations in saposin C and prosaposin gene (Christomanou et al, 1989; Harzer et al, 1989; Schnabel et al, 1991).

1.7 Genotype/phenotype correlation

Some of the phenotypic heterogeneity of Gaucher disease can be ascribed to the heterogeneity in defects in the glucocerebrosidase gene. The homozygosity for N370S seems to preclude neuronopathic involvement, and with one exception of a child with

oculomotor involvement, there seems to be a relation between the presence of at least one allele with the N370S mutation and a non-neuronopathic course of the disease or type 1 form (Beutler, 1993). In addition homozygosity for the N370S mutation confers the mildest form of the disease and it has been estimated that up to two-thirds of such individuals never develop clinical symptoms (Beutler, 1993). However a pair of monozygotic twins, homozygous for the N370S mutation, and presenting the same markedly deficient residual β-glucosidase activity, were reported to show highly divergent phenotypes (Mistry et al, 1995). The twins were shown to be genetically identical by DNA microsatellite fingerprinting. Immunoglobulin gene rearrangement studies revealed no differences in B cell clonality. This emphasises that determinants other than those in the germline DNA influence the clinical expression of GD (Mistry et al, 1995).

It was also reported that most of the splenectomized patients and those who suffered from avascular necrosis of a large joint (as a manifestation of severe skeletal involvement) present the "severe" genotypes N370S/84GG or N370S/IVS2+1, suggesting that both complications are associated with these genotypes (Zimran, 1995a). Another genotype/phenotype correlation was described: although cardiac manifestations are rare in GD, a sample of Spanish and Arab GD patients of 2-20 years of age, homozygous for the rare point mutation D409H (1342C), presented besides systemic symptoms of GD and signs of oculomotor apraxia, a progressive thickening and calcification of the aortic, or mitral apparatus, or both, resulting in valvular stenosis or insufficiency before adulthood (Chabas et al, 1995; Abrahamov et al, 1995). The D409H mutation is the third most common mutation among Spanish Gaucher patients. Arab patients come from an inbred population of Jenin and it is likely that the mutation arose on a single ancestral chromosome. The possibility of a contiguous gene syndrome causing this particularly clinical picture or the alteration by this mutation of the transcript of a convergently transcribed gene was however not excluded (Mistry, 1995).

Yet, other data point to the lack of existence of genotype/phenotype correlation; for instance, homozygosity for L444P mutation is associated with either the acute neuronopathic (type 2), the subacute neuronopathic (type 3) forms, as well as with no development of neurological symptoms at all in Japanese patients (Tsuji et al, 1987; Dahl et al, 1990; Masuno et al, 1990). Despite the L444P homozygosity reported for all Norbotten type 3 patients, it was also found a wide inter-individual variability in disease severity and progression (Dahl et al, 1990). Since this mutation has also been found in patients from other origins often affected with a more severe phenotype (type 2), never seen in Norrbotten patients, in them this mutation must be linked to another genetic

change which causes a milder phenotype. In addition, even within other particular genotype group, a wide variability in severity of disease has been described, even within sibships and between identical twins (Amaral et al, 1994; Mistry and Cox, 1995).

In order to study this presence/absence of genotype-phenotype correlation, the phenotypic diversity within the genotype, the pathogenesis of the disease and also for evaluating therapeutic approaches, an animal model for Gaucher disease was generated. This was done by creating a null allele in embryonic stem cells through gene targeting and using these genetically modified cells to establish a mouse strain carrying this mutation. Mice homozygous for this mutation were found to have <4% of normal glucocerebrosidase activity, died within twenty-four hours of birth and stored glucocerebroside in lysosomes of cells of the reticuloendothelial system (Tybulewicz et al, 1991; Tybulewicz et al, 1992). This transgenic model allowed the recognition of a distinct GD phenotype, a neonatal form (Sidransky et al, 1992a), which consists of a progressing fatal disease, and associated ichthyotic skin (Lui et al, 1988) and/or hydrops fetalis. This specific clinical manifestations have helped in recognising and beginning to emphasise a distinct human phenotype of Gaucher disease (Sidransky et al, 1992a).

Other work point to an eventual additional inter-regulation among glucocerebrosidase and closely linked genes. In an attempt to establish a mouse model for the described mild form of GD (N370S mutated protein), a targeted mutation (A→G in exon 9) was introduced into glucocerebrosidase gene by homologous recombination in embryonic stem cells; for this, a phosphoglycerate kinase-neomycin gene cassete was also inserted into the 3'-flanking region of glucocerebrosidase gene as a selectable marker, disrupting the coding region in the terminal exon of metaxin (Bornstein et al, 1995) and presumably interfering with the processing and/or stability of the transcript, since metaxin mRNA levels became reduced by 50% in mice heterozygotes. Mice homologous for the combined mutations (in the glucocerebrosidase and metaxin gene) die early during gestation (Bornstein et al, 1995). Since the analogous amino acid substitution in human glucocerebrosidase gene is generally associated with a mild form of GD (Horowitz and Zimran, 1994b) and since mice that lack glucocerebrosidase gene do not die until after birth (Tybulewicz et al, 1992), Bornstein and coworkers (1995) suggest that metaxin protein may be required for embryonic development. Metaxin protein was found to be expressed ubiquitously in tissues of the young adult mouse (Bornstein et al, 1995) and preliminary data suggest that metaxin is a component of the outer mitochondrial membrane and may function in mitochondrial import (Armstrong et al, 1995). The metaxin murine gene, spans the 6-kb interval separating the glucocerebrosidase gene from the thrombospondin 3 gene in chromosome 3E3-F1,

and was found to be transcribed convergently with glucocerebrosidase gene. Thrombospondin 3 protein is a member of a family of five homologous, secreted, modular glycoproteins, and in the mouse is present primarily in lung, brain and cartilage but its function is essentially unknown. The organisation of the metaxin/thrombospondin 3/glucocerebrosidase genes in the mouse is conserved on human, with the added complexity that the DNA duplication has led to the creation of a direct repeat containing pseudogenes for glucocerebrosidase and metaxin. In the human genome, metaxin is interposed between thrombospondin 3 and the glucocerebrosidase pseudogene (Figure 1-3). The metaxin and the thrombospondin 3 gene share a common promoter region and are transcribed divergently. On the other hand, metaxin and the glucocerebrosidase pseudogene are transcribed convergently and contain very closely apposed polyadenylation sites. The metaxin pseudogene was characterised and it was reported that it is not transcribed or is transcribed at only a very low level (Long et al, 1996). If coordinate regulation of expression of either two or all three genes can be shown, such regulation may reflect metabolic interrelationships among glucocerebrosidase, metaxin and thrombospondin 3 genes.
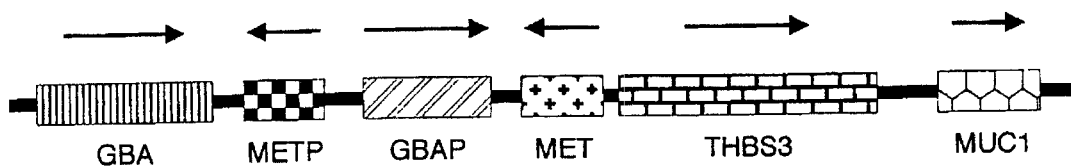
Figure 1-3.

Diagram of genomic region near the glucocerebrosidade (EBA) locus on chromosome 1. MET, THBS3 and MUC1 are the metaxin, thrombospondim 3 and muc 1 oncogene loci, respectively. The METP and GBAP are the MET and GBA pseudogenes. The arrows indicate the direction of transcription of various genes. The MET and THBS3 genomic loci overlap in the promoter region. The region from GBA to THBS3 is about 40 kb in length (Adopted from Grabowski and Horowitz, 1997).

All human disease may be said to result from an interaction of an individual's genetic makeup and the environment (Wachbroit, 1994). Other factos such as genetic background or non-genetic factors may also influence the clinical heterogeneity observed in patients for a given genotype.

1.8   The β-Glucosidases

1.8.1   Isoenzymes and molecular forms

Five β-Glucosidases isoenzymes have been described in humans:

a) Lysosomal glucocerebrosidase (EC 3.2.1.45), the enzyme deficient in Gaucher disease, is a membrane-associated glycoprotein present in all cell types. This enzyme hydrolyses not only the natural substrate, glucosylceramide, but also artificial β-glucosidic substrates and is irreversibly inhibited by conduritol B-epoxide (CBE). The optimum pH of the enzyme is dependent on the assay conditions, varying from 4.0 to 5.5. Glucosylsphingosine is also a substrate for glucocerebrosidase (Vaccaro et al, 1985; Sarmientos et al, 1986).

b) The lactase-phlorizin hydrolase (EC 3.2.1.62-108) is an integral membrane glycoprotein that catalyses the hydrolysis of β-galactosidic and β-glucosidic substractes (Leese and Semenza, 1973; Mantei et al, 1988) and is exclusively present in the microvilli of intestinal epithelial cells (Leese and Semenza, 1973). This enzyme is active towards glucosylceramide and is irreversibly inhibited by CBE (Mantei et al, 1988).

c) The soluble broad-specificity β-glucosidase (EC 3.2.1.21) is not a glycoprotein and is most probably located in the cytosol. This enzyme is active towards β-glucosidic, β-galactosidic, β-fucosidic, β-xylosidic and β-arabinosidic derivatives of 4-methylumbelliferone and p-nitrophenol, but not towards glucosylceramide. The isoenzyme is not inhibited by CBE and its pH optimum is near neutral. This enzyme is present in various tissues, in particular in liver, spleen and kidney, but absent in fibroblasts (reviewed in Glew et al, 1988).

d) A tightly membrane-bound β-glucosidase has been described and referred to as membrane-bound non-specific β-glucosidase (Turner et al, 1977). This isoenzyme is also not able to hydrolase glucosylceramide, has a pH optimum of 5.4, is very strongly and competitively inhibited by D-gluconolactone and is markedly inhibited by detergente (Maret et al, 1981; Salvayre et al, 1982).

e) Finally, a non lysosomal glucocerebrosidase that is not deficient in patients with GD but is also active towards glucosylceramide, was recently described in human skin fibroblasts, Epstein Barr-virus transformed lymphocytes, a erythroblastoid and hepatoma cell lines. This hitherto glucocerebrosidase is located in compartments with a considerably lower density than lysosome and differs in several respects from the lysosomal glucocerebrosidase. The non-lysosomal isoenzyme proved to be tightly membrane-bound, whereas lysosomal glucocerebrosidase is weakly membrane-associated. The optimum pH of the non-lysosomal isoenzyme (5.5) is less acidic than that of lysosomal glucocerebrosidase. The non-lysosomal glucocerebrosidase, in contrast to the lysosomal isoenzyme, was not inhibited by low concentrations of conduritol B-epoxide, was markedly inhibited by taurocholate, and was not stimulated

by the lysosomal activator protein saposin C. This second non-lysosomal glucocerebrosidase seems to be able to catabolize some of the endocytosed glucosylceramide. The physiological function of this enzyme is yet unknown. However it presents the same properties as the membrane-bound non-specific β-glucosidase, when measured in the presence of detergent (van Weely et al, 1993a).

1.8.2 Glucocerebrosidase synthesis, modification, transport and stability

The glucocerebrosidase polypeptide (497 amino acids) (Dinur et al, 1986) is only proteolytically processed for the removal of the leader sequence and presents three disulphide bridges (Martin et al, 1989). The protein has five potential N-glycosylation sites (Tsuji et al, 1986; Martin et al, 1989), but studies of normal and mutated cDNA's in Sf9 and COS-1 cells showed that one of these sites (Asn-462) is not glycosilated (Takasaki et al, 1984). In addition, it was demonstrated that glycosilation of the first asparagine residue (Asn-19) is critical for the synthesis of a catalytically active enzyme (Berg-Fussman et al, 1993) (Figure 1-4).

The purification of glucocerebrosidase and the preparation of monospecific antibodies against the enzyme enable to discriminate the different molecular forms of the enzyme (Ginns et al, 1982; Barneveld et al, 1983b). The enzyme is synthesized in the rough endoplasmic reticulum and the signal peptide is cotranslationally cleaved by signal peptidase. The asparagine residues are core glycosilated (Erickson et al, 1985). In the Golgi apparatus the oligosaccharide chains of glucocerebrosidase are predominantly processed to complex-type structures with terminal sialic acid residues, resulting in an increase of apparent molecular weight of the 62 kDa precursor form to species of approximately 65-68 kDa (van Weely et al, 1990). Glucocerebrosidase membrane association probably occurs between exit from the endoplasmic reticulum and arrival in the trans-Golgi network (Rijnboutt et al, 1991). These forms are transported to lysosomes by a mechanism independent of the mannose-6-phosphate specific receptor pathway (Aerts et al, 1988), probably a transport pathway leading directely from the trans-Golgi network to the lysosomes (Willemsen et al, 1991). There, glucocerebrosidase undergoes further modification of its oligossaccharide chains by the action of exoglicosidases, resulting in mature 59 kDa molecular weight species (Aerts et al, 1986a; van Weely et al, 1990). According to these study the extent of modification does not significantly influence membrane association, half-life or catalytic efficiency of glucocerebrosidase. The enzyme does not contain a sufficiently long stretch of hidrophobic amino acids to allow membrane spanning (Sorge et al, 1986; Beutler and Grabowski, 1995). In particular in the carboxy-terminal part of the enzyme

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a number of short hydrophobic stretches are present; this may underly the tendency of the enzyme to associate with membranes (Furbish et al, 1977; Murray et al, 1985; Willemsen et al, 1987). Glucocerebrosidase spends a major part of its life time in prelysosomal structures, and has a relatively short life span in lysosomes as compared to other acid hydrolases (Aerts, 1988); thus the physiological role of glucocerebrosidase may not be restricted to intralysosomal glucosylceramide degradation (Aerts, 1995).
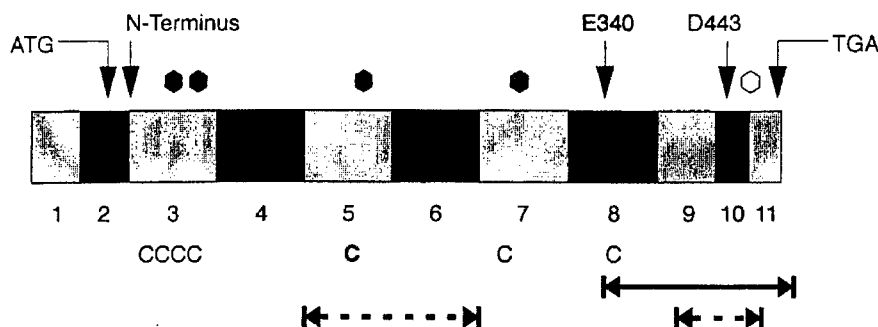
Figure 1-4

A function map of the glococerebrosidade protein. The alternating dark and light regions represent exons that are numbered below. The dark and light hexagons are the occupied and unccupied N-glycosylation concensus sequences. Glycosylation of the first site is essential for proper folding of the enzyme during translation. The Cs are the cysteines with free C129 in bold. All other cysteines participate in disulphide bond formation. The hatched and solid arrows bellow represent regions important to enzyme stability and catalytic activity, respectively. The tree-dimensional structure of the acid β-glucosidase has not been solved (Adopted from Grabowski and Horowitz, 1997).

1.9 Diagnosis and residual activity/phenotype correlation

The diagnosis of the disease can be made by determining β-glucosidase activity in leucocytes (Peters et al, 1976; Raghavan et al, 1980; Wenger et al, 1978), platelets (Nakagawa et al, 1982), skin fibroblasts cultures (Beutler et al 1971; Ho et al, 1972; Mueller and Rosenberg, 1977) or ammonium sulfate-concentrated urine (Aerts et al, 1986b). The amount of β-glucosidase activity in control leukocytes measured with the fluorogenic substrate 4-methylumbelliferyl-β-D-glucopyranoside (if sodium taurocholate is added) is very comparable to the β-glucosidase activity measured with the natural substrate and all GD patients present 8-18% of control β-glucosidase activity using both substrates. Concentrations of pure sodium taurocholate above about 6 mg/ml will give the optimum amount of hydrolysis of the substrate. Replacement of the large amount of taurocholate with a mixture of 2.5 mg/ml taurocholate and 2 mg/ml Triton X-100 resulted in equally high values (Wenger et al, 1978).

Clinical variability is generally believed to be due to differences in the residual activity of the affected enzyme. In GD as well as in other lysosomal storage diseases (Conzelmann and Sandhoff, 1983/84), residual enzyme activity, even in very mild variants or apparently healthy individuals, can be as low as 1-4% of normal activity (Beutler, 1977a). In addition, since it is associated with recessively transmitted disorders, heterozygotes, which usually possess half normal enzyme levels, are clinically normal. The great variability in possible residual activities arising from the type of molecular defect in a single gene locus (e.g., point mutation, base pair insertion or deletion, posttranslational modification) is amplified by the occurrence of heteroallelic compounds, i.e., individuals carrying different mutations in the two alleles of the same gene. Furthermore, the consequences of a reduced enzyme activity for the turnover rate of the substrate *in vivo* are different from that suggested with *in vitro* experiments, where a linear relationship can be observed between the amount or activity of the enzyme and the turnover rate of its substrate. Substrate concentrations *in vivo* are usually far below saturation of the enzyme and may also vary to a great extent. A reduction of the amount of enzyme may therefore within wide limits be compensated for by an ensuing increase in substrate concentration, restoring normal turnover rate. In addition, other factors need to be considered in establishing a correlation between the residual activity of a mutated enyme and the severity of the disease, like removal of the accumulating substrate by other processes (e.g., alternative metabolic pathways or excretion), interaction of enzyme or substrate with other cofactors, and feedback regulation mechanisms (Conzelmann and Sandhoff, 1983/84).

All of the mutated forms of glucocerebrosidase result in a large, but comparable, decrease in glucocerebrosidase activity when measured *in vitro* using a variety of synthetic substrates (Dinur et al, 1984; Osiecki-Newman et al, 1987). For instance, no correlation is observed *in vitro* between the V_{max} towards 4-methylumbelliferyl β-D-glucopiranoside of glucocerebrosidase produced from different GD mutated alleles (Horowitz and Zimran, 1994a). Other authors reported a correlation between levels of residual lysosomal glucocerebrosidase activity, measured *in situ* in fibroblast cells, and the type of Gaucher disease, by making use of lissamine rhodamine sulphonylamidosphingosyl derivatives. Long incubations revealed differences in the ratio of fluorescent ceramide to fluorescent glucosylceramide between type 1 and type 2 or 3 Gaucher fibroblasts (Agmon et al, 1993); similar differences were reported in another study by using a radioactive short-acyl-chain analogue of glucosylceramide, N-(1-[^{14}C]hexanoyl)-D-erythro-glucosylsphingosine (Meivar-Levy et al, 1994). Moreover, these authors claimed that cells with similar mutations had similar specific activities *in situ*. Although in these studies a consistent correlation between enzyme activity and

Gaucher type has been observed, it should be noticed that reticuloendothelial cells are affected to a greater extent in Gaucher disease than skin fibroblasts; in fact, these cells may be an unattractive cell model, since the level of glucocerebrosidase is about 30-fold higher than that in blood cells and most tissues, and thus, even in fibroblasts from type 2 and 3 GD patients that are severely deficient in glucocerebrosidase protein, more enzyme is present per unit of total cellular protein.

However, no strict correlation was noted between GD phenotype and parameters like: specific activity of the enzyme in extracts towards radioactively labelled natural lipid and artificial substrate in the presence of different activators; the enzymatic activity per unit of glucocerebrosidase protein; the rate of synthesis of the enzyme and its stability; and the post-translational processing of the enzyme (van Weely et al, 1991). In subsequent studies, the activity *in situ* of glucocerebrosidase, i.e. the lysosomal activity in the intact cell (fibroblasts and cultured macrophages obtained by isolating monocytes from peripheral blood), was assessed towards exogenous analogue 6-[N-7-nitrobenz-2-oxa-1,3-diazol-4-yl-aminocaproyl] sphingosyl-β-glucoside, (C_6-NBD-glucosylceramide). Again no clear relationship was found between the clinical phenotype, the genotype and the activity *in situ* (van Weely et al, 1991, van Weely et al, 1995) and the authors suggest that the properties of glucocerebrosidase in tissue macrophages are different from those in cultured cells or, alternatively, that other factors besides endogeneous glucocerebrosidase determine the ability of tissue macrophages to manage the influx of glucosylceramide substrate.

Still according to the theoretical model (Leinekugel et al, 1992; Conzelmann and Sandhoff, 1983/84), the correlation between residual activity and clinical variability should exist since small changes in residual enzyme activity below a critical threshold value would significantly affect the rate of degradation of lysosomal substrates; changes in levels of enzyme activity above the threshold would have no effect, since the enzyme is present at higher levels than that required to hydrolyse all the substrate.

1.10 Therapeutics

Among inherited metabolic disorders, the lysosomal storage diseases are the most susceptible to therapeutic attempts through enzymatic correction (de Duve, 1964). Exogenous enzymes may diffuse from circulation to tissues and be transported to lysosomes after passive pinocytosis (Loyd and Griffiths, 1979) or receptor mediated endocytosis (Kornfeld, 1987). Several ways of administering exogenous enzymes have been reported, such as infusion of plasma, leukocytes, or purified enzymes, and transplantation of fibroblasts, organs, or bone marrow (Di Ferrante et al, 1971;

Knudson et al, 1971; Daloze et al, 1977; Dean et al, 1979; Brady et al, 1974; Groth, 1982; Hobbs et al, 1982; Vellodi et al, 1987).

1.10.1 Cell transplantation

Repeated implantations of amniotic epithelial cells, freed from other stromal cells of the amniotic membrane, and maintained in culture some time before the implantation was described in the treatment of several lysosomal diseases includind type 3 GD (Scaggiante et al, 1987; Bembi et al, 1992, Sakuragawa et al, 1992). Although not providing a lifelong source of the deficient enzyme, amniotic epithelial cells implantations can be carried out easily also in outpatient clinics without requiring immunosuppression (Adinolfi et al, 1982; Scaggiante et al, 1988). Under normal conditions epithelial cells of the amniotic membrane lack HLA-A,B,C antigens and β_2-microglobulin on their surface and do not undergo malignant transformation in culture (Adinolfi et al, 1982).

Bone marrow transplantation (BMT) from a normal donor has also been used as a continuous source of enzyme, by replacement of enzyme deficient cells with normal cells of hematopoietic origin. Type 1 GD had been a good candidate for BMT since this type of GD primarily results in the accumulation of glucocerebroside in bone marrow-derived macrophages. Type 3 GD patients revealed a stable neurological status although glucocerebrosidase activity in brain did not increase (Tsai et al, 1992). Curative BMT has however the disadvantages of the limited number of donors (about 25% of the patients will have a compatible donor) and the risk of fatality that is estimated to be between 10-25%.

1.10.2 Enzyme supplementation

Type 1 Gaucher disease is the first inheritable disease of lysosomal storage to be treated successfully by supplementation of the deficient enzyme, glucocerebrosidase (Barton et al, 1991a; Brady and Barton 1994a; Brady and Barton 1994b).

Attempts at enzyme therapy for Gaucher disease began with the development of purification techniques for glucocerebrosidase from human placenta (Pentchev et al, 1973). The initial trials using human placental glucocerebrosidase showed encouraging biochemical results, but the subsequent experience was disappointing (Brady et al, 1974; Brady RO, 1977; Brady et al, 1980; Beutler and Dale, 1982), since uptake studies in rat liver showed that the purified human placental enzyme was predominantly targeted to parenchymal cells instead of nonparenchymal cells (Furbish et al, 1978). However a sequential exoglycosidase digestion of native placental glucocerebrosidase exposes internal mannose residues (Murray et al, 1985; Brady et

al, 1994) and directs enzyme to the targeted mannose-specific lectin receptors on the surface of cells (Achord et al, 1978).

The therapeutic efficacy of the preparation of human glucocerebrosidase, alglucerase (Ceradase®; Genzyme Corporation, Cambridge, MA), was clearly demonstrated, even for the 13% patients who developed antibodies against the enzyme (Richards et al, 1993).

Pharmacokinetic and pharmacodynamic data as tissue, cellular, subcellular distribution, as well as the half-life of alglucerase in Gaucher cells after infusion, is being studied in order to establish the optimum dosage regimen for the better efficiency of the treatment. *In vitro* studies have shown that endothelial cells are more effective in uptake of alglucerase than macrophages (Sato and Beutler, 1993). In addition, some beneficial response to alglucerase therapy has been attributed to the clearance of the storage material from blood circulation (Sidransky et al, 1993). Electromicroscopic studies in mice and rats following intravenously administered alglucerase demonstrated that alglucerase is targeted to the endosomal/lysosomal system of both Kupffer cells and endothelial cells (Willemsen et al, 1995; Bijsterbosch et al, 1996). The uptake and metabolism of mannose-terminated glucocerebrosidase was also studied *in vivo* by ^{123}I- and ^{125}I-labelled placental and recombinant enzymes (Mistry et al, 1995). In this work, the metabolism of the enzyme admnistered by bolus injection to patients with type 1 GD, was followed by analysis of blood, urine and feces; the tissue distribution was followed by γ-scintiscanning. The disappearance of labeled enzyme could be resolved into 4 exponential: 56-66% was removed rapidly with $t_{1/2}$ of 3-6 minutes; the $t_{1/2}$ of the other 3 exponential ranged from 0.25 to 27 hours. There was rapid uptake by the liver (29-37% of the injected dose) and the spleen (8-23% of the injected dose). Bone marrow uptake was demonstrated but reliable quantification was not possible. Serial scintigraphy was performed over 48 hours to estimate $t_{1/2}$ of the tracer in different organs. In the liver and spleen, 40-55% of the tracer in the viscera disappeared with $t_{1/2}$ 1-2 hours; the remainder persisted with a $t_{1/2}$ 49-60 hours. The $t_{1/2}$ in the bone marrow was 17 hours. Analysis of urine over 48 hours revealed that 6.1-8.5% of the injected dose was excreted in trichloroacetic acid-precipitable form and 31-34% of the injected dose in the non-iodide form. Thus 73-78% of the excreted label was bound to proteins or peptide fragments. Cumulative urinary excretions of tracer (% of injected dose) was at 10 hours, 32-38%; 20 hours, 42-46%; 30 hours, 47-49%; 40 hours, 52-53% and 48 hours, 52-54%. Fecal excretion was negligible.

1.10.2.1 Clinical results obtained with different dose/regimen

Several aspects of enzyme replacement therapy remain unresolved and require further investigation, namely, if the disease progression occur when therapy is initiated with low-dose regimens, if highly frequent infusions confer any additional benefits beyond those of dose alone, the minimum dose of enzyme required to produce skeletal improvement, and finally under what circumstances should prophylatic treatments be considered (Barton, 1995). It has been claimed that enzyme supplementation therapy reverse the signs and symptoms of GD if sufficient doses are administered, and that clinical responses are dose dependent and organ system specific. The dosage range administered for most patients has been 10-60U per Kg of body weight, every 2 weeks.

Reversal of skeletal pathology occurs slowly compared with the hematologic and visceral responses to enzyme replacement therapy (Rosenthal et al, 1995). After 6 months of high-dose infusions (60 U/kg every 2 weeks), biochemical improvement in the bone marrow was demonstrable in paired marrow biopsy specimens obtained from two patients; a twofold increase in triglyceride and a fivefold reduction in glucocerebroside content were observed. Noninvasive imaging studies were not informative at this time. However, after 42 months of treatment, dramatic normalization of the marrow fat content, as assessed by quantitative chemical shift imaging, was observed in all 11 patients who participated in the trial. Furthermore, the histologic appearance of the marrow reverted to normal in a single child who was treated in a similar fashion (Parker et al, 1991). In addition to the marrow response, net increases in either cortical or trabecular bone mass, as assessed by combined cortical thickness measurements and dual-energy quantitative computed tomography, respectively, were observed in 10 patients. The latter responses to treatment are particularly important for children, because a normal peak bone mass is achieved by early adulthood if they are adequately treated before fusion of the epiphyseal growth plates. Conversely, although several patients with a previous history of chronic bone pain/avascular necrosis of large joints, noticed an amelioration in intensity/frequency of pain, radiological evidence of bone improvement was not seen within 6-36 months of treatment (Zimran et al, 1995b).

The enzyme replacement therapy in the treatment of four patients with the neutrophil chemotactic defect and suffering from either severe pyogenic infections or recurrent urinary tract infections, allowed the reduction of neutrophil chemotaxis and of incidence of bacterial infections (Zimran et al, 1993).

Eight patients with the type 3 GD (from the Norrbottnian region) aged 4-42 years, and five of them splenectomized, were treated with high dosage at start of enzyme infusions. In the 13 to 29 months follow-up of treatment all patients responded well,

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with decreased liver and spleen volumes, normalized hematological parameters, growth in children, and no further neurological deteoration (Erikson and Mansson, 1995).

With respect to the establishment of the minimum dose, once a stable reversal of disease had been achieved in patients, the dose of enzyme has been empirically reduced by 50% every 9 months to determine the minimum amount required for biochemical control of the illness (Barton et al, 1991a; Brady and Barton, 1994a). All patients remained clinically and biochemically stable for 9 months at an enzyme dose of 30 U/kg every 2 weeks. However, 9 months after the dose was reduced to 15 U/kg every 2 weeks, 3 of 11 patients developed recurrent signs and symptoms of the illness. These included a gradually decreasing platelet count, slowly increasing splenic volume, and recurrent dull pain in the distal femurs. Modest increases in enzyme dose to 20-25 U/kg every 2 weeks provided adequate long-term maintenance therapy in the latter patients.

Altered dosage was accompanied by change in the glucosylceramide concentration. After single enzyme infusions, the level of circulating glucosylceramide was determined during 12 days in two patients. A slight reduction was observed during the first 24 hours and thereafter the concentrations slowly returned to the levels before infusion (Mansson and Erikson, 1995).

A different dosing regimen have been proposed, consisting of frequently administered low doses of alglucerase, either 30U/kg/month (Zimran et al, 1994b) or even 15U/kg/month (Hollak et al, 1995a,b) administered three times a week, which the respective authors claimed to be as effective as the high dose/low frequency, given every two weeks (120U/kg/month). The rationale of frequent administration is that the continuous presence of the enzyme in the macrophages is promoted, since the half-life of endogeneous glucocerebrosidase is approximately 24 hours (and the lysosomal turnover of endogenous glucocerebrosidase seems not to be determined by the glycan composition of the enzyme) (Van Weely et al, 1990). No sub-set of patients responded in a unique way, implying that there is apparently no correlation with age, sex, prior splenectomy, genotype or general phenotypic expression on the extent of response to therapy (Zimran et al, 1995b), and emphasizing the need for individual dosing (Hollak et al, 1995a,b). The high frequency of this administration causes however a burden for the patients, which is partly overcome by the more convenient method of home treatment.

Although therapy with alglucerase is effective, the purification from pooled human placental tissue cannot absolutely guarantee against the risks such as of viral

contamination, or the agent of the Creutzfeldt-Jakob disease. The preparation of human glucocerebrosidase expressed as the recombinant product in engineered Chinese hamster ovary cells, imiglucerase (Cerezyme™; Genzyme Corporation) is becoming available and is as effective as alglucerase (Grabowski et al, 1995; Zimran et al, 1995c). Imiglucerase is a monomeric glycoprotein of 497 amino acids, which differs from placental alglucerase by one aminoacid at position 495 where histidine is substituted for arginine. Kinetics of the labeled recombinant enzyme was also indistinguishable from those of the placental one (Mistry et al, 1995).

1.10.3   Somatic gene therapy

The transfer of genes for somatic gene therapy has been attempted by in vivo transfer of the gene (by either viral or nonviral methods) directly into the affected cell or tissue, or by ex vivo delivery of the gene to targeted host cells followed by autologous cell transplantation. For several disorders allogenic bone marrow transplantation (ABMT) has reversed the visceral complications of the disease. In Hurler disease and adrenoleukodystrophy, clinical results suggest that the complications in the nervous system can be halted, if not reversed, by ABMT (Krivit and Shapiro, 1991; Aubourg et al, 1990). The mechanism of this effect is probably the consequence of the gradual replacement of brain macrophages and microglia that are derived from the allograft. Bone marrow transplantation studies in the mannosidosis cat show that bone marrow-derived cells gain acess to the brain and result in improvement in the neurologic manifestation of the disease (Walkely et al, 1994).

In the development of gene therapy for Gaucher disease, it was demonstrated that ABMT restores hematopoietic stem cells and results in the repopulation of tissues with bone marrow-derived cells (Ginns et al, 1984; Ringden et al, 1988). Since in Gaucher disease the macrophage is the major cell involved in storage of glucosylceramide, bone marrow is, therefore, an appropriate target cell for gene transfer. Furthermore, since the gene product (glucocerebrosidase) is therapeutic when targeted to reticuloendothelial cells and macrophages, gene therapy will be efficient for Gaucher disease, provided that sufficient transfer and expression of the transgene can be achieved.

Several groups have reported protein expression in macrophages differentiated from mouse hematopoietic stem cells that were infected with a retroviral vector containing the human glucocerebrosidase gene (Weinthal et al, 1991; Ohashi et al, 1992; Correl et al, 1992; Krall et al, 1994). Imunohistochemical analysis of tissues of transplanted mice revealed that expression of human glucocerebrosidase was not only restricted to macrophages in visceral tissues, but also in brain (Mowery-Rushton et al, 1993). In the

central nervous system of these mice, human glucocerebrosidase-positive macrophages were observed, predominantly in perivascular spaces, but surprisingly also in microglia in close proximity with blood vessels. These findings suggest that circulating monocytes can enter the brain as perivascular cells and subsequently traverse the basement membrane and enter the brain parenchyma. The reconstitution with mouse glucocerebrosidase-positive macrophages is however only partial i.e. on the average in 20% of Kupffer cells, splenic macrophages and microglia cells by 8 months after transplantation (Krall et al, 1994).

Long-term bone-marrow cultures of hematopoietic cells of Gaucher patients were also successfully transduced with a retroviral vector encoding normal glucocerebrosidase cDNA (Nolta et al, 1992; Xu et al, 1994). Gene therapy is under study for all types of Gaucher disease (Barranger, 1994; Correl and Karlsson, 1994). Allogenic bone marrow transplantation may however have a role, possibly supported by enzyme replacement therapy, in very early stages of type 2 and type 3 GD. However none of the somatic gene therapy attempts till now have led to a permanent correction of the protein deficiencies (Correll and Karlsson, 1994). In addition, the clinical response was slow and required 10-12 months for measurable improvement to be evident.

1.10.4 Splenectomy and partial splenectomy as a therapeutic procedure

Prior to the era of enzyme replacement therapy, full or partial splenectomy was a commonly performed therapeutic procedure since generally it corrects the haematological abnormalities (neutropenia, thrombocytopenia and anaemia), relieves the symptoms of mechanical compression, engenders a compensatory growth spur in children, and improves the nutritional status in adults (Fleshner et al, 1991; Cohen et al, 1992).

However mental deterioration in patients with the type 3 of GD was reported to accelerate after splenectomy (Herrlin and Hillborg 1962; Dreborg et al, 1980). A deposition of circulating glucosylceramide in the adventitial cells of small blood vessels of cerebellum was found, which could be responsible for the accelerated mental retardation before death. The storage of glucosylceramide increased in other organs, e.g. the liver, and the level of circulating glucosylceramide raised (Nilsson and Svennerholm, 1982). Since there have been also reports of significant progression in liver and bone involvement following splenectomy (Ashkenazi et al, 1986), and the increase of risk of serious microbial infection particularly in infants, splenectomy is no longer a treatment of choice in GD. Given the ability of even low-dose enzyme

replacement therapy to relieve splenomegaly and ameliorate cytopenias, splenectomy has been reserved for patients with life-threatening hemorragic diathesis.

1.11 Unresolved questions of GD

Many questions remain unsolved with respect to either the epidemology and the pathophysiology of GD. In the Ashkenazi Jewish population the most commonly encountered mutation is the N370S glucocerebrosidase, which in the homo and heteroallelic form is associated with a non-neuronopathic clinical course. However, it remains to be established the N370S gene frequency which would allow to ascertain the incidence of the homozygosity, and its association with actual disease manifestation. With respect to the pathophysiology of GD, the mechanisms responsible for the variability of the clinical manifestations, especially in type 1 GD, are unclear. Although some authors claimed to obtain differences in glucocerebroside catabolism between the different types of GD (type 1,2 and 3), no correlation was established between the properties of the mutant enzymes and the residual activity in cells of type 1 GD patients, who present the most heterogeneous phenotype of the disease. In addition, the presence of a large number of storage cells can not explain all phenomena observed in GD patients. The storage macrophages are viable cells and it seems likely that chronic, excessive secretion of factors by these cells mediates a number of pathological processes that remain to be established and that probably contribute to the large clinical evolution of the disease. Finally, other factors besides the basic deficiency remain unidentified and may have a role in the clinical presentation of the disease; cellular glucocerebroside and ceramide concentrations may have an effect on key cellular events and by this way determine the involvement of other physiological systems.

Chapter 2
Aims of the experimental work and background information

2.1   Outline of this thesis

The studies described in this thesis were aimed to obtain further insight into the epidemology of Gaucher disease (GD) in Portugal as well as into the pathophysiological aspects of the disease. The nature and aims of the present investigations were the following:

i.   To determine the frequency of the N370S mutation in the Portuguese population and selected areas (Chapter 3).

ii.   To study the possibility of a common origin of the N370S mutated allele in Portuguese and Ashkenazi Jewish patients (Chapter 3).

iii.   To establish an enzyme binding assay for the detection of carriers with unknown mutated glucocerebrosidase alleles (Chapter 3).

iv.   To evaluate the predictive value of genotype for the glucocerebrosidase residual activity and the severity of clinical manifestation of GD (Chapter 4).

v.   To analyze secondary abnormalities of GD patients and their relation with glucocerebrosidase genotype and/or specific clinical phenotype (Chapter 5).

vi.   To study the effect of enzyme supplementation therapy on secondary abnormalities in order to get insights in the pathophysiology of GD (Chapter 5).

2.2   Purposes, approaches and new contributions

For these purposes different approaches were used and the experimental work of this thesis revealed the following new contributions for the study of GD:

(i) Gaucher disease (GD) revealed to be the most frequent inherited sphingolipid lysosomal storage disorder in the Portuguese population, and that when studied at the gene level, the Portuguese GD patients resembled the Ashkenazi Jewish Gaucher patients with respect to the high incidence of the N370S mutated allele. The general Portuguese population was characterized with respect to the frequency of N370S mutated allele:

-The frequency of the N370S mutated allele in the Portuguese population was found to be of 0.0043 (with 95% confidence limits between 0.0023 and 0.0063) (Chapter 3).

-On the basis of the frequency value, the incidence of N370S homozygotes can be calculated to be about 1/55 000 in the total Portuguese population (Chapter 3).

This result pointed to a mild or sub-clinical presentation of this genotype since apparently about 90% of the Portuguese N370S homozygous patients remain undiagnosed.

(ii) On the basis of the occurrence of the N370S mutation in the context of one haplotype of an intragenic polymorphism, the N370S mutation has been claimed to arisen recently and only once. The possibility of a common origin for the N370S mutated allele in the Portuguese and in the Ashkenazim populations was studied by linkage analysis:

-In the Portuguese population the frequency of each intragenic Pvull polymorphic form was found to be of 0.836 and 0.164 (for respectively Pv1.1- and Pv1.1+), which differs from the 0.647 and 0.353 values described as being the average of different racial groups (Chapter 3).

-The N370S mutated allele was always invariably associated with the Pv1.1- form of the intragenic polymorphism (Chapter 3).

Like GD patients of Ashkenazi Jewish ancestry, Portuguese GD patients presented linkage disequilibrium between the N370S mutated allele and the intragenic Pv1.1- haplotype, and thus a common origin for this mutated allele in both populations can not be excluded.

(iii) Due to the relatively high percentage of unknown mutations in the remaining mutated alleles, an enzyme-based assay was applied in the study of affected families presenting non-identified mutated alleles:

-The enzyme based screening assay allowed the detection of carriers for the two most frequent mutated alleles in Portuguese GD patients (N370S and L444P) as well as the identification of obligate carriers of unknown mutated allele(s) (Chapter 3).

Increased frequencies of N370S carriers were demonstrated within the population originated from certain Portuguese geographical areas with incidence of GD.

(iv) Compound heterozygotes for the N370S mutated enzyme were free from neurological symptoms. The clinical presentation of the Portuguese GD patients is however very heterogeneous, which may be related to combinations (at least 12) of different glucocerebrosidase mutated alleles. Yet no genotype/phenotype correlation could be established even among sibs. Glucocerebrosidase residual activity was determined in cells of patients presenting different genotypes:

-The proportion of glucocerebrosidase extracted in aggregated form (enzyme+lipids+activator protein) was found to contribute predominantly to the total glucocerebrosidase activity in leukocytes of patients presenting at least one N370S mutated allele (Chapter 4).

-The dependence of the N370S glucocerebrosidase activity on the aggregated form was reflected by the marked effect of the physiological activators (phosphatidylserine and saposin C) on the specific activity of the monomeric form at pH 4.5 (Chapter 4).

The reactivation ability of the N370S mutated enzyme probably contributes to a higher residual activity in cells of patients carrying at least one N370S mutated allele. The variable percentage of this mutated glucocerebrosidase in the activated state (aggregated form) probably determine the residual activity.

(v) GD patients present increased plasma chitotriosidase activity (which may reflect the overall Gaucher cell accumulation in the body) and tartrate resistant acid phosphatase (TRAP) activity (which may constitute a marker of osteoclastic activity). Plasma glucosylceramide, the peripheral blood T lymphocyte major subsets, serum ferritin and known abnormalities associated with GD were studied as indicators of disease manifestation in order to find any correlation with either genotype or clinical severity:

-A genotype/marker correlation was obtained where N370S homozygous patients were characterized by the absence of significant abnormalities in $CD4^+/CD8^+$ T lymphocyte ratio, significantly less abnormal TRAP and chitotriosidase activity (Chapter 5).

-A phenotype/marker correlation was observed where plasma glucosylceramide concentration was significantly correlated with the clinical severity score. In non-splenectomised GD patients it was observed an association between bone pathology and statistically significant abnormalities in the $CD8^+$ T lymphocyte subset, further substantiated by the existence of a significant correlation between the number of these cells in peripheral blood and plasma TRAP activity (Chapter 5).

The clinically milder phenotype of the N370S homozygotes was characterized by relatively less abnormal secondary markers. The obtained findings on T lymphocyte major subsets suggest the involvement of the immune system in the pathophysiology of GD, providing a useful disease marker.

(vi) Since enzyme supplementation therapy aims the correction and/or prevention of ongoing formation of the glucosylceramide-loaded macrophages, secondary abnormalities were studied in patients submitted to the alglucerase supplementation therapy, as quantitative markers of the amount of storage cells and macrophage activation:

-Chitotriosidase activity decreased in a dose dependent manner and preceding the reduction of glucosylceramide concentration which occurred after 12 months of therapy (Chapter 5).

-No effect was observed in the %$CD4^+$ and %$CD8^+$ T lymphocytes. With respect to the $CD4^+$ and $CD8^+$ T lymphocytes statistically significant changes occurred only after 12 months of treatment (Chapter 5).

-The evolution of TRAP activity agrees with the observed unclear and/or slower reversion of bone pathology in GD patients (Chapter 5).

Chitotriosidase activity seems to reflect the overall Gaucher cells accumulation in the body, whereas glucosylceramide decreased probably reflects the lysed lipid loaded macrophages. The less clear reduction of TRAP activity seems to indicate that the release of this enzyme is probably more restricted to a specific location of slower correction and/or prevention of ongoing formation of the lipid laden GD cells.

2.3   Background information

2.3.1   Genotype and clinical presentation of studied GD patients

All Gaucher disease (GD) patients described in this work were identified during the last 12 years in the Enzymology Department from the Instituto de Genética Médica Jacinto de Magalhães. Biochemical diagnosis was done by demonstration of severely reduced glucocerebrosidase activity in leukocytes, as measured with 4-methylumbelliferyl-β-glucoside artificial substrate in the presence of taurocholate. A total of 40 GD patients were clinically classified as being type 1 (37 patients), type 2 (2 patients) and type 3 (1 patients). Up to the present time none of the type 1 patients investigated has shown pathological involvement of the central nervous system.

At the beginning of this work detection of mutant glucocerebrosidase alleles by DNA analysis was restricted to two mutations. Consequently the initial research focused largely on the frequency and origin of the commonest in Portugal. Identification of more rare glucocerebrosidase mutations in the Portuguese GD patients was the topic of another work presently undertaken at the same Institute. The screening of mutations allowed the identification of about 87% of the mutant alleles among unrelated individuals. The remaining 13% of patients mutant alleles are still unidentified, even when tested for a total of 19 previously described mutations (Amaral et al, 1996).

The patients present age ranges from 10 to 59 years, 11 of them being male and 20 women. In general all non-splenectomized patients suffered from enlargement of the liver and spleen with subsequent pancytopenia and abdominal pain. Ten patients had had total splenectomy which, with the exception of one patient, resulted in the correction of anemia and thrombocytopenia. The accumulation of Gaucher cells in bone marrow is associated with varying degrees of necrosis, fibrous proliferation and resorption of the bony trabeculae, followed by erosion of the endosteal surface of the cortex and modeling deformities. Bone pathology was evaluated by treating physicians, using several methods, e.g. X-ray, magnetic resonance imaging (MRI) and bone tomodensitometry. The lesions detected ranged from diffuse osteoporosis (stage 1), to medullary expansion (stage 2), osteolysis (stage 3), necrosis/sclerosis (stage 4), destruction and collapse (stage 5) (Hermann et al, 1986). Among the earliest signs is the Erlenmeyer flask deformity of the distal femur and the proximal tibia (stage 2) which although not pathognomonic of the disease, is seen in most patients at presentation.

For each patient, the clinical severity was calculated on the basis of the Zimran's severity score index (SSI) (Zimran et al, 1989). This index takes into account the age of onset, the degree of organomegaly, liver function tests, clinical signs of liver disease, cytopenia, bone involvement and other organ involvement (Table 2-1).

The severity score index of each type 1 GD patient was evaluated by Ana Maria Fortuna, M.D. (from the Instituto de Genética Médica Jacinto de Magalhães). Patients' characteristics including age, sex, genotype, clinical presentation with respect to splenectomy, bone involvement, and the overall disease severity, presented as severity score (SSI) on the basis of the respective clinician assessment, are shown in Table 2-2.

As shown in Figure 2-1, a large variability in the severity score seems to exist in the seven most frequent genotype groups (N370S/N370S, N370S/L444P, N370S/IVS2+1, N370S/Rec TL, N370S/? and G377S/G377S); since only one patient was identified in each of the remaining genotype groups (N370S/R463C, N370S/X, X/X,

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N370S/RecNcil, L444P/L444P and L444P/?), nothing can be stated in this respect. In relation to three of these genotypes the clinical variability could also be observed between sibs. The intra-familial heterogeneity is particularly remarkable in the unique family with the N370S/IVS2+1 genotype, SSI varying from 5 to 14.

Table 2-1

Parameters used in the calculation of the severity score index (SSI) (Zimran et al, 1988). Mild, was used when spleen/liver tip does not extend below level of umbilicus; moderate, was used when tip is palpable between level of umbilicus and pelvic rim, and for the spleen, without extension to right side of abdomen; massive, was considered to be when the organ extends to below pelvic rim, and virtually occupies whole abdomen.

Feature	Score	Feature	Score
Age at diagnosis(yr)		**Liver function tests**	
<50	0	Normal	0
26-50	1	Minor abnormalities	1
11-25	2	Severe abnormalities	2
6-10	3	**Clinical signs of liver disease**	4
1-5	4	**Cytopenia**	
<1	6	Unsplenectomised	1
Splenectomy	3	Splenectomised	3
Splenomegaly		**Other organ involvement**	4
None	0	**Bone involvement**	
Mild	1	No signs/symptoms	0
Moderate	2	Radiograph or scan signs	1
Massive	3	Mild pain	2
Hepatomegaly		Chronic pain	3
None	0	Fractures	
Mild	1	Pathological	4
Moderate	2	Post-traumatic	1
Massive	3	Joint replacement	5
		Severe disability	2

When analyzed by genotype, a lower number of N370S/N370S homozygous patients were found to present bone involvement (Table 2-2). As can be observed in Figure 2-1, this is reflected in a comparatively milder overall clinical presentation of the N370S homozygotes, as assessed by the Zimran severity score index (SSI).
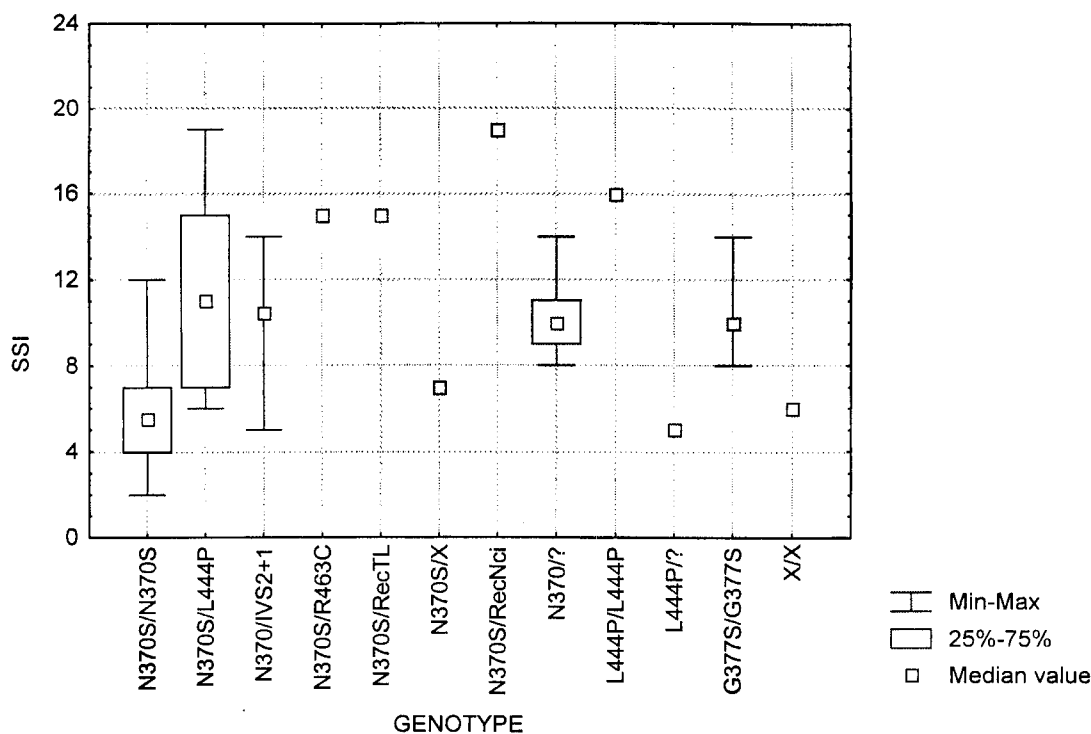
Figure 2-1
Clinical severity (SSI) of genotyped type 1 GD patient.

2.3.2 Gaucher disease patients submitted to the enzyme supplementation therapy

From the 46 studied patients 24 started intravenously administered enzyme supplementation therapy, the large majority with alglucerase (human glucocerebrosidase purified from human placenta and enzymatically deglycosylated to reveal terminal mannose residues, which are recognized by the macrophages membrane mannose-specific receptors, allowing the targeting and intracellular trafficking (Furbish et al, 1981)). Presently all treated patients are receiving the recently available imiglucerase (human glucocerebrosidase expressed as the recombinant product in engineered Chinese hamster ovary cells and enzymatically deglycosylated).

With respect to the dosage and frequency of administration, 21 patients started with high dose (60 U/per kg body weight) given every two weeks (60U/kg/2w). Three patients (patients 23, 31 and 34) started with frequently administered low doses (7U/per kg body weight)), given either weekly or three times a week (30U/kg/4w). Treatment regimens were changed according to the patient's clinical response. Patients 29 and 39 interrupted therapy after 9 and 6 months, respectively.

GD patients submitted to enzyme supplementation therapy presented different genotype and clinical severity (SSI) aged at start ranged from 9 to 53 years, being 10

of them man and 9 women. In addition to skeletal complications, all non-splenectomized patients suffered from excessive enlarged liver and/or spleen with subsequent pancytopenia and abdominal pain. Six patients were submitted to total splenectomy in the past which, with the exception of one patient (patient 41), resulted in the correction of anemia and thrombocytopenia (Table 2-2).

2.3.3 Clinical follow-up of GD patients submitted to enzyme supplementation therapy

With respect to the clinical follow-up, in accordance to the highly heterogeneous clinical presentation of GD, the response to therapy is also variable and organ system-specific (reviewed in Beutler and Grabowski 1995). Sustained clinical improvement was observed in all patients, with the high dose regimen (60U/kg/2w). In contrast patients receiving the low dose regimen (30U/kg/4w) showed a delayed clinical response and for that reason were changed to the high dose regimen.

Objective clinical improvement was obtained as well as a clear increase in the quality of life, with respect to personal performance and well being. After 12 months a significant reduction in the size of the spleen (20-54%) could be observed in 4 patients that presented splenomegaly. In 3 of these patients as well as in other non-splenectomized patient, the resulting reductions in liver size obtained after a 12 month period were between 17-44%. Patients presenting mild organomegaly at baseline, showed completely regress to normal after 12 months of treatment; with respect to the remaining patients in general the excessively large spleens became markedly smaller, but did not completely regress after 12-36 months of treatment.

The bone painful symptoms were drastically reduced in all patients and specially in the patient who required hip replacement during treatment. One patient that presented growth retardation at baseline, increased 14cm in height after 36 months of therapy.

Table 2-2

Characteristics of the studied GD patients. For each patient it is was indicated the case number, sex, age (years), genotype, overall clinical severity (assessed by the Zimran`s severity score index, SSI), the occurrence of splenomegaly, splenectomy date, hepatomegaly, growth retardation and bone pathology. The clinical signs of bone disease were classifyed in stages acording to Hermann et al, 1986. The dose and regimen (at the beginning of treatment) was also indicated for patients submitted to the enzyme suplementation therapy.

	Patient	Sex	Age	Genotype	SSI	Splenomegaly (date of splenectomy)	Hepatomegaly	Growth retardation	Bone involvement	Clinical signs of bone disease	Patients under treatment
1	4	f	57	N370S/N370S	12	moderate	-	-	+	Stage 1	-
2	5	f	49	N370S/N370S	5	moderate	-	-	+	Bone pain	-
3	6	f	47	N370S/N370S	4	moderate	-	-	+	Bone pain	-
4	9	m	35	N370S/N370S	4	moderate	-	-	+	Bone pain	-
5	18	f	51	N370S/N370S	7	Splenectomy in 1993	-	-	-	No signs	60U/kg/2w
6	21a	f	52	N370S/N370S	7	mild	mild	-	-	No signs	60U/kg/2w
7	32a	m	54	N370S/N370S	7	moderate	mild	-	-	No signs	60U/kg/2w
8	38b	f	31	N370S/N370S	2	moderate	-	-	-	No signs	-
9	42	f	22	N370S/N370S	6	moderate	-	-	+	Stage 1	-
10	43b	f	26	N370S/N370S	2	moderate	-	-	-	-	-
11	44c	f	21	N370S/N370S	NA	moderate	-	-	-	-	-
12	45c	f	26	N370S/N370S	NA	moderate	-	-	-	-	-
13	10d	f	13	N370S/L444P	11	massive	moderate	-	+	Stage 1	60U/kg/2w
14	11d	m	17	N370S/L444P	11	moderate	moderate	+	+	Stage 4	60U/kg/2w
15	13	f	76	N370S/L444P	6	moderate	-	-	-	-	-
16	23	m	30	N370S/L444P	6	moderate	mild	-	+	Stage 4	60U/kg/2w
17	28	f	6	N370S/L444P	7	moderate	-	-	-	-	-
18	33	f	25	N370S/L444P	19	massive	Massive	-	+	Stage 5	60U/kg/2w
19	34	f	32	N370S/L444P	11	massive	Moderate	-	+	Stage 1	30U/kg/4w
20	40	m	41	N370S/L444P	15	massive	moderate	-	+	Stage 3	-
22	7e	f	21	N370S/IVS2+1	14	Splenectomy in 1981	Moderate	-	+	Stage 3	-
23	8e	f	44	N370S/IVS2+1	14	Splenectomy in1969	Mild	-	+	Stage 3	-
24	14e	f	37	N370S/IVS2+1	7	mild	mild	-	+	Stage 1	-
25	15e	f	28	N370S/IVS2+1	5	-	-	-	-	-	-
26	1	m	23	N370S/R463C	15	massive	massive	+	+	Stage 5	60U/kg/2w

Table 2-2 (Continuation)

Characteristics of the studied GD patients.

	Patient	Sex	Age	Genotype	SSI	Splenomegaly (date of splenectomy)	Hepatomegaly	Growth retardation	Bone involvement	Clinical signs of bone disease	Patients under treatment
27	3	f	28	N370S/RecTL	15	moderate	mild	+	+	Stage 2	-
28	12	f	58	N370S/RecTL	15	Splenectomy in 1939	mild	-	+	Bone pain	-
29	20	f	26	N370S/X	5	moderate	Moderate	-	+	Stage 2	60U/kg/2w
30	36	m	32	N370S/RecNcil	19	Splenectomy in 1977	moderate	-	+	Stage 5	60U/kg/2w
31	2	f	44	N370S/?	11	+	mild	-	+	Stage 1	-
32	19	f	10	N370S/?	10	massive	moderate	-	+	Stage 2	60U/kg/2w
33	22	f	24	N370S/?	8	+	-	-	+	Stage 1	-
34	24	m	19	N370S/?	10	Splenectomy in 1990	-	-	+	Stage 1	-
35	30	m	36	N370S/?	9	Splenectomy in 1994	moderate	-	-	No signs	30U/kg/4w
36	31	m	45	N370S/?	14	Splenectomy in 1994	moderate	-	+	Stage 1	30U/kg/4w
37	35	f	27	N370S/?	9	massive	mild	-	+	Stage 1	60U/kg/2w
38	39	m	18	N370S/?	12	massive	moderate	-	+	Stage 1	60U/kg/2w
39	46	f	23	N370S/?	10	massive	mild	+	+	Stage 2	60U/kg/2w
40	47	f	29	N370S/?	9	Splenectomy in 1982	moderate	-	+	Stage 5	60U/kg/2w
41	41	m	17	L444P/L444P	16	Splenectomy in 1993	moderate	-	+	Stage 4	60U/kg/2w
42	16	f	27	L444P/?	5	mild	mild	-	-	No signs	-
43	25	m	39	G377S/G377S	8	splenectomy in 1989	mild	-	+	Stage 2	-
45	29	M	43	G377S/G377S	14	Splenectomy in 1962	moderate	-	+	Stage 4	60U/kg/2w
44	49	M	49	G377S/G377S	10	moderate	mild	-	-	No signs	60U/kg/2w
46	37	F	37	X/X	6	massive	mild	-	+	Bone pain	60U/kg/2w

a, b, c, d and e) indicate related patients from different families.

"?" indicates that none of the tested mutations were present.

"X" indicates an unpublished new mutated allele.

Chapter 3
N370S glucocerebrosidase gene mutation in the Portuguese

3.1   Introduction

3.1.1   Occurrence of rare genetic disorders in small populations; The incidence of lysosomal storage diseases in the Ashkenazi Jewish community

The occurrence of one or more rare genetic disorders -in particular autosomal recessive ones- in relatively high frequency, have been reported in isolated communities or small populations, like for instance, the Old Order Amish people, the Ashkenazi Jews, the Finns or the French Canadians. These communities were isolated because of geographical conditions, religion, or by preference. In some cases, the genealogical studies demonstrated a common origin of the carriers, and a founder effect together with genetic drift was proposed to explain the high frequency of the disorders (Diamond and Rotter, 1987).

In the Ashkenazi Jews, who represent the largest Jewish community, several genetic diseases have been reported to occur in relatively high incidence, including Tay Sachs (GM2 gangliosidosis type B), Gaucher type 1, Niemann-Pick type A (1:40 000 and 1:80 000 of type B), familial dysautonomia, Canavan, Bloom, mucolipidosis (ML) type IV, hereditary dystonia, and factor XI deficiency (reviewed in Bonné-Tamir and Adam, 1992; Diamond, 1994). Four of these disorders -Tay Sachs, Gaucher, Niemann-Pick A, B and ML IV- are lysosomal storage disorders. The incidence of lysosomal storage diseases in the general population is very low, similar to those of other autosomal recessive genetic disorders, and thus the existence in high frequency of four of these diseases among the Ashkenazi Jews is unexpected and intriguing, particularly since it

involves the storage of substances of a common nature - the GM_2 ganglioside in Tay-Sachs, the glucocerebroside in Gaucher, the sphingomyelin in Niemann-Pick, and the gangliosides in ML IV, all share the ceramide-lipid backbone (Zlotogora et al, 1988; Diamond, 1994). Also in the analysis of patients with lysosomal storage disorders a similar tendency was observed that is, more than one lysosomal storage disorder in certain large families or communities and despite the different enzymes involved the stored substances are usually of similar or common nature (Zlotogora et al, 1988).

According to the analysis of haplotypes and/or mutations, it may be possible to distinguish 3 groups of disorders frequent in isolated populations. In the first group, all the affected patients have only one frequent mutation, suggesting a founder effect with genetic drift. In the second group, more than one mutation is found among the patients, however, most of the patients are homozygous for one frequent mutation which most probably originated from a common founder; the other patients are compound heterozygous for the common mutation and a rare mutation. In the third group, more than one frequent mutation is found responsible for each disease. This may be due to a selective advantage which allows the expansion of each new mutation in the particular population or to multiple founder effect with genetic drift in smaller communities which thereafter mixed to form the larger population (Zlotogora, 1994).

Using mathematical analysis Jorde (1992) concluded that at any reasonable rate of mutation or migration, and at any effective population size, drift alone is unlikely to account for the elevated frequency of either of the major Tay-Sachs mutations in the Ashkenazi population. In considering elevated frequencies of recessive disease genes, a selective advantage is often invoked for the heterozygote. This can take the form of either increased resistance to a selective agent or an intrinsic fertility advantage (Jorde, 1992). A selective advantage for the carriers of lysosomal storage diseases has been suggested (Zlotogora et al, 1988). It may be that even though carriers of lysosomal diseases are healthy, the partial enzyme deficiency leads to a subtle storage of similar type of substances in the lysosome. This storage may be advantageous to the cell in some particular environmental conditions. For instance, it may confer resistance to some infectious agents to the cell. In a population such as the Ashkenazim, such a selective advantage may explain that not only one disease is caused by more than one frequent mutation, but also that more than one lysosomal storage disease is found in high frequency. It has been suggested that the advantage may have been resistance to pulmonary diseases, in particularly tuberculosis, which were more prevalent in the urban ghettos where the Eastern European Ashkenazi Jews lived, than in their predominantly rural Gentile neighbours (Rotter and Diamond, 1987). So, the selection forces might be environmental (e.g., lung disorders) which would be a situation similar

to that seen in both the resistance to the malaria parasite in heterozygous for the sickle cell-anaemia gene and G6PD deficiency in Africa, or alternatively, the selection could occur at the gamete level; the later hypothesis is however unlike since these disorders are rare in the general population (Zlotogora et al, 1988).

3.1.2 Frequency of Gaucher disease mutated alleles in the Ashkenazi Jewish and the Portuguese populations

The prevalence of the GD mutations has been reported as being different between patients of Ashkenazi Jewish and non- Ashkenazi Jewish origin (Horowitz et al, 1993). The N370S mutation was reported as the most frequent among Ashkenazi Jewish patients, whereas L444P was described as the most prevalent among non-Jewish patients (Beutler et al, 1993). More specifically, the N370S mutation is present in 77% of the mutated alleles in the Ashkenazi Jewish type 1 Gaucher patients (Beutler et al, 1992), where in the few studies that have been performed on non-Jewish of diverse ethnic or non-specific origin patients, the N370S mutated allele accounted to 22-46% of mutated alleles (Beutler et al, 1991; Levy et al, 1991; Choy et al, 1991; Sidransky et al, 1992b).

However DNA analysis of the Portuguese type 1 Gaucher patients showed that the N370S mutation was the most prevalent mutation, accounting for 63% of mutated alleles (being L444P the second most frequent one) (Amaral et al, 1993). The high prevalence of the N370S allele was thus closer to that described for Ashkenazi Jewish patients than the remaining non-Jewish Gaucher patients. Two mutations alone (N370S and L444P) account for as much as 84% of all the mutated alleles in the Portuguese patients.

In the Ashkenazim patients five common mutations collectively were reported as accounting for about 97 per cent of Ashkenazi Jewish GD alleles (Beutler et al, 1992b). The two most frequent mutant alleles had a measured heterozygote frequency of 0.0022-0.032 -far higher than could be sustained at equilibrium (in the face of deaths or debilitation from Gaucher) by recurrent mutations in the absence of any compensating selective advantage. Since three of the mutant alleles occurred in Jews in the context of only a single haplotype, the mutations must havearose in Jews recently, and only once, and have within a short time been pumped up to high frequencies by selection (Diamond, 1994). Another possible explanation for the finding of more than one frequent mutation in a population where a disease is frequent, may be that while nowadays the population seems to be homogeneous, however it is composed of smaller communities which, in the past, were isolated one from the other. In fact, the Eastern European Jewry were dispersed in small communities since the Middle Ages,

and only recently are considered as the Ashkenazim ethnic group (Goodman and Motulsky, 1979). The occurrence of mutations in some of the smaller communities may have been followed later by the spread of each mutation within the community. The spread may have been because of a selective advantage or a founder effect (which is essentially a form of genetic drift) in each of the smaller communities (Zlotogora, 1994).

The noted high frequency of the N370S mutated allele in the Portuguese population raise the question that this could be due to the historical major influx of Sephardic Jewish genes in the Portuguese country.

3.1.3 Cultural and genetic roots of Jewish communities - Sephardic Jewish ancestry of the Portuguese

During the process of their formation and settlement in a given territory, human populations are subjected to the influences of various evolutionary factors, such as migration, admixture with people from other populations, random differentiation, and specific selective process. Despite the complicated interactions of these factors in the ethnic groups populating modern Europe, evidence of the effects of single processes, such as directional patterns caused by migration, as well as of stochastic differentiation, may be demonstrated (Sokal et al, 1989).

Jewish communities trace back both culturally and genetically to some extent to a population occupying a small geographic area in Eastern Mediterranean, the land of Israel, several thousand years ago (Dellapergola, 1992). Jewish populations were dispersed from there in the main between 600 B.C. and the Second Diaspora (dispersion of Jews from the land of Israel after the destruction of the Second Temple by the Romans in 70 A.D.). The history of their subsequent residence in and movements between various countries in Europe, North Africa, and the Middle East has resulted in a nearly unique pattern of genetic relationships among Jewish populations and between them and the non-Jewish peoples among whom they live.

Modern Jewish population includes: The Sephardic Jews who live in and around the Mediterranean basin; the Oriental Jews which came from Asia; the Eastern European Jewry, whose story began probably after the Crusades (1200 A.D.) with the arrival of a modest number of Jewish immigrants (most probably Sephardic Jews from Western Europe) to Middle (corresponding to Austria, Hungary, Czech and Eslovakia Republic) and then Eastern Europe (Poland and Russia) (Petersen et al, 1983) where they have lived for centuries, and only recently are considered as the Ashkenazim ethnic group (Goodman and Motulsky, 1979). Nowadays they comprise about 40% of the Jewish population.

The Sephardic Jews may be described briefly as the descendants of that group of Jews who lived in Spain during the Middle Ages. It is not known when Jews first entered Spain, but the expressed intention of the Apostle Paul (Romans 15: 24) to visit that country almost certainly implies the presence there of Jewish communities in the first century of the Christian era. However, long before the Muslim invasion in 711 A.D., the Christianised Visigoths, who had succeeded the Romans in power, had completely suppressed the open practice of the Jewish religion (reviewed in Mourant et al, 1978). The Jews who entered Spain in large numbers immediately after the original Muslim invasion came from North Africa. The Jews of this region, while including many who were of Berber descendent, were mostly the descendants of Palestinian Jews who had entered through Egypt. The descendants of these Egyptian Jews had in turn been driven further west by persecution, especially by the Romans, first pagan and then Christian, as long as they controlled north Africa. The freedom of the Jews in Spain (during the Muslim control) and their growing intellectual and religious status attracted Jews from communities of the eastern Mediterranean and especially from Babylon, which finally, in the eleventh century, yielded the religious and cultural leadership of the Jewish world to Spain. The reconquest of Spain by the Catholic forces of Ferdinand and Isabel resulted at first in intolerance and for a short time in conciliating position to Judaism, but in 1492 under papal influence Jews were expelled by decree (unless they accepted Christianity they were forced to leave the country). Those who reached the Portuguese frontier achieved a short rest but, one of the conditions for the marriage of the Portuguese king with the daughter of the Spanish Catholic sovereigns was that all Jews should be expelled. Repressive laws and massacres began to cause Jews in Portugal to conversion to Christianity, but large numbers of those who did so became "Marranos", publicly professing conversion but continuing to practise the Jewish religion in secret. While, however, these "New Christians" (as converted Jews were called) continued to occupy positions which they had held as Jews, many even married into "Old Christian" families and any suspicion that their conversion was not genuine exposed them to the heresy-hunting Inquisition (reviewed in Mourant et al, 1978). To this day, however, it also remains, among the Catholics of Portugal the Belmonte community who, hardly knowing the reason why, continue to perform some of the superficial rites of the Jewish religion (Prinz, 1974; Garcia, 1993).

In the XVIII century important groups of Portuguese Sephardic Jews were settle down in the USA (reviewed in Sequeiros, 1989), most of them coming from Holland to where they had emigrated due to the Inquisition persecutions. In fact American Jewish communities of New Amsterdam (actually New York) were founded by Portuguese Jews (Silveira Cardozo, 1976; San-Payo, 1985). In 1677 a new group of Portuguese

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Jews coming from Barbados were established in Newport (San-Payo, 1985); several other groups joined them later on coming from Curaçao, Holland, Brazil and Portugal (Silveira Cardozo, 1976). In 1734 David Mendes Machado, born in Lisbon, was rabbi of the New York Jewish community (Silveira Cardozo, 1976), and the most ancient synagogue in the USA, which was builded in 1759 and located in Newport, was founded by the Portuguese Jew Aaron Lopes.

The Azores islands are constituted by 9 islands in the North Atlantic which were discovered in 1432 and colonised 7 years later. The first colons were in the great majority from Southern Portugal (in particular the Algarve region), and included both "new" Christians (Jewish and Muslim) and "old" Christians; the remaining population was originated from Britain, Scotland, Ireland and Italy (Guill, 1972). Nowadays the Azores population is constituted by about 250 thousands inhabitants, and in the USA live about 1,5-3 millions of people with Portuguese ascendance and most of them from Azores Islands (Guill, 1972). They are distributed mostly in the New England and California states, special in the South of Massachusetts, Rhode Island, as well as in the North of California and San Francisco bay (U.S. Department of Commerce, 1983).

3.1.4   Genetic affinities of Jewish populations to each other and to their non-Jewish neighbours

Whereas the factors molding the genetic structure of the typical European population are spatial differentiation, migration, and amalgamation of native gene pools (Sokal et al, 1989), Jewish populations have the potential for exhibiting a more dendritic or hierarchic structure due to their migration and branching history. Spatial differentiation based on isolation by distance is possible for them only in situations where they were numerous and widespread enough and resident for a sufficient period to permit the effects to become manifest (Livshits et al, 1991). Computer simulation studies have shown that the percentage of shared polymorphic alleles declines very rapidly in groups of populations after their splitting, even when daughter populations are of as large an effective size as 10,000 individuals (Li and Nei, 1977; Fuerst, 1985). Because the sizes of Jewish populations frequently were small, there is evidence (Carmelli and Cavalli-Sforza 1979; Morton et al, 1982) of the additional significant effects of chance sampling of the gene pool (genetic drift, bottleneck effects). Finally, although religious law and custom proscribed intermarriage with the surrounding non-Jewish populations, there is little doubt that some genetic admixture took place (Livshits et al, 1991).

With respect to the genetic affinities of Jewish populations to each other and to their non-Jewish neighbours, Mourant et al (1978) maintained that each major Jewish community as a whole bears some resemblance to indigenous peoples of the region

where it first developed, while Morton et al (1982) using the modified kinship function of Malécot, concluded that there was substantial intermixture between Jewish and neighbouring non-Jewish populations. Other studies (e.g., Karlin et al, 1979; Kobyliansky et al, 1982; Bonne-Tamir, 1985) reported a considerably greater genetic similarity for most pairs of Jewish populations than between Jewish and non-Jewish communities. It has also been shown by blood groups ABO and typing that inbreeding within Jewish populations as measured by F_{is} is higher than in non-Jewish populations, whereas Wright's standardised genetic variance (F_{st}) among Jewish groups is lower than among non-Jewish communities (Kobyliansky and Livshits, 1983). Other studies concluded that European Jews and non-Jews form two distinct and closely knit clusters, and that North African and Iraqi Jews are closer to the European Jews than to the corresponding non-Jews (Rao and Boudreau, 1984). In other studies (Wijsman, 1984) it was found that Ashkenazi Jews have a low to moderate non-Jewish genetic component, consistent with both the low estimates suggested by Karlin et al (1979) and higher estimates suggested by others (Carmelli and Cavalli-Sforza, 1979; Morton et al, 1982). Computing the level of admixture separately for the different loci, no admixture was found in HLA-A and HLA-B loci, but various amounts of admixture for other loci (Cavalli-Sforza and Carmelli, 1979). Similar variability across loci was reported (Motulsky, 1980).

By assembling the largest combination of gene frequencies based on independent loci for matched Jewish and non-Jewish populations reported to date, and the largest number of loci for individual Jewish populations, and also by introducing a new analytical distance by means of Mantel tests against design matrices, the results of the study of Livshits et al (1991) consistently showed lower distances among Jewish populations than with their non-Jewish neighbours, most simply explained by the common origin of the former. This findings are easiest to explain by deriving the modern Jewish populations from a common original gene pool which underwent relatively few changes during the dispersion of the Jewish people. Unless differential evolutionary rates are postulated, the common origin of the Jewish populations must be more recent than that of the non-Jews (Livshits et al, 1991). Yet, there was evidence also of genetic similarity between Jewish and corresponding non-Jewish populations, suggesting reciprocal gene flow between these populations or convergent selection in a common environment. The study also indicated that stochastic factors are likely to have played a role in masking the descent relationships of the Jewish populations (Livshits et al, 1991).

Modern genetic structure of Jewish populations do not reflect the history of dispersal, placing all but two of the Jewish populations in a common cluster, and the fine structure

of this cluster does not mirror the known history of these populations (Livshits et al, 1991). Starting out as a single entity (comparable to other ethnic groups of that time), the Jewish people at the beginning of the Diaspora were subdivided into numerous populations which became dispersed in the course of dozens of generations to various parts of the world. Within each country in which they came to reside the Jews usually became nonintermarrying subpopulations. Consequently, a significant contribution of stochastic differentiation between populations and inbreeding within populations could be expected. The reduction of effective population size in such a set of populations may lead to a rapid increase of genetic distances between them, primarily due to the decrease of heterozygosity in each of these populations (Chakraborty and Nei, 1977). The effect of a bottleneck on average heterozygosity may last for hundreds or even thousands of generations after the recovery of population size (Nei, 1987). Thus estimates of the descent and branching relationships of populations may be seriously distorted by bottlenecks. One way to study this problem is to examine the relationship between genetic distance and the average heterozygosity over pairs of populations (Livshits and Nei, 1990). The negative correlation between genetic distance and heterozygosity of pairs of populations would be expected on condition that the populations were derived from the same ancestral stock and at the same historical time. But it is also necessary to assume absence of migration, as well as of selection after separation (Livshits et al, 1991). Despite the fact that human populations can hardly satisfy the above conditions, especially regarding the total absence of migration, genetic distance values are strongly negatively correlated with heterozygosity estimates. Statistically significant negative correlations between genetic distance and heterozygosity were found in separate studies of Caucasoid, Amerindian, and Far Eastern Mongoloid populations (Livshits and Nei, 1990). Therefore stochastic factors are likely to have played a role in masking the descent relationships of the Jewish populations. However, when populations representative of different major races were combined, an almost negligible correlation was found, implying that the time after splitting of the human species into the major races was long enough to restore equal heterozygosity in each stock (Livshits and Nei, 1990). So, the complex structure of the cluster of Jewish populations must also be due to the differences in the amount of admixture with neighbouring non-Jewish populations, because of both different lengths of contact and different rates of admixture.

3.1.5 Aims and approaches of the study

Molecular studies of haplotypes and mutations are useful to try to understand the mechanisms which lead to the high frequency of genetic disorders in some

populations. However, it is difficult in some cases to determine whether the principal cause is related to genetic drift or selection. The analysis of mutations and their diffusion in isolated populations will help to understand what were the mechanisms which lead to the high frequencies of genetic diseases in isolated populations. This knowledge should be useful for understanding the cause of the high frequency of diseases in larger populations (Zlotogora, 1994).

Gaucher disease is the most frequent inherited sphingolipid lysosomal storage disorder in the Portuguese population. DNA analysis of Portuguese type 1 Gaucher patients showed that the N370S mutation accounted for 63% of the glucocerebrosidase mutated alleles (Amaral et al, 1993), which was more similar to the described in Ashkenazi Jewish type 1 Gaucher patients (72-75%) (Levy et al, 1991; Beutler et al, 1991), than in the remaining non-Jewish type 1 Gaucher patients, where it accounted for 46% (Levy et al, 1991), 39% (Walley et al, 1993) or 25% (Beutler et al, 1991) of the mutated glucocerebrosidase alleles. The estimation of the N370S gene frequency in the Portuguese population, based on the prevalence of the mutation in patients, would be misleading since homozygous for this mutation can present a mild or asymptomatic clinical phenotype and escape detection (Beutler et al, 1993).

- In order to accurately determine the N370S frequency, for the first time in a non-Ashkenazi population, the DNA of 2000 individuals randomly sampled from the Portuguese new-born PKU screening program were genotyped.

- In order to find hot spots in the Portuguese population, the N370S gene frequency was also determined in two restricted populations, Azores Islands and Belmonte village.

It was previously shown that the N370S glucocerebrosidase mutation was invariably associated with one haplotype of the intragenic PvuII polymorphism (Zimran et al, 1990; Beutler et al, 1991). In contrast, mutation L444P (Tsuji et al, 1987), was found to be in linkage equilibrium with both forms of this polymorphism (Zimran et al, 1990; Beutler et al, 1991).

- The frequency of each PvuII polymorphic form was first determined in the general Portuguese population (where a Sephardic but not an Ashkenazi Jewish background is probable) by studying an ethnically homogeneous sample of unrelated individuals. Portuguese type 1 Gaucher patients and carriers where then studied with regard to linkage between mutations and PvuII polymorphism.

DNA analysis of Portuguese type 1 Gaucher patients showed that L444P was the second most frequent mutation in the Portuguese patients, accounting for 22% of the glucocerebrosidase mutated alleles (Amaral et al, 1993). Moreover, although some of

the most frequent mutations described in type 1 Gaucher patients had been tested, 15% of Portuguese patients mutated alleles remained unidentified (Amaral et al, 1993), contrasting with the 4% described in Ashkenazi Jewish patients (Beutler, 1992).

- An enzyme based assay, in which the enzymatic activity was determined per unit of cross-reactive glucocerebrosidase protein bounded to a monoclonal antibody (Aerts et al, 1991), was applied in the study of Portuguese affected families, including the ones carrying known mutations. The reliability of this assay in the detection of N370S and L444P mutated proteins was confirmed by DNA analysis.

3.2   Materials and methods

3.2.1   Materials

3.2.1.1   Samples

Subject population studied for the N370S frequency determination: The Portuguese Neonatal Screening Program covers 95% of all the new-borns in Portugal, and all the analysis are done at the Instituto de Genética Médica Jacinto de Magalhães. In this program blood spots are collected on Guthrie cards and for the present research work they were used as a source of DNA.

For ethical reasons the sampled individuals were not identified.

Subjects studied with respect to the PvuII polymorphism: DNA was extracted from white blood cells or from cultured cells (skin fibroblasts or lymphoblasts) according to standard methods (Maniatis et al, 1982; Miller et al, 1988), from the following subjects.

Type 1 Gaucher patients: Fifteen patients presenting five different genotypes, namely N370S/N370S (n=4). N370S/L444P (n=5), N370S/? (n=4), L444P/? (n=1) (Amaral et al, 1993), as well as N370S/R463C (n=1).

Members of Gaucher families: Thirty-eight obligate or possible carriers, being 27 carriers of the N370S mutation, 4 carriers of the L444P mutation and 7 obligate carriers in which the mutated allele remained unidentified.

Controls: Sixty-four healthy individuals unrelated to Gaucher families, none presenting the N370S glucocerebrosidase mutation.

Subject population studied with the immunobinding assay: Urine was collected from 15 patients (2 males and 13 females with ages ranging from 7 to 74 years), 41 family members of Gaucher patient's families of both sexes (including 22 obligate carriers and

19 relatives designated as "putative carriers") with ages ranging from 6 to 82, and 81 healthy control subjects of both sexes with ages ranging from 4 to 62 years.

3.2.1.2   Reagents

The 4-methylumbelliferyl-β-D-glucoside substrate was purchased from Koch Light (Colnbrook, UK) and sodium taurocholate (grade A) was purchased from Calbiochem (San Diego, CA).

Monoclonal anti-placental glucocerebrosidase antibodies were a kind gift from Prof. J. Aerts.

[γ-^{32}P]ATP was purchased from Amersham, T4 polynucleotide kinase was purchased from New England Biolabs, and Zeta probe membrane was from Bio-Rad. The oligonucleotide primers used for DNA amplification where chosen on the basis of the published gene and pseudogene sequence (Horowitz et al, 1989), and were synthesized by Centro de Citologia Experimental, Porto. Taq polymerase and PCR buffer were from Clontech. XhoI and PvuII restriction enzymes and buffer used in the digestion reaction were from Pharmacia. Electrophoresis agarose gel NuSieve 3:1 from FMC BioProducts was used. All other reagents were of pure grade quality.

3.2.2   Methods

3.2.2.1   Portuguese population sampling for the N370S frequency determination

The whole Portuguese population comprised 9.6 in 1993 million persons; a Sephardic Jewish ancestry is known (Schmelz, 1970), but not an Ashkenazi one.

To accurately determine the N370S mutated frequency in the Portuguese population, DNA was obtained from dried blood spots randomly collected from the Portuguese Neonatal Screening Program. The information on each card is kept in a database, and from a total of about 75000 cards, corresponding to 79000 new-borns in the whole country for a period of 8 months, a simple random sample of 2000 cards was selected through a random number generator. The frequency of individuals selected from the different regions in Portugal was compared with the population distribution through a chi-squared test. This sampling was executed by Prof. Pedro Oliveira, from the Department of Production and Systems Engineering, of Minho University.

3.2.2.2   Portuguese Azores islands population sampling

The Azores islands comprise about 250 thousands inhabitants and are relatively free from admixture population.

To determine the frequency of the N370S mutation in the Azores islands population, DNA was obtained from dried blood spots randomly collected from the Portuguese Neonatal Screening Program. The information on each card is kept in a database, and from a total of 2148 cards, corresponding to about 2285 newborns in this region for a period of 8 months, a simple random sample of 279 cards was selected through a random number generator. The frequency of individuals selected from the different islands was compared with the population distribution through a chi-squared test.

3.2.2.3   Belmonte population sampling

Belmonte village comprises 2008 individuals, 95 of them claiming to be part of an ancient closed Sephardic Jewish community (Garcia, 1993).

To determine the frequency of the N370S mutation in Belmonte population, DNA was obtained from dried blood spots collected from the Portuguese Neonatal Screening Program. The information on each card is kept in a database, and a total of 219 newborns in a period of 3 years were genotype, corresponding to all the newborns in that period.

3.2.2.4   DNA analysis for the N370S mutation

The genomic DNA amplification was performed directly from autoclaved dried blood specimens on filter paper (reviewed in McCabe 1991). The detection of the N370S mutation was done using primers chosen on the basis of gene versus pseudogene sequence (Horowitz et al, 1989), the 5`-primer being mismatched at one nucleotide so as to create a XhoI restriction site (Beutler et al, 1990, with minor modification) (Figure 3-1).

A 4 mm^2 piece of dried blood spot on Guthrie cards was cut from each filter and added to a 100 µl reaction mixture containing 100 pmol of each oligonucleotide primer, 10 mmol/l Tris-HCl (pH 8.8 at 25°C), 50 mmol/l KCl, 1.5 mmol/l $MgCL_2$, 0.1% Triton X-100, 5% DMSO (dimethyl sulfoxide) and 0.2 mmol/l of each deoxynucleotide triphosphate (Saiki et al, 1988). The contents of the reaction tube were denatured at 96 °C for 15 minutes in a Bio-Med Thermocycler 60. Two units of Taq polymerase were then added to each tube, and 30 cycles of PCR amplification consisting of 94°C for 1 min, 55°C for 2 min and 72°C for 3 min were done. The products were cooled and next, 38.9µl aliquots of each reaction mixture were digested by 16.5 units of XhoI restriction

enzyme in a 50μl reaction mixture containing 20 mmol/l Tris-acetate (pH 7.5), 20 mmol/l magnesium acetate, and 100mmol/l potassium acetate. The digested fragments were then subjected to electroforeses in 3% NuSieve 3:1 agarose gels in 1 x TBE (Tris-Borate-EDTA, pH 8.0) for 3 hours at 60 V. The PCR products were then visualised by ethidium bromide staining and exposure to U.V. light.

Amplified fragment of 118 base pairs from the genomic nucleotide 5820 to 5938:

5820→Upstream primer←5840 5919→Dowstream primer←5938
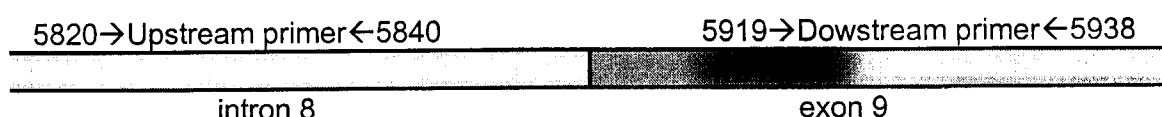
intron 8 exon 9

Oligonucleotide antisense primer (20):

5`GATGGGACTGTCGACAAAGT3` Downstream 5919-5938 (exon 9) fragment

Oligonucleotide sense primer (21 mer):

5`TGCCTTTGTCCTTACCCTCGA3` Upstream 5820-5840 (intron 8) fragment

↑
A to C mismatch PCR

Wild sequence 5`TGCCTTTGTCCTTACCCTAGA3`

Amplified sequence by mismatched PCR 5`TGCCTTTGTCCTTACCCTCGA3`

Restriction digestion:

XhoI cut
⇓
C TCGAG XhoI recognised sequence
↑
A to G substitution in mutated alleles

Figure 3-1

DNA analysis of the N370S mutation. Amplified fragment, oligonucleotyde primers and XhoI restriction digestion.

3.2.2.5 DNA analysis for the PvuII polymorphism

The genomic DNA was amplified in the region between nucleotides 3003 and 4086 with genomic sense primer 3003-3026 and antisense primer 4068-4086. The amplified sequence contains two PvuII restriction sites as well as the polymorphic site at position 3931 (Zimran et al, 1990) (Figure 3-2).

Amplified fragment of 1083 base pairs from the genomic nucleotide 3003 to 4086:

3003→Upstream primer←3026   4068→Dowstream primer←4086

intron 4   intron 6

Oligonucleotide antisense primer (19 mer):

5`TCCACTTTCTTGGCCGGAT3`   Downstream 4068-4086 (exon 4) fragment

Oligonucleotide sense primer (23 mer):

5`CTCGGACTACCATATCTTGATCA3`   Upstream 3003-3026 (intron 6) fragment

Restriction digestion:

PvuII cut
⇓
CAG CTG   PvuII recognised sequence
↑
G to A substitution in polymorphic 3931 nucleotide

Figure 3-2

DNA analysis of the PvuII polymorphysm. Amplified fragment, oligonucleotyde primers and PvuII restriction digestion.

Genomic DNA (1 μg) was added to 100 μl of reaction mixture containing 100 pmol of each oligonucleotide primer, 10 mmol/l Tris-HCl (pH 8.8 at 25°C), 50 mmol/l KCl, 1.5 mmol/l $MgCL_2$, and 0.2 mmol/l of each deoxynucleotide triphosphate (Saiki et al, 1988). The contents of the reaction tube were denatured at 94°C for 6 minutes in a Bio-Med Thermocycler 60. Three units of Taq polymerase were then added to each tube, and 30 cycles of PCR amplification consisting of 94°C for 45sec and 66°C for 6 min were done. The products were cooled and next, 2000ng of amplified DNA of each reaction mixture were digested by 5 units of PvuII restriction endonuclease enzyme in a 30μl reaction mixture containing 20 mmol/l Tris-acetate (pH 7.5), 20 mmol/l magnesium acetate, and 100mmol/l potassium acetate. The digested fragments were then subjected to electroforeses in 3% NuSieve 3:1 agarose gels in 1 x TAE (Tris-Acetate-EDTA, pH 8.0) for 3.5 hours at 2 V/cm. The PCR products were then visualized by ethidium bromide staining and exposure to U.V. light.

3.2.2.6   Statistical analysis

In order to estimate the overall prevalence of N370S allele in each of these restricted areas, it was weighted the observed prevalence in each area by the population of the area based on the census.

3.2.2.7   Concentration of urine samples

Freshly voided urine from healthy individuals and patients with type 1 Gaucher disease was collected in the morning, cooled to 0 °C and concentrated 20-fold by ammonium sulfate precipitation, as follows. To the urine, solid $(NH_4)_2SO_4$ was added to 70% saturation. After stirring for 0.5 hours, the suspension was centrifuged at 50,000xg during 0.5 hours at 4°C, and the precipitate was taken up in distilled water and dialyzed overnight against distilled water. Any precipitate formed was removed by centrifugation at 10,000xg for 0.5 hours (Aerts, 1988).

3.2.2.8   Measurement of glucocerebrosidase activity per amount of antigen

The relative enzymatic activity of glucocerebrosidase, defined as the enzymatic activity per unit of antigen, was determined according to the following: An identical amount of rabbit anti-(mouse IgG) prepared in PBS (phosphate buffered saline) was coated to each well (0.45μg/well) of a microtitre plate by incubation for 1 h at 37°C. Next, an identical amount of anti-glucocerebrosidase monoclonal antibody 8E4 (Barneveld et al, 1983) prepared in PBS was coated on each well (62.5 ng/well) by incubation for 16 hours at 4°C. Unbound antibody was removed by washing in PBS containing 0.05% (v/v) Tween-20. Concentrated urine containing excess amount of glucocerebrosidase protein was previously diluted in 50/100 mmol/l citric acid/sodium phosphate buffer (pH 5.2) containing 10g/l ovalbumin and 0.25% (v/v) Triton X-100, to a α-glucosidase activity of 300 nmol/h/ml. The enzyme binding assay was made by incubation overnight at 4°C and for 2 hours at room temperature the antibody with serial dilutions of the excess amount of glucocerebrosidase added to the first wells. After washing the unbound material, the maximal bound glucocerebrosidase activity for each extract was measured by incubating the wells for 2 hours at 37°C with 4-MU-β-glucoside (3.7 mmol/l), at pH 4.5 and 5.2 (100/200 mM citric acid/sodium phosphate buffer), in the presence of 0.2% (w/v) sodium taurocholate and 0.1% Triton X-100 (Aerts et al, 1990a). The reaction was stopped by addition of excess glycine/NaOH (pH 10.0) and the 4-methylumbelliferone formed was measured fluorimetrically. The released fluorescence was compared with that of standards, being measured with a excitation and emission wavelength of respectively 365 and 445nm (fluorescence read on a Titertek plate spectrofluorometer). Standards used for the calibration curve were

prepared from a solution of 10mmol/l 4-MU in cloroform/methanol (2:1). For each sample the bound activity was determined as the mean of triplicate assays. Results of each serious of measurements were algebrically calculated by using the linear regression parameters corresponding to the calibration curve.

Since the affinity of N370S mutant and control glucocerebrosidase for binding to 8E4 is similar (as checked by mixing experiments) (Aerts et al, 1990a) the activity that is bound to identical amounts of antibody is directly proportional to the enzymatic activity per identical amount of antigen.

3.3 Results

3.3.1 Frequency of the N370S mutated glucocerebrosidase gene in the general Portuguese population

In order to accurately determine the N370S frequency, for the first time in a non-Ashkenazi population, the DNA of 2000 individuals randomly sampled from the Portuguese newborn PKU screening program were genotype by direct PCR amplification of DNA obtained from dried blood spots on Guthrie cards. The digestion of the 118 bp PCR amplified fragment encompassing an A to G substitution in exon 9, yields 101 bp and 17 bp fragments (Table 3-1).

Table 3-1

DNA analysis of the N370S mutation: Expected fragments upon XhoI digestion.

	Undigested	Digested		
		Wild type	**Homozygous mutant**	**Heterozygous mutant**
Expected	118bp	118bp		118bp
fragments			101bp	101bp
			17bp	17bp

In Figure 3-3 the detection of this mutation is exemplified. Non-carriers present the non-digested 118 bp band and carriers display the 118 bp band corresponding to the normal allele and the products of the cleaved band (101 and 17 bp fragments). Each set of 40 PCR amplifications and digestion reactions included a double heterozygote patient; PCR amplification was done using master mix reaction mixture.
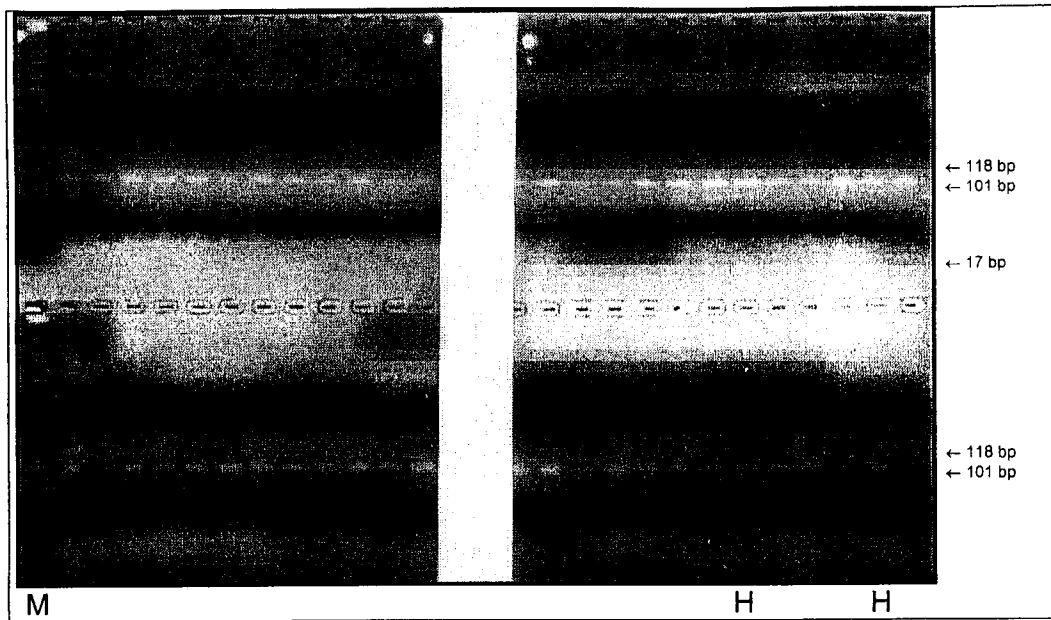
Figure 3-3

Ethidium bromide-staining patterns of DNA fragments obtained by PCR amplification genomic DNA blood spots. Master mixture without DNA and the double heterozygote patient DNA amplifications are also included in each experiment. Fragment sizes are shown on right. DNA samples from normal individuals show only the uncut 118bp fragment. Heterozygous for the N370S mutated allele shows both the 118bp and 101 bp fragments. M, molecular weight markers; H, heterozygote for the N370S mutated allele.

Seventeen heterozygous and no homozygous for the N370S mutation were identified (Table 3-2). The frequency of this allele was found to be 0.0043 with 95% confidence limits between 0.0023 and 0.0063. On the basis of the obtained gene frequency the number of homozygous predicted by the Hardy-Weinberg (Emery, 1976) equation should be 1 in 55000, i.e. about two hundred homozygous when considering a total Portuguese population of 10^7 individuals.

Table 3-2

Frequency of N370S mutated allele in the Portuguese population

	Observed	Expected*
Total individuals	2000	2000
Heterozygous	17	16.9
Homozygous	0	0.036
Gene frequency	0.0043	

* Calculated by the Hardy-Weinberg equation for 0.0043 gene frequency.

55

3.3.2 Frequency of the N370S mutated allele in relatively closed populations: Azores islands and Belmonte

To test whether major differences of the N370S allele frequency in Portuguese sub-populations exist, the incidence of this mutated allele in individuals from two selected areas was examined: the Azores islands and the Belmonte village (which have an ancient Sephardic Jewish community).

Among 379 randomly sampled Azores islands new-borns, 4 heterozygous and no homozygous for the N370S mutated allele were identified (Table 3-3). The frequency of this mutated allele was found to be 0.0053, with 95% confidence limits between 0.0001 and 0.0104. Due to the overlapping of confidence intervals this frequency is not statistically significantly different from the frequency of the general population.

Table 3-3

Frequency of the N370S mutated allele in the Azores islands.

	Studied individuals	Mutated alleles/ total alleles	Frequency
Azores islands	379	4/758	0.0053
Total Portuguese	2000	17/4000	0.0043

All newborns from Belmonte were genotyped for this mutated allele during a period of 3 years and 4 non-related heterozygous were found. As it can be observed in the Table 3-4, in the second studied year it was observed a relative high number of N370S mutated alleles. If assumed that in this population the N370S frequency is the same as that in the general Portuguese population, this number is significantly different, since the probability of observing a value equal or higher is 4.9%. However, this value must be taken with caution due to the variation over the other studied years.

Table 3-4

Frequency of the N370S mutated allele in the newborns of Belmonte .

	Total newborns	Mutated alleles/total alleles	Frequency
1st year	83	0/166	0
2nd year	93	3/186	0.0161
3rd year	43	1/86	0.0116

3.3.3 Linkage analysis between the N370S mutated allele and the intragenic PvuII polymorphism

A restriction fragment length polymorphism for the enzyme PvuII, due to a G→A substitution at genomic DNA nucleotide 3931 in intron 6 of the glucocerebrosidase gene, is responsible for the presence (Pv1.1$^+$) or absence (Pv1.1$^-$) of a 1.1 kb genomic glucocerebrosidase DNA fragment upon PvuII digestion (Sorge et al, 1985a; Zimran et al, 1990).

In order to determine the PvuII haplotype of the Portuguese GD mutated alleles, genomic DNA was genotyped by PCR amplification and restriction enzyme analysis. The digestion of the 1083 bp PCR amplified fragment yields a 983, a 72 and a 28 bp fragments (designated in literature as Pv1.1$^-$ haplotype); samples encompassing an G→A substitution in intron 6 (the PvuII polymorphic site) yields the 857 bp and 126 bp digestion fragments instead of the 983 bp band (here designated as Pv1.1$^+$ haplotype) (Table 3-5).

Table 3-5

DNA analysis of the PvuII polymorphism: Expected fragments upon PvuII digestion

	Undigested	Digested		
		Pv1.1$^-$/ Pv1.1$^-$	Pv1.1$^-$/ Pv1.1$^+$	Pv1.1$^+$/ Pv1.1$^+$
Expected fragments	1083bp	983	983	
			857	857
			126	126
		72	72	72
		28	28	28

In Figure 3-4 it is exemplified the detection of this mutation. Pv1.1$^-$ samples present the non-digested 983 bp band; in Pv1.1$^+$ samples it can be observed the products of this cleaved band (857 and 126 bp fragments).
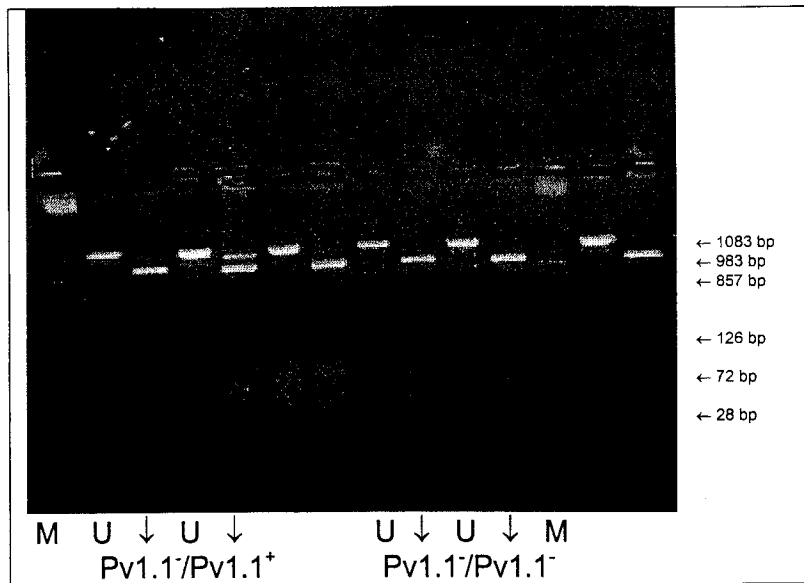
Figure 3-4

Ethidium bromide-staining patterns of DNA fragments obtained by PCR amplification genomic DNA samples. Master mixture without DNA and the double Pv1.1-/Pv1.1+ genotype amplification are also included in each experiment. Fragment sizes are shown on right. DNA from Pv1.1- haplotypes show only the uncut 983 bp fragment. Pv1.1-/Pv1.1+ haplotypes show both the 983 bp and the 857 bp fragments. M, molecular weight markers; U, undigested 1083 bp fragment.

Table 3-6 presents the PvuII genotype of 15 type 1 Gaucher patients and 38 carriers. The frequency of the Pv1.1⁻ and Pv1.1⁺ alleles in individuals carrying the N370S or the L444P mutation in one allele is compared with the frequency determined in the Portuguese control. As can be observed in the table, the four homozygous for the N370S mutation presented a Pv1.1⁻/Pv1.1⁻ genotype; all the heterozygous patients (n=10) for this same mutation presented at least one Pv1.1⁻ allele. The R463C mutation present in a compound heterozygote is therefore presumably associated with the Pv1.1⁺ haplotype. The L444P mutation found to be present in six of the patients studied appeared either in Pv1.1⁺ (n=1) or Pv1.1⁻ (n=5) alleles. Mutated alleles with as yet unidentified glucocerebrosidase mutations were either Pv1.1⁺ or Pv1.1⁻. The results obtained with the PvuII polymorphism study in the Portuguese controls (64 Caucasian individuals, not carrying the N370S mutation) showed 0.836 and 0.164 frequencies for the Pv1.1⁻ or Pv1.1⁺ alleles, respectively, in contrast to the corresponding 0.647 and 0.353 allele frequencies previously described as an average of different racial groups (Sorge et al, 1985a). Frequencies of 0.92 and 0.080 for the Pv1.1⁻ or Pv1.1⁺ alleles, respectively, were obtained in the 37 individuals carrying the N370S mutation in one allele (10 double heterozygous and 7 carriers) (Table 3-6).

Table 3-6

Pvull haplotype of genotype of type 1 Gaucher patients, carriers and controls.

		Pvull genotype			Gene frequency	
		-/-	+/-	+/+	Pv1.1⁻	Pv1.1⁺
Patients per genotype						
4	N370S/N370S	4	0	0	1.00	0.00
5	N370S/L444P	5	0	0	1.00	0.00
4	N370S/?	2	2	0	0.75	0.25
1	L444P/?	0	0	1	0.00	1.00
1	N370S/R463C	0	1	0	0.50	0.50
Carriers per genotype						
27	N370S	24	3	0	0.94	0.06
4	L444P	2	2	0	0.75	0.25
7	?	3	3	1	0.64	0.36
N370S heterozygous per genotype						
37	N370S/X	31	6	0	0.92	0.08
L444P heterozygous per genotype						
5	L444P/Y	2	2	1	0.60	0.40
Unrelated individuals						
64	Controls	43	21	0	0.836	0.164
	Expected [b]	44.7	17.6	1.7		

"?" indicates that none of the tested mutations were present.

"X" indicates normal or mutated alleles with the exception of L444P.

"Y" indicates normal or mutated alleles with exception of N370S.

[b] The expected number of cases based on Hardy-Weinberg equilibrium.

3.3.4 Identification of Gaucher disease carriers in affected families and control population of the same geographic area: glucocerebrosidase antigen and DNA analysis

The activity of hydrolases in urine samples, like those in blood cells and tissues, differ between individual control subjects, but the pattern of hydrolase activities was very constant in morning urine (Aerts et al, 1986b). Urinary glucocerebrosidase was found to originate almost entirely from turnover of proximal tube epithelial cells rather than being directly secreted in a precursor form into urine (Aerts et al, 1986c).

Serial dilutions of a concentrated urine sample were added to wells containing identical amounts of immobilised anti-glucocerebrosidase monoclonal antibody 8E4. After

washing, the activity of the bound glucocerebrosidase was measured. In Figure 3-5 the bound glucocerebrosidase activity is related to the input of urine sample expressed as equivalents of acid α-glucosidase activities in the incubations. Curve 1 represents a urine sample of a control subject and curve 2 that of a carrier for N370S glucocerebrosidase. It can be seen that at similar input of α-glucosidase activity a maximum is reached for glucocerebrosidase binding in both samples. This indicates that a similar amount of glucocerebrosidase antigen is present in both preparations. In the case of the carrier sample the maximal bound activity is about half of that obtained for the control subject. This difference is due to the fact that about half the glucocerebrosidase antigen of the carrier sample represents enzyme with very low specific activity. Curve 3 represents a sample of a carrier for the L444P mutation which leads to a rapidly degraded enzyme. It can be seen that a higher input of urine (α-glucosidase equivalents) is required to reach maximal binding, indicating that indeed less glucocerebrosidase antigen is present in the sample of the L444P glucocerebrosidase carrier. The maximal bound activity can be, however, near normal due to the fact that the sample contains only glucocerebrosidase molecules with normal specific activity.
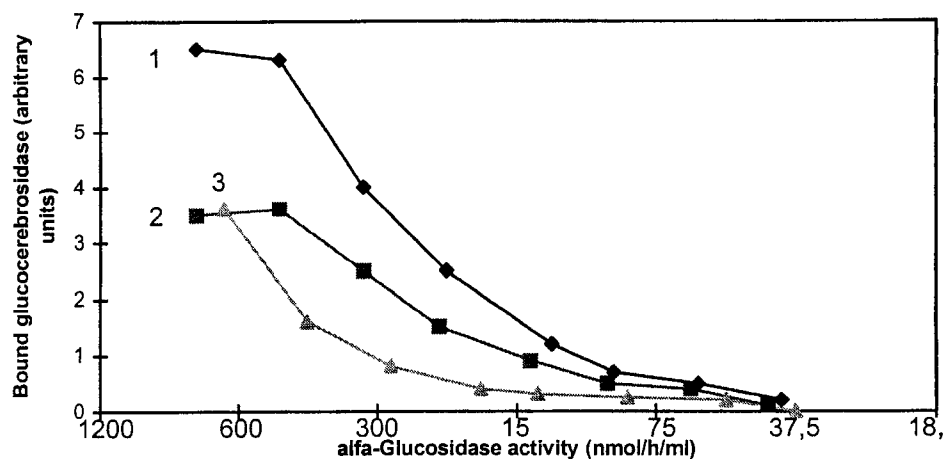
Figure 3-5

Glucocerebrosidase activity per amount of antigen of controls and carriers for the N370S and L444P mutations. Urines were concentrated equally by volume and the different lines represent serial dilutions of a control (curve 1), a N370S carrier (curve 2) and a L444P carrier (curve 3). α-Glucosidase activity corresponding to each dilution is marked along the X axis.

By investigating enzyme binding curves from a large number of obligate carriers, we established that when an input of urine equivalent to 300 nmol/h of α-glucosidase activity is used, the discrimination between control subjects and carriers for the two

mutations is optimal. However it must be noticed that the ranges of fixed glucocerebrosidase activity values for both carriers and controls, may depend on the described (Aerts et al, 1991) individual heterogeneity in the amount of α-glucosidase secreted into urine. This was overcome by using serial dilutions of each sample.

Urine samples from 15 type 1 Gaucher disease patients, 41 relatives (22 obligate carriers and 19 putative carriers), and 83 controls were tested with the enzyme binding assay (EBA) procedure. In Figure 3-6 the frequency distribution of the results is depicted. It can be seen that the data distribution is non-Gaussian. This phenomenon has been observed earlier (Aerts et al, 1991) and is most likely due to the heterogeneous α-glucosidase secretion. All patients are clearly distinguishable from the healthy controls. An overlapping zone can be observed between control and type 1 Gaucher families (obligate and putative carriers) EBA values.
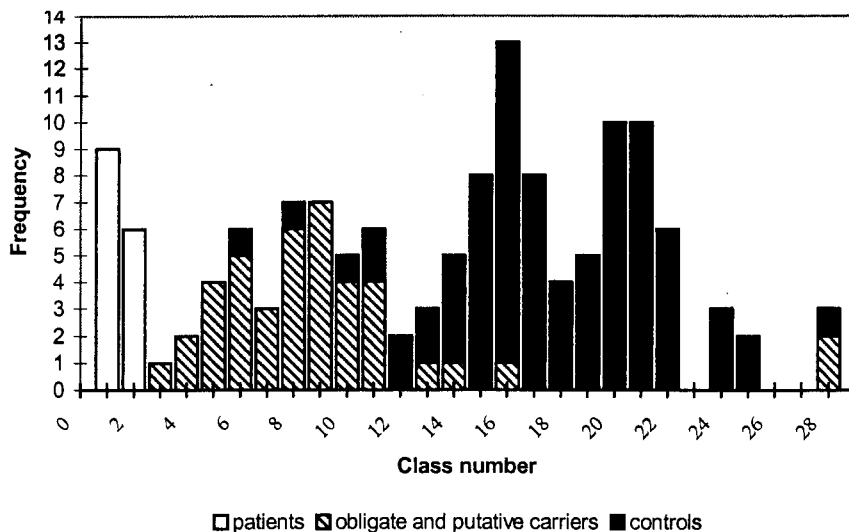
Figure 3-6

Histogram of glucocerebrosidase activity per amount of antigen of urine from controls, GD patients, obligate and putative carries. Every class has a range of 50 units of glucocerebrosidase activity, which is within 2x the mean range of the triplicate readings.

Obligate and putative carriers as well as controls presenting low EBA values were screening for the N370S and L444P glucocerebrosidase mutations. The DNA analysis results are presented in Figure 3-7 and Figure 3-8. With the exception of one obligate carrier for an unidentified mutation (class 16) all the others ranged between classes 4 and 13 (Figure 3-7).

Four of the nine healthy controls presenting EBA values within and below class 13 proved to be carriers for the N370S mutation (marked with green in Figure 3-8). The

remainder did not present either of the two mutations. On the basis of these results the overlapping range was considered to comprise EBA frequency classes 11-13.

Figure 3-8 also shows the DNA results of 19 putative carriers. From the 14 cases presenting EBA values in the obligate carrier range (below class 11), 9 carried the N370S glucocerebrosidase mutation. The remainder (all from families where an unknown allele was present) had neither of the tested mutations.

Obligate carriers

◪ N370S carrier ☐ L444P carrier ■ neither mutation present
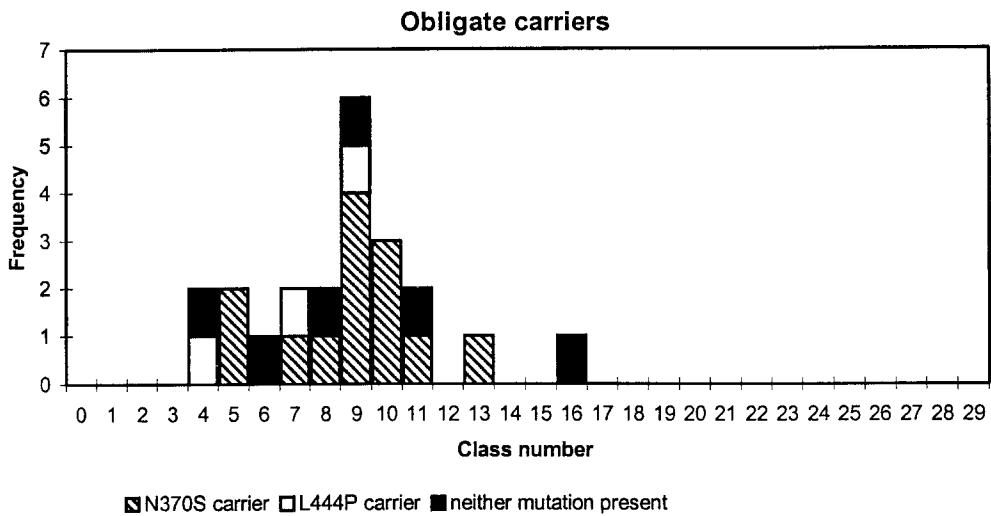
Figure 3-7

Comparison of genotype results obtained by DNA analysis of the N370S and L444P mutations and the results obtained with the enzyme binding assay of obligate carriers.

Putative carriers and controls

◪ N370S carrier ☐ N370S carrier "control" subject ■ neither mutation present
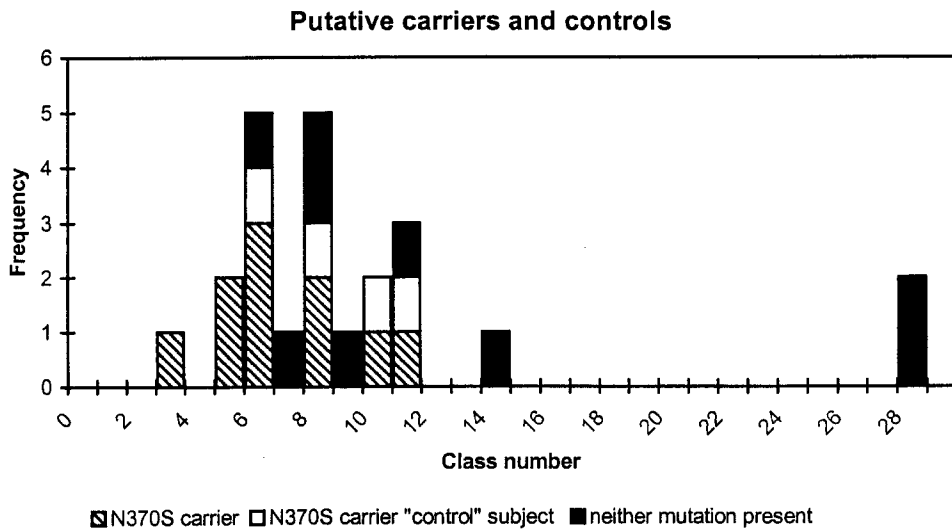
Figure 3-8

Comparison of genotype results obtained by DNA analysis of the N370S and L444P mutations and the results obtained with the enzyme binding assay of GD putative carriers and control subjects.

Healthy individuals sampled in the general population, but in which the N370S mutation was found to be present, are also indicated.

The results obtained with carrier identification using EBA procedure and DNA analysis showed that when considering an EBA cut-off value corresponding to class 13 (Figure 3-7 and Figure 3-8), all carriers for N370S and the L444P mutations were detected. Five controls can be considered to be misclassify as carriers on the basis of the two glucocerebrosidase gene mutations analysed. No definitive statement can be made when unknown glucocerebrosidase gene mutations are also considered. Nevertheless, one can say that by using class 13 as cut-off value, five obligate carriers for unknown mutation(s) were detected and only one was lost. Therefore, it can be expected that a significant proportion of the 9 putative carriers for these mutations are also "true" carriers. Considering the probability density functions corresponding to confirmed carriers (carriers of either N370S or L444P and obligate carriers for unidentified mutations) and controls, a probability below 1% of misclassifying a control as a carrier can be calculated for results below class 11, and so these individuals should be considered as carriers; a probability below 2% of misclassifying a carrier as a control can be calculated for results above class 13. Eleven individuals presented results between classes 11 and 13 - one obligate and one putative carriers for unidentified mutation(s), four N370S carriers, and five controls not carrying either N370S or L444P mutations. In this class range the probability of misclassifying a control as a carrier varies from 1% (class 11) to 8% (class 13) and the probability of misclassifying a carrier as a control varies from 18% (class 11) to 2% (class 13).

3.4 Conclusions

Glucocerebrosidase activity per amount of cross-reacting activity recognised by a monoclonal antibody, was determined in urine samples from type 1 Gaucher disease patients, obligate carriers, putative carriers and controls. The reliability of this enzyme binding assay (EBA) for the detection of carriers, in a population where 15% of mutated alleles remain unidentified, was tested by DNA analysis for two most frequent mutated alleles in Portuguese patients (N370S and L444P). All N370S and L444P carriers were distinguished from healthy controls but an overlapping zone was observed between values of controls, obligate carriers and putative carriers for unknown mutations. Of the six obligate carriers for an unknown mutant allele, only one was not identified. So, this EBA assay may be used in the identification of carriers for most unidentified mutant alleles, since it should detect carriers for any mutation affecting the glucocerebrosidase enzymatic activity or the amount of protein (cross reacting material). Individuals

presenting a result in the overlapping range comprising classes 11-13 must however be extensively studied at the DNA level (tested for other mutations, SSCP analysis, sequencing) to exclude the presence of a mutated glucocerebrosidase allele.

The frequency of the causual type 1 GD most common mutated allele (N370S) was determined in the Portuguese population. On the basis of the obtained gene frequency of 0.0043, the number of homozygotes calculated by the Hardy-Weinberg equation should be about 1 in 55 000 individuals, although only 15 N370S homozygous patients have been identified till now. The obtained value for the N370S frequency in the general Portuguese population was also found to be about 7-fold lower than that described in the Ashkenazim, but the survey of the general Portuguese population could not detect "hot spots" with a high frequency of carriers. The EBA screening of control individuals from areas with known incidence of GD revealed however a remarkably high local frequency of the N370S mutated allele (0.024, value which is close to the 0.032 described in the Ashkenazi Jewish population. However the number of tested individuals (n=83) was small. By this reason, the hypothesis was tested that, like in Ashkenazi Jews, in the Portuguese population within restricted areas, a high frequency of the N370S mutation could have been maintained due to a certain degree of inbreeding. The results obtained in DNA analysis of the restricted area of Azores islands population, showed however that although the obtained frequency value is slightly higher than is the one observed for the general Portuguese population, there were no significant differences (respectively 0.0053 and 0.0043). Regarding the Belmonte population study (which included the closed small community of individuals claiming a Sephardic Jewish ancestry) the determined N370S allele frequency was found to be higher than that in the general population, but still not statistically significant (0.0091). No conclusion can however be drawn with respect to the Jewish ancestry to the newborns presenting a mutated allele, since for ethical reasons this study employed only coded samples.

The incidence of the N370S mutation is also interesting in the light of the origins of the Portuguese population, where a Sephardic rather than an Ashkenazi background has been ascribed. In this study, the N370S glucocerebrosidase mutated allele in the Portuguese type 1 Gaucher patients was found to be invariably associated with the Pv1.1⁻ form of the intragenic PvuII polymorphism, as it was reported for other patients. A common Jewish origin for the N370S glucocerebrosidase allele in Ashkenazi Jews and in Portuguese can, theoretically, be advanced although the possibility that this mutation occurred by an independent event cannot be excluded since the Pv1.1⁻ haplotype was found to be the most common in the Portuguese population.

Chapter 4
Activity of glucocerebrosidase extract from cells of type 1 Gaucher disease patients with different genotypes

4.1 Introduction

4.1.1 The glucocerebrosidase protein

Heterologous expression in eukaryotic cells of human cDNAs encoding normal and mutant glucocerebrosidases was performed to characterize these enzymes with respect to catalytic properties, stability, inhibitor binding and modifier interactions (Ohashi et al, 1991; Grace et al, 1994). Kinetic studies have indicated effector binding sites on the enzyme for negatively charged lipids and protein activators. These effectors induce conformational changes and enhance catalytic activity. Based on the effects of the mentioned modifiers of glucocerebrosidase activity it was proposed that the active site of glucocerebrosidase consists of three subsites: the hydrophilic glycon-binding site; the aglycon-binding site for interaction with the fatty acid-acyl chain of glucosylceramide; and a site for interaction with the sphingosyl moiety of glucosylceramide (Osiecki-Newman et al, 1987) (Figure 4-1). Glu^{340} was identified as the catalytic nucleophile in the active site of glucocerebrosidase (Miao et al, 1994). The critical importance of the catalytic nucleophile and the surrounding structure for enzyme activity is also reflected in the fact that Glu^{340} is located in the sequence within the longest stretch of 100% amino acid identity (315-375) between the human and murine enzymes (O'Neil et al, 1989).
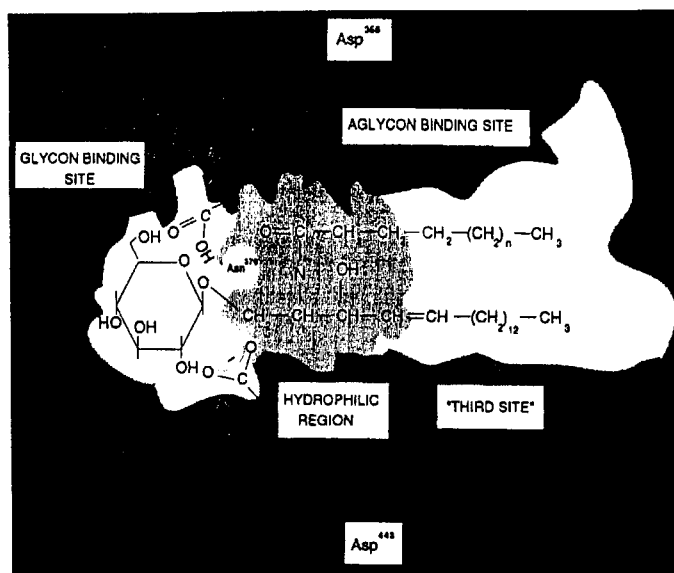
Figure 4-1

Proposed schematic for the active site of acid β-glucosidase containing the major natural substrate, glucosylceramide. The designated amino acids may function as the proton donor (Asp[358], putative) and the Br-conduritol B epoxide binding site (Asp[443]). Asn[370] is important for determining the specificity of interaction at the active site and N370S is the most frequent substitution in Gaucher disease genes. The sphingosyl moiety is usually an octadecene but chain lengths vary from 16 to 22. The fatty acid acyl moiety chain length varies from 16 to 24 carbons and is dependent on the tissue source. Cleavage of the bond at the anomeric carbon releases glucose with retained β configuration. (Adopted from Beutler and Grabowski, 1997).

Mutations in genes encoding lysosomal hydrolases like glucocerebrosidase gene may result in the reduction of the *in vivo* enzyme activity in a number of ways. Missense mutations in the structural gene may affect substrate binding (K_m variants) or the turnover rate of the substrate (V_{max} mutations) or both. Base pair insertions or deletions in the DNA may lead to frame shift mutations that potentially result in the formation of an incomplete protein devoid of any catalytic activity. Other mutations may result in enhanced degradation of newly synthesized protein in the rough endoplasmic reticulum or in lysosomes. Some other mutations may alter the solubility properties of a polypeptide (Proia and Neufeld, 1982) or may result in impaired post-translational modification leading to incorrect compartmentalization or secretion, enhanced degradation, or impaired function in the lysosomes (Ginns et al, 1982). Disturbances of posttranslational modification may, of course, also result from an abnormality of one of the enzymes required for this process (Conzelmann and Sandhoff, 1983/84). Mutations may also result in an altered transcription rate, splicing or stability of the mRNA.

4.1.2 Properties of the mutated glucocerebrosidases presented by the Portuguese Gaucher disease patients

4.1.2.1 The N370S mutated glucocerebrosidade

The properties of the N370S mutated glucocerebrosidase have been investigated by site-directed mutagenesis of human cDNA followed by transfection in insect or murine cells (Grace et al, 1990; Ohashi et al, 1991; Grace et al, 1994). It was shown that this mutant enzyme is synthesised in near normal amounts, is efficiently transported to lysosomes, shows a normal intralysosomal stability, and is as stable to heating as the normal enzyme. However, the kinetic properties are altered, presenting low activity toward an artificial or natural substrate (Grace et al, 1990) and abnormal interaction with active site-directed inhibitors (which localizes this residue to the glycon binding region of the active site) (Grace et al, 1994). The concentration of inhibitor which results in a 50% decrease of initial enzyme activity is increased 2.5-7 fold. In studies where the expression of this mutant cDNA was performed in human HeLa cells using a vaccinia virus derived expression system, the K_m of the enzyme towards the artificial substrate 4-MU-β-D-glucopyranoside was shown to be close to that of the normal enzyme (0.35 versus 0.67mmol/l, respectively). However, V_{max} of the mutant enzyme was 0.029 compared to 0.4 mmol/h/mg of protein of the normal enzyme (Horowitz and Zimran, 1994a) and the glucocerebrosidase activity against the artificial substrate was 13-25% of the normal activity (Pasmanik-Chor et al, 1995). Furthermore, when compared to the normal enzyme this mutant enzyme was shown to be poorly stimulated by the physiological activators saposin C while being stimulated to a greater extent by phosphatidylserine (Ohashi et al, 1991). However other authors reported that the catalytic efficiency was highly dependent on the association with a combination of saposin C and phosphatidylserine. In these conditions and provided this mutated glucocerebrosidase is able to operate at a sufficiently acid pH, it can be reactivated *in vitro* to near-normal activity (Aerts et al, 1990b; van Weely et al, 1993b).

4.1.2.2 The L444P mutated glucocerebrosidade

Analysis of the human glucocerebrosidase with the L444P mutation expressed in murine fibroblasts has revealed that the mutant enzyme is synthesised in normal amounts, but is unstable (Ohashi et al, 1991) being the most part degraded prelysosomally (Willemsen et al, 1987). Overexpression of the mutant gene in Sf9 insect cells resulted in the synthesis of enzyme in amounts comparable to normal; the observation of several additional smaller bands was interpreted as degradation products and the enzyme was described as unstable. In addition the authors reported

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very low catalytic activity and poor stimulation by saposin C and phosphatidylserine, which was considered to indicate that the enzyme sites for interaction with these activators were within the carboxyl terminal of the protein (Grace et al, 1994). In contrast, expression of this mutated enzyme cDNA in human HeLa cells using a vaccinia virus derived expression system, resulted on a stable mutant protein presenting an enzyme activity against the artificial substrate with 13-25% of normal activity (Pasmanik-Chor et al, 1995). Its Km toward the artificial substrate was found to be close to that of normal enzyme (0.35 and 0.67 mmol/l, respectively), the Vmax being however much lower for the mutant enzyme (0.03 versus 0.4 mmol/h/mg of protein for the normal).

4.1.2.3 The IVS2+1 mutated glucocerebrosidade

The IVS2+1 mutation is a splice site mutation. Due to a G to A transition of the first nucleotide of the second intron of the active glucocerebrosidase gene, the 5'donor splice site is destroyed. This mutant allele directs synthesis of low levels of aberrant mRNAs. One of these mRNAs lacks exon 2 while the other lacks exon 2 and 115 bases of the third exon. These mRNAs do not direct synthesis of active enzyme (He and Grabowski, 1992).

4.1.2.4 The R463C mutated glucocerebrosidade

The R463C mutant protein is less thermostable but presents an active site with similar behavior to that observed for the normal glucocerebrosidase (as indicated by Ki for glucosphingosine and the concentration of the irreversible inhibitor conduritol B epoxide (CBE) required to achieve 50% inhibition of enzyme activity). Being the specific activity of this mutant protein about 40% of normal, and since the activity is poorly stimulated by saposin C and phosphatidylserine, this was considered to be the first example of a refractory response to saposin stimulation as a cause for GD (Ohashi et al, 1991).

4.1.2.5 The RecNcil and RecTL mutated glucocerebrosidades

The expression of the complex alleles recNcil (L444P, A456P and V460V) and recTL (D409H, L444P, A456P and V460V) mutated cDNAs in human HeLa cells using the vaccinia virus derived expression system, produced enzymes with very low enzymatic activity against an artificial substrate (4.3% of normal activity) (Pasmanik-Chor et al, 1995). Since the D409H, L444P and A456P mutations are very unfavorable aminoacid substitutions, this may lead to the observed decrease in proteolytic stability which

results in transient presence or rapid degradation of the expressed mutant enzymes (Grace et al, 1994).

The properties of the above mentioned mutated enzymes are summarised in Table 4-1.

Table 4-1

Properties of mutated proteins obtained by site-directed mutagenesis of human cDNA with naturally occurring mutations. Transfection in murine fibroblasts (a), Sf9 insect cells (b) or human HeLa cells (c).

	Amount of protein	Stability	kinetic properties	Interaction with active site-directed inhibitors	Stimulation by physiological activators		Residue location
					Saposin C	Phosphatidylserine	
N370S	(b) normal	(a) stable	(c) low activity (13-25%) normal KM abnormal Vmax	(b) abnormal	(a) Poor	(a) higher	glycon binding region of active site
L444P	(a) normal	(a,b) unstable (c) stable	(c) low activity (13-25%) normal KM abnormal Vmax		(b) Poor	(b) Poor	interaction with activators
IVS2+1	no protein						
R463C	(a) normal	(a) thermolabile	(a) reduced activity (40%)	(a) normal	(a) Poor	(a) Poor	interaction with saposin C
RecTL	(c) normal	(c) unstable	(c) very low activity (4.3%) abnormal Vmax				
RecNcil	(c) normal	(c) unstable	(c) very low activity (4.3%) abnormal Vmax				

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4.1.3 Enzymatic activity: physiological, nonphysiological activators and inhibitors

Glucocerebrosidase is optimally active at pH values below 6.0; however the exact pH optimum strongly depends on the nature of activators and detergents present (Aerts et al, 1990b; Sá Miranda, 1992). Glucocerebrosidase is reversibly inactivated by extraction with neutral detergents (e.g. Triton X-100); negatively charged detergents such as bile salts (e.g. taurocholate, a potent stimulator of many enzymes that hydrolise glycosphingolipids) (Wenger et al, 1978) or anionic lipids (e.g. phosphatidylserine) are required for its reactivation *in vitro* (Aerts et al, 1990b; Sá Miranda, 1992).

The small heat-stable glycoprotein saposin C (Radin and Berent, 1982) is abundant in GD patients spleen and immunoelectronmicroscopic studies on the subcellular localization demonstrated association of the this activator protein with the lysosomal membrane (Paton et al, 1990). This protein has been shown to activate purified glucocerebrosidase *in vitro* provided that acidic phospholipids such as phosphatidylserine are also present in the assay; evidence exists that this protein does not facilitate enzymatic activity of glucocerebrosidase by dispersion of substrates or products, but functions via direct interaction with the hydrolase (Radin and Berent, 1982; Aerts et al, 1990; Sá Miranda et al, 1990; Sá Miranda, 1992). Acidic lipids are also capable of inducing aggregation and activation of glucocerebrosidase, although to a lesser extent than saposin C. Using phosphatidylserine-containing vesicles it has been found that saposin C is also capable of destabilizing and fusing phospholipid-containing bilayers, thereby creating a situation by which glucocerebrosidase can insert into the membranes and subsequently be enzymatically active towards its substrate (Vaccaro et al, 1994)

Conduritol B-epoxide (CBE) is an effective and irreversible inhibitor of lysosomal glucocerebrosidase that bind via an ester bond with aspartate residues in the active site and can be used to inactivate the enzyme both *in vitro* and in the intact cell (Grabowski et al, 1985) (Figure 4-2 and Figure 4-1). This inhibitor is analogous in structure to the transition state of gluconolactone formed during hydrolysis of glucocerebroside and binds with 1:1 stoichiometry (Dinur et al, 1986). The use of this inhibitor allows the discrimination between the degradation of substrate by lysosomal and nonlysosomal glucocerebrosidase.
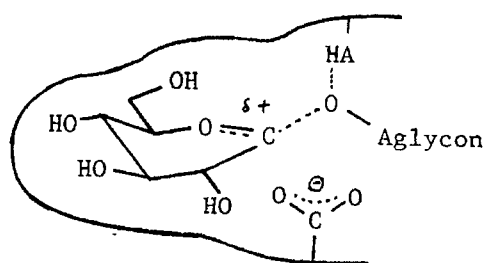
Figure 4-2

Model for the transition state of the first step of β-glucoside hydrolysis by β-glucosidases. (Adopted from Legler, 1988).
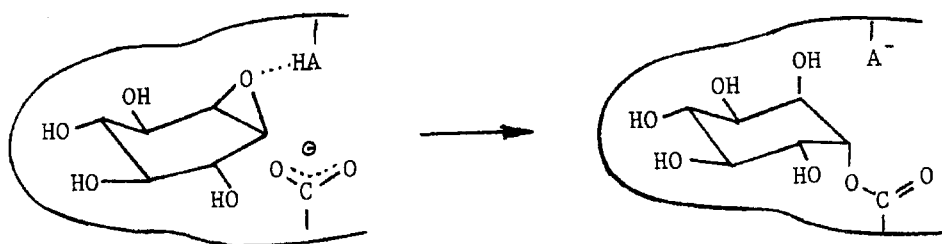
Figure 4-3

Reaction of conduritol B epoxide with β–glucocidase. (Adopted from Legler, 1988).

4.1.4   Aims and approaches of the study

No correlation has been found between the clinical manifestation of type 1 Gaucher disease and the residual activity of glucocerebrosidase in extracts from various cell types and tissues, when measured in the presence of the activator sodium taurocholate (method currently used in diagnosis) (Peters et al, 1976; Daniels et al, 1981).

Complexes of glucocerebrosidase and SAP-2 have previously been detected in extracts from cells and tissues (Aerts et al, 1985; Aerts et al, 1987; Sá Miranda et al, 1988). In extracts from spleen from control subjects the major part of glucocerebrosidase activity is due to the monomeric state of the enzyme (called form I) and a minor part to the enzyme present in aggregated form associated with SAP-2 (form II) (Aerts et al, 1987; Sá Miranda et al, 1988). Monomeric (form I) enzyme was found to be poorly active and strongly stimulated by the activator taurocholate, whilst form II glucocerebrosidase was present in an activated state and was hardly stimulated further by taurocholate. Forms I and II differed also in mobility on cellulose acetate gel electrophoresis (Sá Miranda et al, 1988) and reaction with anti-(glucocerebrosidase) antibodies (Aerts et al, 1985; Aerts et al, 1986d). Sá Miranda and coworkers (1988) were able to show a complete conversion of this enzyme into an aggregate which presented identical electrophoretic mobility to that of form II (band 2) β-glucosidase of tissue extracts. Finally, it was observed that the proportion of form II

glucocerebrosidase was tissue-specific and that it was clearly less reduced in spleens from type 1 Gaucher disease patients (Aerts et al, 1985; Aerts et al, 1987; Sá Miranda et al 1990).

Since form II glucocerebrosidase activity may contribute significantly to the total glucocerebrosidase activity *in vivo*, further information about the occurrence of glucocerebrosidase-activator protein complexes seems essential in order to be able to assess the actual residual glucocerebrosidase activity in genotyped GD patients. For this purpose, in the present study the following experiments were performed:

- The amount of glucocerebrosidase protein as well as the presence of the different molecular weight species in cultured skin fibroblasts extracts from patients presenting different genotypes was analyzed by Western blotting.

- Glucocerebrosidase residual activity was measured in peripheral blood total leukocytes and fibroblasts from genotyped GD patients.

- Glucocerebrosidase residual activity of peripheral blood total leukocytes and fibroblasts from genotyped GD patients.was determined under conditions favoring the existence of the form II.

- Extracts of leukocytes and fibroblasts from individual GD patients presenting different genotypes were studied with respect to the aggregation state of the glucocerebrosidase form II.

- The modulation of the enzymatic activity per unit of cross-reactive glucocerebrosidase (form I) by of taurocholate, SAP and phosphatidylserine activators, was studied.

4.2   Materials and methods

4.2.1   Materials

4.2.1.1   Samples

Blood and skin biopsies were obtained from Portuguese patients and control subjects. Spleens from Portuguese type 1 GD patients were collected after therapeutic splenectomy. All tissues were kept frozen at -70°C. Tissues requiring transportation were shipped in solid carbon dioxide.

4.2.1.2 Reagents

Polyclonal antiserum against homogeneous glucocerebrosidase that was raised in a rabbit (Ginns et al, 1982) as well as monoclonal antibodies (8E4 and 2C7) that were obtained from the culture medium of hybridoma cells (Barneveld et al, 1983b), were provided by Dr. J Aerts.

Cell culture reagents were from Gibco BRL. CNBr-activated Sepharose 4B-beads were from Pharmacia.

4-Methylumbelliferyl-β-D-glucoside substrate and bovine serum albumin (BSA) was purchased from Sigma. Sodium taurocholate (grade A) was obtained from Calbiochem and conduritol B epoxide from Biomol.

Acrylamide and N,N`-methylenebisacrilamide was purchased from Sigma. Nitrocellulose membrane was from Schleicher and Schuell. The alkaline phosphatase substrate was the ImunoPure NBT/BCIP Substrate Kit from Pierce.

All other reagents were from Merck (proanalysis grade).

4.2.2 Methods

4.2.2.1 Preparation of cells

Leukocytes were isolated from heparinised peripheral blood by differential sedimentation in dense isotonic dextran (Skoog and Beck, 1956; Magalhães et al, 1984b). Contaminating erythrocytes were removed by hypotonic lysis and leucocytes were washed with 0.9% (w/v) sodium chloride. Pellets were stored at -70°C until used.

Human skin fibroblasts were obtained by skin biopsy and cultured in Dulbecco`s modified Eagle`s medium supplemented with 20% foetal-calf serum, 10 unit/ml penicillin, 10 μg/ml streptomycin and 20 mM glutamine. Cells were grown at 37°C under 5% (v/v) CO_2 in the gas atmosphere. Until 5 days after confluence cells were cultured with HEPES buffered Dulbecco`s modified Eagle`s medium. Cells were harvested by trypsinization and using a rubber policeman and then washed with phosphate-buffered saline (PBS) at pH 7.2.

4.2.2.2 Extraction of cells

Cell pellets, (leukocytes and fibroblasts), were suspended in 4 volumes of 10mM Tris-HCl buffer pH 7.0 containing 0.1% (v/v) Triton X-100, 1mM EDTA, 0.287 mM PMSF and 0.004 mM leupeptine. Cells were extracted by sonication with 30 cicles of 0.6 second, 50 watts, with pauses of 0.4 second (Weat Systems W 375). The homogenate

was centrifuged for 30 min at 15 000xg and the supernatant (referred to as extract) was collected. All these procedures were done at 4°C.

For SDS-PAGE and in order to desaggregate form II glucocerebrosidase, the extraction buffer contained 0.25% instead of 0.1% (v/v) Triton X-100. Under that conditions all form II is converted into form I as juged by acetate cellulose electrophoresis as well as by imunoprecipitation studies using a monoclonal antibody.

4.2.2.3   Isolation of activator protein SAP-2 preparation

Activator protein SAP-2 was isolated from the spleen of a Gaucher disease patient. The spleen tissue (25g) was homogenised in 4 volumes of 50 mmol/l potassium phosphate buffer (pH 6.5) containing 0.25% (v/v) Triton X-100 by 6 cycles of 30 seconds at 4000 rpm (Polytron homogenizer). Cells were disrupted by sonication with 30 cycles of 0.6 seconds, 50 watts, with pauses of 0.4 seconds (Weat Systems W375). The homogenate was then centrifuged at 40 000xg for 0.5 hours (Sorval centrifuge). The supernatant was boiled for 4 minutes and again centrifuged at 18 000xg for 0.5 hours. The supernatant was adjusted to pH 4.4 with 1 mol/l acetic acid and a similar centrifugation was performed. After addition of 5 volumes of ice-cold absolute ethanol to the supernatant the preparation was centrifuged at 100 000xg for 1 hour and the pellet was dissolved in 2% trichloroacetic acid (TCA). The procedure was repeated and the obtained precipitate was dissolved in distilled water. This preparation is usually referred to as crude activator protein preparation (Peters et al, 1977).

4.2.2.4   The β-Glucosidase activity: Glucocerebrosidase and cytosolic broad-specific β-glucosidase activity

The fluorometric assay was performed using the 4-methylumbelliferyl (4-MU) derivatized artificial substrate basically according to Galjaard (1980). The reaction was stopped by the addition of 1mol/l sodium glycinate buffer, pH 10.0. The released fluorescence was compared with that of standards, using an excitation and emission wavelenght of 365 and 445nm, respectively (fluorescences read on an Aminco SPR spectrofluorometer). Standards used for the calibration curve were prepared from a solution of 10mmol/l 4-MU in chloroform/methanol (2:1). Results of each series of measurements were algebrically calculated by using the linear regression parameters corresponding to the calibration curve.

β-glucosidase activity: The assay was carried out by incubating during 120 minutes at 37°C the cell extract with 3.7 mmol/l 4-MU-β-D-glucopyranoside substrate prepared in 0.1/0.2 mol/l citrate/phosphate buffer (pH 5.2) and 0.02% (v/v) Triton X-100. Sodium taurocholate (0.6% (w/v)) was included where indicated.

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Glucocerebrosidase activity: The cell extract was pre-incubated during 0.5 hours in parallel with and without 1 mmol/l conduritol B-epoxide (CBE). This pre-incubation results in maximal irreversible inhibition. Glucocerebrosidase activity was calculated as being the CBE-inhibitable β-glucosidase activity:

Cytosolic broad-specificity β-glucosidase: Calculated as the β-glucosidase activity non-inhibited by CBE.

4.2.2.5   Measurement of protein

Protein was measured according to a modification (Herbert et al, 1974) of the Lowry's method (Lowry et al, 1951). Bovine serum albumin was used as a standard.

4.2.2.6   Activity of glucocerebrosidase monomeric and aggregated forms: Enzymatic activity per amount of antigen

Form I glucocerebrosidase is precipitated by the monoclonal antibodies 8E4 and 2C7, whereas form II is not. Kinetic parameters, such as Km values for artificial substrates and stimulation by activators, were identical for the enzyme in solution and immobilized. Since the affinity of the mutant and control glucocerebrosidase for binding to the antibodies 8E4/2C7 is similar (as checked by mixing experiments) bounded activity to identical amounts of antibody is directly proportional to the enzymatic activity per identical amount of antigen.

An identical amount of rabbit anti-(mouse IgG) prepared in PBS (phosphate buffered saline) was coated to each well (0.45µg/well) of a microtitre plate by incubation for 1 h at 37°C. Next, an identical amount of anti-glucocerebrosidase monoclonal antibodies 8E4/2C7 prepared in PBS were coated on each well (62.5 ng/well) by incubation for 16 hours at 4°C. Unbound antibody was removed by washing in PBS containing 0.05% (v/v) Tween-20. The enzyme binding assay was made by incubation overnight at 4°C and for 2 hours at room temperature an excess amount of antibody with cell extracts.

Form II glucocerebrosidase activity: The supernatant was removed from wells and the unbounded form II glucocerebrosidase activity was measured as the CBE inhibitable β-glucosidase activity.

Form I glucocerebrosidase activity: Bounded glucocerebrosidase activity (form I) was measured by

-incubating the wells for 2 hours at 37°C with 3.7 mmol/l 4-MU-β-glucoside, prepared in 100/200 mM citric acid/sodium phosphate buffer (at the indicated pH), with 0.2% (w/v) sodium taurocholate and 0.1% Triton X-100.

-incubating the wells for 2 hours at 37°C with 3.7 mmol/l 4-MU-β-glucoside prepared in 50mmol/l acetate buffer (at the indicated pH), with 0.04 μg/μl phosphatidylserine (PS) and 0.138 μg/μl activator protein (Aerts et al, 1990a).

The reaction was stopped by addition of excess glycine/NaOH (pH 10.0) and the 4-methylumbelliferone formed was measured fluorimetrically. The released fluorescence was compared with that of standards, being measured with a excitation and emission wavelength of respectively 365 and 445nm (fluorescence read on a Titertek plate spectrofluorometer). Standards used for the calibration curve were prepared from a solution of 10mmol/l 4-MU in chloroform/methanol (2:1). For each sample the bound activity was determined as the mean of triplicate assays. Results of each serious of measurements were algebrically calculated by using the linear regression parameters corresponding to the calibration curve.

4.2.2.7   SDS-PAGE and immunoblotting of glucocerebrosidase

The glucocerebrosidase present in extracts was immunoprecipitated by incubating extracts overnight at 4°C with monoclonal antibodies 8E4 and 2C7 anti-glucocerebrosidase immobilized to CNBr-activated Sepharose 4B-beads. β-Glucosidase activity was measured in the supernatants to ascertain that all activity was imunoprecipitated. Subsequently, beads were washed by 2 cycles with PBS, one cycle with 30% ethylenoglycol in PBS, followed by another cycle with PBS. After centrifugation for 5 minutes at 10 000xg samples were ressuspended in 2x concentrated SDS-PAGE sample buffer (125mmol/l Tris/HCl, pH6.8, containing 2.3% glicerol, 3.8% SDS, 0.0048% bromophenol blue), and heated at 95°C for 2 minutes. After pelleting the beads, supernatants containing glucocerebrosidase were removed and β-mercaptoethanol was added to the final concentration of 2%. Samples were then subjected to SDS-PAGE performed on vertical slab gels. The ratio of acrylamide to N,N`-methylenebisacrilamide was 29.2:0.8. Total monomer concentrations were 10% and 5% in the separating and stacking gels, respectively. Electrophoresis was stopped 1cm before the bromophenol blue dye marker reached the bottom of the gel. Afterwards protein was transferred electrophoretically into nitrocellulose filters using a semi-dry blotting apparatus at 0.8mA/cm^2 for 2.5 hours, and with 48 mmol/l Tris/HCl (pH 8.3) containing 39mmol/l glicine and 20% (v/v) methanol. After electroblot, nitrocellulose filters were washed 2x with PBS containing 0.05% Tween 20 and 0.5% BSA during 30 minutes. Next, nitrocelulose filters were incubated overnight with rabbit anti-glucocerebrosidase polyclonal antibody diluted 1:2000 in PBS with 0.05% Tween and 0.5% BSA. Filters were next washed 3 times with 0.05% Tween in PBS during 10 minutes, and afterwards incubated during 1 hour with the alkaline phosphatase-second

antibody conjugate. Next, nitrocellulose filters were washed 1 hour with 0.05% Tween in PBS, followed by another washing with PBS and revealed by adding the alkaline phosphatase substrate.

4.3 Results

4.3.1 Molecular weight forms and amount of glucocerebrosidase protein in patients presenting different genotypes

Molecular weight determinations of glucocerebrosidase protein from control fibroblasts revealed processing forms ranging from 58 to 68 Kda, with three major forms with appearant Mr of 66, 63 and 59 kDa (lane p). The observed heterogeneity in the relative proportion of the different glucocerebrosidase processing forms is due to the highly dependency of subcellular distribution (and thus, molecular mass species patterns) on the external pH of culture medium, in physiological range of 7.0-7.4 (Aerts, 1988). Minor differences in the amount of glucocerebrosidase protein were observed in patients that present both or at least one N370S mutated allele. In the G377S and X homozygotes (respectively patients 29 and 37 in Figure 4-4) no differences from controls were observed in the presence of processing forms and stability of glucocerebrosidase. Thee L444P homozygote patient is the only one presenting a markedly reduced amount of protein (patient 41 in Figure 4-4). In the lane corresponding to patient 16, the L444P/? compound heterozygote, a relatively higher amount of protein is detectable, presenting apparently normal processing forms, which points to the presence of protein codified by the unknown mutated allele.
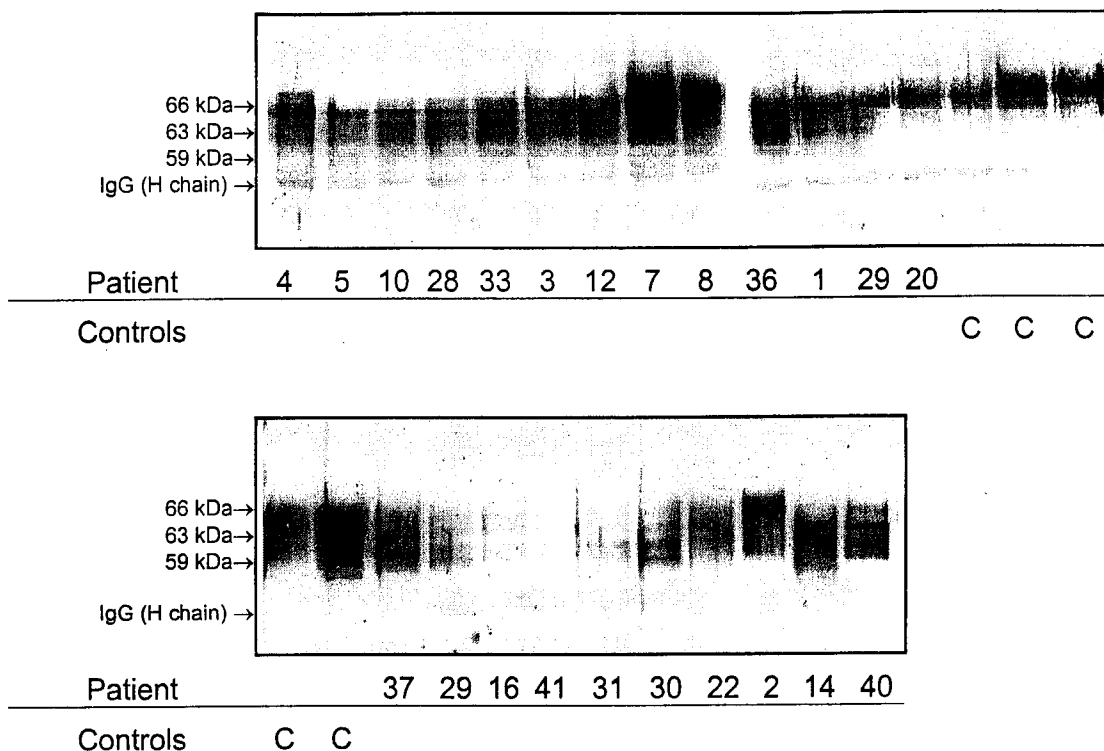
Figure 4-4

Pattern of molecular mass species of glucocerebrosidase protein extracted from cultured skin fibroblasts genotyped type 1 GD patients. Prior to SDS-PAGE and immunoblotting, glucocerebrosidase was purified by immunoprecipitation from an aliquot of extracts containing 150 µg of total protein. Patients 4 and 5 are N370S homozygotes; patients 10, 28 and 33 are N370S/L444P compound heterozygotes; patients 3 and 12 are N370S/rec TL compound heterozygotes; patients 7 and 8 are N370S/IVS2+1 compound heterozygotes; patient 36 is a N370S/recNcil compound heterozygote; patient 1 is a N370S/R463C compound heterozygote; patient 29 is a G377S homozygote; patient 29 is a X homozygote; patient 37 is a X homozygote; patient 16 is a L444P/? compound heterozygote; patient 41 is a L444P homozygote; patients 31, 30 and 22 are N370S/? compound heterozygotes; patient 14 is a N370S/IVS2+1 compound heterozygote; patient 40 is a N370S/L444P compound heterozygote.

4.3.2 Glucocerebrosidase residual activity in peripheral blood total leukocytes and cultured skin fibroblasts of GD patients and controls

The mean glucocerebrosidase activity measured in the presence of taurocholate was significantly reduced in extracts of peripheral blood total leukocytes and cultured skin fibroblasts from GD patients being less than 11 % of control mean (Table 4-2). The activity of the non-specific β-glucosidase was however not significantly different from controls in any cell type studied (Table 4-2).

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Table 4-2

Glucocerebrosidase and non-specific β-glucosidase activity (nmol/h/mg protein) extracted from peripheral blood total leukocytes and cultured skin fibroblasts type 1 GD patients. Glucocerebrosidase activity was determined with taurocholate.

ENZYME	CELL-TYPE					
Glucocerebrosidase		**Activity in controls**				
		Mean±std	Range	n		
	Leukocytes	9.5±3.8	2.8-19	18		
	Fibroblasts	232±135	103-552	11		
		Activity in patients				
		Mean±std	Range	n	%control	p
	Leukocytes	0.77±0.65	0.00-2.8	33	8.1	<0.01
	Fibroblasts	13±15	1.46-74	22	5.6	<0.01
Non-specific β-glucosidase		**Activity in controls**				
		Mean±std	Range	n		
	Leukocytes	0.99±1.0	0.08-3.6	18		
	Fibroblasts	6.1±0.61	0.10-18	5		
		Activity in patients				
		Mean±std	Range	n	%control	p
	Leukocytes	0.75±0.65	0.09-2.7	32	76	n.s.
	Fibroblasts	5.3±5.0	0.33-20	23	90	n.s.

Figure 4-5 shows that no significant correlation was observed between the leukocytes glucocerebrosidase activity, determined in the presence of taurocholate (T+) and clinical severity (SSI) of GD patients.
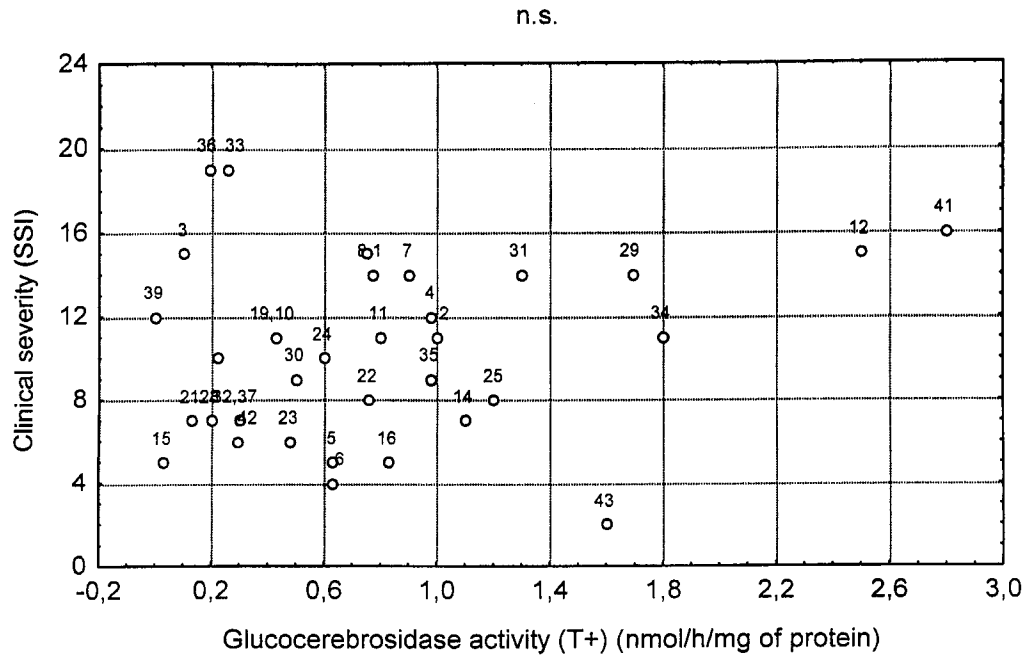
Figure 4-5

Peripheral blood total leukocytes glucocerebrosidase activity (nmol/h/mg of protein), measured in the presence of taurocholate (T+) and clinical severity (SSI) of type 1 GD patients.

In order to have a first insight on the amount of the residual activity corresponding to the aggregated form of glucocerebrosidase (form II), the activity in extracts of leukocytes and fibroblasts was measured in the absence of taurocholate. Table 4-3 shows that in leukocytes, the glucocerebrosidase activity determined in the absence of taurocholate was not significantly different from controls (78% of control activity).

In this table it is also shown the cell type specific effect of taurocholate on the extracted glucocerebrosidase activity. Taurocholate stimulated glucocerebrosidase activity extracted from fibroblasts of controls and GD patients. In contrast, the glucocerebrosidase activity extracted from total leucocytes of GD patients was clearly inhibited by the bile salt.

Table 4-3

Comparison between the glucocerebrosidase activity (nmol/h/mg protein) determined in the absence (T-) and in the presence (T+) of sodium taurocholate of peripheral blood total leukocytes and cultured skin fibroblasts from type 1 GD patients.

		Activity (nmol/h/mg protein)			
		Leukocytes		Fibroblasts	
		T-	T+	T-	T+
Controls	n	n=18	n=18	n=11	n=11
	mean±std	1.8±0.73	9.5±3.8	36.1±33.0	232±135
	range	1.1-4.3	2.8-19	8.87-124	103-552
T+/T-		5.3		6.4	
Patients	n	N=37	n=37	n=26	n=26
	mean±std	1.4±1.1	0.77±0.65	4.67±5.32	13.1±14.7
	range	0.0-4.0	0.0-2.8	0.0-19	1.5-74
T+/T-		0.88		2.8	
p		n.s.	<0.01	<0.01	<0.01
Patients % of control		78%	8.1%	13%	5.6%

4.3.3 Glucocerebrosidase residual activity in peripheral blood total leukocytes and cultured skin fibroblasts of genotyped GD patients

Glucocerebrosidase activity measured in the absence (T-) of taurocholate was compared with that measured in the presence of taurocholate (T+), in both extracts of total leukocytes and fibroblast from individual GD patients presenting different genotypes. Table 4-4 presents the mean, standard deviation and range of glucocerebrosidase activity (measured in the presence and in the absence of taurocholate) for each genotype group of GD patients.

The median and range for each genotype group is also graphically depicted in Figure 4-6 for total leukocytes and in Figure 4-7 for skin fibroblasts. As shown in Figure 4-6, in total leukocytes of patients presenting at least one N370S mutated allele, the glucocerebrosidase activity is higher when measured in the absence of the effector taurocholate (with the exception of patient 36, N370S/RecNcil). This effect is not observed the activity of skin fibroblasts (Figure 4-7). On the contrary, the glucocerebrosidase activity of patients without the N370S mutated allele is higher when determined in the presence of taurocholate; the glucocerebrosidase residual activity of the patient presenting the X/X genotype is not affected by this effector.

Table 4-4

Comparison between the glucocerebrosidase activity (nmol/h/mg of protein) determined in the absence (T-) and in the presence (T+) of sodium taurocholate of peripheral blood total leucocytes and cultured skin fibroblasts from type 1 GD patients with different genotypes.

| | | Activity (nmol/h/mg protein) at pH 5.2 | | | |
| | | Leukocytes | | Fibroblasts | |
		T-	T+	T-	T+
N370S/N370S	Patients	n=10	n=10	n=6	n=6
	Mean±std	1.3±1.3	0.60±0.47	7.1±6.1	18±9.5
	Range	0-3.8	0-1.6	2.2-19	6.4-34.0
N370S/L444P	Patients	n=7	n=7	n=5	n=5
	mean±std	1.6±1.2	0.57±0.60	1.7±0.89	13±7.9
	Range	0.69-4.0	0-1.80	0.49-2.7	4.2-25
N370S/IVS2+1	Patients	n=4	n=4	n=2	n=3
	mean±std	1.7±1.6	0.70±0.47	1.3±0.70	4.4±3.0
	Range	0.0-3.2	0.03-1.1	0.81-1.8	1.5-7.4
N370S/R463C	Patient 1	2.0	0.75	2.5	10
N370S/Rec TL	Patients	n=2	n=2	n=2	n=2
	mean±std	2.7±0.57	1.3±1.7	1.8±2.1	5.7±5.0
	Range	2.3-3.1	0.1-2.5	0.26-3.3	2.2-9.3
N370S/X	Patient 20	NA	NA	1.5	6.5
N370S/RecNcil	Patient36	0.56	0.19	3.7	5.05
N370S/?	Patients	n=6	n=7	n=3	n=3
	Mean±std	1.2±0.95	0.77±0.36	0.96±0.95	3.6±1.13
	Range	0.07-2.5	0.22-1.3	0-1.9	2.4-4.66
L444P/L444P	Patient 41	0.59	2.8	7.0	13
L444P/?	Patient 16	0.53	0.83	17	74
G377S/G377S	Patients	n=2	n=2	n=1	n=1
	mean±std	0.70±0.28	1.45±0.35	15.9	14.9
	Range	0.50-0.89	1.2-1.7		
X/X	Patient 37	0.29	0.28	9.3	7.5
Controls	n	n=18	n=18	N=11	n=11
	mean±std	1.79±0.73	9.53±3.77	36.1±33.0	232±135
	Range	1.10-4.28	2.80-19.1	8.87-124	103-552

NA means that samples are not available.
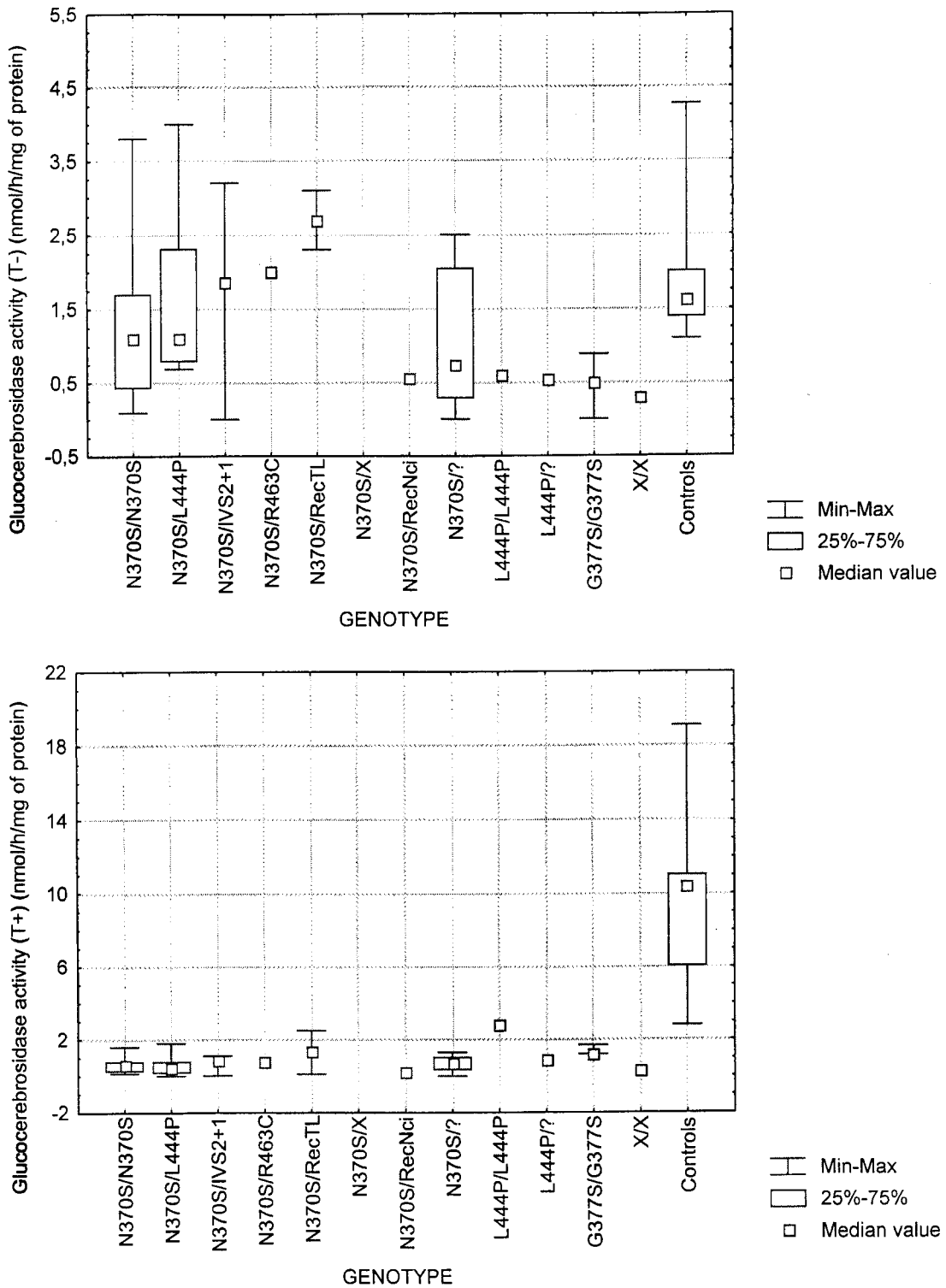
Figure 4-6

Genotype median and range values of glucocerebrosidase activity (nmol/h/mg of protein) measured in the presence (T+) or in the absence (T-) of taurocholate from peripheral blood total leucocytes of type 1 GD patients.
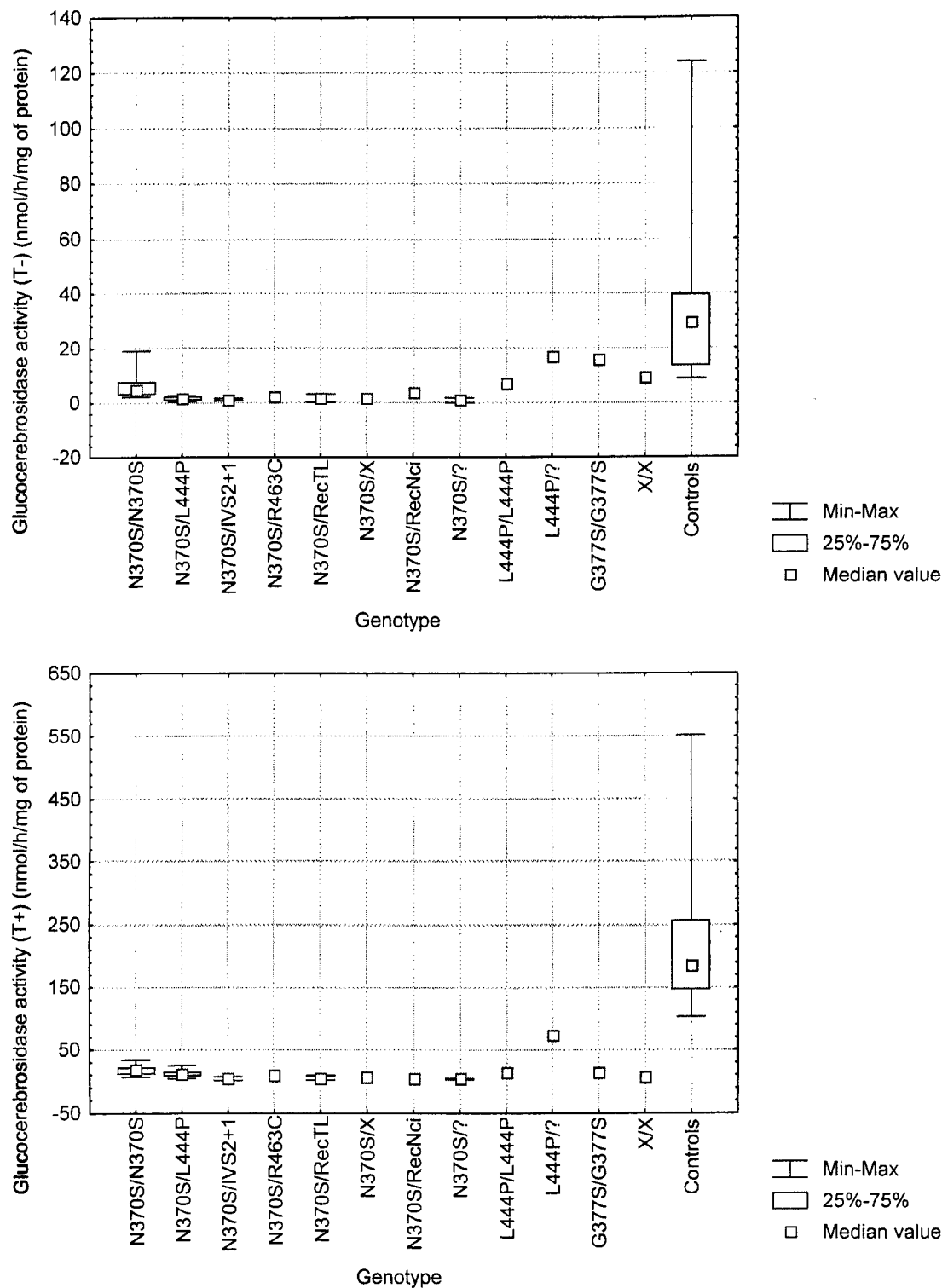
Figure 4-7
Genotype median and range values of glucocerebrosidase activity (nmol/h/mg of protein) measured in the presence (T+) or in the absence (T-) of taurocholate, from cultured skin fibroblasts type 1 GD patients.

The glucocerebrosidase mean activity corresponding to each genotype group expressed as percentage of the control mean is graphically depicted in Figure 4-8 for

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total leukocytes. As it can be observed, the residual glucocerebrosidase activity, determined in the absence of taurocholate (T-), was almost normal in total leukocytes (ranging from 69-151% of control mean), in patients presenting at least one N370S mutated allele (being an exception the N370S/RecNcil patient, whose activity represents 31% of control mean). In genotypes were the N370S mutation was not present, the glucocerebrosidase residual activity in leukocytes, determined in the absence of additional activators was lower and ranged from 16-39% of mean control value. For all genotype groups, the mean glucocerebrosidase activity measured in the presence of taurocholate is always lower than about 30 % of the control mean activity.
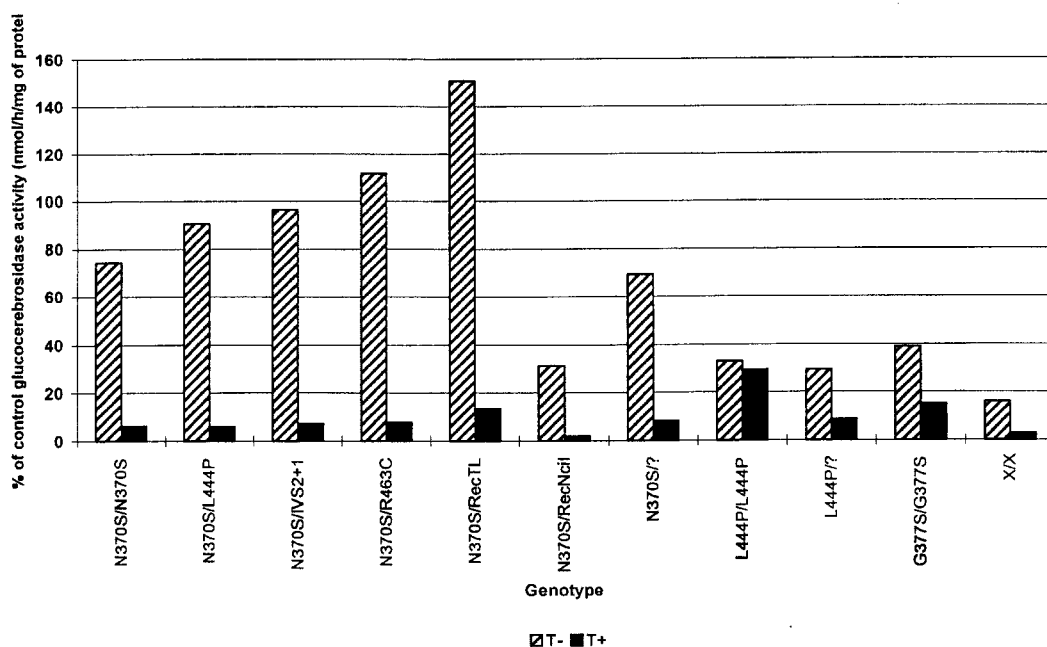
Figure 4-8

Percentage of control mean glucocerebrosidase activity of each genotype group, measured in the presence (T+) or in the absence (T-) of sodium taurocholate, from peripheral blood total leucocytes of type 1 GD patients.

4.3.4 Quantification of the aggregated form of glucocerebrosidase in total leukocytes and fibroblasts extracts

Form I glucocerebrosidase (monomeric) and form II (aggregate of enzyme+activator protein+phospholipids) were separated by immunotitration with monoclonal anti-glucocerebrosidase antibodies. Table 4-5 shows the contribution of non-immunoprecipitable (form II) glucocerebrosidase to total glucocerebrosidase activity, measured in the presence and in the absence of taurocholate, in extracts of total leukocytes and fibroblasts from patients and controls.

In fibroblasts, for both patients and controls, the percentage of form II glucocerebrosidase related to total glucocerebrosidase activity is similar either when measured with or without taurocholate, being in general respectively 12% and 16% of the corresponding total glucocerebrosidase. In contrast, in total leukocytes, the percentage of form II glucocerebrosidase related to total glucocerebrosidase activity is significantly higher than in fibroblasts, specially when measured in the absence of taurocholate:

In controls and when measured in the absence of taurocholate, the percentage of form II glucocerebrosidase accounts for about 75% of total glucocerebrosidase activity. In patients presenting at least one N370S mutated allele, in the absence of taurocholate, the percentage of form II activity is highly variable, ranging from 21-100%, even ranging within the same genotype from 21 to 77% (e.g. the results found in patients presenting the N370S/L444P genotype).

With respect to patients that do not have the N370S mutated allele the following was observed:

Although the G377S homozygote presents also an high percentage of agregated form activity (76% of total glucocerebrosidase), the remaining genotypes showed a comparatively lower amount of form II; patient 37, the X homozygote, presents a low percentage of aggregated form activity (27% of total glucocerebrosidase activity) and the L444P homozygote shows a very low percentage of form II activity (6.5%).

The effect of taurocholate on the aggregated form II activity can be also seen in Table 4-5. With the exception of patient 36 (the N370S/RecNcil), the activity of form II from all compound heterozygotes for the N370S mutated enzyme is dramatically reduced by taurocholate. With respect to the taurocholate effect on form II activity of the other mutated enzymes, it can be observed that form II activity is slightly decreased, not affected and markedly increased for respectively the G377S, X and L444P mutated enzymes.

Table 4-5

Percentage of aggregate glucocerebrosidase activity (form II) from total glucocerebrosidase activity of peripheral blood total leucocytes and cultured skin fibroblasts of genotyped type 1 GD patients.

| | | % of form II glucocerebrosidase activity | | | |
| | | Total leukocytes | | Fibroblasts | |
Genotype	Patients	T-	T+	T-	T+
N370S/N370S	21	100	10	0	3.4
	42	87	7.1	15	1.7
N370S/L444P	10	29	0	1.55	5.0
	23	21	7.4	9.7	6.0
	28	77	29	16.3	5.2
N370S/IVS2+1	8	100	9	6.4	7.3
	14	100	11	16	36
N370S/R463C	1	73	6.5	5.7	12
N370S/Rec TL	3	100	12	35	12
N370S/RecNcil	36	23	20	2.2	2.0
L444P/L444P	41	6.4	20	0.95	1.3
G377S/G377S	29	76	57	2.2	2.0
X/X	37	27	27	2.4	0
Controls	range	50-75	10-13	8-15	5-6
	n	3	3	4	4

No correlation was obtained between the glucocerebrosidase residual activity measured in conditions that do not lead to the destruction of the aggregated form (determined in the absence (T-) of taurocholate) and clinical severity (SSI) of GD patients (Figure 4-9).
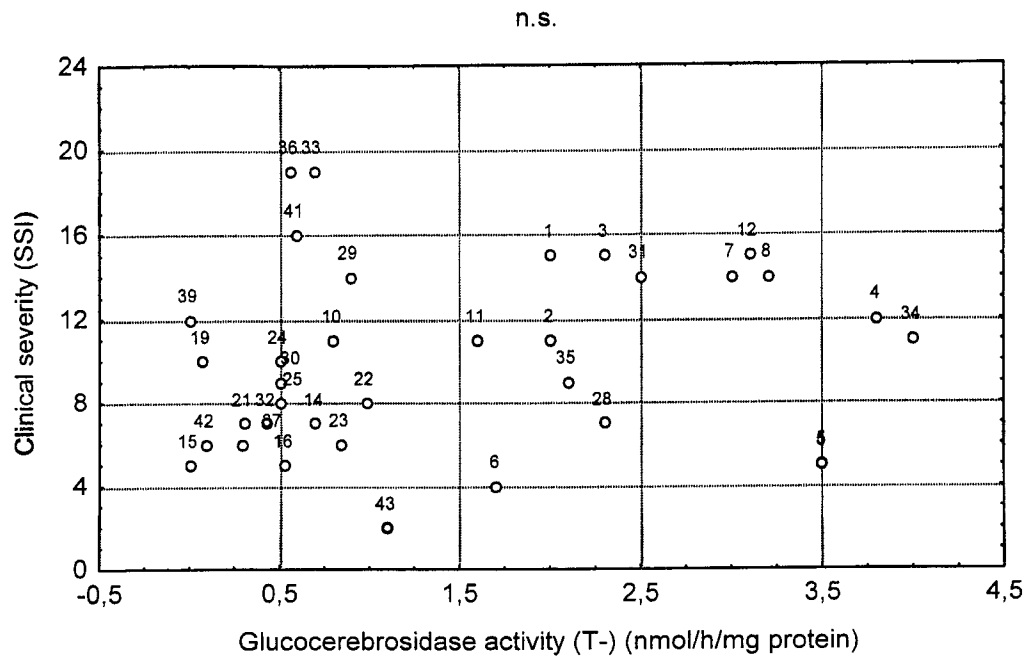
Figure 4-9

Glucocerebrosidase activity measured without taurocholate (T-) (nmol/h/mg of protein) of peripheral blood total leukocytes and clinical severity (SSI) of type 1 GD patients.

4.3.5 Modulation of glucocerebrosidase monomeric form specific activity

The effect of taurocholate (T+), saposin C (SAP+) and phosphatidylserine (PS+) was studied on the enzymatic activity of bounded glucocerebrosidase monomeric form.extracted from fibroblasts of GD patients and controls was determined at ph 4.5 and 5.2 (Table 4-6).

In the presence of taurocholate, the activity from patients presenting at least one N370S mutated allele was in general lower than 25% of control mean value, when measured at pH 4.5 or 5.2. Similar findings were observed for the G377S and X mutated enzymes. The specific activity of the L444P mutated enzyme was however 42% and 54% of control mean value, at pH 4.5 and 5.2 respectively. The specific activity might be however underestimated due to the fact that little amount of protein was available in the extracts, and it can not be excluded that no complete saturation of the antibody was accomplished (which is required for obtaining the correct specific activity value).

In the presence of phosphatydilserine and activator protein saposin C the properties of form I mutanted enzyme were quite different. In contrast to the L444P and X mutant enzymes, the specific activity of the N370S and G377S mutated enzymes became close to normal in the presence of the physiological activators.

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It is of interest to note the effect of pH on specific activity of the mutant enzymes when measured with the physiological activators. In compound heterozygoptes of the N370S mutated enzyme the pH dependence was inter individually different: in some cases the specific activity is higher at lower pH and in some other cases it is lower. In the case of the G377S mutant enzyme the specific activity is clearly higher at pH 4.5 than at pH 5.2 (Table 4-6). The (PS+SAP+)/T+ ratio showed that the reactivation effect of the PS+SAP (related to the activity measured with taurocholate) is clearly higher at pH 4.5, for the genotypes presenting at least one N370S mutant allele.

Table 4-6

Saposin C, phosphatidylserine and taurocholate modulation effect on form I glucocerebrosidase activity (nmol/h/amount of antigen). Glucocerebrosidase was extracted from cultured skin fibroblasts of type 1 GD patients and the activity of monomeric (form I) glucocerebrosidase bounded to the monoclonal antibodies 8E4/2C7 was measured in the presence of taurocholate (T+) or phosphatidylserine (PS+) and activator protein saposin C (SAP+).

Patient	Genotype	Glucocerebrosidase specific activity (nmol/h/CRIM molecule)					
		T+		PS+SAP+		(PS+SAP+)/T+	
		pH 4.5	pH 5.2	pH 4.5	pH 5.2	pH 4.5	pH5.2
4	N370S/N370S	0.07	0.17	1.1	1.9	16	11
21		0.12	0.16	2.0	0.23	18	1.4
10	N370S/L444P	0.18	0.29	3.9	1.5	22	5.2
13		0.18	0.31	1.2	2.0	6.7	6.4
23		0.23	0.32	0.55	0.64	2.4	2.0
28		0.19	0.19	1.5	0.21	7.9	1.1
7	N370S/IVS2+1	0.05	0.15	1.1	1.9	21	13
8		0.23	0.45	3.5	3.2	15	7.1
14		0.28	0.38	3.1	3.4	11	8.9
1	N370S/R463C	0.21	0.55	4.0	4.6	19	8.4
3	N370S/RecTL	0.06	0.41	2.5	0.47	42	1.1
36	N370S/RecNcil	0.10	0.16	2.2	0.71	22	4.4
2	N370S/?	0.05	0.11	0.83	1.3	18	12
41	L444P/L444P	0.59	1.3	1.1	0.66	1.9	0.51
16	L444P/?	0.40	0.88	1.4	2.1	3.5	2.4
29	G377S/G377S	0.32	0.69	3.0	1.7	9.4	2.5
37	X/X	0.14	0.35	0.62	0.76	4.4	2.2
Control mean ± std (n=6)		1.4±0.14	2.4±0.18	3.3±0.22	4.2±0.26	2.3±0.17	1.8±0.08

4.4 Conclusions

In portuguese type 1 GD patients, glucocerebrosidase processing forms ranging from 58 to 68 kDa (Jonsson et al, 1987), with three major forms with appareant Mr of 66, 63 and 59 kDa, were detected in fibroblasts cell homogenates of patients presenting at

least one N370S mutated allele and the G377S and X mutant homozygotes. For these two last reported mutant enzymes (Amaral et al, 1996), the presence and the apparently normal amount of glucocerebrosidase molecular forms allows to expect that normal processing occurs of a mature enzyme (probably presenting reduced catalytic activity), instead of either a rapid lysosomal degradation, obstruction of transport or loss by secretion. Only the cell extracts of the L444P homozygote presented a significative reduction in the amount of glucocerebrosidase protein.

Glucocerebrosidase residual activity was determined in peripheral blood total leukocytes and fibroblasts cell extracts of GD patients and since the detergent extraction (with Triton X-100) of this membrane associated enzyme results in its inactivation, taurocholate has been currently used for the *in vitro* enzyme reactivation. In these conditions glucocerebrosidase activity was found to be markedly reduced in patients cell extracts; however when the activity was determined in the absence of the bile salt taurocholate, patients presented an almost normal glucocerebrosidase activity in total leukocytes, as opposed to the clear deficiency found in fibroblasts.

Genotyping revealed that this cell type specific residual glucocerebrosidase activity existed specifically for patients having at least one N370S mutated allele, being the glucocerebrosidase residual activity in total leukocytes higher than 69% of control mean. In contrast, in the remaining genotypes, the residual glucocerebrosidase activity in leukocytes ranged from 16-39% of control mean activity. Previous studies with purified mutant enzyme provided a simple explanation for the remarkable cell type dependency of the N370S glucocerebrosidase activity (Aerts et al, 1990b). The mutant enzyme in monomeric form exhibits a severely reduced activity either in the absence or in the presence of taurocholate. The activity of the mutant enzyme in reconstituted complexes of glucocerebrosidase with activator protein saposin C (SAP) and phosphatidylserine (PS) was, however, almost as high as in controls. Addition of taurocholate leaded to dissociation of the complex and reactivation of the monomeric form of glucocerebrosidase, but the reactivation is much greater in control glucocerebrosidase than in the mutant enzyme (Sá Miranda, 1992).

Thus it was proposed that the cell-type specific differences of this mutated glucocerebrosidase activity were due to differences in the proportion of glucocerebrosidase extracted in aggregated, activated form (form II) from different cell types (Sá Miranda et al, 1990). The present study confirms and extends the findings of the early investigations since the results obtained in the form II quantification showed also that, when compared to controls, in the absence of taurocholate, form II enzyme contributes predominantly to the total glucocerebrosidase activity shown by extracts of total leukocytes of GD patients presenting at least one N370S mutated allele (from 21

to 100%). A similar finding was obtained for the G377S and to a lesser extend for the X homozygote. In contrast, the L444P homozygote presented only a low percentage of form II glucocerebrosidase.

The different features of the mutant enzymes studied in this work are illustrated in Table 4-7.

Table 4-7

Features of mutant glucocerebrosidases extracted from cells of type 1 GD patients.

		Mutant enzymes			
	Control enzyme	N370S	L444P	G377S	X
Stability (based on CRIM)	Normal	Normal	Reduced	Near normal	Near normal
Activation state (based on form II)	Intermediate	High	Low	Intermediate	Intermediate
Taurocholate effect (on form II activity)	Marked activation	Marked reduction	Marked activation	Slight reduction	Not significantly affected
Specific activity (with taurocholate)	High	Very low	High	Very low	Low
Specific activity (with PS+SAP)	High	High	High	High	Low

Firstly, the activity of the N370S glucocerebrosidase aggregate form (form II) was decreased to about 10% of that measured without taurocholate and only slightly reduced in the G377S homozygote; in contrast the activity of the X form II glucocerebrosidase was not significantly affected by taurocholate but was markedly activated in the case of the L444P homozygotes. Secondly, the dependence of the mutated enzymes on the aggregated form was shown by the marked effect of the physiological activators PS and SAP on the specific activity (enzymatic activity per amount of cross-reactive glucocerebrosidase). For the N370S mutated enzyme the specific activity was found to be very low when measured in the presence of taurocholate, and in general near normal when measured in the presence of the physiological activators PS and SAP. Similar findings were obtained for the G377S. In contrast there was only a low activation effect for the X and L444P homozygotes. In general for both the N370S compounds and the G377S homozygote this activation effect was higher at pH 4.5. It was also observed that although the L444P homozygote cell extracts have a low amount of glucocerebrosidase protein, this mutant enzyme was

found to present the highest specific activity (about 50% of control mean) when measured in the presence of taurocholate.

Relatively high residual β-glucosidase activity in leukocytes from Gaucher patients had been observed previously (e.g. Beutler and Kuhl, 1970; Raghavan et al, 1980) and were explained by assuming the occurrence of non-specific β-glucosidases in the preparations. The findings obtained in this study raise the question of whether the residual capacity for glucocerebrosidase hydrolysis might be much higher in some blood cell types of GD patients (presenting at least one N370S mutated allele) than was generally assumed.

Since the activity of the N370S mutated form seems to be highly dependent on the existence in the aggregated form, the results obtained with compound heterozygotes of the N370S mutated allele, where the percentage of form II activity was found to range from 21 to 100% of total glucocerebrosidase, lead to the tentative search of correlation between the amount of the aggregated form and the clinical severity. Still no clear correlation was obtained but some observations can be made:

The specific activity of the glucocerebrosidase monomeric form extracted from cells of the N370S homozygote patient 21 was very low when measured with taurocholate (T+), being however highly reactivated by the physiological activators PS+SAP at pH 4.5 (18-fold the specific activity measured with taurocholate). This patient presented a mild to moderate clinical phenotype (SSI of 7) and all glucocerebrosidase activity was extracted from total leukocytes in the activated state, the aggregate form.

In contrast to that observed for the N370S mutated enzyme, but similarly to that observed for the control enzyme, the specific activity of the glucocerebrosidase monomeric form extracted from cells of the L444P homozygote patient was very high when measured with taurocholate (T+), although the amount of CRIM was found to be very low; this patient presented a severe phenotype (SSI of 16) and the glucocerebrosidase activity extracted as aggregate form was almost absent (6.4%).

Patient 36, the N370S/RecNcil compound heterozygote, presents a severe clinical phenotype (SSI=19), and although the specific activity of the extracted monomeric form of glucocerebrosidase was found to be highly modulated by the physiological activators PS+SAP at pH 4.5 (reactivation related to T+ of 22-fold), a low amount of glucocerebrosidase was extracted in the activated aggregate form (23%). With respect to the N370S/RecTL patient 3, although all glucocerebrosidase was extracted as aggregate form (100%), and the specific activity of the extracted monomeric form of glucocerebrosidase was highly modulated by the PS+SAP at pH 4.5 (reactivation

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related to T+ of 42-fold), this patient presents a less severe phenotype, but still a moderate score (SSI of 15).

On the other hand, the large clinical heterogeneity observed in patients presenting the N370S/L444P genotype group (SSI ranging from 6 to 19) could be explained by the existence of variable proportions of the unstable L444P mutated enzyme, which presents high specific activity per amount of cross reacting material. This was suggested by the observation that from cells of patient 23 a low amount of glucocerebrosidase was extracted as aggregate form (21%), and accordingly the specific activity of the monomeric form was poorly modulated by the PS+SAP at pH 4.5 (reactivation related to T+ of 2.4-fold). This data suggested the presence of higher proportion of L444P mutated enzyme and the patient presented a mild to moderate score of clinical severity (SSI of 6). However, patient 28 although presenting a similar score of clinical severity (SSI of 7), showed comparatively higher quantity of glucocerebrosidase extracted as aggregate form (77%) and the specific activity of the monomeric form of glucocerebrosidase was showed an higher reactivation by PS and SAP (reactivation related to T+ of 7.9-fold).

It is likely that the G377S and X mutations resemble more the N370S mutation than the L444P. The G377S homozygote (patient 29) has a moderate to severe SSI of 14 and although an high amount of the glucocerebrosidase activity was extracted as aggregate form (76%), the specific activity of the glucocerebrosidase monomeric form was only moderately activated by the PS and SAP activators (reactivation related to T+ of 9.4-fold). The X homozygote patient 37 presents a mild to moderate index of clinical severity (SSI of 6) and although the reactivation effect by PS and SAP on the specific activity of this mutated glucocerebrosidase monomeric form was relatively low (reactivation related to T+ of 4.4-fold) and a considerable amount of the glucocerebrosidase activity was extracted as aggregate form (27%).

Chapter 5
Enzyme supplementation therapy in the study of underlying pathogenic mechanisms of Gaucher disease

5.1   Introduction

5.1.1   Secondary abnormalities in GD patients

5.1.1.1   Glucosylceramide   accumulation   and   other   glycolipid abnormalities

Glucosylceramide is an intermediate in the degradation of complex glycosphingolipids such as globosides and gangliosides that are mainly present in cell membranes. In macrophages of GD patients due to the deficiency of glucocerebrosidase, the breakdown of glucosylceramide in the lysosome is impaired. The resulting Gaucher cells (lipid loaded macrophages) occur in almost every organ, but are especially present in the spleen, liver and bone marrow. Glucosylceramide was found to be increased in plasma of GD patients (Strasberg et al, 1983), being transported as a component of lipoprotein complexes (Dawson and Oh, 1977). The lipid level was reported as being about three times increased in type 3 Gaucher patients and twice in type 2 patients. In type 1 patients, glucosylceramide was described as being only slightly elevated, (Dawson et al, 1982), although the level has been found to rise after splenectomy (Nilsson et al, 1982; Svennerholm et al, 1982). The origin of the elevated plasma glucosylceramide is not precisely clear. It has been suggested that storage material might leak from lysed Gaucher cells into the extracellular space (Pennelli et al, 1969; Hibbs et al, 1970). Concomitant with the increase in glucosylceramide, abnormalities have been reported in the levels of at least two other glycosphingolipids. Increased concentrations (5 fold) of the ganglioside G_{M3} have been found in tissues of patients with GD (Philippart and Menkes, 1964). In brain, the levels of lactosylceramide

and oligohexaosylceramides were found to be slightly raised as well as an increased proportion of G_{M2} and G_{M3} gangliosides (Nilsson and Svennerholm, 1982). Type 2 GD brain was characterized by a decreased galactocerebroside content in addition to the increased glucocerebroside content (Sudo, 1977; French et al, 1969).

The neurotoxin lysosphingolipid glucosylsphingosine (a deacylated analogue of glucocerebrosidase also designated as psychosine) which is not detectable in normal human brain, is another compound that accumulates in brain from GD patients (Figure 5-1). This substance is increased at least 100 to 1000 fold in the neuronophathic forms being the concentration higher in type 2 than in type 3, and higher in cerebellar than in cerebral cortex (Nilsson and Svennerholm, 1982). Glucosylsphingosine has been reported to inhibit protein kinase C (Hannun and Bell 1987), albeit at rather high concentrations. It has been suggested that the accumulation of this toxic substance is the basis for the extensive neuronal cell loss in the neuronopathic forms of GD.
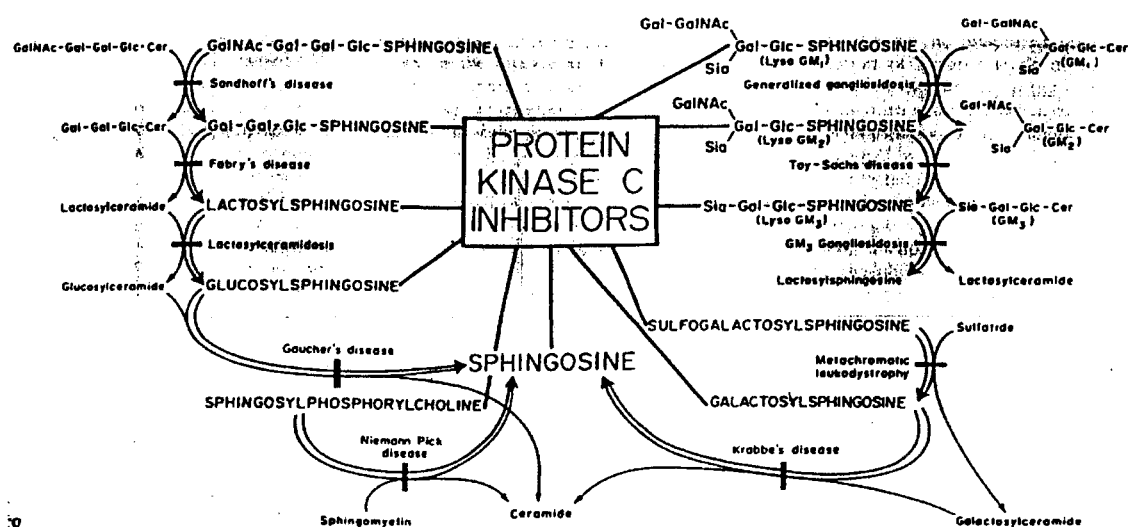
Figure 5-1

Proposed metabolic pathways for the degradation of sphingolipids and lysosphingolipids. The pathways of sphingolipid catabolism are illustrated (thin arrows). Deficiency of enzymes in these pathways (solid bars) lead to the indicated diseases. It is proposed that the same enzymes also act on lysosphingolipids (double arrow), and therefore, both the parent sphingolipid and the lysosphingolipid would accumulate in enzyme deficient states. Sphingosine and lysosphingolipids differ from the parent sphingolipids in their ability to strongly inhibit protein kinase C, thus interfering in the function of important pathways of signal transduction and cell regulation. Also the cytotoxicity of lysosphingolipids may ultimately lead to permanent tissue damage and cell death. (Adopted from Hannun and Bell, 1987).

5.1.1.2   Activity of lysosomal enzymes

The clinical manifestation of GD is reported to be generally accompanied by abnormalities in plasma levels of the some lysosomal hydrolases (Ockerman and Kohlin, 1967; Hultberg et al, 1980). The cause of elevated serum activities of lysosomal

enzymes in Gaucher disease is not known, although Crocker and Landing (1960) suggested that spillage of lysosomal enzymes occurs from tissues rich in Gaucher cells. However, since the elevated levels of acid phosphatase and β-hexosaminidase activities were not accompanied by elevation of lactic dehydrogenase activity (Nagakawa et al, 1983), gross cellular injury appears not to be the cause of increased amounts of these enzymes. Furthermore, *in vitro* studies of murine macrophage cultures showed that the addition of glucocerebroside at concentrations that had no detectable effect on lactic dehydrogenase, resulted in release of substantial amounts of β-hexosaminidase into the medium (Gery et al, 1981). This suggests that the accumulation of glucocerebroside may stimulate exocytosis of large amounts of lysosomal hydrolases.

5.1.1.2.1   Tartrate resistant acid phosphatase (TRAP)

The acid phosphatase (EC 3.1.3.2.) is one of the six isoenzymes of acid phosphatase that in many tissues is a minor component of total acid phosphatase activity and in contrast to the major lysosomal acid phosphatases is not strongly inhibited by L-(+)-tartrate. This tartrate-resistant acid phosphatase (TRAP) is produced in large quantities by osteoclasts and macrophages (Moss, 1992) being secreted during bone resorption (Chambers et al, 1987). Osteoclasts attach to the bone surface so as to form a sealed extracellular pocket, which is acidified by the action of a plasma membrane proton pump, thereby forming an acidic extracellular space into which lysosomal enzymes are secreted. TRAP of skeletal osteoclasts partially dephosphorylates the bone matrix phosphoproteins, osteopondin and bone sialoprotein and once dephosphorylated osteoclasts no longer bind to them (Ek-Rylander et al, 1994). This suggests that secretion of TRAP from osteoclasts into the resorption area could modulate attachment or motility of these cells on bone surface and affect the development of ruffled borders in bone tissue.

TRAP has been mapped to the short arm of human chromosome 19 (Lord et al, 1990; Moss, 1992). The 5' flanking region of the TRAP gene contains an iron responsive element being the expression regulated by iron at the level of gene transcription (Alcantara et al, 1994). The mRNA is expressed at high levels in osteoclasts and in cells of monocyte/macrophage lineage. During *in vivo* osteoclast formation, it appears that TRAP expression first occurs in mononuclear cells near the bone surface prior to the appearance of osteoclasts. These mononuclear, TRAP-positive cells are considered to be osteoclast precursors (Lacey et al, 1994). The presence of iron in the TRAP active site is required for enzymatic activity (Lord et al, 1990).

Acid phosphatase increased activity in GD was known before the identification of β-glucosidase deficiency as the primary defect of the disease and was the most prominent secondary biochemical abnormality (Tuchman et al, 1956; Tuchman et al, 1959; Chambers et al, 1977,1978; Robinson and Glew R, 1980). The serum activity of acid phosphatase was also found to be age dependent in controls and GD patients (Magalhães et al, 1984a). Although plasma TRAP activity in symptomatic GD patients can be about 10-fold increased, in asymptomatic patients this activity was found to be within the control range (e.g. Hollak et al, 1994).

5.1.1.2.2 The β-hexosaminidase activity

Gaucher disease patients also have a two to three-fold elevation in plasma levels of β-hexosaminidase (Moffitt et al, 1978). This activity is also known to increase markedly and progressively during pregnancy, as well as being moderately elevated in patients with *diabetes mellitus* (Bomback et al, 1976). Although the elevation of total β-hexosaminidase activity in serum of patients with Gaucher disease and pregnant women is similar, the quantitative distribution of β-hexosaminidase isoenzymes is distinctly different. In control subjects β-hexosaminidase A is the major isoenzyme activity followed by β-hexosaminidase I and β-hexosaminidase B. All isoenzymic forms are elevated in pregnant women, but there is a characteristic increase of the I isozyme or β-hexosaminidase P (Stirling, 1972; Nagakawa et al, 1983). Patients with insulin-dependent *diabetes mellitus* also have moderate elevation in all three isoenzymes (Bomback et al, 1976).

In GD, although all hexosaminidase isoforms are elevated, when compared to control serum, β-hexosaminidase B activity was increased approximately 3 fold while β-hexosaminidase A activity was increased nearly 2 fold (Moffitt et al, 1978). Spleen and liver from patients were found to contain a 5-fold increased in β-hexosaminidase activity (Ockerman and Kohlin, 1969; Moffitt et al, 1978). Separation of splenic β-hexosaminidase A and B activities on DEAE-Sephadex chromatography showed that the activity of β-hexosaminidase B isoenzyme was increased 12 fold, whereas β-hexosaminidase A activity was increased slightly more than 3 times relative to control spleen (Moffitt et al, 1978).

5.1.1.2.3 Abnormalities in other lysosomal enzymes

In GD it was observed a marked elevation in plasma levels of β-glucuronidase and nonspecific esterase activities (Moffitt et al, 1978). Spleen extracts contained increased activity in α-fucosidase (Ockerman and Kohlin, 1969), 10 fold increase in galactocerebrosidase activity (lactosylceramidase I) but not in GM1-ganglioside β-

galactosidase (lactosylceramidase II), and a 2 fold increase in β-glucuronidase and nonspecific esterase activity (Ockerman and Kohlin, 1969; Moffitt et al, 1978). Similar increases were also observed in liver α-fucosidase. Only slight increases in brain α-fucosidase have been observed (Ockerman and Kohlin, 1969). Levels of splenic alkaline phosphatase, arylsulfatase A and B, α-arabinosidase, sphingomyelinase, α-mannosidase, and G_{M1}-ganglioside β-galactosidase activities were similar to those of control tissue (Moffitt et al, 1978; Aerts et al, 1990a). Arylsulfatase A activity was found to be markedly reduced (20% of control) in brain homogenates from a type 2 GD (Moffitt et al, 1978).

5.1.1.2.4   Chitotriosidase activity

A striking elevation of the activity of chitotriosidase, an endo-β-glucosaminidase, was described in plasma from Gaucher patients (Hollak et al, 1994). This activity in samples from type 1 (n=491), type 2 (n=4) and type 3 patients (n=11) was in average 1033, 199, and 650-fold respectively the median value of the control subjects (Aerts et al, 1995b). The same work reported that in asymptomatic Gaucher disease patients the elevation was only 4.5-9.7 fold. Besides the observation of only mild elevations in chitotriosidase levels in asymptomatic Gaucher patients, no correlation was found between this activity and the severity of clinical manifestations (as assessed by the use of the Zimran's severity score index, SSI), or with patients genotype (Hollak et al, 1994). Chitotriosidase activity was found to be absent in about 6% of the population due to a recessively inherited deficiency (Hollak et al, 1994). Recently a single mutation has been identified in homozygous form in chitotriosidase deficient individuals. The mutation, a duplication in the chitotriosidase gene, causes formation of mRNA that encodes for an enzymatically inactive chitotriosidase protein. The mutation has been detected with a high frequency in individuals from various ethnic groups, suggesting an ancient origin (Boot et al, 1995). Since Gaucher patients lacking chitotriosidase activity show the usual clinical symptoms, it is unlikely that chitotriosidase itself contributes to the clinical presentation of Gaucher disease (Hollak et al, 1994).

Chitotriosidase activity was found to be about 12-fold increased in brain (Aerts et al, 1995a) and 50-fold increased in spleen of a type 1 Gaucher patient as compared with control subjects. Two major isoforms of chitotriosidase with isoelectric points of 7.2 and 8.0 and molecular masses of 50 and 39 KDa, respectively, were purified from spleen and the N-terminal amino acid sequence of the two forms proved to be identical (Renkema et al, 1995a). Isoelectric focusing revealed minor forms of pI 5.5-6.0 and pI about 6.5. In plasma, the apparent isoelectric point of chitotriosidase was predominantly 7.2 with minor amounts of 6.0 and 8.0, being comparable to that

observed in controls (Renkema et al, 1995a). An antiserum raised against the purified 39-KDa chitotriosidase precipitated all isoenzymes and 98% of the activity in the Gaucher spleen extract.

Both the N-terminal sequence and an internal sequence of chitotriosidase proved to be homologous to sequences in proteins that are members of the chitinase family (Hakala et al, 1993). This family consists of proteins from various organisms, with strong homology in several domains including the region that is involved in the catalysis of chitin (a polymer of β-1,4-linked N-acetylglucosamine moieties) and the artificial substrate 4-MU-chitotrioside (Watanabe et al, 1993). The human chitotriosidase showed chitinolytic activity toward artificial substrates as well as chitin (Renkema et al, 1995a) and may therefore be considered to be a chitinase that shares homology with chitinases from non-mammalian organisms, e.g. the nematode Brugia malayi (Fuhrman et al, 1992) or the fungus Aphanocladium album (Blaiseau and Lafay, 1992).

The biological function and the relationship between the several hundred-fold increased plasma levels of chitotriosidase and the pathophysiology of Gaucher disease is however unknown. It was described that during monocyte morphological differentiation into macrophage, cells begin to produce and secrete increasing amounts of chitotriosidase (Hollak et al, 1994) as well as acid phosphatase (Moss, 1992). Northern blot analysis showed that the remarkably strong expression of chitotriosidase mRNA occurred only at a late stage of differentiation of monocytes to activated macrophages in culture (Boot et al, 1995).

The finding that chitotriosidase was a chitinase was found to be very important since the human body is still believed to contain no chitin (Raghavan et al, 1994). The chitotriosidase isolated from Gaucher spleen clearly differed from the other mammalian members of the chitinase protein family which have been found to be devoid of chitinolytic activity; thus it appeared to be more closely related to the chitinases of non-mammalian organisms. The unexpected occurrence of a human chitinase lead to the search of other proteins produced by macrophages that could interact with chitin. Renkema and co-workers found that macrophages synthesise large amounts of a chitin binding protein, that was early described as the human chondrocyte glycoprotein (HC gp-39) (Hakala et al, 1993). This protein has a high degree of similarity with the chitinase protein family. HC gp-39, in contrast to chitotriosidase, was not able to degrade the natural or the artificial chitinase substracts (Hakala et al, 1993). A comparison between the amino acid sequences of chitotriosidase and HC GP-39 showed that the presumed catalytic center of chitotriosidase is almost completely conserved in HC gp-39, but two critical acidic amino acids, required for the hydrolytic activity, were found to be substituted, probably leading to the chitin lectin properties of

this protein (Renkema et al, 1995b). So human macrophages were found to synthesise a functional chitinase, a highly conserved enzyme with a strongly regulated expression. This human chitotriosidase is thought to be involved in defence against and in degradation of chitin-containing pathogens such as fungi, nematodes and insects, and can be used as a marker for specific disease states (Boot et al, 1995).

5.1.1.3   Iron distribution

In adult type 1 Gaucher patients, the accumulation of glucocerebroside in reticuloendothelial cells is in most cases accompanied by a varying degree of iron accumulation (Lorber, 1960; Lorber, 1965; Lorber and Nemes 1967; Lee et al, 1967). The existence of abundant iron was demonstrated histochemically (Lorber, 1960; Lorber and Nemes 1967), by electron microscopy (Lorber and Nemes 1967; Lee et al, 1967), immunochemical procedures (Lorber and Nemes, 1967), chemical analysis (Lorber and Deren, 1968), and by abnormal iron kinetics (Lorber, 1965; Lee et al, 1967). The presence of iron in the Gaucher cells may suggest a derivation of iron and lipid following the phagocytosis of erythrocytes (Pick, 1933). However, the observation that lipid and iron accumulation do not necessarily parallel each other, and that cells of infants with the disorder do not present iron accumulation (Pick, 1933) indicates that this is not basic to the disorder and that it only occurs after prolonged time. The water soluble non-heme iron is present associated with the ferritin protein (Rothen, 1944). Large aggregates of cross-linked ferritin molecules that comprise the water-insoluble non-heme iron particles are usually termed hemosiderin (Nixon and Olson, 1968). In Gaucher cells such aggregates are rare. The general cytoplasmic dispersion of ferritin molecules is not unique for Gaucher cells but has also been noted in normal macrophages (Karrer, 1958) and in hemochromatosis (Bessis and Caroli, 1959). In spleen of adult Gaucher patients, splenic parenchyma total iron (heme iron, ferritin and hemosiderin) is elevated between 3 and 28 times the normal level; heme iron is usually increased and the ferritin:hemosiderin partition varies from 35:65 to 60:40. In children, despite splenomegaly, total iron and ferritin:hemosiderin ratio is normal (Lorber and Deren, 1968).

A dynamic equilibrium has been described between plasma iron and ferritin iron (Bessis, 1962). In normal subjects, much of the plasma iron enters the reticuloendothelial system and the hepatic parenchyma (Granick and Hahn, 1944; Fineberg and Greenberg, 1953). In Gaucher cells an appreciable number of ferritin micelles occurs in the periphery of the cytoplasm, particularly in and near the numerous pseudopods that at times form the external surface of the cell. This, together with the rapid disappearance of plasma radioiron, might indicate that iron in the Gaucher cells

was derived not only from ingested red blood cells (Lorber and Nemes, 1967; Lee et al, 1967), but also from the surrounding plasma or interstitial fluid which normally contains a significant labile iron pool (Wasserman et al, 1964; Wasserman et al, 1965).

The presence of iron in Gaucher cells is another example of iron accumulation in tissues with lipid accumulation, as for example also in xanthomata. It is unlikely that iron accumulation in Gaucher cells is only caused by the phagocytic nature of these cells, since although granular hemosiderin may be present in the trabeculae, sinus walls and Gaucher cells of the spleen, the Kupffer cells of the liver usually appeared to be normal despite their phagocytic function (Popper and Schaffner, 1957). In addition, many or most Niemann-Pick cells (Bloom, 1925) may develop from reticuloendothelial cells, yet appear to be devoid of iron.

5.1.1.3.1   Cell-mediated immunity and iron metabolism

Maintenance of cellular iron homeostasis is not only a general prerequisite for growth and proliferation of all cells but it also is of central importance for the regulation of the immune system. Both iron deficiency and iron overload can exert subtle effects on immune status by altering the proliferation of T and B cells (reviewed in De Sousa et al, 1991; Brock, 1994). This is partially due to the central role of iron as a cofactor for critical enzymes, such as mitochondrial aconitase and ribonucleotide reductase. Furthermore, the availability of cellular iron may even have a specific influence on the proliferation of T helper 1 (Th1) and Th2 subsets, thus modulating the activities of different lymphocyte subpopulations and their subsequent effector mechanisms. In addition, iron plays a critical role in macrophage-mediated cytotoxicity by contributing to the production of several reactive oxygen species. As a consequence of all this, imbalances of iron metabolism seem to strongly affect immune effector function (reviewed in Weiss et al, 1995).

There are several literature reports that indicate the very complex relationships between monocytes, macrophages, T lymphocytes, cytokines and cellular iron homeostasis. For example, the macrophage-derived cytokines interleukin 1(IL-1) and TNF-α are known to induce hypoferraemia by increasing iron uptake into monocytes/macrophages. Additionally, the Th1-type cytokines IFN-γ and IL-2 were shown to enhance strongly the expression of transferrin receptor, the essential protein for iron uptake in macrophages. IL-6 may also increase ferritin synthesis, thus contributing to efficient storage of the acquired iron (reviewed in Weiss et al, 1995).

The impact of iron on cell-mediated immunity was further substantiated by reports demonstrating that iron-loaded macrophages lose their ability to kill intracellular

pathogens by IFN-γ-mediated pathways. However, the diversion of iron traffic by increased uptake and incorporation of iron into ferritin by macrophages is thought to cause growth limitation of microorganisms and tumor cells by restricting iron availability. Furthermore, it is thought that enhances the production/release of cytokines such as IFN-γ or TNF-α by monocytes/macrophages, promoting host defence capacity. Finally, activated macrophages are able to synthesize transferrin, which then promotes and enhances lymphocyte proliferation in a paracrine manner, thus overcoming the depressive effects of hypoferraemia (reviewed in Weiss et al, 1995).

T lymphocytes migrate to places of either physiological or pathological iron overload (De Sousa, 1978, 1981); it was postulated that immunological system cells may actively participate in the protection of potential iron toxicity (De Sousa, 1981; Nishiya et al, 1980). Porto (1993) demonstrated, for the first time in man, a remarkable stability of the total numbers and relative proportions of T lymphocytes in peripheral blood, and a correlation between the relative expantion of the CD4+ (helper) and CD8+ (killer) populations and iron metabolism.

5.1.1.4   Osteoclastic activity and T lymphocytes

The occurrence of the lipid loaded macrophages (Gaucher cells) in tissues and organs, underlies the GD most common signs of pancytopenia, organomegaly and skeletal deterioration (reviewed in Cox and Schofield, 1997). Infiltration of normal bone marrow by lipid-laden Gaucher cells results in apparently progressive displacement of the fat-rich marrow proper with a consequent shift in haematopoietic activity from proximal to more distal sites (Rosenthal, 1995). The pathophysiology is however not clearly understood. In particular, the balance between the impact of altered numbers and increased activity of osteoclasts (whose progenitors in the marrow are of haematopoietic lineage), the possibly suppressed osteoblast activity (Siffert and Platt, 1982) as well as the mechanism of bone infarction (caused by impaired collateral bone micro- and macrovascular supply). Enzyme supplementation therapy (Barton et al, 1991a), which has as its principal aim the correction and/or prevention of ongoing formation of the lipid loaded macrophages, has proven to be safe and effective in improving the haematological parameters and reducing the organomegaly in GD patients. However, the general experience is that the skeletal response to therapy lags behind all other symptoms (Elstein et al 1996). The relatively slow skeletal response to enzyme therapy does not necessarily imply poor delivery of enzyme to the skeleton. Tissue distribution studies with tracer doses of radio-iodinated mannose-terminated

enzyme showed avid uptake in the marrow, in proportion to the putative cellular pool of macrophages in this organ (Mistry et al, 1996).

Recent reports of studies in vitro point to the involvement of T lymphocytes in bone homeostasis via the regulation of osteoclast differentiation (John et al, 1996; Horwood et al, 1998). Decreased CD8^{+} T lymphocyte numbers in diseases associated with increased bone resorption have been previously reported (e.g. Consolini et al, 1987; Nagasawa et al, 1995). In addition in vitro studies showed that depletion of cells expressing the CD8 antigen, from bone marrow cell populations that contain osteoclast-precursor cells, significantly increased the number of osteoclasts generated in vitro (John et al, 1996). More recently (Horwood et al, 1998), a mechanism was proposed for the osteoclastogenesis inhibition, in which IL-18 (produced by stromal cells or other cells of the bone microenvironment) act on T cells (either CD8^{+} or CD4^{+} subsets) via an unidentified receptor, to increase GM-CSF production, which ultimately inhibits osteoclastogenesis. However, this newly inhibitory pathway where IL-18 acts as a local inhibitory agent, may be able to be compensated for by other mechanisms, since GM-CSF-/- and GM-CSF R-/- deficient mice lack major skeletal abnormalities.

5.1.1.5 Immunoglobulin abnormalities

Nearly half of the tested patients presented hypergammaglobulinemia (Schoenfeld et al, 1980), possibly due to chronic stimulation of the immune system, which may also infer a connection to the relatively high incidence of multiple myeloma reported in Gaucher disease (Zimran et al, 1995a). The subset of patients at risk for these abnormalities is not known.

5.1.1.6 Cytokines

The functional consequences of the massive accumulation of glucocerebroside in tissue macrophages is not completely known, but the addition of glucosylceramide to cultured normal circulating monocytes was shown to stimulate lymphokine interleukin-1 release, and augmented lipopolysacharide-induced interleukin-1 release by cells (Gery et al, 1981). In patients, the cytokine macrophage colony stimulating factor (M-CSF), the soluble form of CD14 (sCD14) and IL-8 were reported to be elevated (respectively 2-8 fold, 2-5 fold and 2-20 fold) (Hollak 1996). CD14 is a monocyte/macrophage differentiation antigen and has been reported to be a receptor for LPS-LBP complex. Although the biological role of the soluble form of this receptor (sCD14) remains to be clarified, elevated levels might be associated with an increased number or activation of cells from the monocyte/macrophage lineage. Concentrations of IL-6 and IL-10 (which

are important regulators of lymphocyte growth and differentiation) were also found to be significantly elevated in sera from patients with GD (respectively 2 and 8-fold that of controls). Interleukin-1β was not detectable in sera obtained from patients and no difference from controls was observed in the concentrations of TNF-α. Interleukin-6 concentration in the serum of patients showing monoclonal or biclonal gammopathy has also been found significantly elevated. Since IL-6 has been implicated in the development of localized osteolysis in multiple myeloma and osteoporosis, enhanced release of these cytokines provides a functional link between GD and its associated lymphoproliferative and skeletal manifestations (Cox et al, 1997). The increased secretion of cytokines by stimulated macrophages as a response to extensive storage may thus contribute to the pathogenesis of the disease (Moffitt et al, 1978; Gery et al, 1981).

5.1.1.7 Monocyte dysfunction

A significant monocyte dysfunction (but not in granulocytes) was most consistently expressed by markedly glucocerebroside dose-dependent suppressed superoxide generation. Consequently, NBT (nitroblue tetrazolium test) reduction and *Staphylococcal* killing were considerably reduced in monocytes (and normal in granulocytes) (Liel et al, 1994). This abnormality was observed in a large majority of GD patients, regardless of the clinical severity of the disease (Liel et al, 1994). Superoxide generation, or respiratory burts is produced by an enzymatic complex, the NADPH oxidase, in response to various microbial pathogens and by soluble inducers (Babior, 1984; Rossi, 1986). It constitutes the principal mechanism responsible for the killing of invading microbial pathogens. Available evidence implies an important role for the activation and translocation from the cytosol to the cell membrane of protein kinase C (PKC) in the process of phosphorylation of the p47 component and stimulation of the NADPH oxidase complex (Okamura et al, 1988, Rotrosen et al, 1990). Since granulocytes and monocytes from GD patients display normal activity of the NADPH oxidase system, it is unlikely that components of the NADPH oxidase system are inherently, quantitatively or qualitatively abnormal in GD. It is most likely that glucocerebrosidase interferes with the signal transduction at, or distal to PKC. The precise mechanism remains to be defined (Liel et al, 1994).

Phagocytosis, which is unrelated to superoxide production, was also described as being decreased in monocytes but normal in granulocytes of GD patients (Liel et al, 1994). Chemotaxis (monocyte motility) was also found to be impaired in 50% of the patients (Liel et al, 1994). Although the neutrophil is not classically considered to be a site of sphingolipid storage, a neutrophil chemotactic defect was also reported to

occurs in 29% of GD patients (Aker et al, 1993; Liel et al, 1994). However this could be the result of secretory products of mononuclear phagocytes, eg, tumor necrosis factor and interleukin-2 (Zimran et al, 1994a).

Bacterial infections have a prominent role by ocurring repeatedly and being life-threatening in patients with severe GD (Zimran et al, 1994a). Pyogenic infections are recognized as one of the three major causes of death in GD patients (Beutler, 1991), especially in the pediatric population (Zimran et al, 1994a). Osteomyelitis in GD patients, particularly by anaerobic pathogens (Finkelstein et al, 1992) could represent specific local failure of the reticuloendothelial system to eliminate invading pathogens. Susceptibility toward systemic infections is probably less likely due to the relative integrity of granulocytes function (Liel et al, 1994). Zimran and coworkers claim to have found a direct relationship between the phenotypic expression of early age of onset, severity score index, and tendency to bacterial infections and the chemotaxis defect (Zimran et al, 1994a).

5.1.2 Aims and approach of the study

Many questions remain unsolved with respect to the pathophysiology of GD. Factors influencing the clinical expression are unknown and a clear correlation between phenotype, genotype and residual activity of the enzyme is lacking. Analysis of secondary plasma abnormalities may help to increase insight into the complicated pathophysiology of the disease and could also provide useful disease markers. Some of these abnormalities are probably directly due to the mechanisms via which lipid-loaded Gaucher cells affect their surroundings, e.g. hydrolases release and abnormal cytokine production. A number of hydrolases that might originate from macrophages (e.g. TRAP, β-hexosaminidase and chitotriosidase) are usually found to be elevated in plasma of symptomatic Gaucher patients. If the lipid-loaden macrophages are indeed involved in the release of those enzymes, their plasma levels may serve as quantitative markers for the amount of storage cells. The observation that administration of corticosteroids to patients with sarcoidosis (disease in which the macrophage is also involved in the pathology) leads to a rapid reduction in plasma chitotriosidase activity, seems to indicate that the synthesis and release of this enzyme is also highly dependent on the macrophage state of activation (Hollak, 1996).

It is conceivable that both the amount of storage cells as well as their degree of activation contributes to the (interindividually different) clinical expression of the disease. In order to get further insights in the amount of Gaucher cells and/or their activation state, the following studies were performed:

- Glucosylceramide concentration was determined in plasma of Portuguese GD patients presenting different genotypes and level of clinical severity. Plasma concentration of other membrane lipids (GM3 ganglioside and ceramide) was also studied in order to determine if the storage of glucosylceramide affects the metabolism of these compounds.

- Serum ferritin, the activity of TRAP, β-hexosaminidase and chitotriosidase was determined in plasma of GD patients, in an attempt to find any correlation with the severity of clinical manifestations or genotype.

- The T lymphocyte major subsets were also studied in GD patients in order to verify if abnormalities in the immune system could also be associated to the clinical expression of the disease.

- Finally, this work reports the assessment of all these abnormalities in patients submitted to the enzyme supplementation therapy in order to get further insight in the pathophysiology of the disease.

5.2 Materials and methods

5.2.1 Materials

5.2.1.1 Samples

Blood and tissues were obtained from Portuguese GD patients (both under treatment and untreated) and control subjects. Separated plasmas were stored frozen at -70°C. Control spleens were collected from surgery of organ transplantation donors. Spleens from Portuguese patients with type 1 GD were collected after therapeutic splenectomy. All tissues were kept frozen at -70°C. Tissues requiring transportation were shipped in solid carbon dioxide.

5.2.1.2 Reagents

4-methylumbelliferone (4-MU), 4-Methylumbelliferylphosphate, 4-MU-β-D-N,N´,N´´-triacetylchitotriosidase and 4-MU-N-acetylglucosamine (4 MU-GlcNac) were purchased from Sigma. The 4-MU-GlcNac sulphatide derivative (4-MU-glcNacS) was obtained from Moscerdam (The Netherlands).

Standards of cholesterol, ceramide, non-hydroxylated and hydroxylated galactosylceramide, phosphatidylethanolamine, lactosylceramide, phosphatidylcholine, phosphatidylserine, phosphatidylinositol, sphingomyelin and ganglioside III were all from Sigma and of bovine origin.

Glucosylceramide standard was from Sigma and of Gaucher spleen origin. C18 Bond Elut columns (2.8ml column volume and 500mg sorbent mass) were from Analytichem International. 10x10cm and 10x20cm HPTLC plates precoated with silica gel 60 (0.25mm), were from Merck.

All the others chemicals and solvents were from Merck.

5.2.2   Methods

5.2.2.1   Lipid analysis

Extraction: Sphingolipids and phosphoglycerides are of amphipatic nature and were extracted from their biological sources with chloroform/methanol mixtures (Folch et al, 1957).

Total lipid extract was obtained by 2 cycles of vortexing 1 minute the sample with 6 volumes of chloroform/methanol (1:2, v/v). The total volume of solvent extraction was evaporated to dryness and the dryed lipid content was further dissolved in chloroform/methanol/water (2:8:4).

Fractionation: Prepacked reversed-phase C18 columns were used to remove non-lipid contaminants (Kirklund, 1987) and to separate by polarity differences into acidic and neutral lipids. The column bounded lipids were fractionated into acidic (eluted with methanol/water, 12:1) and neutral (eluted with chloroform/methanol, 1:2); water-soluble lipids namely gangliosides were recovered in the acidic fraction (Kirkund, 1987). The procedure was carried out at room temperature. Both fractions were evaporated to dryness at 40°C under a stream of nitrogen.

Alkaline hydrolysis: To half of the neutral fraction, a mild hydrolysis was done as an additional purification step which allows the elimination of some contaminating phosphoglycerides that interfere in thin layer chromatography (TLC) with sphingolipids by presenting similar Rfs (Esselman et al, 1974)). The ceramide moiety is made of sphingosine, a long chain amino alcool, and an amide linked fatty acid (being human sphingosine essentially of C18 nature). Phosphoglycerides have O-linked fatty acids which do not occur in sphingolipids. A way to distinguish between these two types of lipids is performing alkaline hydrolysis. This treatment affects only lipids containing esterified fatty acids (Vance and Sweeley, 1967).

After being redissolved by chloroform/methanol (1:2, v/v) one half of the neutral lipid fraction was subjected to mild alkaline hydrolysis in methanolic sodium hydroxide at 37 °C during 30 minutes (Vance and Sweeley, 1967). Gangliosides being rather hydrophilic due to their content of glycosides are lost in the aqueous phase during

alkaline hydrolysis procedure (see flow scheme of the previously described lipid analysis procedure in Figure 5-2).
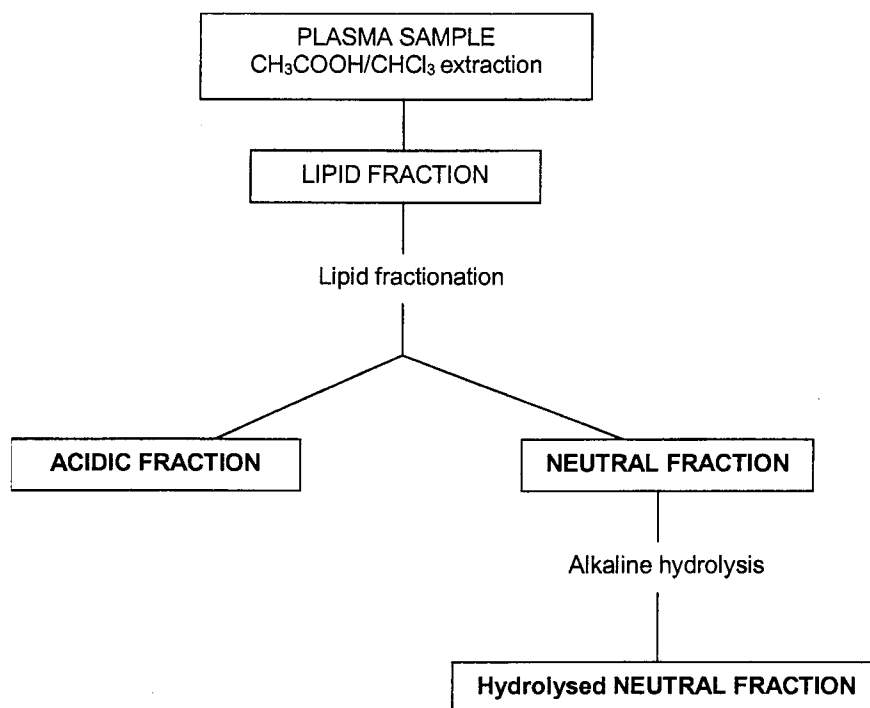
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                    ┌─────────────────────────────┐
                    │      PLASMA SAMPLE           │
                    │  CH3COOH/CHCl3 extraction    │
                    └─────────────────────────────┘
                                 │
                       ┌──────────────────┐
                       │  LIPID FRACTION   │
                       └──────────────────┘
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                         Lipid fractionation
                                 │
                    ┌────────────┴────────────┐
           ┌────────────────┐        ┌──────────────────┐
           │ ACIDIC FRACTION │       │ NEUTRAL FRACTION │
           └────────────────┘        └──────────────────┘
                                             │
                                    Alkaline hydrolysis
                                             │
                              ┌──────────────────────────────┐
                              │ Hydrolysed NEUTRAL FRACTION   │
                              └──────────────────────────────┘
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Figure 5-2
Flow scheme of the lipid analysis procedure.

Thin layer chromatography (TLC): Phosphoglycerides, sphingolipids and sterols were then fractionated by TLC and visualized with specific staining reagents.

Dried fractions were redissolved in chloroform/methanol (1:2, v:v) and immediatly applied to HPTLC plates previously activated at 100°C for 1 hour before use. An equivalent to 100 µl of plasma and appropriate amounts of lipid standards (0.25-2 µg) were applied to the plates. Several developing solvent mixtures were used for lipid separation; The monohexoside fraction was separated into glucosylceramide and galactosylceramide on TLC plates previously impregnated with a 1% $Na_2B_4O_7$ solution (Kean, 1966) before sample application and the development was performed with chloroform/methanol/water (70:30:5). To separate phosphatidylserine from phosphatidylinositol the plate was firstly developed with a mixture of chloroform/methanol/acetic acid/formic acid/water (35:15:6:2:1) to 4.5cm from the application point in the plate. The plate was then redeveloped with a mixture of n-hexano/diisopropilic ether/acetic acid (65:35:2) in the same direction and to 6.0cm from the application point of the plate (Macala et al, 1983). The chloroform/methanol/15mmol/l $CaCl_2$ (55:45:10) system was used for the resolution of

gangliosides and chloroform/methanol/NH$_3$ 25% (95:5:0.8) for the resolution of non-polar lipids such as free ceramide and esterified cholesterol.

After development plates were dried under air stream and the lipids were visualised with 0.4% anisaldehyde and 2% (v/v) sulphuric acid in acetic acid, followed by 30 minutes at 100°C in the oven. Gangliosides were visualised with 0.3% resorcinol, 80% (v/v) sulphuric acid and 0.25 mmol/l cupric sulphate.

The doublet observed in some of the sphingolipid spots was caused by the diferential migration of the lipid molecules. This is due to either the content of hydroxy fatty acids in the ceramide moiety (being the lower band due to the hydroxylated one), or to differences in fatty acid lenghts in the ceramide moiety (Merrill and Jones, 1990).

Quantification: The optical density of each band was scanned with a Transidyne General Corporation Densitometer, model 2500, in the transmittance mode, at 500nm wavelenght, and the area of each peak was determined by integration. Lane widths were kept constant within each comparison set. The quantification of the lipid band was made by comparing with the area of the respective standard applied on the same plate with a known concentration. The recovery of the sphingolipid following the complete procedure ranged from 72 to 81%. With respect to sensitivity, the smallest amount of sphingolipid that could be detected by TLC was 250ng and the maximal amount quantitatively detected was 2000ng.

5.2.2.2   Plasma separation

Plasma was obtained from heparinised peripheral blood, by centrifugation at 310 rpm during 10 minutes (Skoog and Beck, 1956).

5.2.2.3   Preparation of tissue homogenates

Tissues (4ml/g of tissue) were homogenised in 10 mmol/l Tris-HCl buffer ph 7.0 containing 0.1% (v/v) Triton X-100, 1mmol/l EDTA, 0.287 mmol/l PMSF and 0.004 mmol/l leupeptin, by 6 cycles of 30 seconds at 4 000 rpm (Polytron homogeniser). Cells were disrupted by sonication with 30 cicles of 0.6 second, ≈50 watts, with pauses of 0.4 second (Weat Systems W 375). The crude homogenates were then subjected to centrifugation at 100 000 xg for 1 h (Sorval centrifuge). The entire procedure was performed at 4°C. The resulting supernatant, referred to as homogenate, served as the source of enzyme in all assays.

5.2.2.4   Enzyme assays

Fluorometric assays were performed using 4-methylumbelliferyl (4-MU) derivatized artificial substrates, essentially according to procedures described by Galjaard (1980). All assays were carried out at 37°C. Reactions were stopped by the addition of 1mol/l sodium glycinate buffer, pH 10.0. The released fluorescence was compared with that of standards, using an excitation and emission wavelenght of 365 and 445nm, respectively (fluorescence read on an Aminco SPR spectrofluorometer). Standards used for the calibration curve were prepared from a solution of 10mmol/l 4-MU in cloroform/methanol (2:1). Results of each series of measurements were algebrically calculated by using the linear regression parameters corresponding to the calibration curve.

Chitotriosidase activity: The assay was carried out by incubating during 15 minutes the previously acidified plasma or cell/tissue extract with 0.0260 mmol/l 4-MU-β-D-N,N`,N``-triacetylchitotriose substrate, prepared in 0.1/0.2 mol/l citrate/phosphate buffer, pH 5.2.

Total β-hexosaminidase activity: The assay was carried out by incubating during 15 minutes the previously acidified plasma or cell/tissue extract with 1.7 mmol/l 4-MU-N-acetyl-β-D-glucosaminide substrate, prepared in 0.1/0.2 mol/l citrate/phosphate buffer, pH 4.4.

β-hexosaminidase A activity: The assay was carried out by incubating during 60 minutes the previously acidified plasma or cell/tissue extract with 1 mmol/l 4-MU-6-sulpho-β-D-glucopyranoside substrate, prepared in 0.1/0.2 mol/l citrate/phosphate buffer pH 4.0.

Tartrate resistant acid phosphatase (TRAP) activity: assay was carried out by incubating during 15 minutes the previously acidified plasma or cell/tissue extract with 5mmol/l 4-MU-phosphate substrate prepared in 0.1 mol/l sodium citrate/citric acid buffer, pH 6.0, and 0.057 mol/l tartaric acid.

5.2.2.5   Protein determination

Protein was measured according to a modification (Herbert et al, 1974) of the Lowry`s method (Lowry et al, 1951). Bovine serum albumin was used as a standard.

5.2.2.6 Cellulose acetate electrophoresis of β-hexosaminidase isoenzymes

Samples were applied with an LRE applicator on cellulose acetate strips 5.7cmX14.0cm (Cellogel) previously equilibrated in 40mmol/l potassium phosphate, pH 6.0, electrophoresis buffer. The strips were placed in the electrophoretic tank (EP-166 Medizin Technik) and a constant voltage of 200 V was applied for 1.5 hours, at 4°C. Strips were incubated at 37°C, for 0.25 hours, with 5mmol/l 4-MU-N-acetyl-β-D-glucosaminide, in 0.1/0.2 mol/l citrate/phosphate buffer, pH 4.75. The reaction was stopped with 1mol/l sodium glycinate buffer, pH 10.0. The fluorescent bands of β-hexosaminidase activity were visualized at 366nm and photographed with a type 667 Polaroid film (Poenaru and Dreyfus, 1973; Ribeiro et al, 1995).

5.2.2.7 Ion exchange chromatography of β-hexosaminidase

Columns (0.5x5.0cm) of DEAE-Sephadex were prepared by equilibrating the resin in 0.1 mol/l sodium phosphate buffer, pH 6.0, and next in 0.01 mol/l sodium phosphate buffer, pH 6.0. To the above columns, 0.2 ml of homogenate containing 0.1-0.5 mg of protein was applied. β-Hexosaminidase B activity was eluted in the first fractions of the equilibrating buffer wash with a flow rate of 0.2-0.3 ml/minute. Following elution of the B isoenzyme activity, β-hexosaminidase A activity was eluted with 0.01 mol/l sodium phosphate buffer, pH 6.0, containing a gradient of 0-0.3 mol/l NaCl. The enzymatic activity of 600 μl fractions was measured with 4-MU-GlcNAc substrate as previously described. The identity of the resolved isoenzyme activities was confirmed as being hexosaminidase A and B, respectively, either by the heat denaturation procedure of incubation at 0°C and 50°C prior to the enzyme activity measurement, as well as by cellulose acetate electrophoresis. The thermo-resistant activity was expressed as percentage of the total activity (Nagakawa et al, 1977).

5.2.2.8 Statistical analysis

Data were analyzed using the SPSS (Statistical Program for the Social Sciences). All testing was performed at the level of significance of 0.05. Correlation coefficients (Spearman rank correlation) were calculated to infer the possible association between the variables under study. The statistical analysis focused on the demonstration of significant differences found between group means was analysed by student t-test or by a non-parametric test (Kruskal-Wallis test) when data do not showed a normal distribution.

It was performed non-linear trend analyses to detect changes in the mean number of T lymphocyte cells in those patients treated with alglucerase/imiglucerase enzime supplementation therapy for at least 24 months. For the analyses from a single patient, multiple regression models were formed in a hierarchical steps-down fashion. The response variable was percent of initial activity for $CD4^+$ (or $CD8^+$) cells and indicator variables represented whether a change did, or did not, occur during each specified month of therapy. All possible subsets of indicator variables (i.e. months) were considered using themethod known as Leaps and Bounds (Furnival et al, 1974). Thus, models with one or more change(s) in the mean response were compared to that of the null model (no change in the mean response during therapy). A minimum risk criteria was applied for model selection (Eubanks, 1988). Specialized programs for these trend analyses were written using the statistical programming language S-PLUS 4.5 (MathSoft, Inc., Seattle, Washington). This analyses was perfomed by CE McLaren from the Division of Epidemiology, Department of Medicine, University of California.

5.2.2.9 Iron metabolism and T lymphocyte analysis

The hematological and biochemical parameters of iron metabolism were performed by the Department of Hematology, at the St. António General Hospital, in Porto. The CD4+ and CD8+ T cell analysis was performed by the Department of Molecular Immunology and Pathology of the Abel Salazar Institute for Biomedical Sciences, of Porto University.

5.3 Results

5.3.1 Plasma lipid analysis

The neutral lipid fraction is mainly constituted of glucosylceramide, galactosylceramide, lactosylceramide, ceramide, sphingomyelin, free and esterified cholesterol and the phospholipids phosphatidylethanolamine and phosphatidylcholine. The separation of the glucosylceramide using the chloroform/methanol/water (70:30:5) system showed an increased concentration of this lipid in the majority of the GD patients; with respect to the remaining separated lipids (galactosylceramide, lactosylceramide, trihexosylceramide and sphingomyelin) no significant differences were found between patients and controls (Figure 5-3).
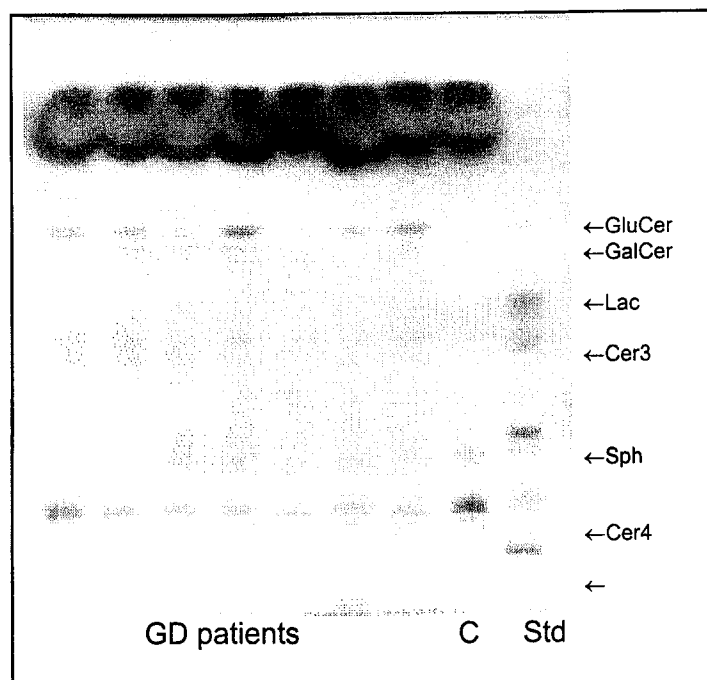
Figure 5-3

TLC profile of glucosylceramide extracted from plasma of type 1 GD patients. The neutral lipid fraction of plasma samples was submited to alkaline hidrolysis and the plates were developed with the chloroform/methanol/water (70:30:5) system. Applied standards (std) are glucosylceramide (GluCer) hydroxilated and non hydroxilated, galactosylceramide (GalCer) hydroxilated and non hydroxilated, lactosylceramide (Lac), trihexosylceramide (Cer3) and sphingomyelin (Sph) C, control plasma sample.

Statistical analysis showed that plasma glucosylceramide concentration was significantly different (p<0.01) between controls (mean=0.843±0.292 mg/100 ml plasma, n=17) and GD patients (mean=2.21±0.966 mg/100ml plasma, n=29). Although plasma glucosylceramide concentration could be 6-fold the control mean value, there is an overlap between the concentration range of patients (1.08-5.00 mg/ml of plasma) and controls (0.32-1.19mg/ml of plasma) (Table 5-1).

No significantly difference (p=0.981) was observed in glucosylceramide concentration between splenectomised and non-splenectomised GD patients.

Table 5-1

Plasma glucosylceramide concentration (mg/100 ml plasma) of type 1 GD patients. The genotype, clinical severity score (SSI) and the spleen status (patients subjected to splenectomy) are also indicated.

Genotype	Patient	SSI	Splenectomy	Glucosylceramide (mg/100ml plasma)
N370S/N370S	5	5	-	1.28
	6	4	-	1.78
	9	4	-	1.49
	18	7	+	1.25
	21	7	-	2.32
	32	7	-	1.81
	Mean			1.66
	Range			1.25-2.32
N370S/L444P	10	11	-	3.30
	11	11	-	2.68
	23	6	-	1.40
	28	7	-	1.58
	33	19	-	3.18
	34	11	-	2.33
	Mean			2.43
	Range			1.40-3.30
N370S/R463C	1	15	-	4.98
N370S/IVS2+1	7	14	+	2.17
	8	14	+	2.45
	15	5	-	1.14
	Mean			1.92
	Range			1.14-2.45
N370S/?	19	10	-	1.13
	22	8	-	2.65
	24	10	+	2.78
	30	9	+	2.12
	31	14	+	1.81
	35	9	-	1.17
	39	12	-	3.62
	47	9	+	1.08
	Mean			1.82
	Range			1.08-2.78
N370S/Rec TL	12	15	+	3.82
N370S/Rec NciI	36	19	+	3.35
L444P/L444P	41	16	+	1.71
G377S/G377S	29	14	+	2.43
X/X	37	6	-	1.81

Glucosylceramide		Patients	Controls	p
n		29	17	
mean±std		2.21±0.966	0.843±0.292	<0.01
Range		1.08-5.00	0.32-1.19	

Student't test was used to study differences between patients and controls. Probability (p) values above 0.05 are considered non-significant (n.s.).

No statistically significant difference was also observed in plasma glucosylceramide concentration between the different genotype group of patients (Figure 5-4).
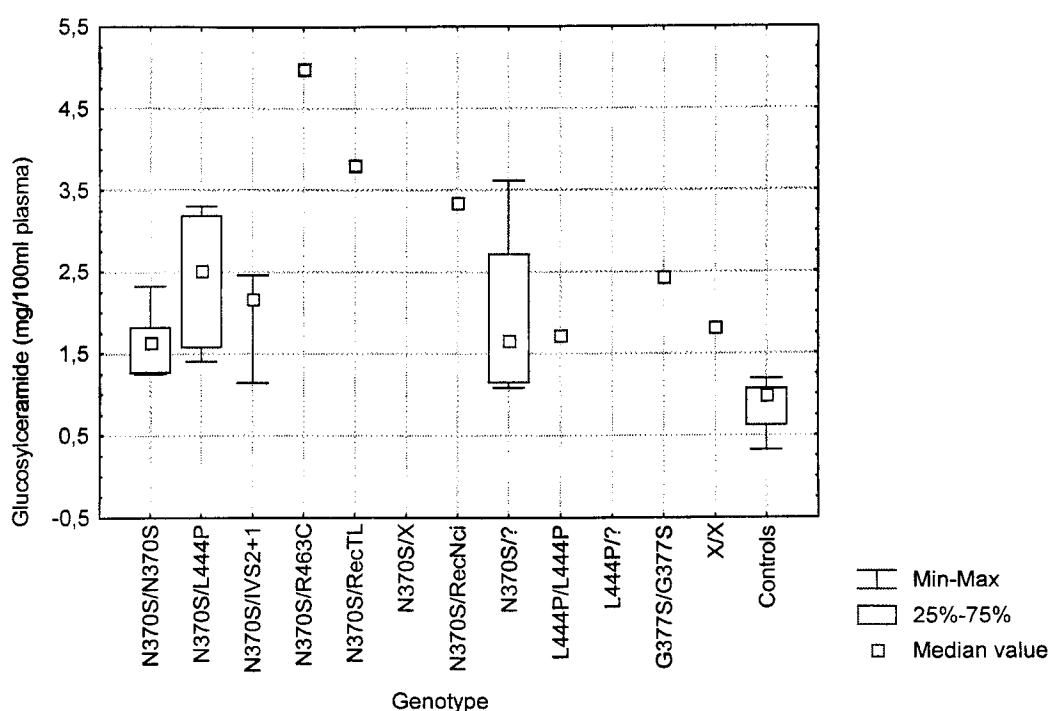
Figure 5-4

Plasma glucosylceramide concentration (mg/100 ml of plasma) of genotyped type 1 GD patients (splenectomized and non-splenectomized).

As it can be observed in Figure 5-5, a significant positive correlation (r=0.60) was observed between plasma glucosylceramide concentration and clinical severity score (SSI) of GD patients (Figure 5-5). As presented in the previous table, within the same genotype group, clinically more severe patients (showing higher SSI) had higher plasma glucosylceramide concentration (Table 5-1). More specifically, patient 15 (N370S/IVS2+1) presented a relatively mild clinical phenotype (SSI of 5) and was clinically asymptomatic until very recently. In contrast, patient 7 and 8 showed about twice the concentration of plasma glucosylceramide and are comparatively clinically more severe (both had an early onset and present a SSI of 14). Patient 12, 36 and 29 present a relatively moderate plasmatic glucosylceramide accumulation and have a moderate to severe SSI (15, 19 and 14 respectively). Within the N370S/L444P genotype, patients 10, 11, 33 and 34 present a higher level of circulating glucosylceramide than patients 23 and 28. Accordingly these two last patients present a moderate clinical phenotype (SSI of respectively 6 and 7) while patient 10, 11, 33 and 34 have a more severe clinical phenotype (SSI of respectively 11, 11, 19 and 11) (Table 5-1).
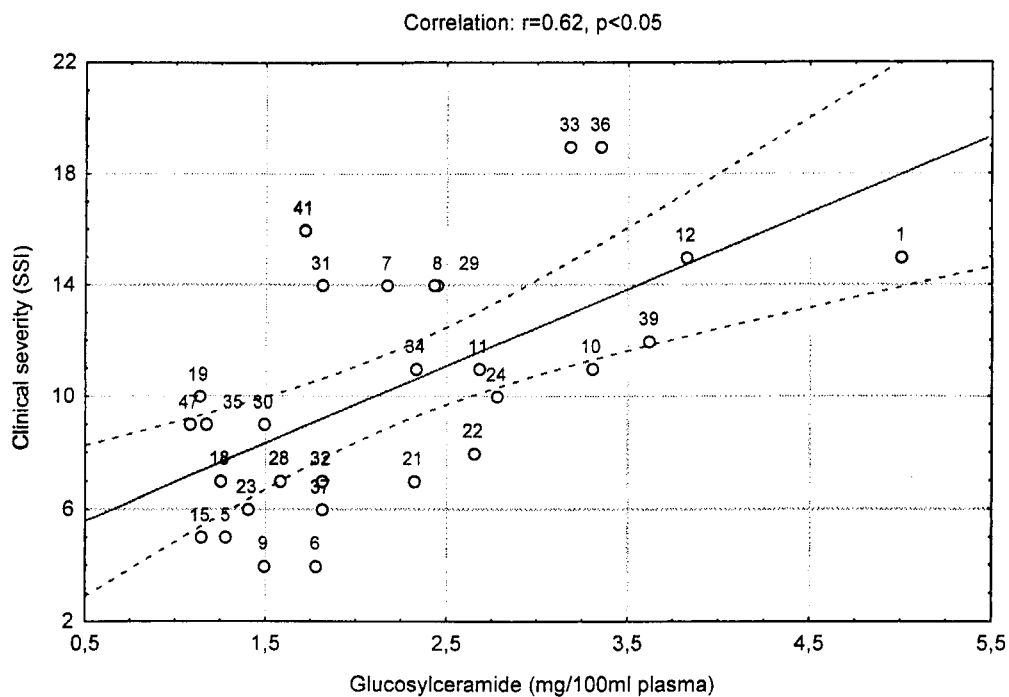
Figure 5-5

Plasma glucosylceramide concentration (mg/100 ml of plasma) and clinical severity (SSI) of type 1 GD patients.

In order to quantify plasma ceramide concentration, the TLC analysis was performed using the chloroform/methanol/NH3 25% (95:5:0.8) development system (Figure 5-6). No significant difference ($p > 0.05$) was observed in ceramide concentration between controls (1.42 ± 1.30, range 0.394-4.95 mg/100ml of plasma, n=12) and GD patients (1.95 ± 1.45, range 0.212-4.68 mg/100ml of plasma, n=20).
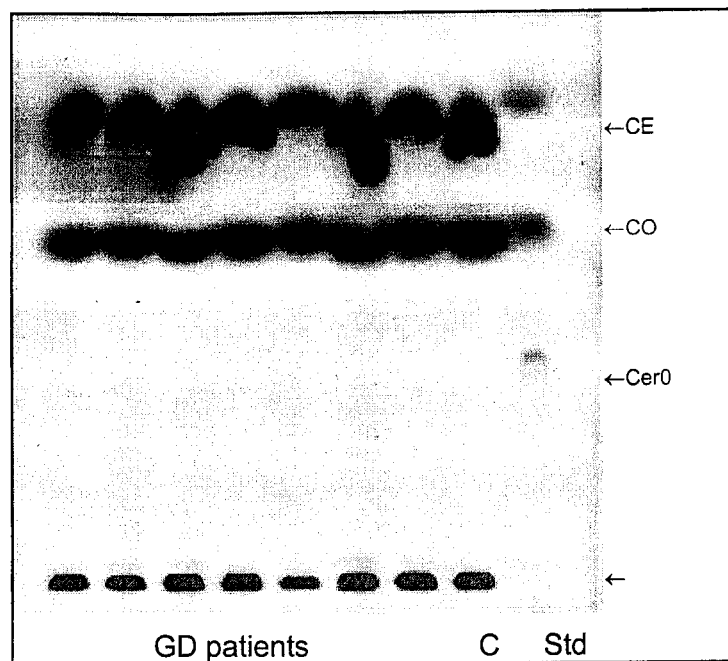
Figure 5-6

TLC profile of ceramide extracted from plasma of type 1 GD patients. To separate cholesterol and ceramide from the neutral lipid fraction of plasma samples the plate was developed with a mixture of chloroform/methanol/NH3 25% (95:5:0.8). Applied standards (std) are colesterol esters (CE), colesterol (CO) and 4 μg of ceramide (Cer0). Triglycerides (TG) migrate with CE. C, control plasma sample.

In order to quantify plasma gangliosides concentration, the TLC analysis was performed using the chloroform/methanol/15mM calcium chloride (55:45:10) development system (Figure 5-7). No significant difference (p>0.05) was observed in GM3 ganglioside concentration between controls (1.19±0.38, range 0.870-1.91 mg/100ml of plasma, n=6) and GD patients (1.48±0.502 range 0.650-2.90 mg/100ml of plasma, n=20).
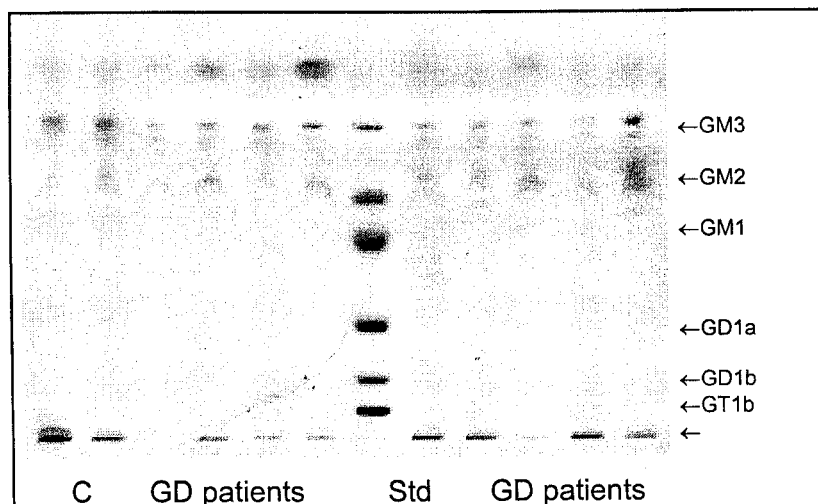

Figure 5-7

TLC profiles of GM3 ganglioside extracted from plasma of type 1 GD patients. To separate different gangliosides from the acidic lipid fraction of plasma samples plates were developed with the

chloroform/methanol/15 mmol/l calcium cloride (55:45:10) system. Applied standards (std) are monosialoganglioside GM3 (GM3), monosialoganglioside GM2 (GM2), monosialoganglioside GM1 (GM1), disialoganglioside GD1a (GD1a), disialoganglioside GD1b (GD1b) and trisialoganglioside GT1b (GT1b). The lipid band that migrates above GM3 ganglioside representes a mixture of acid phosphoglicerides. C, control plasma sample.

5.3.2   Plasma tartrate resistante acid phosphatase (TRAP) activity

Plasma TRAP activity is significantly different ($p < 0.01$) between adult and infantile population. A statistically significant difference ($p < 0.01$) was observed between the activity of TRAP in adult controls (mean=371.4±170.8 nmol/h/ml of plasma, n=68) and GD patients (mean=2459±1727 nmol/h/ml of plasma, n=24). Although the TRAP activity of patients could be increased 22-fold the control mean value, there was an overlap between the range activity from patients (507-7781 nmol/h/ml of plasma) and controls (54.1-968 nmol/h/ml of plasma). There is no overlap between plasma TRAP activity from the 2 infantile patients and the corresponding age matched control range (37.1-1591 nmol/h/ml of plasma) (Table 5-2).

Table 5-2
Plasma tartrate resistant acid phosphatase (TRAP) activity (nmol/h/ml of plasma) of type 1 GD patients.

| | Plasma tartrate resistant acid phosphatase activity (nmol/h/ml) | | | |
| | Adults (age >12 years) | | Infants (age ≤12 years) | |
	Patients	Controls	Patients	Controls
n	26	68	2	76
Mean±std	2367±1697	371.4±170.8	2586±21.21	636.5±316.0
Range	507-7781	54.1-968	2571-2601	37.1-1591
p	<0.01		<0.01	

Student`t test was used to study differences between patients and controls. Probability (p) values above 0.05 are considered non-significant (n.s.).

The analysis of plasma TRAP activity for each genotype group showed that the activity presented by the N370S homozygote group (1121±486.3, n=7) (being all of them adults) is significantly lower ($p < 0.05$) than that presented by the remaining adults patients (2886±1759, n=19). Moreover, the overlap between plasma TRAP activity of GD patients and the control range was specially observed for the N370S homozygotes (Figure 5-8).
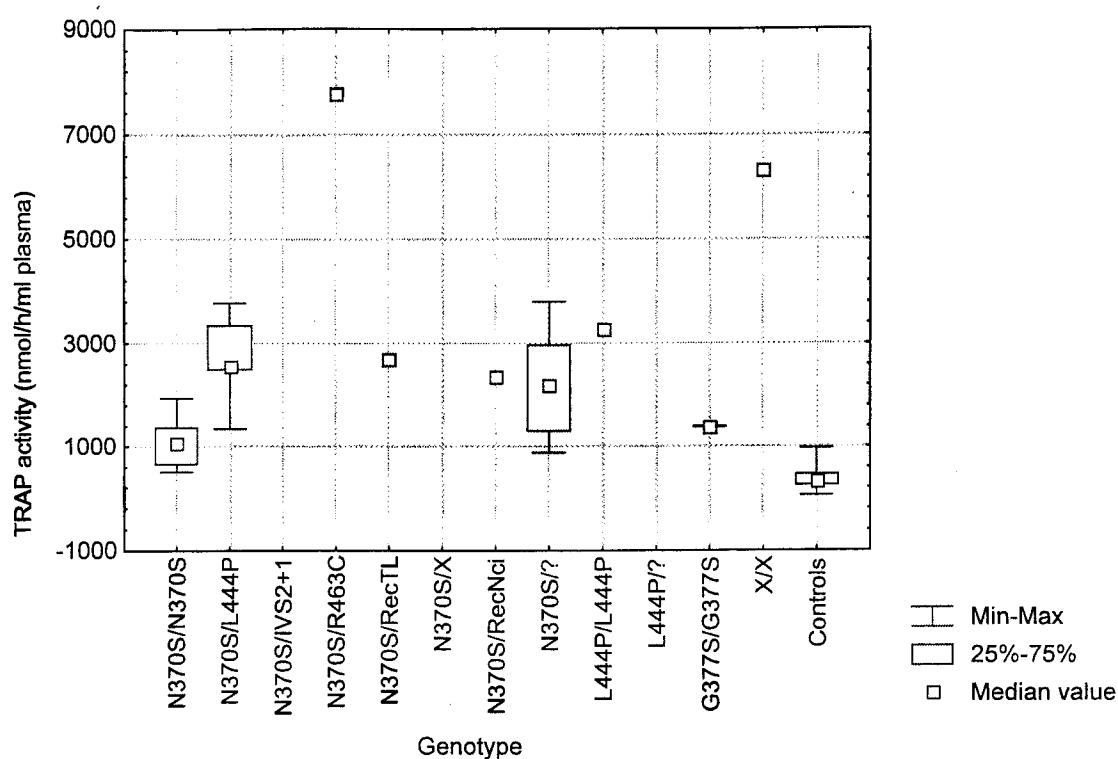
Figure 5-8

Plasma tartrate resistant acid phosphatase (TRAP) activity (nmol/h/ml of plasma) of genotyped type 1 GD patients.

No significant correlation (r=0.28) was obtained between plasma TRAP activity and clinical severity score (SSI) of adult GD patients (Figure 5-9). However normal activity values were observed in patients without skeletal disease (patients 18, 21, 32, 38). Exceptions were patients 30 and 49, who present abnormal TRAP activity values without imagiological (by Rx and RMN) signs of bone disease.
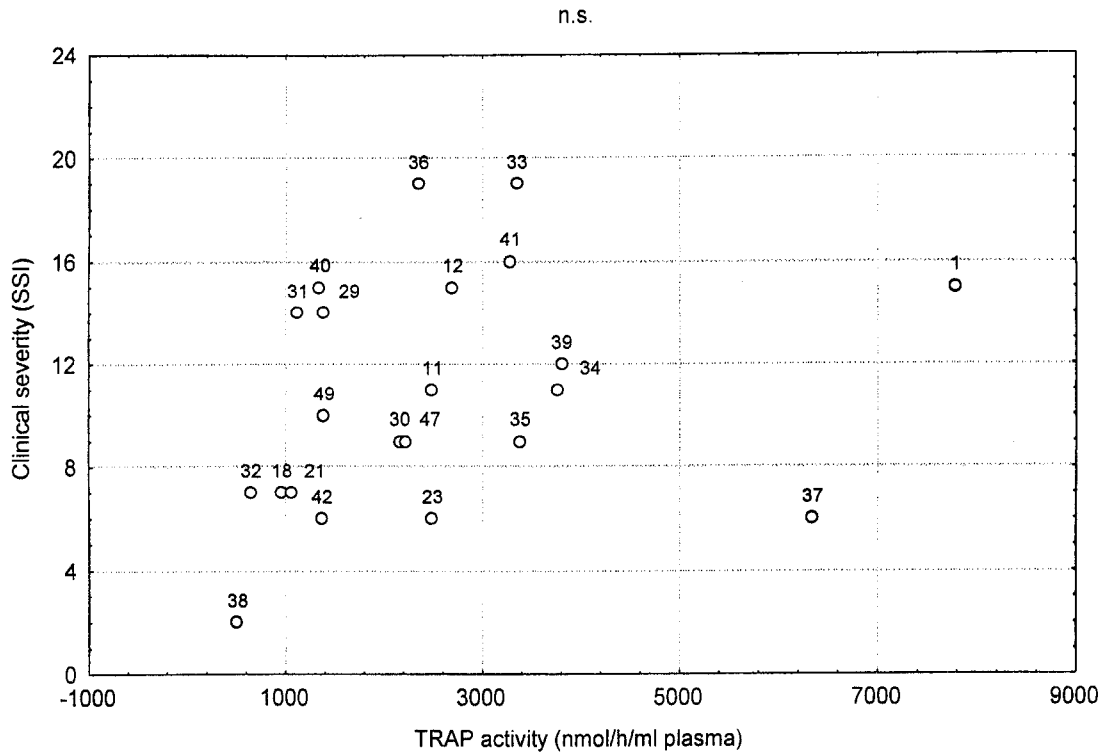
Figure 5-9
Plasma tartrate resistent acid phosphatase (TRAP) activity (nmol/h/ml of plasma) and clinical severity (SSI) of type 1 GD patients.

5.3.3 The β-Hexosaminidase activity

5.3.3.1 The β-hexosaminidase activity in plasma

A statistically significant difference ($p<0.01$) was observed between total β-hexosaminidase activity in controls (657 ± 250 nmol/h/ml of plasma, n=821) and GD patients (1819 ± 1411 nmol/h/ml of plasma, n=36). Although β-hexosaminidase activity of patients could be increased 9-fold the control mean value, there was an overlap between the activity range from patients (187-7102 nmol/h/ml of plasma) and controls (140-1434 nmol/h/ml of plasma) (Table 5-3).

Similar results were observed with respect to β-hexosaminidase A isoenzyme activity. Although the activity of patients could be increased 2-fold the control mean value, there was an overlap between the activity range from patients (78.0-610 nmol/h/ml of plasma) and controls (28.45-351 nmol/h/ml of plasma) (Table 5-3).

Table 5-3

Plasma total β-hexosaminidase and β-hexosaminidase A activity (nmol/h/ml of plasma) of type 1 GD patients.

	Plasma β-hexosaminidase activity (nmol/h/ml)			
	Total β-hexosaminidase		β-hexosaminidase A	
	Patients	Controls	Patients	Controls
n	36	821	31	394
mean±std	1819±1411	657.1±250.3	244.1±132.9	111.3±57.79
range	187-7102	140.4-1434	78.0-610	28.45-351
p	<0.01		<0.01	

Student't test was used to study differences between patients and controls. Probability (p) values above 0.05 are considered non-significant (n.s.).

No statistically significant difference was observed in plasma β-hexosaminidase activity between the different genotype groups. The overlap between plasma β-hexosaminidase activity of GD patients and the control range was observed for several genotype groups (Figure 5-10).

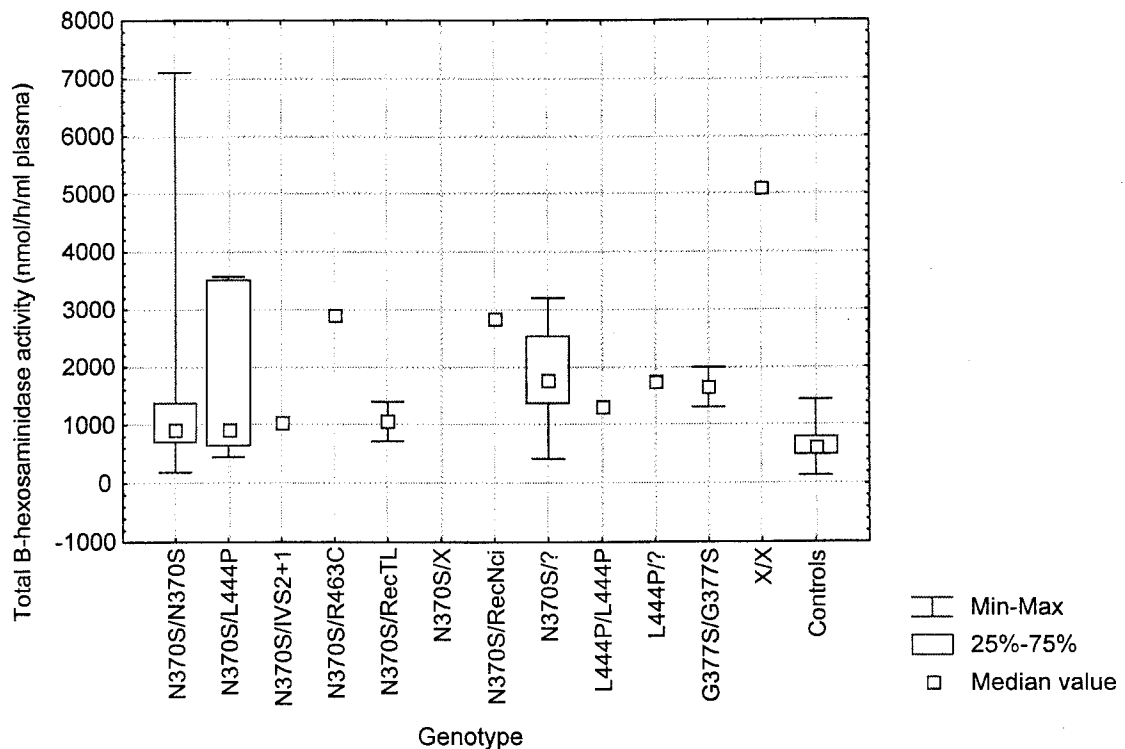**Figure 5-10**

Plasma total β-hexosaminidase activity of genotyped type 1 GD patients.

No significant correlation (r=0.005) was obtained between plasma total β-hexosaminidase activity and clinical severity score (SSI) of GD patients (Figure 5-11).
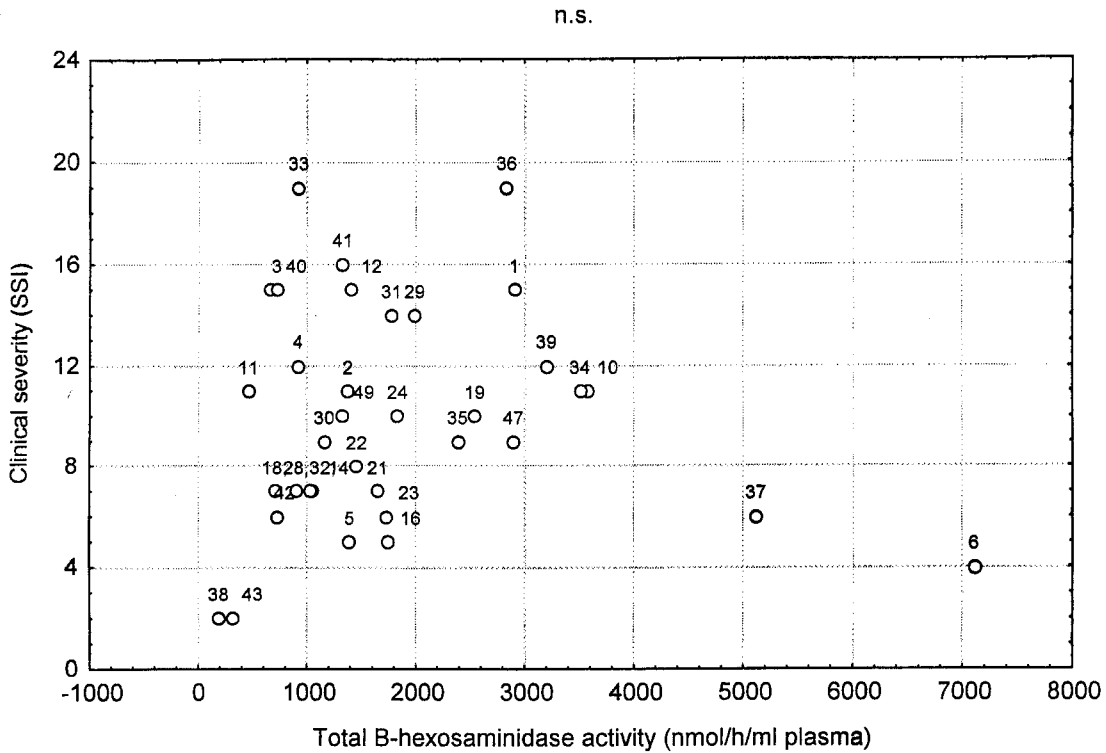
Figure 5-11

Plasma total β-hexosaminidase activity (nmol/h/ml of plasma) and clinical severity (SSI) of type 1 GD patients.

5.3.3.2 The β-Hexosaminidase activity in spleen

In spleen extracts from patients presenting different types of GD, the activity of total β-hexosaminidase was found to be increased 3-17 fold (Table 5-4). In addition to the deficiency in glucocerebrosidase activity, in all types of GD it can also be observed a 2 to 4-fold increase in β-glucuronidase, α-fucosidase and acid phosphatase. The activity of sphingomyelinase and α-mannosidase is however slightly decreased.

Table 5-4

Total β-hexosaminidase and other lysosomal enzymes activity (nmol/h/mmg of protein) from spleen of GD patients. Studied spleens were from two type 1 GD patients (being one of them from Ashkenazi Jewish ancestry, A) a type 2 and a type 3 GD patient. Glucocerebrosidase was measured in the presence of sodium taurocholate, at pH 5.2.

Enzyme	Patient's spleens								Control's spleens	
	Activity (nmol/h/mg of protein)				% of control mean				Activity (nmol/h/mg of protein)	
	1	1(A)	2	3	1	1(A)	2	3	Mean (n=2)	Range
Total β-Hexosaminidase	18 480	31 248	10 004	54 677	563	963	305	1 667	3 280	1 781-4 780
Glucocerebrosidase	1.68	0.57	1.17	1.77	8.4	2.8	5.8	8.8	20.0	18.6-21.4
β-Glucuronidase	557	373	518	590	366	245	341	388	152	114-190
α-Fucosidase	830	597	376	596	449	323	203	322	185	159-212
β-Galactosidase	172	67.9	113	156	73	29	48	67	234	223-246
α-Galactosidase	40.2	22.3	44.4	43.3	93	52	103	100	43.3	33.9-52.7
Acid phosphatase	9 982	7 124	3 916	12 596	192	137	75.5	243	5 185	3 713-6 657
Sphingomyelinase	14	7.7	7.5	8.1	93	51	50	54	17.5	15-20
α-Mannosidase	101	56.5	103	94.1	71	39	72	66	143	127-159

Cellulose acetate gel electrophoresis of β-hexosaminidase activity extracted from spleen showed that in contrast to controls, GD patients present a major form of β-hexosaminidase B activity (Figure 5-12).
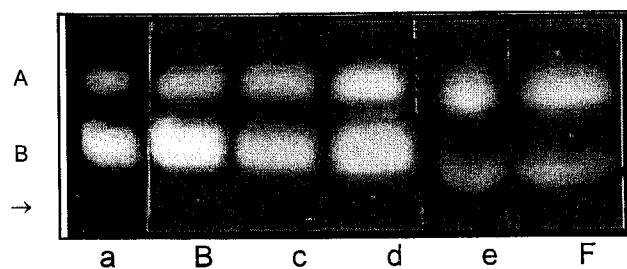

Figure 5-12

Cellulose-acetate gel electrophoretic pattern of total β-hexosaminidase activity extracted from spleen of GD patients. Studied spleens were from patients presenting different clinical types. Lane a, type 1 GD patient (N370S/?); lane b, type 1 GD Ashkenazi patient (N370S/84GG); lane c, type 2 GD patient (L444P/?); lane d, type 3 GD patient (L444P/?); lane e, and f controls. All samples contained 10µg of protein.

The quantification of β-hexosaminidase isoenzymes was done after isoenzymes separation by ion exchange DEAE-cellulose chromatography (Figure 5-13). Recovered fractions were identified by termostability test at 50°C (Table 5-5) and migration pattern on cellulose acetate electrophoresis. β-Hexosaminidase activity contained in the low salt eluate was heat-resistant (and migrates as β-hexosaminidase B of the total applied extract) whereas that in the high salt was heat-labile (and migrates as β-hexosaminidase A of total extract). The recovery of total hexosaminidase activity from the ion exchange column was greater than 90% and the ratio of the β-hexosaminidase A to that of the β-hexosaminidase B in control spleen was on average 3. Similar chromatographic elution patterns were obtained with spleen from GD patients, with the exception that a larger proportion of the total hexosaminidase activity appeared in fractions corresponding to hexosaminidase B (Figure 5-13).
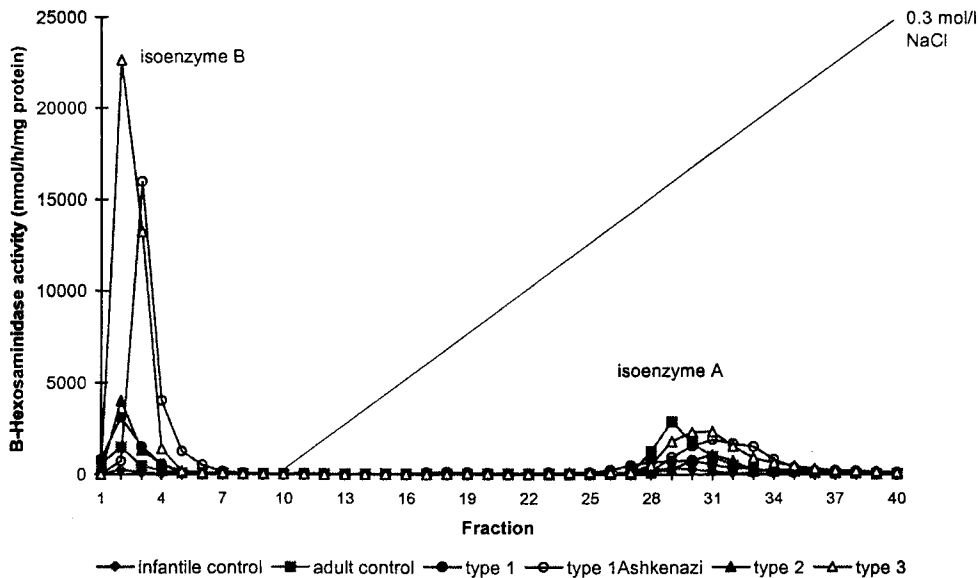

Figure 5-13
Ion-exchange chromatographic profile of β-hexosaminidase activity from spleens of controls, type 1, type 1 Ashkenazi, type 2 and type 3 Gaucher disease patients.

The β-hexosaminidase A/B ratio extracted from spleens of GD patients was on average 0.38 (range 0.28-0.47). In GD patients, β-hexosaminidase B activity was found to be increased 5 to 24-fold whereas hexosaminidase A activity was only 2 times increased (only in the type 3 and type 1 Ashkenazi patient) (Table 5-5).

Table 5-5

The β-hexosaminidase A and B isoenzymes activity and the percentage of termostable activity extracted from spleens of type 1, 2 and 3 GD patients.

| Spleens | β-Hexosaminidase | | | | A/B |
| | % Termostable activity at 50°C | | Activity (nmol/h/mg protein) | | |
	Isoenzyme A	Isoenzyme B	Isoenzyme A	Isoenzyme B	
Control	11	91	4690	1566	3.0
type 1 patient	1.5	75	3813	8039	0.47
type 1 Ashk.	35	85	8666	22582	0.38
type 2	18	87	4298	11074	0.39
type 3	14	89	10434	37243	0.28

5.3.4   Plasma chitotriosidase activity

Chitotriosidase activity was studied in a total of 345 individuals, including subjects without a diagnosis for any of the tested lysosomal storage diseases, type 1 GD patients, GD obligate carriers, GD putative carriers for unkown mutations, patients presenting other lysosomal storage diseases than GD and finally patients presenting Idiopathic Hemochromatosis.

Subjects without diagnosis (n=229) presented a plasma chitotriosidase activity in the range of 1.00-413 nmol/h/ml (median=34.5 nmol/h/ml). Type 1 GD patients (n=29) presented a marked elevation of plasma chitotriosidase activity but ranged from 1.50 to 68 394 nmol/h/ml (median=13 169 nmol/h/ml). GD obligate carriers (n=6) presented a plasma chitotriosidase activity in the range of 2.59-79.1 nmol/h/ml (median=43.8 nmol/h/ml); similarly GD putative carriers for unidentified mutations (n=11) had an activity in the range of 19.00-149.2 nmol/h/ml (median=58.10 nmol/h/ml). Idiopathic Hemochromatosis patients (n=23) presented a plasma chitotriosidase activity in the range of 1.128-569.8 nmol/h/ml (median=70.26 nmol/h/ml) (Table 5-6).

Table 5-6

Plasma chitotriosidase activity of patients with and without lysosomal storage disorders.

	Chitotriosidase activity (nmol/h/mnl plasma)		
	Median	Range	n
Subjects without diagnosis	34.5	1.00-413	229
Type 1 GD patients	13 169	1.50-68 394	29
GD obligate carriers	43.8	2.59-79.1	6
GD putative carriers for unknown mutations	58.10	19.00-149.2	11
Patients with a lysosomal storage disorder	53.34	11.43-1216	23
Idiopathic Hemochromatosis patients	70.26	1.128-569.8	23

The logarithm (log 10) of chitotriosidase activity value was used instead of the observed values, since the distribution of the values after this transformation is approximately normal (Kolmogorov-Smirnov test, p=0.66), allowing to analyse interferences regarding extreme values and outliers. The histogram of log 10 of chitotriosidase activity of all cases allows the perception of extreme values, on both tails of the distribution. GD patients distribute above de 3.00 class of log 10 of chitotriosidase activity (Figure 5-14).

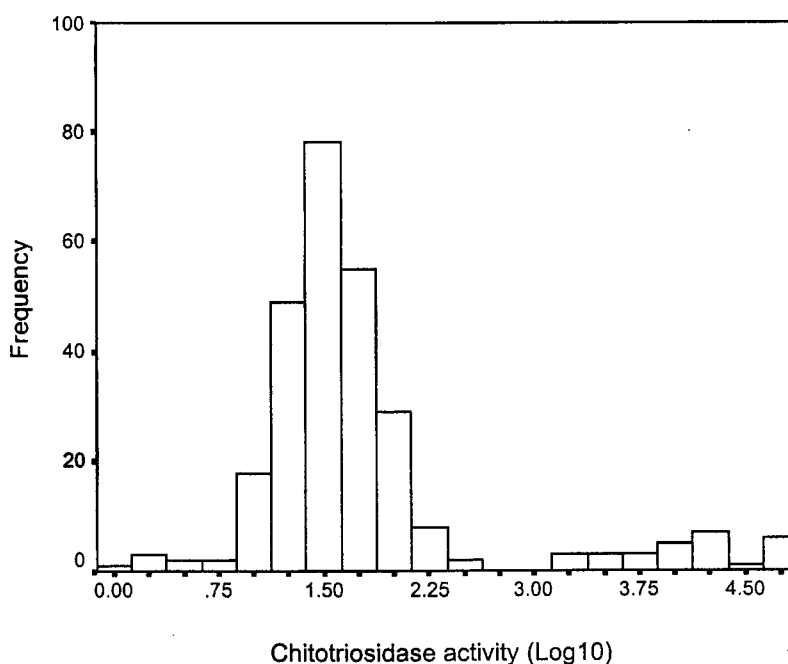
Chitotriosidase activity (Log10)

Figure 5-14

Histogram of plasma chitotriosidase activity (log 10) of type 1 GD patients, obligate and putative carriers (relatives) of GD families, and controls. The X axis shows the logarithm (base 10) of chitotriosidase activity.

The calculated mean and standard deviation for all the 229 control cases (individuals without diagnosis) was respectively 1.516±0.352. In Figure 5-15 it can be observed that there is also no overlap in plasma chitotriosidase activity between GD patients, GD carriers, putative carriers and controls (individuals without diagnosis).
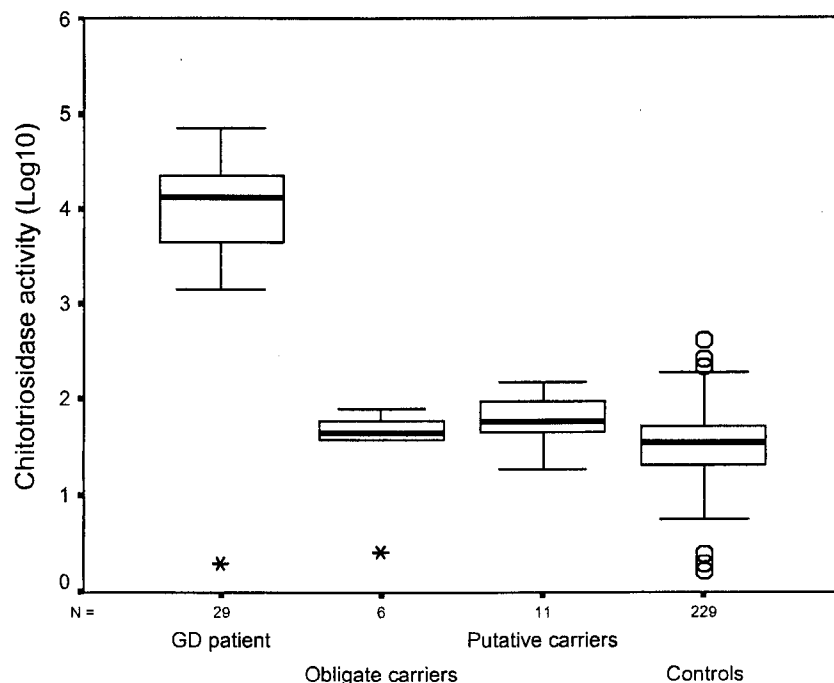

Figure 5-15

Plasma chitotriosidase activity (log 10) of type 1 GD patients, obligate carriers, putative carriers of affected GD families. The Y axis represents the logarithm (base 10) of chitotriosidase activity. For each status median values are plotted within boxes limited by the 1st and 3rd quartil. Smallest and greatest values are also indicated outside boxes. Outlier values are indicated by symbols.

One possible way to deal with the extreme values is to consider them as outliers, i.e., values that occur so far, without a biological explanation and that lay far away from the centre of the distribution. The criteria was to exclude all cases that lie away from the mean three standard deviations, i.e., cases below 0.462 and above 2.571; according to this criteria 9 cases were discarded. Independently of these outlyer values, GD patients are clearly distinguished from other lysosomal or non-lysosomal diseases by presenting a marked elevation of plasma chitotriosidase activity without overlap (Figure 5-16).
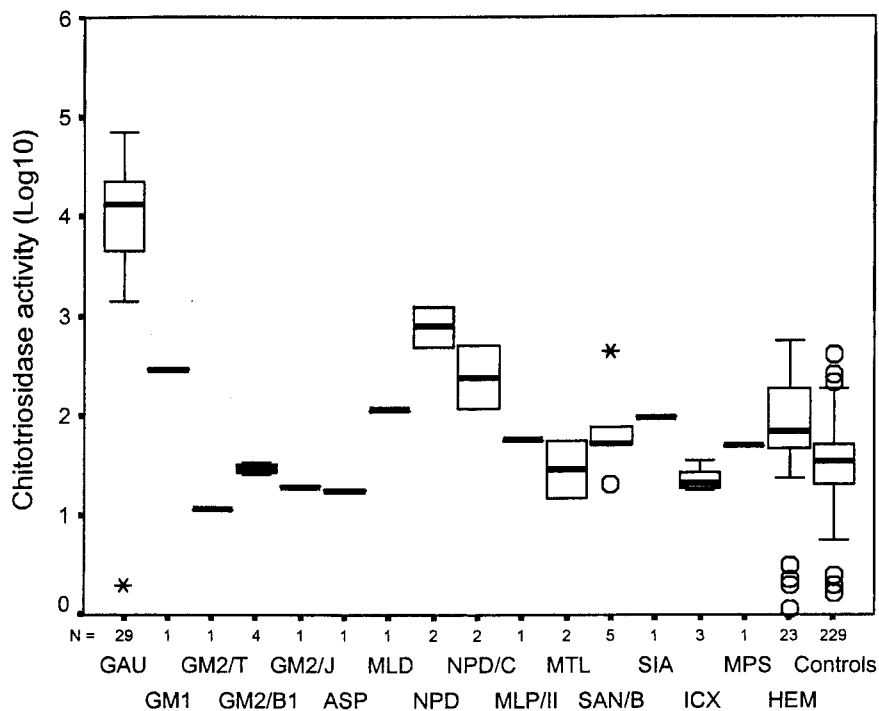
Figure 5-16

Plasma chitotriosidase activity (log 10) of patients with hemochromatosis (HEM), X-linked ictiosis (ICX) and different lysosomal storage diseases. Gaucher disease (GAU), GM1 Gangliosidosis (GM1), Tay-Sachs disease (GM2/T), B1 variant of GM2 Gangliosidosis (GM2/B1), juvenile form of GM2 Gangliosidosis (GM2/J), Aspartylglucosaminure (ASP), Metachromatic leucodistrophy (MLD), Niemman-Pick (type A and B), Niemman-Pick type C (NPD/C), mucolipidosis type 2 (MLP/II), Maroteaux-Lamy (MTL), Sanphilippo type B (SAN/B), Sialidosis (SIA), and mucopolysacharidosis (MPS). The Y axis represents the logarithm (base 10) of chitotriosidase activity. For each status median values are plotted within boxes limited by the 1st and 3rd quartile. Smallest and greatest values are also indicated outside boxes. Outlier values are indicated by symbols.

The new calculated logarithm mean and standard deviation were 1.534 ± 0.300, and the 95% confidence limits of 0.947-2.122, allow the conclusion that approximately 3% of the cases do lie outside these limits (being 2% below limits). All together, independently of status with respect to GD or any other studied disease, according to that reported (Hollak et al, 1994), cases that lie below the limits seem to present the inherited deficient of chitotriosidase activity. In fact, patient 33 was found to present this inherited chitotriosidase deficiency (chitotriosidase genotype analysis performed by the laboratory of Prof. J. Aerts). The non-logarithm mean, standard deviation and range calculated without the chitotriosidase deficient is presented in Table 5-7.

Table 5-7

Plasma chitotriosidase activity of type 1 GD patients.

| | Plasma chitotriosidase activity (nmol/h/ml) | |
	Patients	Controls
N	40	818
mean±std	15 815±16 439	52.83±67.39
Range	* 1 336-68 394	* 8.85-132

* Chitotriosidase deficient individuals were excluded.

The analysis of chitotriosidase activity for each genotype group showed that the activity presented by the N370S homozygotes patients (3 751±2 807, n=11) is significantly lower (p<0.01) than that presented by the remaining patients (21 507±18 208, n=29) (Figure 5-17). Patient 33 did not have chitotriosidase activity and presented the inherited deficiency in the chitotriosidase gene (personal communication of J. Aerts).
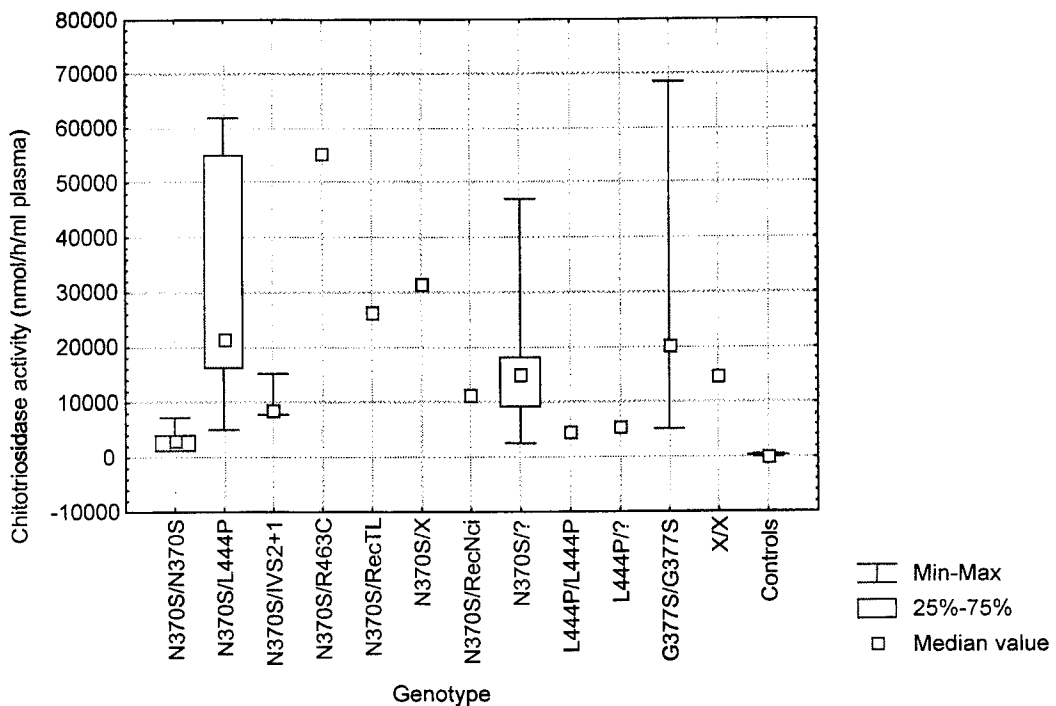

Figure 5-17

Chitotriosidase activity of genotyped type 1 GD patients. Considered control range (8.85-132 nmol/h/ml of plasma) is not presented in the graph.

No significant correlation (r=0.15) was observed between plasma chitotriosidase activity and clinical severity score (SSI) (Figure 5-18).
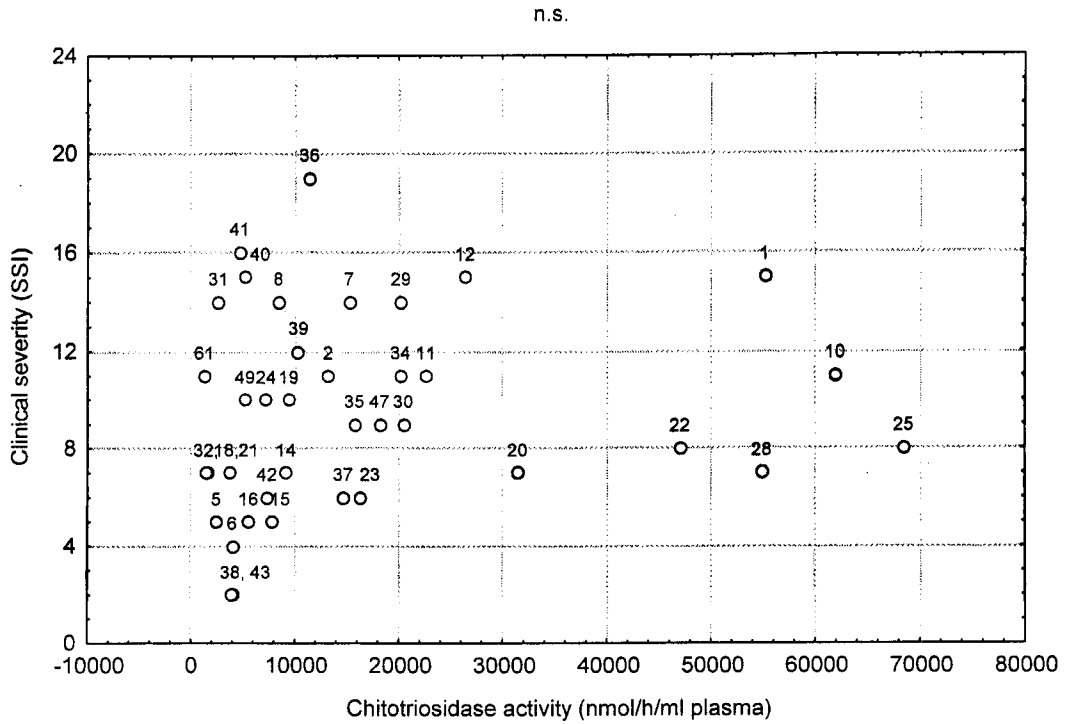
Figure 5-18

Plasma chitotriosidase activity (nmol/h/ml of plasma) and clinical severity (SSI) of type 1 GD patients.

5.3.5 Serum ferritin

When compared to the sex and age matched control range, 25 out of the 30 studied GD patients presented serum ferritin values abnormally high (7-fold maximum). Decreased serum iron and transferrin saturation were observed in 7 patients, 4 of them in association to the abnormal serum ferritin. Two of these were infantile patients, one of them (patient 19) presenting also abnormal serum ferritin (Table 5-8).

Table 5-8

Serum ferritin (ng/ml), transferrin saturation, TS, (%) and serum iron (μg/dl) presented by type 1 GD patients.

Patient	Sex	Age	Genotype	SSI	Splenectomy	Iron (μg/dl)	TS (%)	Serum ferritin
29	M	43	G377S/G377S	14	+1962	113	37	**503**
49	M	49	G377S/G377S	10	-	136	45	**1609**
41	M	17	L444P/L444P	16	+1993	106	35	**441**
19	F	10	N370S/?	10	-	59	18	**319**
24	M	23	N370S/?	10	+	111	44	**835**
30	M	36	N370S/?	9	+1994	96	36	**2714 e)**
31	F	45	N370S/?	14	+1994	84	25	**682**
35	F	27	N370S/?	9	-	83	41	**445 e)**
46 a)	F	23	N370S/?	10	-	48	15	117
47	F	29	N370S/?	9	+1982	123	44	**950**
7 b)	F	21	N370S/IVS2+1	14	+1981	49	**14**	**238**
8 b)	F	44	N370S/IVS2+1	14	+1969	97	29	**1163**
14 b)	F	37	N370S/IVS2+1	7	-	50	18	**359**
34	F	32	N370S/L444P	11	-	104	39	**321 e)**
23	M	30	N370S/L444P	6	-	81	27	**647**
40	M	45	N370S/L444P	15	-	91	30	**637**
10 c)	F	13	N370S/L444P	11	-	**40**	**14**	95 a)e)
11 c)	M	17	N370S/L444P	11	-	69	25	89 a)e)
33	F	25	N370S/L444P	19	-	76	28	**387**
38	F	31	N370S/N370S	2	-	116	47	**1888**
39	M	18	N370S/N370S	12	-	70	23	**710**
32 d)	M	54	N370S/N370S	7	-	57	19	340
18	F	51	N370S/N370S	7	+1993	149	61	261
21 d)	F	52	N370S/N370S	7	-	79	44	**761**
5	F	53	N370S/N370S	5	-	**16**	**5**	**485**
43	F	30	N370S/N370S	2	-	65	25	**899**
1	M	23	N370S/R463C	15	-	93	28	**1892**
36	M	32	N370S/recNcil	19	+1977	78	NA	**1389**
12	F	58	N370S/recTL	15	+1939	70	25	**1273**
20	F	26	N370S/X	5	-	92	26	**579**
37	F	37	X/X	6	-	78	35	**262**
Control range	Male					50-181	19-62%	e)
	Female					44-160	15-59%	

Abnormal values were presented at bold.

a, b, c) and d) indicate related patients from different families.

e) For these patients there were not available serum ferritin values before therapy.

f) Serum ferritin is sex and age dependent (Porto et al 1992) and considered reference values for the Portuguese population were presented in appendix section.

No statistically significant difference was observed in serum ferritin between the different genotype groups (Figure 5-19). Patients without abnormal serum ferritin were representative of the 3 major genotype groups, being namely 1 N370S/? juvenile

patient (patient 46), 2 N370S/L444P infantile patients (patient 10 and 11) and 2 N370S homozygous adult unrelated patients (patients 31 and 32) (Figure 5-19).
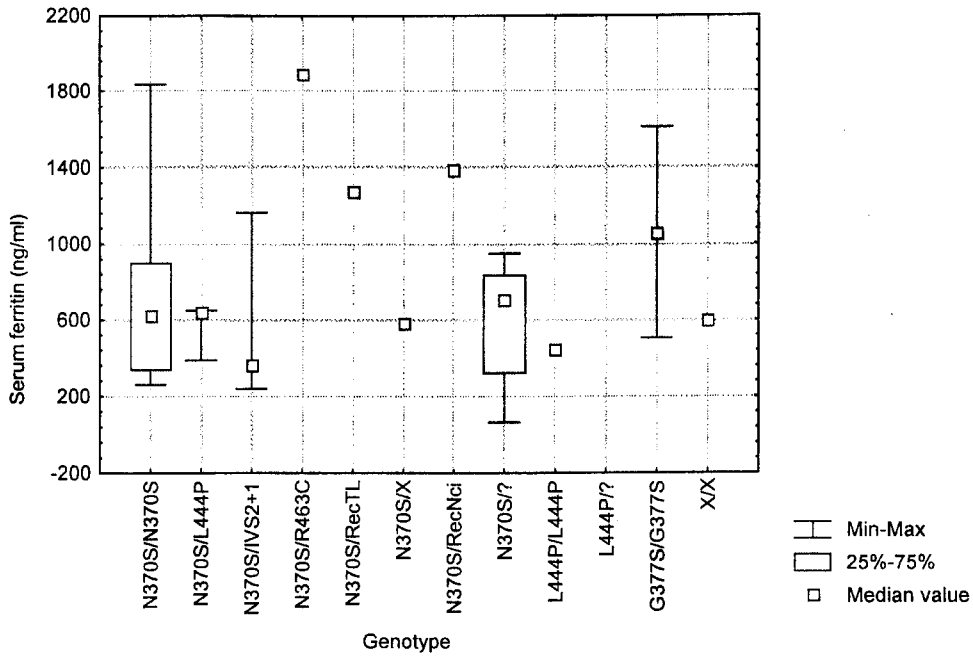

Figure 5-19

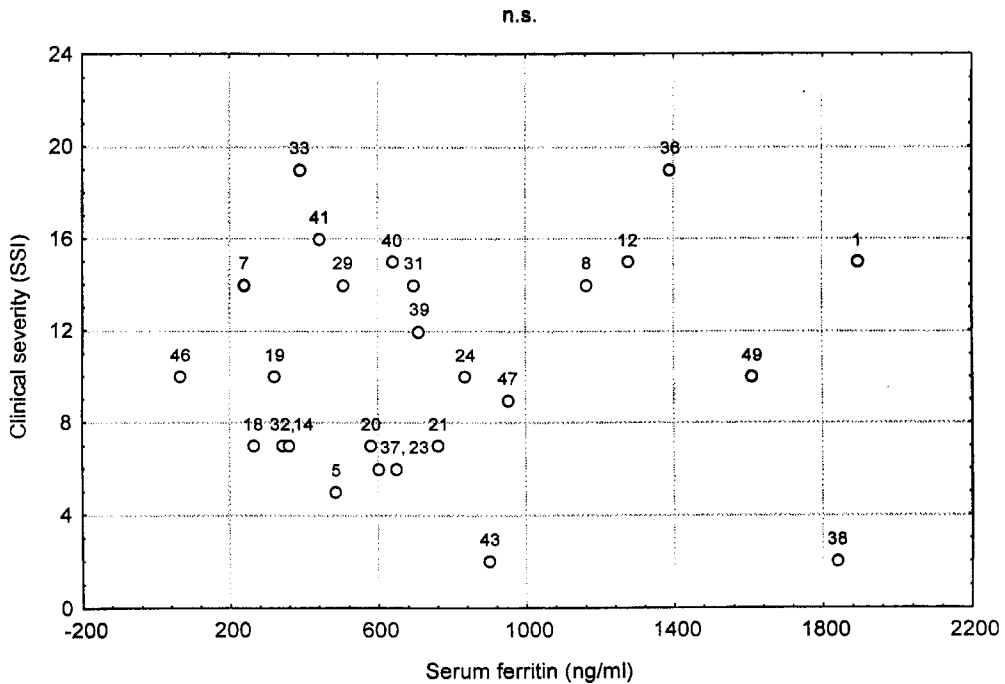**Serum ferritin of genotyped type 1 GD patients.**

Figure 5-20

Serum ferritin (ng/ml of serum) and clinical severity (SSI) of type 1 GD patients.

No significant correlation was also observed between serum ferritin level and clinical severity (SSI) of GD patients (Figure 5-20).

5.3.6 Peripheral blood CD4$^+$ and CD8$^+$ T lymphocyte subsets

The study of the peripheral blood T lymphocytes showed that GD patients presented significantly ($p<0.05$) decreased percentage of the CD4$^+$ T lymphocyte subset (%CD4$^+$) and %CD4$^+$/%CD8$^+$ T cell ratio (Table 5-9).

Table 5-9

Peripheral blood %CD4$^+$ and %CD8$^+$ T lymphocyte subsets of type 1 GD patients.

	Patients mean±std (n)	Controls mean±std (n)	p
%CD4$^+$	39.64±9.05 (28)	45.15±7.40 (67)	0.02
%CD8$^+$	26.80±7.10 (28)	24.58±7.25 (71)	n.s.
%CD4$^+$/%CD8$^+$	1.58±0.533 (28)	2.01±0.834 (67)	0.01

Values represent the mean±sd of the percentage of each population gated on total lymphocytes. The number of analysed individuals is indicated between brackets. Student't test was used to study differences between patients and controls. Probability (p) values above 0.05 are considered non-significant (n.s.).

The analysis of T lymphocyte %CD4$^+$/%CD8$^+$ ratio by genotype showed that when compared to the other patients, the N370S homozygotes present relatively higher ratio (Figure 5-21).
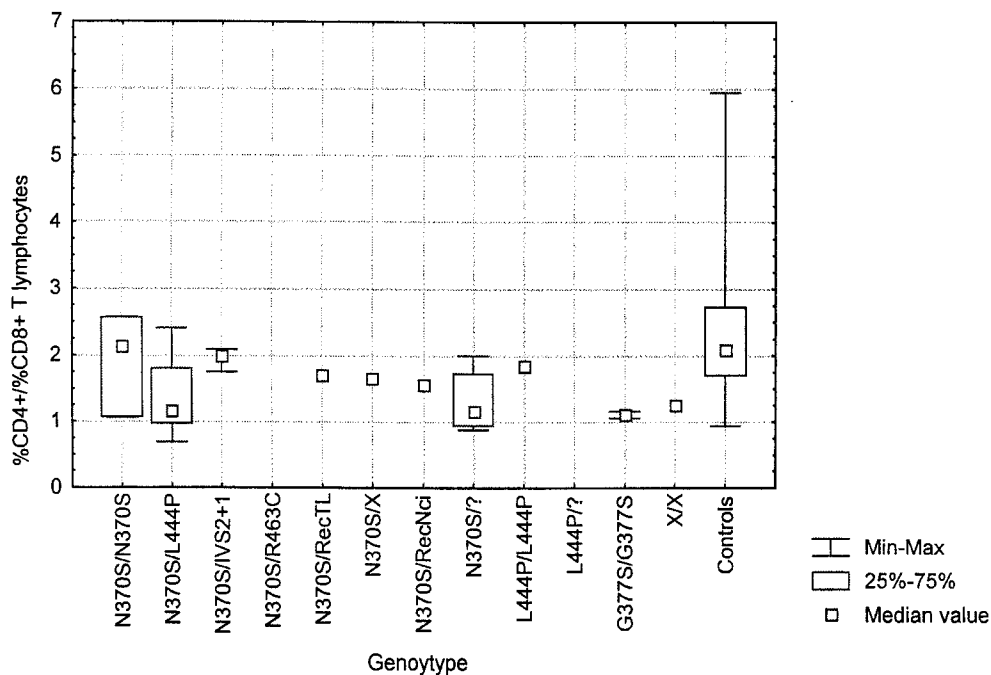

Figure 5-21

Peripheral blood T lymphocyte %CD4$^+$ and %CD8$^+$ subsets ratio of genotyped type 1 GD patients.

There was no significant correlation (r=-0.29) between the %CD4$^+$/%CD8$^+$ ratio and the clinical severity (SSI) of patients (Figure 5-22).
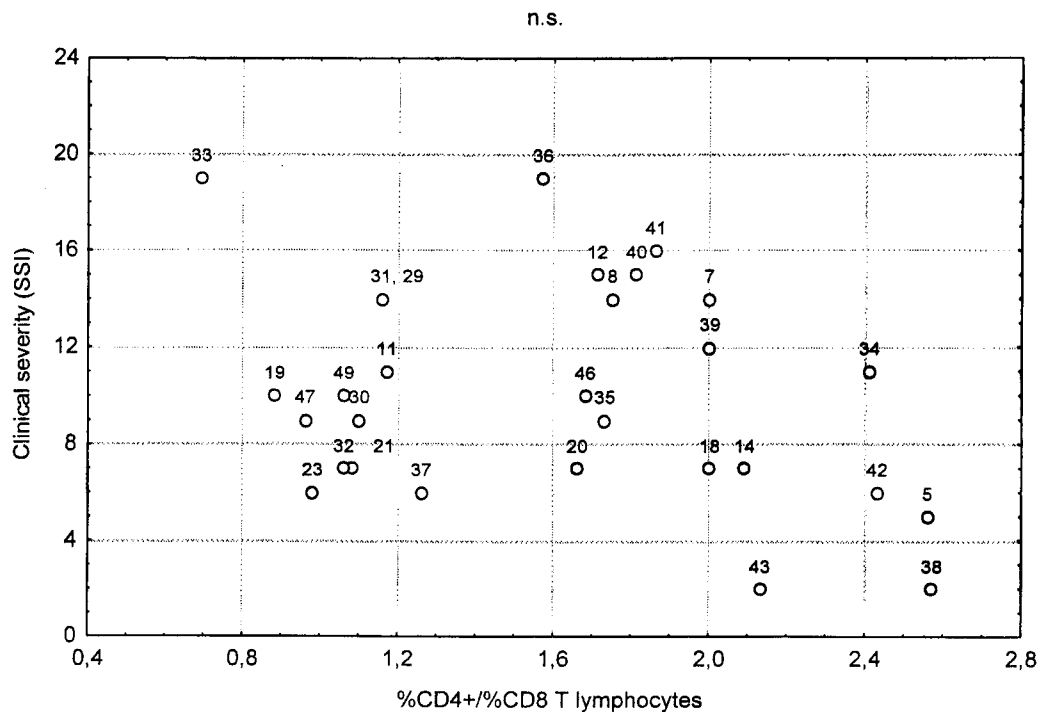

Figure 5-22

Ratio of peripheral blood %CD4$^+$ and %CD8$^+$ T lymphocyte subsets and clinical severity (SSI) of type 1 GD patients.

Statistical analysis showed that the %CD4$^+$ and %CD8$^+$ T lymphocyte subsets in the N370S homozygous patients were not significantly different from controls (Table 5-10). The statistically significant decreased %CD4$^+$ T lymphocytes was observed in patients presenting the remaining genotypes.

Table 5-10

Peripheral blood %CD4$^+$ and %CD8$^+$ T lymphocyte subsets of N370S homozygous type 1 GD patients.

	N370S homozygotes Mean±std (n)	p	Controls Mean±std (n)	p	Other genotypes than N370S homozygotes Mean±std (n)
%CD4$^+$	44.88±8.29 (8)	n.s.	45.15±7.40 (67)	<0.01	**37.54±8.65 (20)**
%CD8$^+$	24.38±6.07 (8)	n.s.	24.58±7.25 (71)	n.s	27.76±7.39 (20)
%CD4$^+$/%CD8$^+$	1.94±0.543 (8)	n.s.	2.01±0.834 (67)	<0.01	**1.43±0.464 (20)**

Values represent the mean±sd of the percentage of each population gated on total lymphocytes. The number of analysed individuals is indicated between brackets. Student't test was used to study differences between patients and controls. Probability (p) values above 0.05 are considered non-significant (n.s.).

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The analysis of results considering two groups of patients, the splenectomized and non-splenectomized, showed that the %CD4$^+$ T lymphocytes was significantly decreased in splenectomized patients. In the non-splenectomized patients the low CD4$^+$/CD8$^+$ was due to the increased circulating population of the CD8 T$^+$ cells (Table 5-11).

Table 5-11

Peripheral blood %CD4$^+$ and %CD8$^+$ T lymphocyte subsets in splenectomised and non-splenectomised type 1 GD patients.

	Non-splenectomised		Controls		Splenectomised
	Mean±std (n)	p	Mean±std (n)	p	Mean±std (n)
%CD4$^+$	41.80±7.59 (16)	n.s.	45.15±7.40 (67)	<0.01	33.40±7.12 (11)
%CD8$^+$	29.33±8.01 (16)	p<0.05	24.58±7.25 (71)	n.s.	23.40±4.22 (11)
%CD4$^+$/%CD8$^+$	1.56±0.573 (16)	p<0.05	2.01±0.834 (67)	a) n.s.	1.47±0.423 (11)

a) Almost significant.

Values represent the mean±std of the percentage of each population gated on total lymphocytes. The number of analysed individuals is indicated between brackets. Student't test was used to study differences between patients and controls. Probability (p) values above 0.05 are considered non-significant (n.s.).

The study of the peripheral blood total leukocytes showed that the large majority of non-splenectomized GD patients presented leukopenia (3 995 ± 1 721 cells x10^6/l, n=20) whereas the splenectomized ones presented in general leukocytosis (13 240 ± 4 901, n=10, p<0.01).

Non-splenectomised patients where analysed separately and no statistically significant differences were observed in the CD4$^+$ and CD8$^+$ T lymphocytes (cells x10^6/l) between the different genotype groups of the non-splenectomized GD patients (Figure 5-23). However, a significant negative correlation (r=-0.64) was observed between the CD4$^+$ T lymphocytes (cells x10^6/l) and the clinical severity (SSI) of the these patients (Figure 5-24).
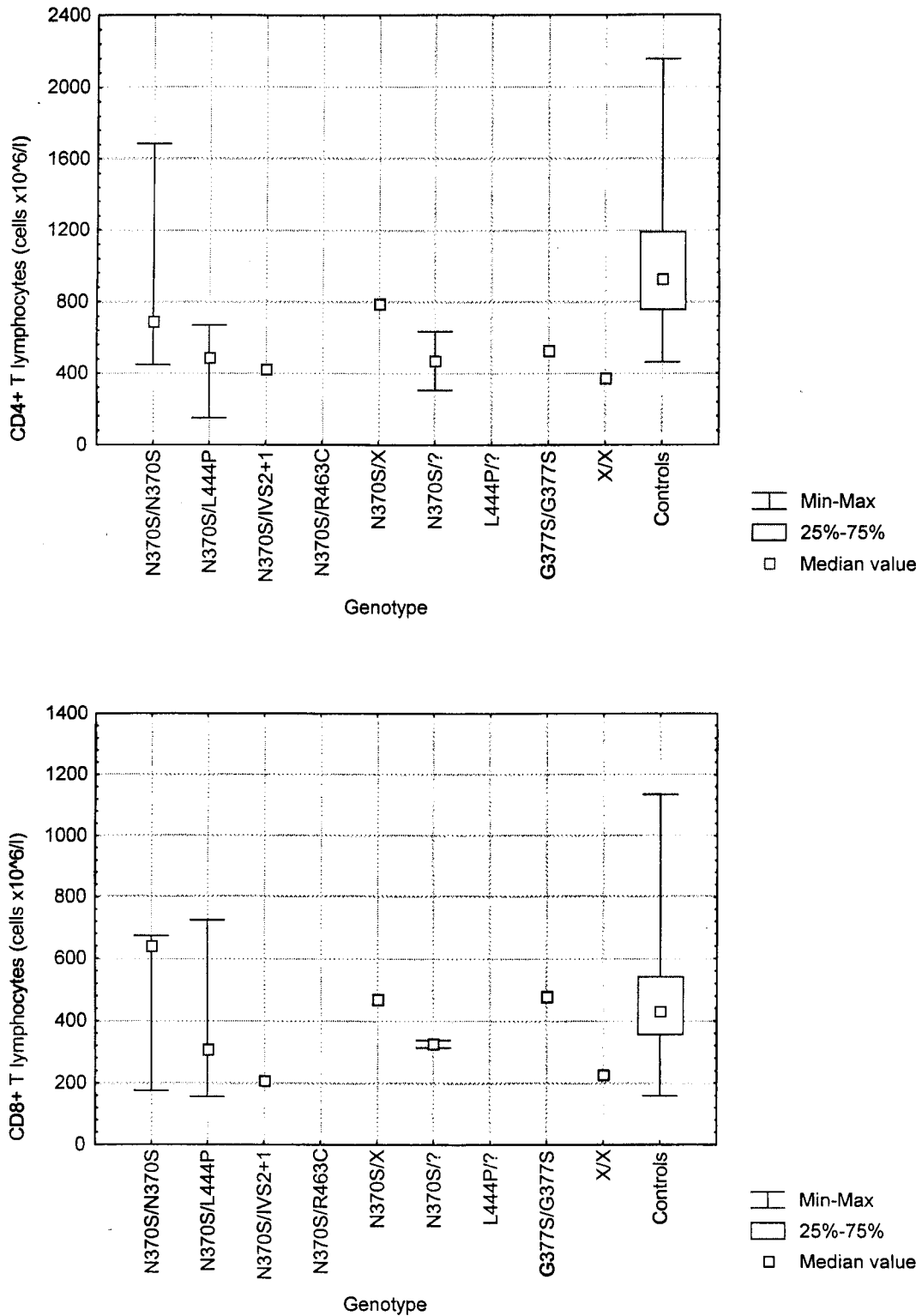
Figure 5-23

Peripheral blood CD4^{+} and CD8^{+} T lymphocyte subsets (cells $\times 10^6$/l) of genotyped non-splenectomized type 1 GD patients.
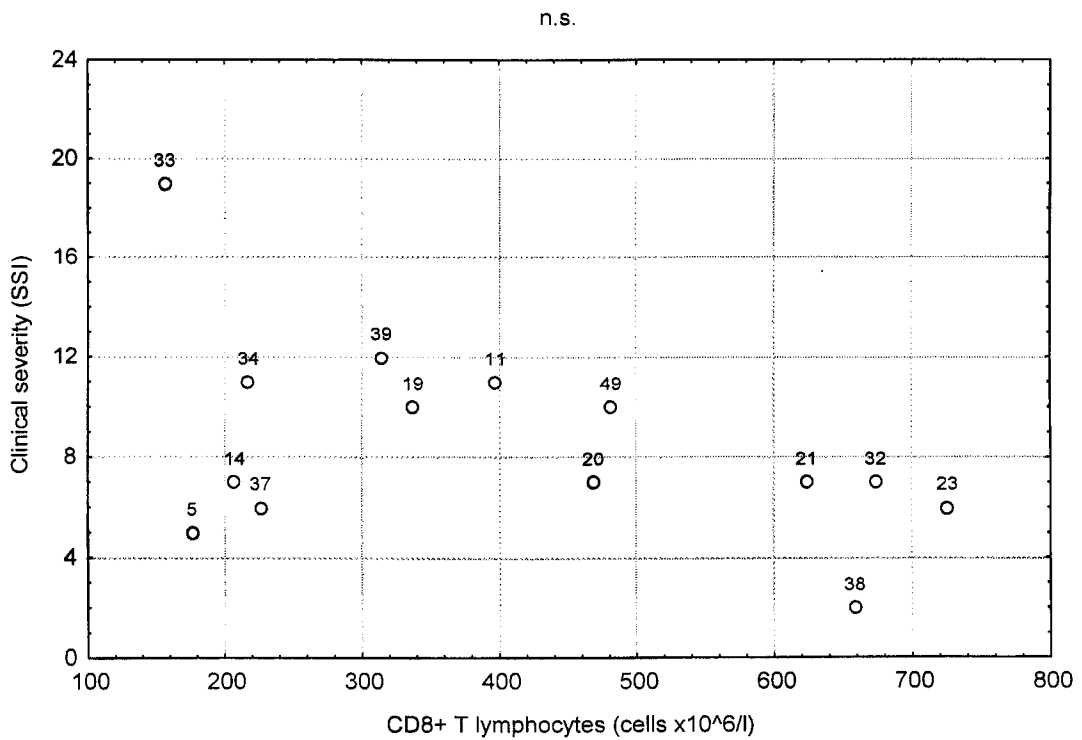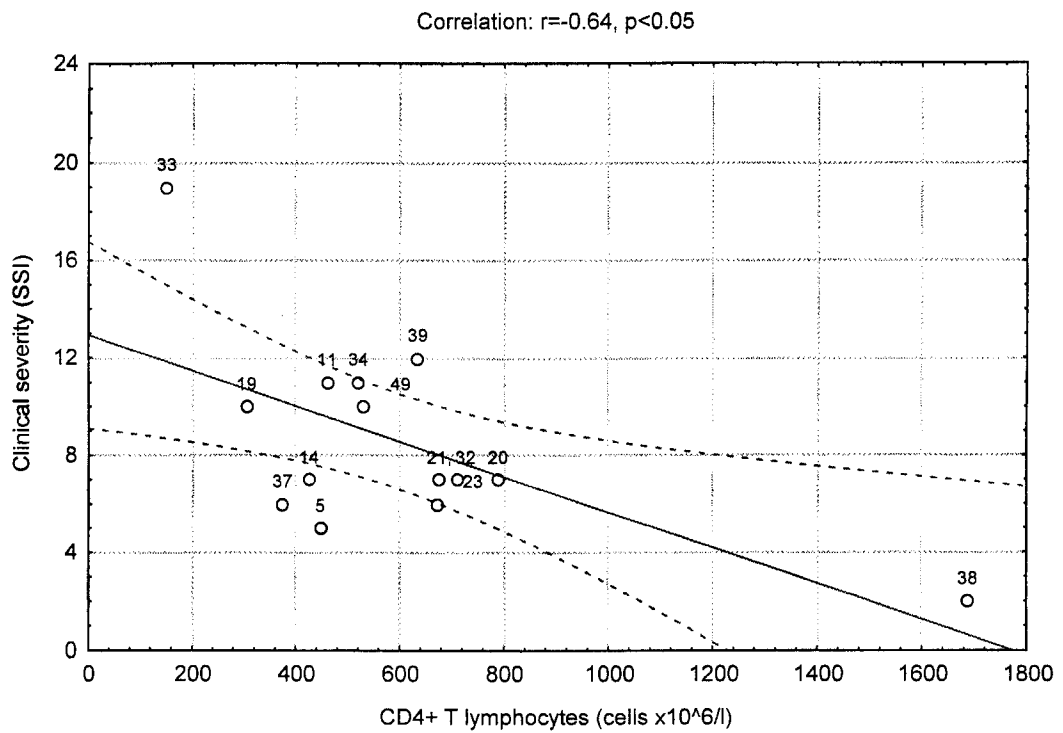
Correlation: r=-0.64, p<0.05

n.s.

Figure 5-24

Peripheral blood CD4^{+} and CD8^{+} T lymphocyte subsets (cells $x10^6/l$) and clinical severity (SSI) of non-splenectomized type 1 GD patients.

Moreover, when compared with controls, with one exception all GD patients presented decreased numbers of CD4+ T lymphocytes. However, although the number of GD patients without bone involvement is relatively low, the CD8+ T lymphocytes tended to be distinct on the basis of the presence or absence of bone disease (Figure 5-25).
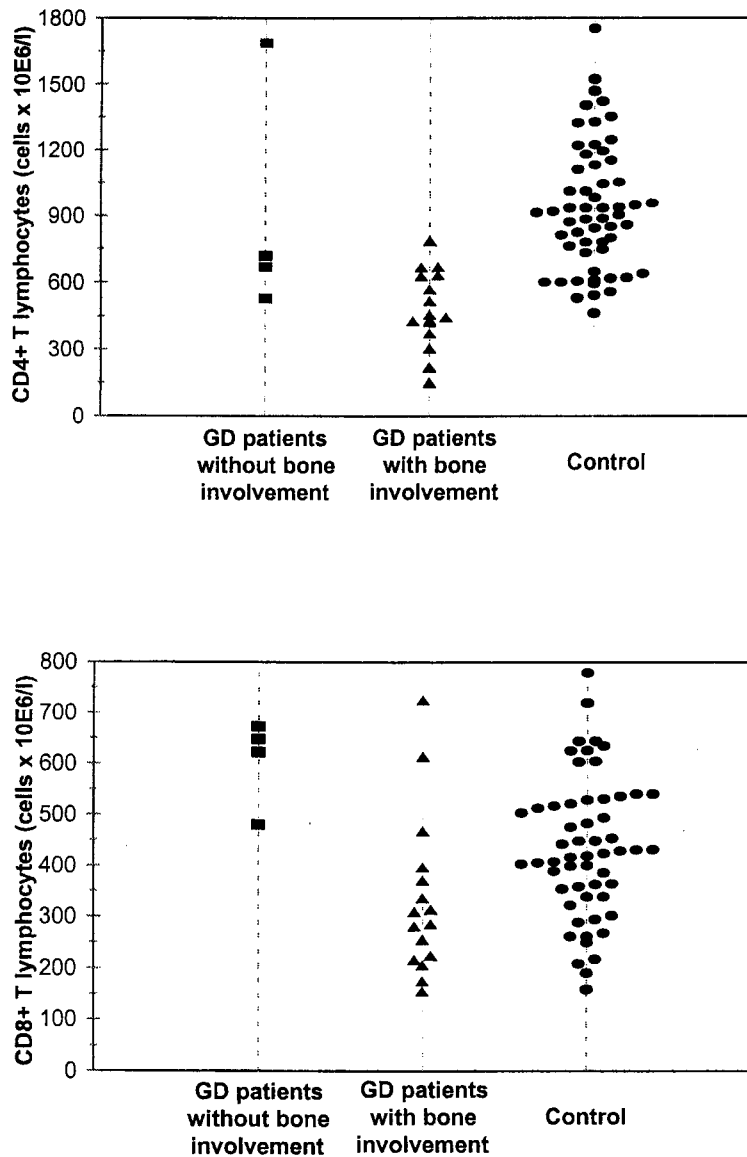

Figure 5-25

Peripheral blood CD4$^+$ and CD8$^+$ T lymphocyte subsets (cells x10^6/l) in non-splenectomised type 1 GD patients with and without bone involvement.

The statistical analysis was done in order to test whether the differences in the numbers of T cell subsets were significantly different between GD patients and controls. A significant ($p<0.05$) difference between the number of both CD4$^+$ (483.1±173.4) and CD8$^+$ (333.9±156.0) T lymphocyte subsets (cells x10^6/l) was

observed in non-splenectomised patients presenting bone involvement (n=16), as compared to controls (respectively 941.2±289.6 and 435.0±136.1, n=56) (Table 5-12). The available number of GD patients without bone disease (the non-splenectomised patients 21, 32, 38 and 49) was however not sufficient to substantiate the differences in T cell numbers between patients with and without bone involvement.

Table 5-12

Peripheral blood CD4$^+$ and CD8$^+$ T lymphocyte subsets (cells x10^6/l) of non-splenectomised type 1 GD patients.

	GD patients with bone involvement	p	Controls
CD4$^+$ T lymphocytes	483.1 ± 173.4 (16)	p<0.01	941.2 ± 289.6 (56)
CD8$^+$ T lymphocytes	333.9 ± 156.0 (16)	p<0.05	435.0 ± 136.1 (56)

Values represent the mean±SD of each population gated on total lymphocytes. The number of analyzed individuals is indicated between brackets. Probability (p) values above 0.05 are considered significant.

5.3.7   Relationship between secondary abnormalities

5.3.7.1   Peripheral blood T lymphocyte CD4+ and CD8+ subpopulations and plasma tartrate resistante acid phosphatase (TRAP) activity

To study whether T lymphocyte numbers could be associated with osteoclastic activity, plasma TRAP enzymatic activity was measured in these patients, and the correlation coeficient was calculated by the Spearman rank correlation. As shown in Figure 5-26, in non-splenectomised GD patients with bone involvement, a significant negative correlation was observed between TRAP activity and CD8+ T lymphocyte cell numbers (unilateral test r=-0.508, p<0.05, and for the CD4$^+$ T lymphocyte subset r=-0.279, being non-significant). Different linear regression models (e.g. linear, cubic, exponential) were tried but further data is needed in order to elucidate the poor fitness encountered.
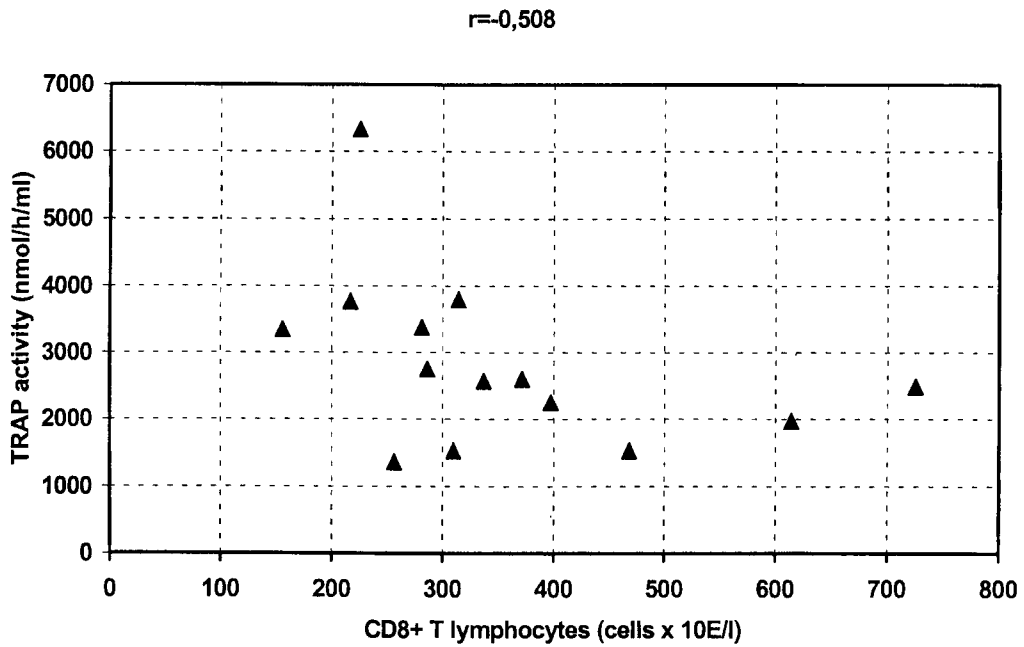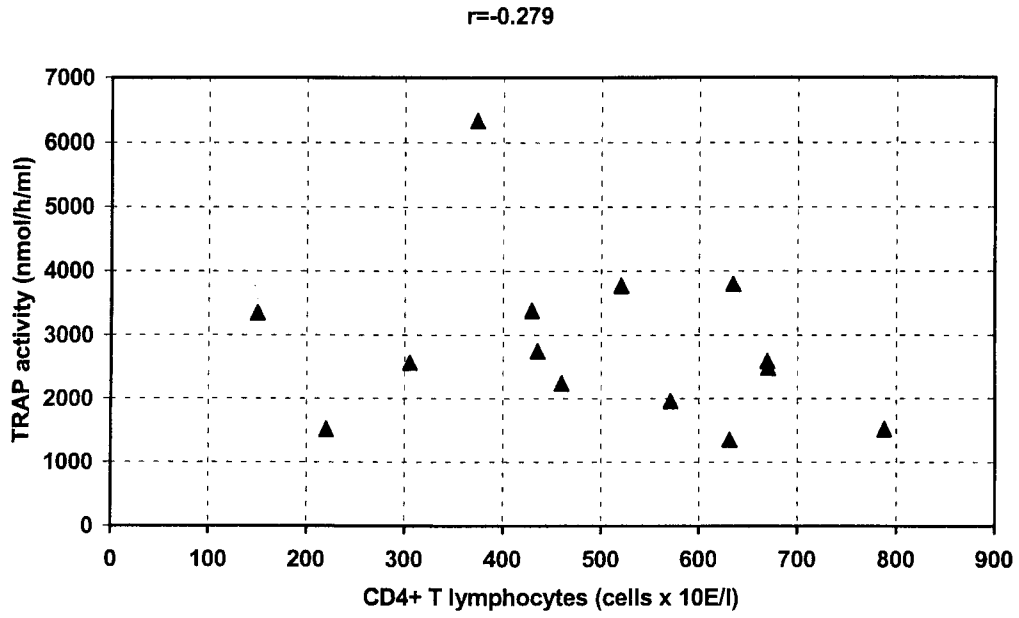
Figure 5-26

Peripheral blood CD4^{+} and CD8^{+} T lymphocyte subsets (cells $X10^6$/l) and plasma tartrate resistante acid phosphatase, TRAP, activity (nmol/h/ml) of non-splenectomised tye 1 GD patients.

5.3.7.2 Correlation between the increase in plasma glucosylceramide concentration and lysosomal enzyme activities

To study whether plasma glucosylceramide concentration could be associated with the increased activity of plasma lysosomal enzimes the correlation coefficient was calculated between these parameters.

As shown in Figure 5-27 a significant positive correlation was observed between plasma glucosylceramide concentration (mg/100ml of plasma) and chitotriosidase activity (nmol/h/ml of plasma) ($r=0.65$, $p<0.05$).
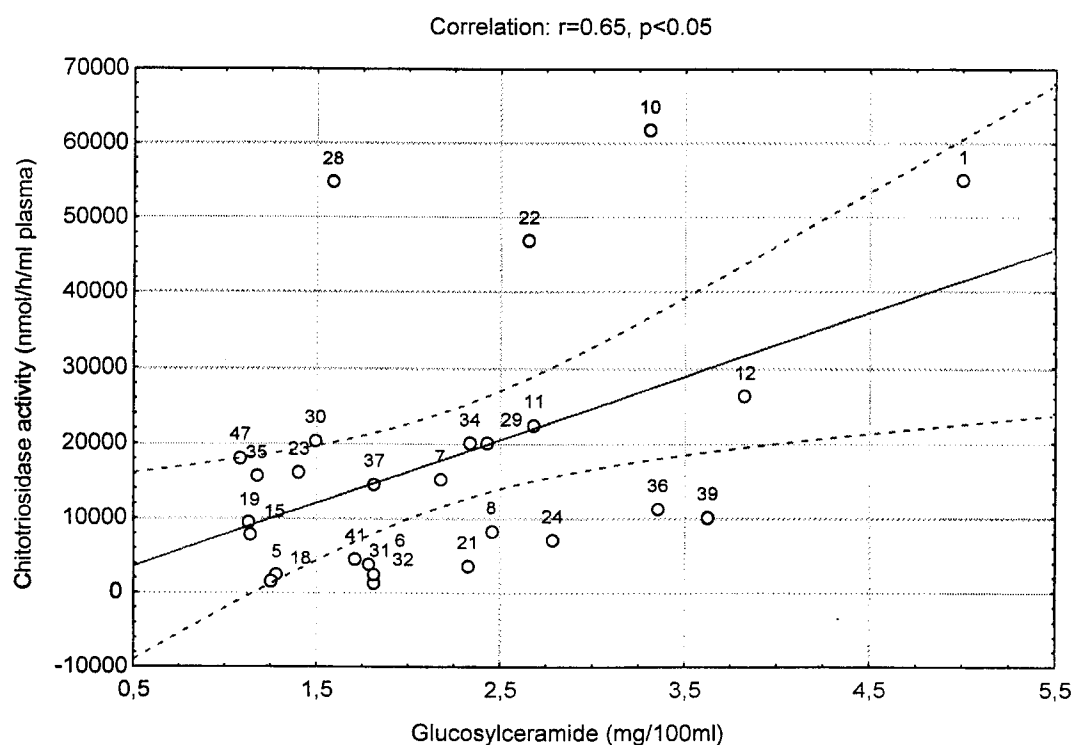
Correlation: $r=0.65$, $p<0.05$

Figure 5-27

Plasma glucosylceramide concentration (mg/100 ml of plasma) and chitotriosidase activity (nmol/h/ml of plasma) of type 1GD patients

A significant positive correlation was also observed between plasma glucosylceramide concentration (mg/100ml of plasma) and TRAP activity of adult GD patients (nmol/h/ml of plasma) ($r=0.50$, $p<0.05$) (Figure 5-28).
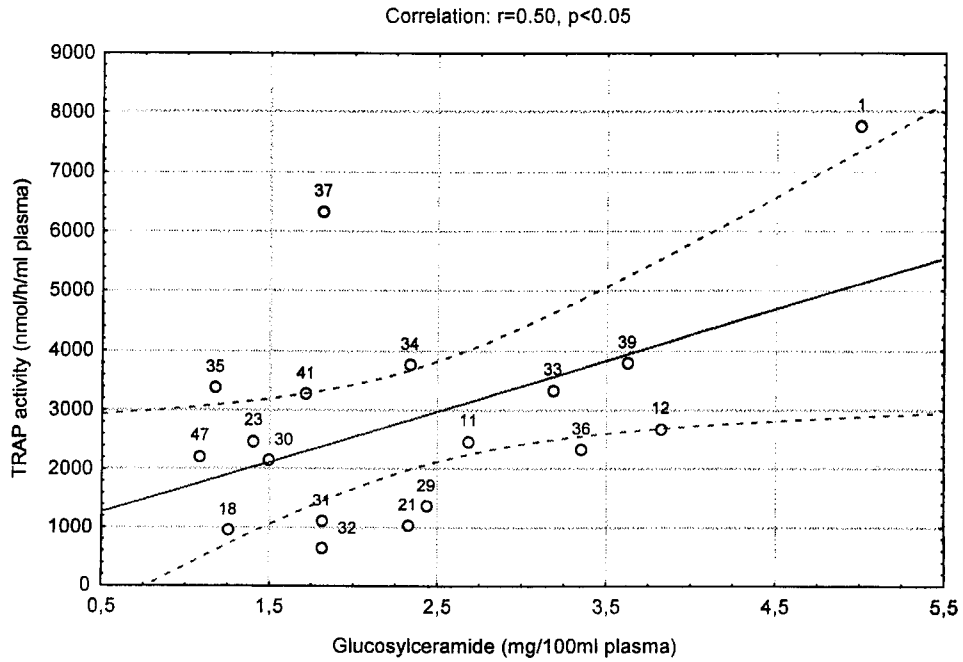
Figure 5-28

Plasma glucosylceramide concentration (mg/100 ml of plasma) and TRAP activity (nmol/h/ml of plasma) of type 1 GD patients

Plasma total β-hexosaminidase activity was only found to be significantly correlated with TRAP activity of adult GD patients (r=0.70, p<0.05) (Figure 5-29).
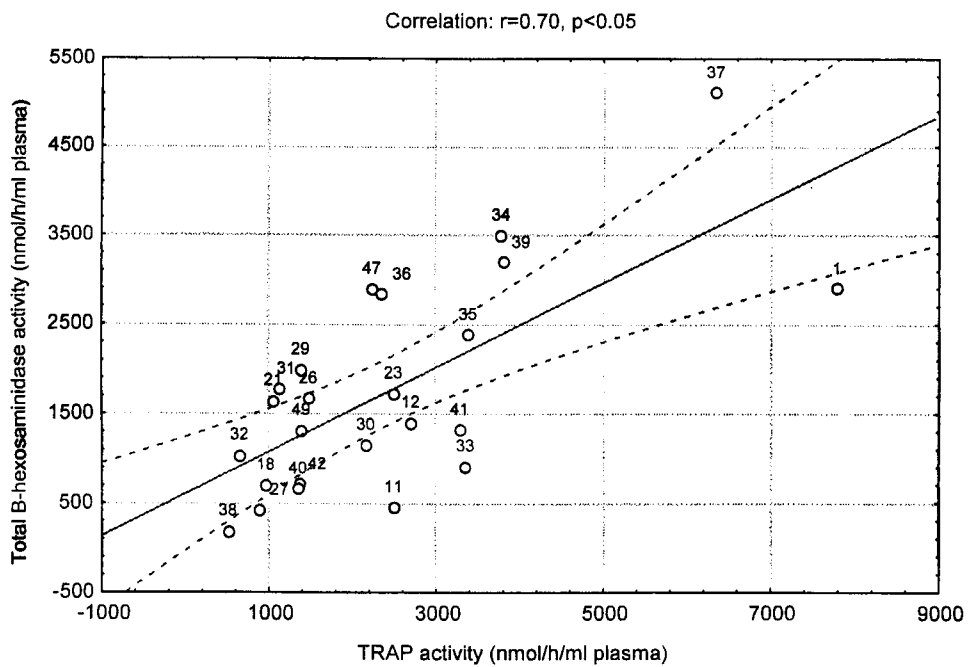

Figure 5-29

Plasma total β-hexosaminidase activity (nmol/h/ml of plasma) and TRAP activity (nmol/h/ml of plasma) of type 1 GD patients

Plasma TRAP activity of GD patients was also found to be significantly correlated with between the activity of chitotriosidase (r=0.71, p<0.05) (Figure 5-30).

Correlation: r=0.71, p<0.05

Figure 5-30
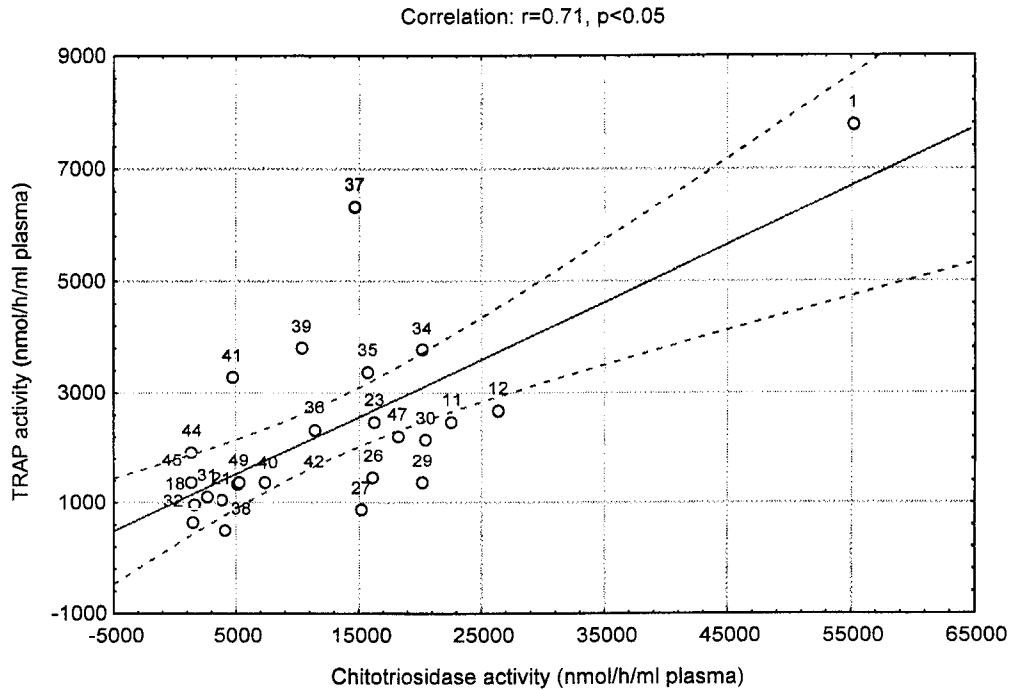**Plasma TRAP activity and chitotriosidase activity of type 1 GD patients.**

5.3.8   Enzyme supplementation therapy

5.3.8.1   Plasma glucosylceramide concentration of GD patients submitted to the enzyme supplementation therapy

For the large majority of the patients, after 12 months of treatment, plasma glucosylceramide concentration decreased to the control range (Figure 5-31).
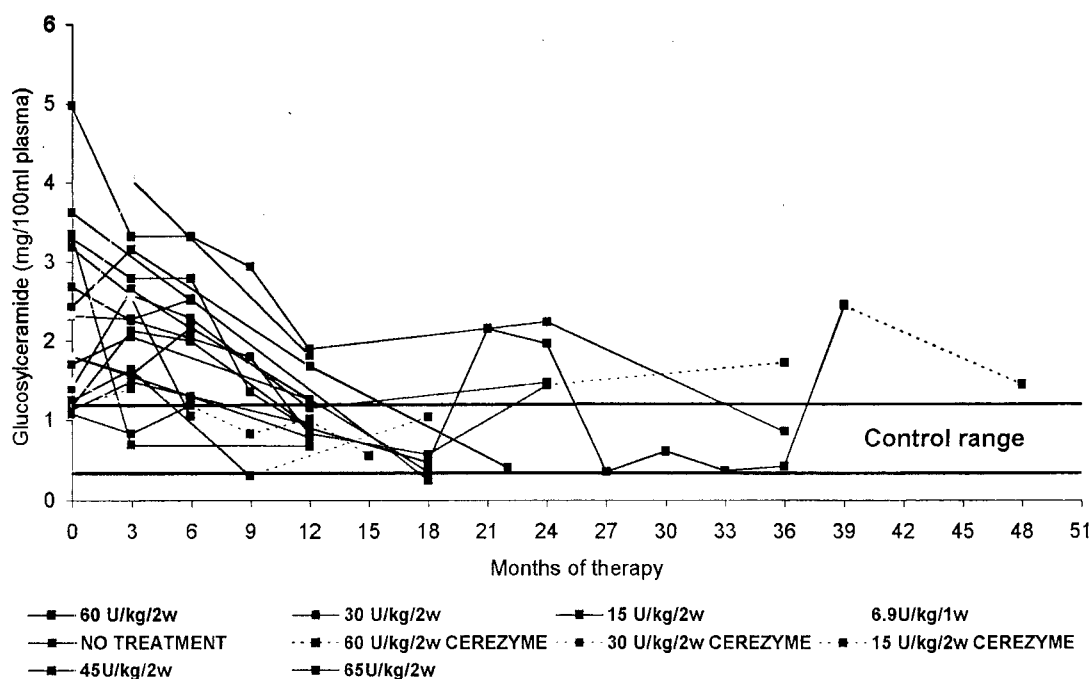
Figure 5-31

Plasma glucosylceramide concentration (mg/100ml of plasma) of type 1 GD patients submitted to the enzyme supplementation therapy.

5.3.8.2 Plasma chitotriosidase activity of GD patients submitted to the enzyme supplementation therapy

The decrease in plasma chitotriosidase activity was depicted as percentage of the initial value since Plasma chitotriosidase activity was found to decline rapidly during therapy, starting already within the first month. In contrast, patients that started treatment with the low dose/high frequency regimen showed a comparatively slower reduction in chitotriosidase activity. Since these patients were not showing clinical improvement (e.g. with respect to hematological parameters) the administrated regimen was changed to the high dose/low frequency of 60U/kg/2w (Figure 5-32).

Figure 5-32

Plasma chitotriosidase activity (expressed in percentage of the initial value) of type 1 GD patients submitted to the enzyme supplementation therapy.

For the generality of the patients, the maximal decrease of chitotriosidase activity was observed in about 12 months of treatment. In this period, complete normalization of chitotriosidase levels was almost achieved for patients presenting less abnormal initial values. In contrast, plasma chitotriosidase activity of the patient receiving therapy for the longest period of time decreased to about 10% of the initial value, which still accounted for about 40-fold the maximal value of the control range (not shown).

5.3.8.3 Plasma TRAP activity of GD patients submitted to the enzyme supplementation therapy

Plasma TRAP activity showed a slow and irregular tendency to decrease, and for the majority the patients this activity remained borderline of the maximal values of control range. Patients that presented normal TRAP activity before treatment revealed sustained activity within control range (Figure 5-33).

Some observations were made for some patients: Patient 1 (which presented the highest TRAP activity level) decreased till 25% of the initial value after 15 months of treatment, but at 31 months returned to the initial activity value, without interruption of treatment. The patient submitted to the larger period of therapy (patient 10) decreased only till 35% of baseline, showing an irregular decreasing tendency. Patient 29

interrupted treatment and after 6 months an increase of TRAP from 56% to 238% of the initial activity (Figure 5-33).
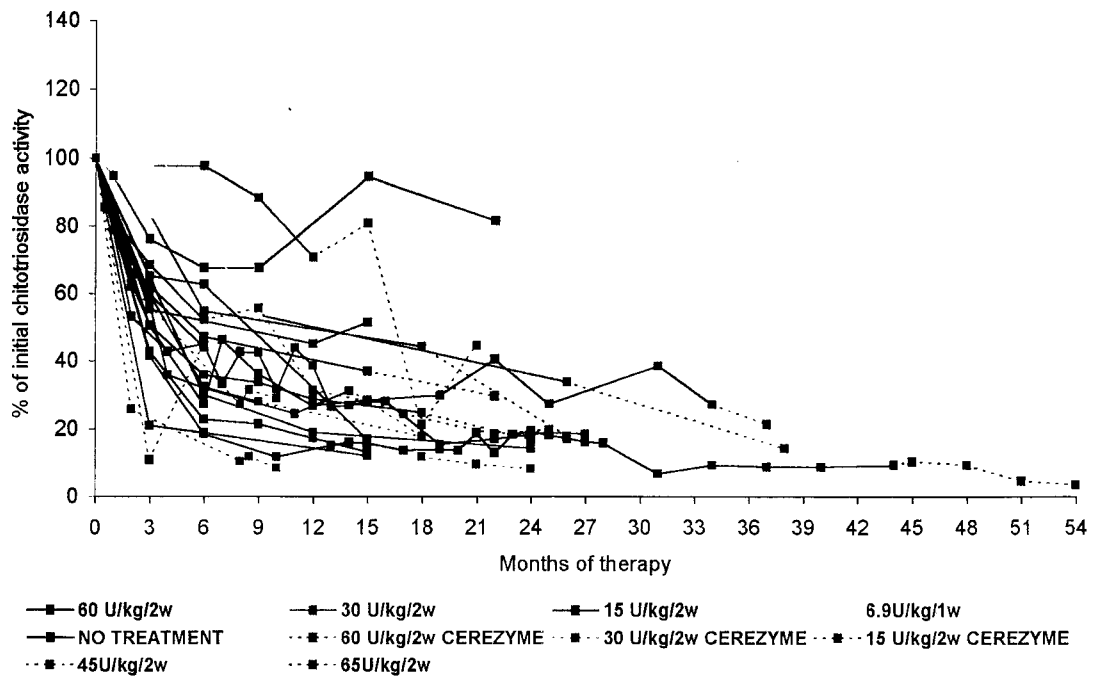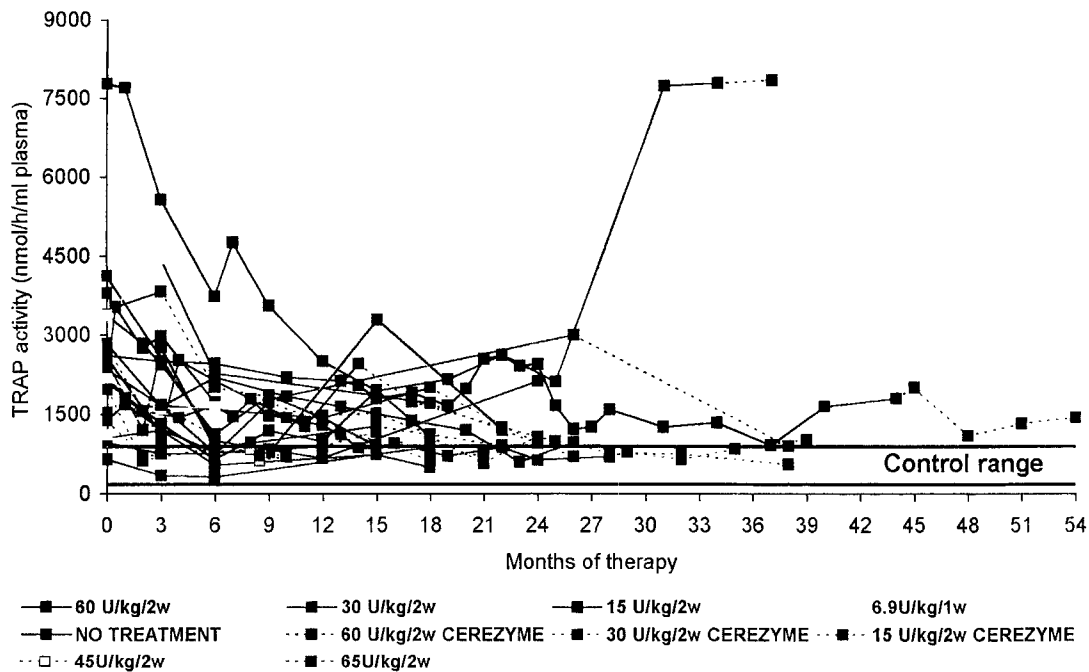

Figure 5-33

Plasma tartrate resistante acid phosphatase (TRAP) activity (nmol/h/ml of plasma) of type 1 GD patients submitted to the enzyme supplementation therapy.

5.3.8.4   Plasma β-hexosaminidase activity of GD patients submitted to the enzyme supplementation therapy

With the exception of patient 1, all patients that presented abnormally high total β-hexosaminidase activity before treatment showed a decreased to control range in 3 to 6 months of enzyme supplementation therapy (Figure 5-34).
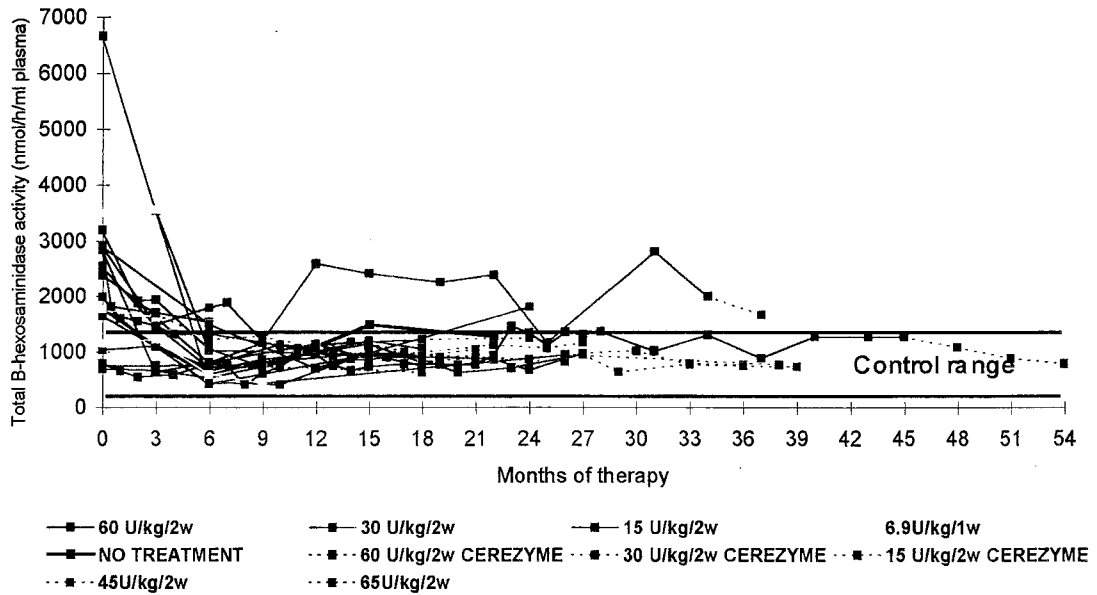
Figure 5-34

Plasma total β-hexosaminidase activity (nmol/h/ml of plasma) of type 1 GD patients submitted to the enzyme supplementation therapy.

5.3.8.5 Serum ferritin of GD patients submitted to the enzyme supplementation therapy

With the exception of the case that presented the most abnormal serum ferritin, the remaining patients that presented abnormally high serum ferritin before treatment showed a decreased to control range in 6 to 12 months of enzyme supplementation therapy (Figure 5-35).
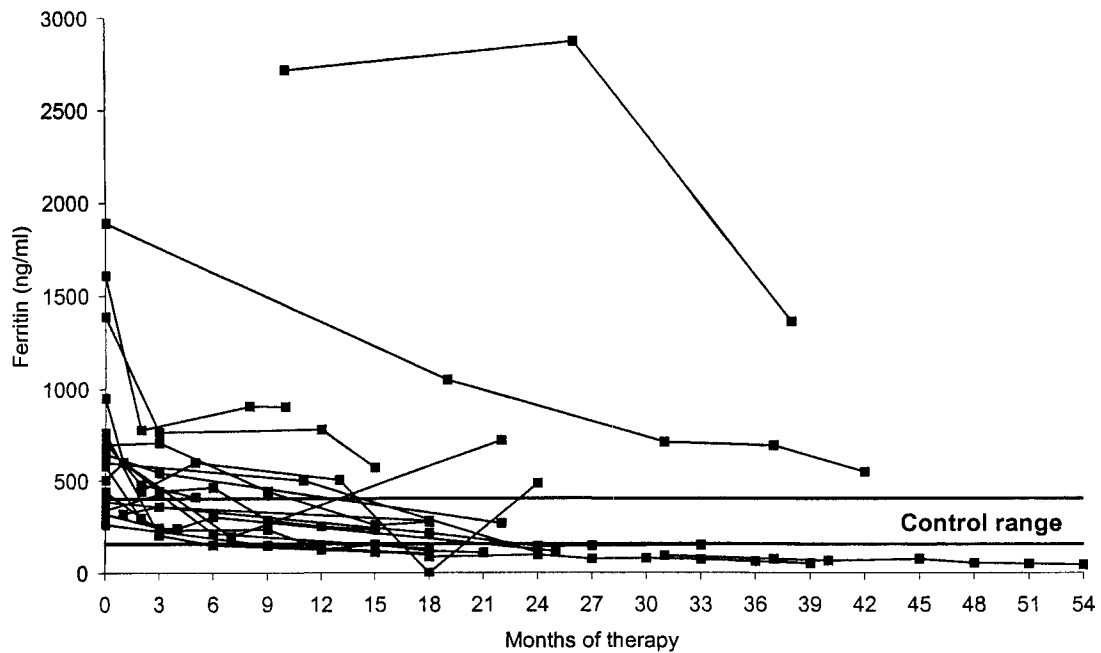
Figure 5-35

Serum ferritin (ng/ml) of type 1 GD patients submitted to enzyme supplementation therapy.

5.3.8.6   Peripheral blood CD4⁺ and CD8⁺ T lymphocyte subsets of GD patients submitted to the enzyme supplementation therapy

No significant changes were observed in the relative proportions of T lymphocyte major subsets (Figure 5-36). The coefficients of variation (CV) of %CD4⁺ and %CD8⁺ T cells were constantly lower than 20% in patients subjected to the enzyme supplementation therapy (being exceptions patients 31 and 11 with a covariance of respectively 25% and 36% for CD8⁺ determinations).
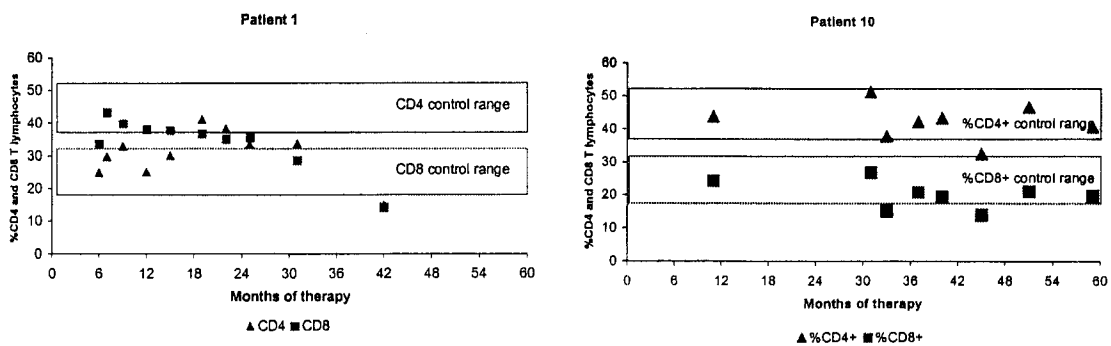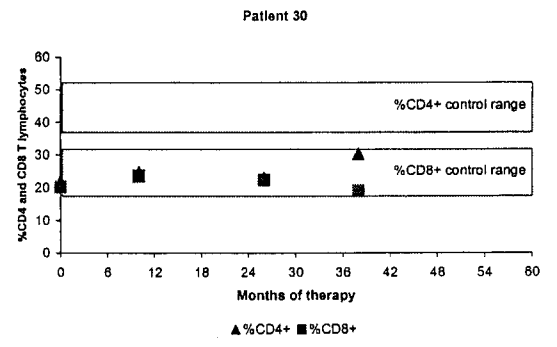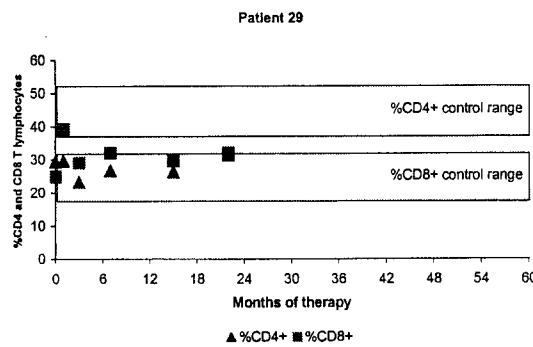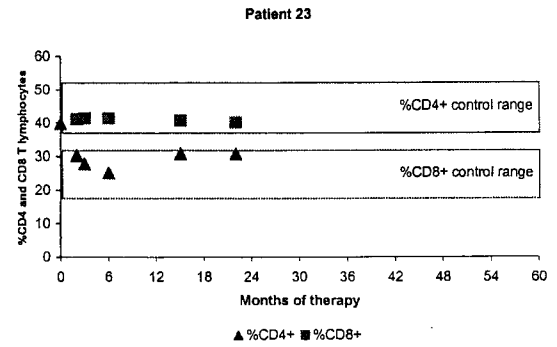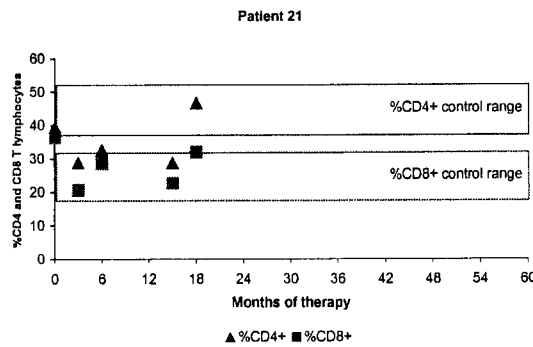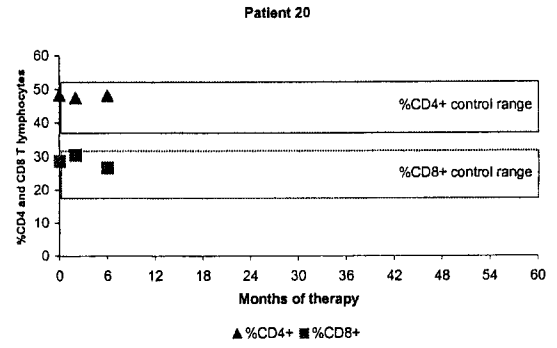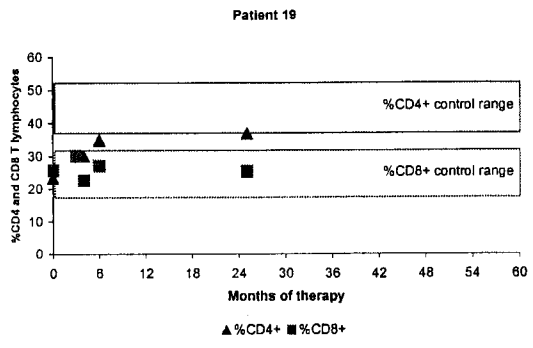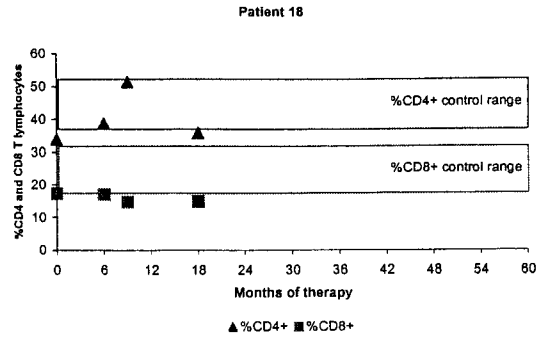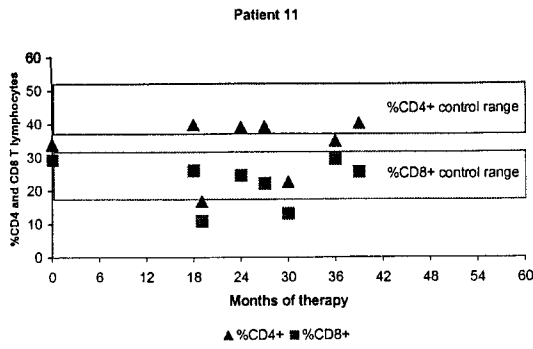
Patient 11

Patient 18

Patient 19

Patient 20

Patient 21

Patient 23

Patient 29

Patient 30

Patient 31

Patient 32

Patient 33

Patient 34

Patient 35

Patient 36

Patient 39

Patient 41

Patient 43
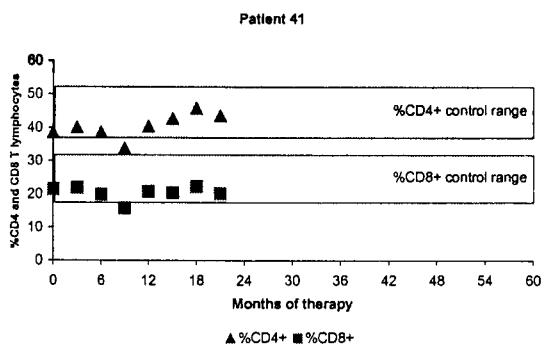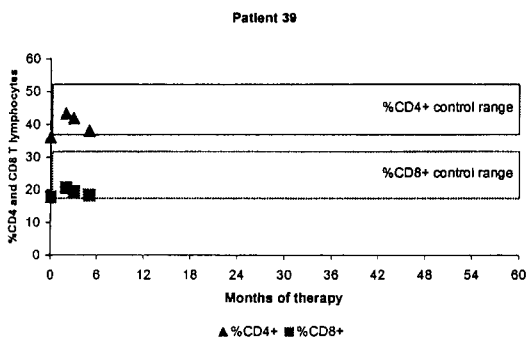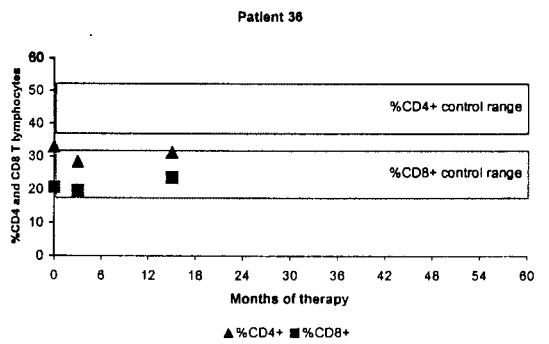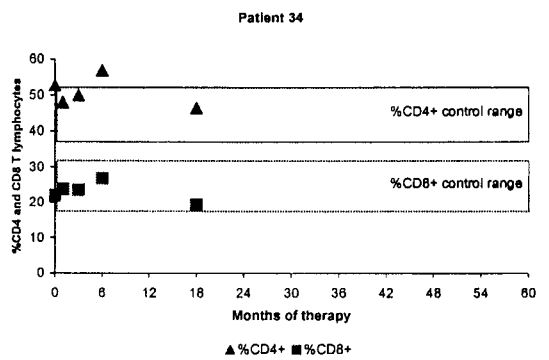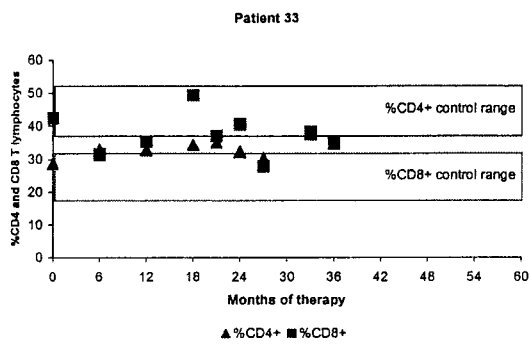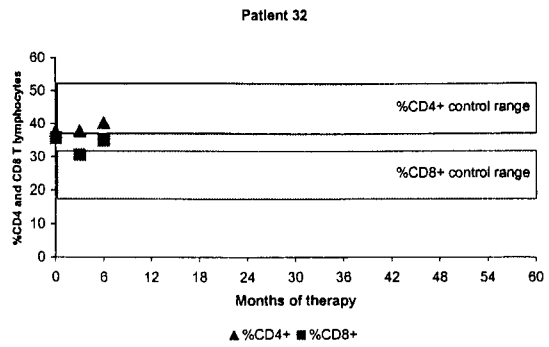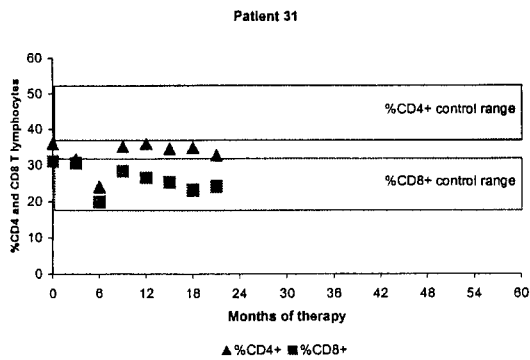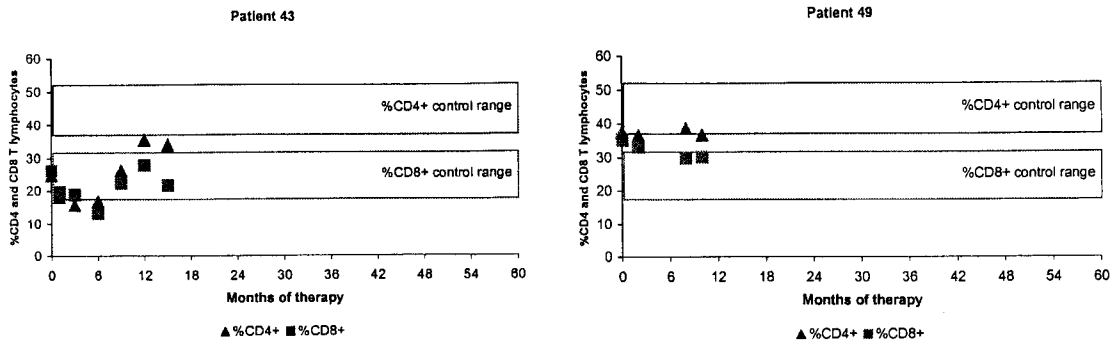
Patient 49

Figure 5-36
Peripheral blood T lymphocyte %CD4$^+$ and %CD8$^+$ subsets of type 1 GD patients submitted to the enzyme supplementation therapy.

To address the question whether the abnormally low T cell numbers presented by patients with bone involvement reflected disease activity, the peripheral blood T lymphocyte subset numbers were followed in 5 patients undergoing enzyme supplementation therapy for at least 24 months. As shown in Figure 5-37, for 4 of 5 patients, non-linear trend analysis showed that statistically significant changes in T lymphocyte cells occurred only after 12 months of therapy. The coefficient of determination for these 4 individual analysis varied from 79% to 100% for CD4$^+$ and from 88% to 100% for CD8$^+$ indicating a good fit of trend models to the data. Using this trend analysis, the change in T cells of patient 19, observed at the 3rd month of therapy was not significant. In contrast, with exception of patient 33 (who presents the inherited deficiency in chitotriosidase activity), therapy has the effect of progressively lowering chitotriosidase activity of these patients to a sustained level. With respect to TRAP activity, some of these patients presented a comparatively slower and irregular decrease (patients 41, 1 and 19), observation that agrees to the unclear evolution of bone disease in these patients. Clinically it must be stated that after about 24 months of treatment, patient 33 showed imagiological signs of improvement (e.g. closed pathological fracture) whereas patient 1 started to present vertebral compression.

Patient 41

Patient 1

152
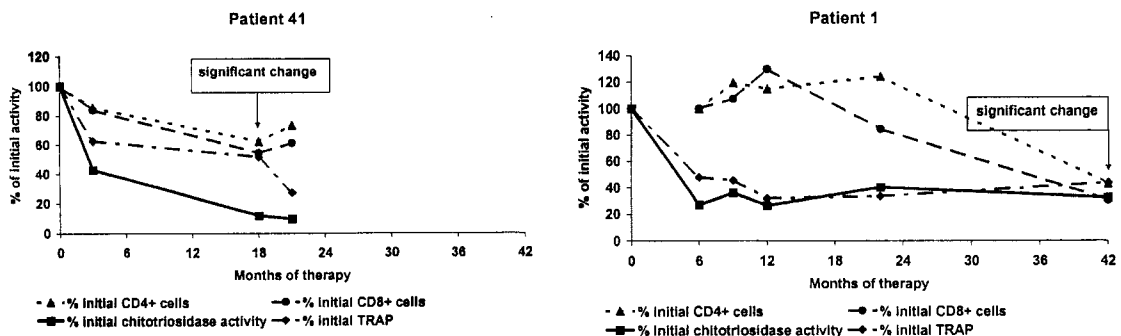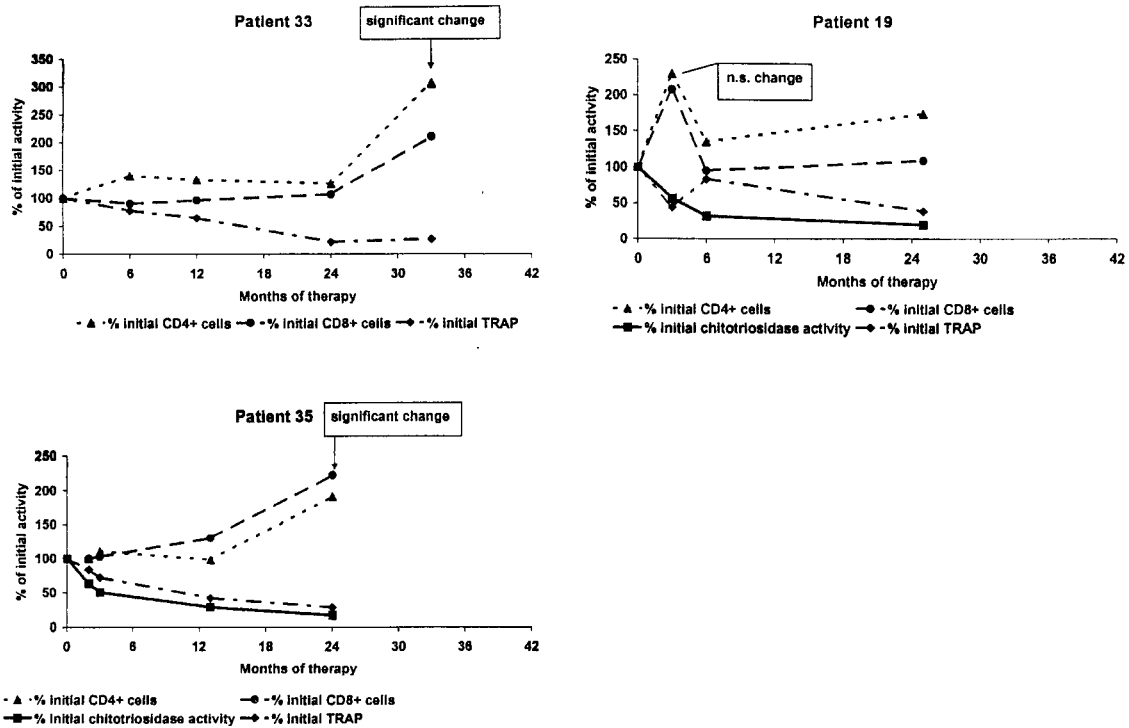
Figure 5-37

Peripheral blood CD4$^+$ and CD8$^+$ T lymphocyte subsets (% of initial number of cells), plasma chitotriosidase and TRAP activity (% of initial activity) of type 1 GD patients submitted to the enzyme supplementation therapy. Arrows indicated the occurrence of significant changes of peripheral blood T lymphocytes.

5.4 Conclusions

The main conclusions of this work are presented in Table 5-13.

Plasma glucosylceramide which is thought to be originated from lysed lipid loaded macrophages was measured in type 1 GD patients presenting different genotypes and clinical severity. In contrast to the reported slight elevation for type 1 GD patients (Dawson et al, 1982), plasma glucosylceramide concentration of the studied type 1 GD patients was found to be higher (2.7-fold the control mean) and similar to that reported for neuronophatic forms of the disease. However since the studied patients presenting the N370S/N370S, N370S/IVS2+1 and N370S/? genotypes had on average onl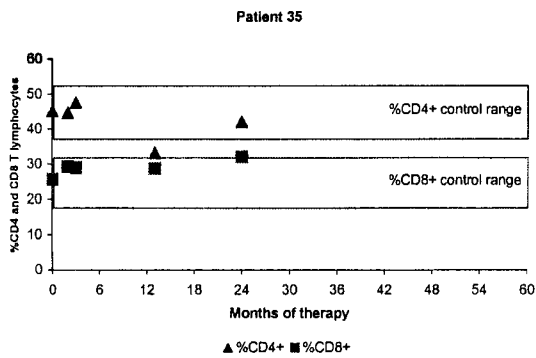y slightly increased levels of plasma glucosylceramide concentration, it is possible that some genotypes were over-represented in the few type 1 GD patients reported in the literature. Importantly, in the studied type 1 GD patients a significant correlation was observed between plasma glucosylceramide concentration and clinical severity, as assessed by the severity score index (SSI). Also in disagreement to that previously reported (Nilsson et al, 1982; Svennerholm et al, 1982), plasma glucosylceramide

concentration of non-splenectomized patients was not significantly different from that presented by the splenectomized patients.

Although it has been reported a 5-fold increased GM3 concentration in spleen and brain of GD patients, in this study no significant changes were demonstrated in plasma of patients.

With respect to the previously reported increased activity of plasma chitotriosidase, a human chitinase and a putative marker of macrophage activation (Hollak et al, 1994), which may reflect the overall Gaucher cell accumulation in the body (reviewed in Aerts and Hollak, 1997), and the tartrate resistant acid phosphatase (TRAP) activity (Ek-Rylander et al, 1991), which may constitute a marker of osteoclastic activity (Ek-Rylander et al, 1994), the results of the studies described in this chapter confirmed and extended the information about these secondary abnormalities in type 1 GD patients:

Firstly, although on average GD patients presented significantly increased plasma TRAP activity (6.6-fold the control mean), some overlap was observed between controls and the N370S homozygous GD patients. TRAP activity was statisticall significantly lower in GD patients presenting the N370S/N370S genotype, but no significant correlation was observed between TRAP activity and the clinical severity (SSI) of all GD patients. In spleen extracts from type 1 GD patients TRAP activity was however only 1.4 to 2.4-fold increased. TRAP activity was also analyzed as a marker of bone involvement and with two exceptions, normal activity values were observed in patients without skeletal disease. The hypothesis can be raised that the occurrence of abnormal TRAP activity in plasma of these two patients without imagiological signs probably constitutes an earlier marker of developing bone disease. This will need future confirmation.

In contrast to TRAP, β-hexosaminidase activity was significantly increased in spleen (a tissue rich in Gaucher cells) and as reported mainly due to the β-hexosaminidase B isoenzyme (5 to 24-fold elevation). In plasma, where β-hexosaminidase was only increased 2.8-fold the control mean, a large number of patients presented activity values within control range and no significant correlation was observed between total β-hexosaminidase activity and genotype or clinical severity score.

Plasma chitotriosidase activity of GD patients was found to be on average 300-fold the mean control value, being the only secondary abnormality in GD that does not overlap with control range. In accordance to that reported by others (Guo et al, 1995) no overlap was also observed between chitotriosidase activity of type 1 GD patients and the activity of patients affected by other lysosomal or non-lysosomal diseases. Importantly, in the present study chitotriosidase activity was found to be significantly

lower in GD patients presenting the N370S/N370S genotype. No significant correlation was observed between this activity and the disease clinical severity score, but since 2% of the control population was found to be deficient in chitotriosidase activity, the carrier status of GD patients for this deficiency needs to be established.

The majority of the studied GD patients presented the previously described abnormally increased serum ferritin and no significant correlation was observed between this abnormality and the patients genotype or severity score index.

This study contributed to further characterisation of GD with respect to novel secondary abnormalities:

Leukopenia is one of the haematological abnormalities that can be associated with GD, and the study of the peripheral blood T cell major subsets showed that a subgroup of GD patients presented abnormal $CD4^+/CD8^+$ T lymphocytes ratio. No significant abnormalities were observed in the N370S homozygotes. In splenectomized patients the number of leukocytes, including T lymphocytes was increased but the %$CD4^+$ T cell subset was decreased. In contrast, non-splenectomised GD patients presented significantly lower number of the two major T lymphocyte subsets. Importantly, lower numbers of CD8+ T cells appeared associated with the subgroup of patients showing bone involvement. Although few patients were classified without bone involvement, this was specially observed in GD patients homozygous for the N370S glucocerebrosidase gene mutation (4 out of 6).

Factors influencing the clinical expression are unknown and a clear correlation between the disease severity and patient's genotype is lacking. In summary, the findings obtained in the study of the disease secondary markers showed that with respect to genotype/marker correlation, the clinically milder N370S homozygous patients were characterized by the absence of significant abnormalities in $CD4^+/CD8^+$ T lymphocyte ratio. When compared to the other GD patients the N370S homozygotes presented also significantly less abnormal TRAP and chitotriosidase activity. With respect to the phenotype/marker correlation, plasma glucosylceramide concentration of GD patients was significantly correlated with clinical severity score. Moreover, in non-splenectomised GD patients it was observed an association between TRAP activity, the number of peripheral blood CD8+ T lymphocytes and the presence of clinical signs of bone disease. The association between bone pathology and statistically significant abnormalities in the $CD8^+$ T lymphocyte subset, was further substantiated by the existence of a significant correlation between the peripheral number of these cells and TRAP enzymatic activity, the putative marker of osteoclastic activity. The analysis of these secondary plasma abnormalities may thus provide useful disease markers and

contribute to obtain more insights into the complicated pathophysiology of the disease. A significant correlation was observed between TRAP, chitotriosidase activity and glucosylceramide concentration. The presence of these abnormalities in plasma may thus be directly due to the mechanisms via which the lipid-loaded Gaucher cell releases plasma glucosylceramide. If the lipid-loaded macrophages are indeed involved in the release of the hydrolases that were elevated in plasma of symptomatic GD patients, their plasma levels may serve as quantitative markers for the amount of storage cells. Follow-up studies of patients submitted to the enzyme supplementation therapy showed that plasma β-hexosaminidase activity normalized within 3 to 6 months of therapy and glucosylceramide decreased to normal level in about 12 months of treatment. This probably reflects the absence of lysed lipid loaded macrophages. In contrast, TRAP activity showed a slow and irregular tendency to decrease and for the large majority of patients becoming borderline with the control range. The persistence of abnormal TRAP activity level in treated patients probably reflects the slower reversion of the bone disease. In this trial, chitotriosidase activity level was from all tested parameters, both the most abnormal prior to the therapy as well as the most responding to the alglucerase/imiglucerase supplementation. This response was specially observed for patients submitted to the high dose/low frequency regimen. Although there was a decrease in serum ferritin, no effect was however observed in the relative percentage of $CD4^+$ and $CD8^+$ T lymphocytes. With respect to the absolute numbers of these cells statistically significant changes occurred however after 12 months of treatment. In the corresponding time period, the irregular decrease of TRAP activity agrees with the observed unclear and/or slower evolution of bone pathology in these patients.

The results obtained with the enzyme suplementation therapy allow to conclude that the lipid-loaded macrophages are indeed involved in the release of chitotriosidase and total β-hexosaminidase activities; their plasma levels may thus serve as quantitative markers for the amount and/or activation of storage cells. The less clear reduction of TRAP activity seems to indicate that the release of this enzyme is probably more restricted to a specific location of slower correction and/or prevention of ongoing formation of the lipid laden GD cells.

Table 5-13

Overview of findings observed in type 1 GD patients with respect to plasma glucosylceramide concentration, enzymatic activity of TRAP, total β-hexosaminidase and chitotriosidase, serum ferritin and peripheral blood T lynphocyte subpopulations.

	Glucosylceramide	TRAP	Total β-hexosaminidase	Chitotriosidase	Serum ferritin	Peripheral blood T cell subpopulations
General patient features	Overlap with controls Range 1.13-5.0 mg/100 ml plasma Mean 2.3 mg/100 ml plasma Mean elevation 2.7-fold	Overlap with controls range 507-7 781nmol/h/ml mean 2 413 nmol/h/ml mean elevation 6.6-fold	Large overlap with controls Range 187-7 102 nmol/h/ml Mean 1 523 nmol/h/ml mean elevation 2.8-fold	No overlap with controls range 1 336-68 394 nmol/h/ml mean 16 585 nmol/h/ml mean elevation 299-fold	Abnormal increase in 25 out of 30	Abnormal $CD4^+/CD8^+$ in 12 out of 28. Decreased number of $CD4^+$ and $CD8^+$ T lymphocytes in non-splenectomised patients.
Genotype correlation	No	Yes -Lower level in N370S/N370S genotype (p<0.05)	No	Yes -Lower level in N370S/N370S genotype (p<0.01)	No	Yes -No significant abnormalities in N370S/N370S genotype
Phenotype correlation	Yes Positive correlation with clinical severity	Normal activity in patients without bone involvement	No	No	No	Negative correlation of CD4+ T cells and clinical severity. Lower number of CD8+ T lymphocytes in patients with bone involvement
Correlation with other abnormalities	Significant positive correlation with: -TRAP activity (r=0.50) -chitotriosidase activity (r=0.65)	Significant positive correlation with: -glucosylceramide concentration (r=0.50) -β-hexosaminidase activity (r=0.70) -chitotriosidase activity (r=0.71)	Significant positive correlation with: -TRAP activity (r=0.70)	Significant positive correlation with: -glucosylceramide concentration (r=0.65) -TRAP activity (r=0.71)	No	Significant negative correlation between CD8+ T cells in non-splenectomized patients and TRAP activity (-0.508)
Response to therapy	Decreased to normal in about 12 months	Irregular decrease to borderline normal values	Normalised in 3-6 months. Patient 1 did not normalised	Clear decrease in association to dose/regimen and slow tendency to stabilization or normalization	Decreased to normal in ≈ 12 months	Significant changes in T lynphocytes after 12 months. No significant changes in the relative percentage of subsets.

Chapter 6
Discussion and future prospects

At the start of the investigation described in this thesis the molecular nature of the defects underlying glucocerebrosidase deficiency in Gaucher disease (GD) patients was still very poorly understood. It had became clear from enzymatic and immunological studies that many of the Portuguese non-neuronopathic (type 1) patients showed a considerable amount of residual glucocerebrosidase protein with abnormal kinetic properties (Sá Miranda, 1992), and this fact was related with the presence of the N370S mutated glucocerebrosidase in these individuals. This mutation was considered to be characteristic of Ashkenazi Jewish Gaucher patients. In the Portuguese type 1 GD patients the N370S glucocerebrosidase mutation accounted for 63% of the mutated alleles (Amaral et al, 1993). This frequency was remarkably high since for non-Jewish type 1 GD patients it had been reported to be 22-46% (Beutler et al, 1991; Choy et al, 1991; Levy et al, 1991; Sidransky et al, 1992), in sharp contrast to the value of 77% observed in Ashkenazi Jewish populations studied (Beutler et al, 1992).

By ascertaining the N370S carrier frequency in the Portuguese population (0.0043), the incidence of the homozygosity for the most common causal mutation of GD was in this population calculated to be of 1 in 55 000 births. Considerably higher frequency of this mutation was meanwhile also determined in the Ashkenazi Jewish population; two studies encompassing 2121 presumably normal Ashkenazi Jewish subjects showed a gene frequency of 0.032 for the N370S mutation, and expected incidences of 1.03 N370S homozygous per 1000 births in this population (Zimran et al, 1991; Beutler et al, 1993). The existence of hot spots in specific restricted areas of Portugal, with high frequency of carriers that could have been maintained due to a certain amount of inbreeding, might have escaped this survey, in which a randomly selected sample representative of the general Portuguese population was used. However, among the new-borns in the restricted area of Azores islands the frequency of the N370S mutated

allele was found to be of 0.0053, value that was not significantly different from the general population (0.0043). The frequency determined for the Belmonte population (including the closed small community of individuals of Sephardic Jewish ancestry) was found to be of 0.0091, which was higher but not statistically different from that obtained for the general Portuguese population. This result would be interesting if a Sephardic Jewish ancestry could have been ascribed to the identified carriers.

In the study of the origin of the N370S mutated allele in the Portuguese population, linkage analysis between this mutation and the previously described intragenic PvuII polymorphism showed that as observed for other patients, Portuguese GD patients presented complete linkage disequilibrium between the N370S mutated allele and the Pv1.1⁻ haplotype. One possible explanation for this finding is that the N370S mutation has more than once been introduced, in the context of a Pv1.1⁻ allele, the most common haplotype in most different racial groups (Sorge et al, 1985) as well as in the general Portuguese population. Another possible explanation would be that the mutation was only once introduced in this haplotype. At present it is not possible to exclude one of the two possibilities. A common origin for this mutation in the Portuguese and the Ashkenazim, could be raised due to the Jewish ancestry of the Portuguese; it is known that in XVI century Sephardic Jews living in Portugal were obliged to mix with the remaining catholic population. At present, in the Portuguese, Spanish and Dutch patients (with nonestabhlished Jewish ancestry), it is the most frequent mutation that is always associated with type 1 GD. However, according to Beutler (Beutler, 1992) the N370S mutated glucocerebrosidase allele has a recente Ashkenazi Jewish origin due to the strict linkage between the N370S mutation and the Pv1.1⁻ allele. In his view, it would be likely that when the mutation would be ancient, no perfect linkage between the N370S mutation and the Pvu II polymorphism would be expected due to the occurrence of crossovers in the glucocerebrosidase gene. Not favouring this hypothesis is the fact that the N370S mutated glucocerebrosidase allele has been observed (at relative high frequency) in the remaining Caucasian patients. Moreover, when considering the hypothesis of being ancient, it is not known whether crossovers affecting the N370S codon and the Pvu II polymorphic site occurred with the expected general statistic frequency; it has been observed that the glucocerebrosidase allele is continuous with other essential genes (Long et al, 1996) which might restrict the propagation of alleles with specific crossovers. Thus it seems more likely that the relative high frequency among Ashkenazim is caused by a common founder effect and limited mixing of this ethnic group with other populations. In conclusion, since at present the precise origin of the frequent N370S

glucocerebrosidase allele is unclear, further studies are necessary in order to ascertain the origin of the N370S mutation; in addition to standard restriction site polymorphisms, RFLPs derived from minisatellite probes (variable number of tandem repeats, or VNRTs) are much more sensitive and possibly the question may finally be answered with a reasonable degree of precision. Nevertheless, when comparing the frequency of other sphingolipidosis in the Portuguese and Ashkenazim populations there are clear differences in relative frequencies, apparently suggesting differences in general genetic background. Gaucher disease, B1 variant of GM2 gangliosidosis, metachromatic leukodystrophy and type C Niemann-Pick disease, are in this order the most frequent in the Portuguese; in the Ashkenazim the more frequent ones are also Gaucher disease, but followed by Tay-Sachs and Niemann-Pick type A diseases (Bach et al, 1992), although an high frequency of metachromatic leukodystrophy was also described but in Habbanite Jews (Zlogotora et al, 1980). Additionally the two more frequent mutations present in Tay-Sachs patients of Askenazi Jewish origin are rare in Portuguese GM2 gangliosidosis patients and the most frequent B1 Variant mutation in the Portuguese patients has not been described in Ashkenazi patients.

Since about 15% of mutated alleles in Portuguese type 1 Gaucher patients remain still unidentified (Amaral et al, 1996), being most probably private mutations, the determination of the urinary glucocerebrosidase activity per amount of cross reacting material showed that obligate carriers for known and as well as unknown mutated alleles could be detected in patients families. Although a combination of the enzymatic assay and DNA analysis had still to be used to ascertain those individuals whose values were falling in the cut-off range of high probability of misclassification, this enzyme based assay (EBA) may be particularly useful to screen carriers in non-Ashkenazi populations, where a large number of mutations can be associated with GD. The identification of as yet unidentified glucocerebrosidase mutations in the Portuguese population will enable the revision of the EBA cut-off range proposed and lead to a better insight on its sensitivity and specificity, when mutations other than N370S or L444P are considered.

In this study an exceptionally large number of N370S carriers were identified within the control population from where GD patients were originated (4 in 83, frequency of 0.024), pointing to a geographically restricted N370S allele frequency similar to the reported in the Ashkenazi Jewish community (0.032). A similar study was also carried out in the Netherlands and allowed the detection of most obligate carriers, but no carriers were found in the tested control sample (n=86). Although the frequency of the N370S mutation has not been determined, the results obtained with this study suggests

a comparably lower frequency of N370S mutated allele in Dutch population (Aerts et al, 1991). More recently, the study of the relative incidence of glucocerebrosidase mutations in Dutch GD patients confirms that results by showing that the N370S mutation was present in only 41% of the patients mutated alleles who belong to families that have lived in The Netherlands for at least several generations (Boot et al, 1996).

The application of the urinary EBA allowed the identification of subclinical cases of GD in the study of affected GD families, i.e. individuals with markedly impaired glucocerebrosidase activity but without clear clinical symptoms. The determination of the N370S mutated allele frequency in the Portuguese population gave an insight on the incidence of N370S glucocerebrosidase homozygosity and its association with actual disease manifestation. On the basis of the obtained N370S gene frequency, the difference between the predicted number of homozygotes and that identified until now in the Portuguese population supported the idea that a large number of the GD patients with this genotype developed only mild symptoms or no symptoms at all. However, the clinical presentation of the N370S homozygous Portuguese patients ranged from mild to moderately severe forms of GD. Thus, accurate prediction of these patient's clinical course is not feasible on the basis of the N370S homoallelic glucocerebrosidase genotype, but till now the homo or heteroallelic presence of the N370S mutated glucocerebrosidase is linked with a non-neuronopathic course of the disease. Thus, detailed studies on the residual activity modulation of this mutant glucocerebrosidase are of great importance to understand the factors that prevent the pathological features of GD.

In this thesis work, immunoprecipitation of the glucocerebrosidase residual activity from peripheral blood total leukocytes and skin fibroblasts, showed that comparatively more glucocerebrosidase was extracted from leukocytes than from fibroblasts in the aggregate form (complexes of glucocerebrosidase monomers, saposin C and lipids) from patients presenting at least one N370S mutated allele. This could not be attributed to the existence of an higher amount of intralysosomal activator protein in these cells, since comparative studies between lymphoblasts and fibroblasts (Van Weely, 1992) reported that the ratio of cross-reactive activator protein related to cross-reactive glucocerebrosidase varied considerably between individual lysosomes, but not significantly different between lymphoblasts and fibroblasts of either controls or patients. Comparable post-translation modifications of the glucocerebrosidase oligosaccharide moieties, predominant localisation in compartments with high density characteristic of lysosomes, and a similar degree of interaction with membranes was

also observed between these cell types (van Weely, 1992). Further analysis are needed on the physical-chemical properties of the N370S glucocerebrosidase aggregate/complex form by either activity modulaters or conformational changes introduced by mutations. *In situ* radiation activation experiments in which the molecular mass of the complexe of catalitically active glucocerebrosidase was determined, showed that glucocerebrosidase in this physiological aggregated state is probably present as a dimer (Choy et al, 1986). It is dificult to predict which properties of glucocerebrosidase will be presented by dimers of different mutant proteins, hampering the prediction of the outcome of particular combinations of mutations in genetic compounds. In this regard the present study did not evidence conformational influence on the aggregation of the enzyme in N370S compound heterozygotes. In particular, the observation of similar glucocerebrosidase specific activity of N370S homozygotes and the N370S/IVS2+1 or N370S/L444P compound heterozygotes was not surprising since it can be expected that in these compound heterozygotes the measured enzymatic activity is almost exclusively contributed by the N370S mutated enzyme, given that the L444P allele encodes an unstable glucocerebrosidase (Ohashi et al, 1991) and the IVS2+1 splice mutation directs synthesis of low levels of aberrant mRNAs, not resulting in synthesis of active enzyme. No significant differences were also observed between the molecular activity presented by N370S homozygotes and compounds for the N370S mutation and RecTL, RecNcil or R463C, being necessary to study these proteins by mutant expression since no homoallelic patients were identified till now for those mutations. However, with respect to the L444P mutated enzyme the findings obtained in patients cell extracts differed from the reports on features of the mutant enzyme obtained in the overexpression systems. Cells of the L444P homozygote patient showed little enzyme with almost normal properties (high specific activity per amount of cross reactive molecule in the presence of taurocholate), whereas the L444P mutant enzyme obtained by mutant expression in human HeLa cells was reported to be normally stable and catalitically abnormal, with an activity ranging between 13-25% of the control (Pasmanik-Chor et al, 1995). So far it is unclear why these differences exist. It should be considered that the overexpression system markedly influences the properties of the enzyme. In contrast to the normal situation, in a overexpression system there is a less efficient control of the quality of the newly formed enzyme (correct folding). Furthermore, it is known that in overexpression systems a large proportion of protein molecules differ in conformation, a post-translational modification from the regularly produced proteins. Interestingly, the predictive value of this mutated allele for patients phenotype has also been limited;

usually caucasian L444P homozygotes have been developing neuronopathic type 2 or type 3 forms of GD, but in contrast Japanese L444P homozygotes do not develop neurological disease. The Portuguese L444P homozygote (patient 41) has not showed so far characteristic neurologic symptoms of Gaucher disease, although suffering from behaviour abnormalities.

The study of patients presenting different genotypes allowed to conclude that the glucocerebrosidase molecular activity extracted from cells of patients presenting at least one N370S mutated allele was found to be considerably higher when measured in the presence of the physiological activators phosphatydilserine and saposin C, being this effect markedly pH dependent. Previous studies on the purified spleen N370S mutated enzyme had already showed that the activity of this mutated enzyme in the presence of activator protein and phospholipids, had a very sharp pH-optimum (in contrast with the control enzyme) (Aerts, 1988; Sá Miranda, 1992). Other studies had also reported that at higher pH values this mutated enzyme was dramatically less sensitive to CBE inactivation (reflecting a decrease of the substrate affinity) (van Weely, 1992). According to these findings, it is conceivable that the degree of activation of the glucosylceramide-loaden macrophage (Gaucher cell) determine intralysosomal conditions and thus modulate differently the highly pH dependent catalytic activity of the N370S mutated enzyme. Thus it can be speculated that the variability in intralysosomal pH may underlie the heterogeneity in severity of the clinical expression of Gaucher disease in individuals presenting the N370S mutated glucocerebrosidase.

Given that the increased secretion of lysosomal hydrolases by stimulated macrophages seems to be a response to extensive storage (Moffitt et al, 1978; Gery et al, 1981), the hypothesis was raised that they may also represent a measure for the total body burden and activation of lipid loaded macrophages. In fact, chitotriosidase activity was recently demonstrated in isolated Gaucher cells and *in situ* hybridisation experiments with a specific probe for chitotriosidase RNA revealed unequivocally that the storage cells massively synthesised the enzyme (Boot, 1997). In this thesis work, plasma chitotriosidase activity was found to be significantly less abnormally increased in patients presenting the N370S/N370S genotype. The results otained in the measurement of plasma hydrolases activity levels were not found to reflect the overall patient clinical severity score but may represent a measure of the lipid loaded macrophages of unknown specific sources. In fact, the clinical manifestation of GD is heterogeneous and has a multi-systemic nature due to the presence of pathological storage cells in different body locations. In accordance, some of the splenectomized

patients showed similar plasma chitotriosidase activity level as patients presenting splenomegaly. It therefore seems likely that in splenectomized patients, the presence of Gaucher cells take place in other different body locations, like liver and bone marrow compartments. The currently used quantification of the extent of GD clinical presentation is based upon standard laboratory measurements, radiographs and physical examination, being thus mainly compiled by the extent of cytopenia, organomegaly and both subjective and objective bone abnormalities. However, the degree of liver and spleen enlargement is not an ideal measure for the local amount of storage cells since the presence of fibrosis and altered vascularization also adds to the increase in volume. Although the accumulation of glucosylceramide occurs specially within lysosomes of macrophages, in this study a significant correlation was observed between plasma glucosylceramide and patients clinical severity (as assessed by the severity score index, SSI), suggesting that plasma glucosylceramide may reflect the overall Gaucher cells accumulation.

Alglucerase supplementation therapy further substantiated that plasma hydrolases are directly released by lipid-laden Gaucher cells and most likely that they are originated from storage cells in different tissues. Whereas patient's total β-hexosaminidase activity decreased to normal range in about 3 to 9 months of treatment, TRAP activity showed a slowly and irregular tendency to decrease, and in the large majority of patients did not achieved control range. According to these results, TRAP activity which has been suggested to reflect osteoclastic activity, would thus probably correlate better with bone involvement and constitute a marker for the slow and unclear evolution of bone disease. Hollak however claims to have found a better correlation between this enzymatic activity and the degree of organomegaly, in particular liver pathology (Hollak, 1996). Chitotriosidase activity was found to decrease in a dose dependent manner since patients submitted to the low dose/high frequency regimen showed a delayed response. Based on this observation it may be speculated that plasma chitotriosidase levels do not reflect the severity of one specific clinical sign but the overall severity as expressed in the total body burden of lipid loaded cells. According to our findings this may be the best secondary marker of the total Gaucher cells, and thus constitute a useful and convenient marker to monitor the efficacy of alglucerase therapy with respect to the overall correction and/or prevention of storage cells. In conclusion, if TRAP activity level could be correlated with bone disease, whereas β-hexosaminidase and chitotriosidase activity levels with a combination of organomegaly and bone disease, this would greatly help in the evaluation of the response of the different compartments to alglucerase treatment.

The study of peripheral blood T cell major sub-sets showed that when compared with controls, non-splenectomised GD patients presented significantly lower number of the two major T lymphocyte subsets. The mechanisms via which Gaucher cells affect their surroundings, e.g. locally abnormal cytokine production, may contribute to the abnormal ratio of the T lymphocyte $CD4^+$ and $CD8^+$ sub-populations observed in the great majority of GD patients. However, no correlation was observed between the level of chitotriosidase activity, which seems to reflect the degree of macrophage activation, and patients $CD4^+$ and $CD8^+$ T lymphocyte abnormalities. In IH patients the relative proportion of $CD8^+$ cells were correlated with the degree of iron overload (patients with high $CD4^+/CD8^+$ ratios have higher iron store than patients with normal or low ratios). Such correlation was not observed in GD patients.

Evidence as been reported that GM1-CD4 interaction triggers dissociation of p56lck from CD4 as well as CD4 internalization and degradation. Concomitant with the increase in glucosylceramide, a five-fold increase in GM3 ganglioside has also been reported in the GD tissues (Philipart and Menkes, 1964). Since CD4 expression may be regulated by other sialyated gangliosides (Grassi et al, 1990) it may be expected that the abnormal presence, proportion and redistribution of these compounds on the surface of Gaucher cells may also change the expression of CD4 in T cells of these patients. Importantly, lower numbers of CD8+ T cells appeared associated with the subgroup of patients with bone involvement. Although few patients were classified without bone involvement, the association between bone pathology and the number of CD8+ T lymphocytes was further substantiated by the existence of a significant correlation between the number of these cells and TRAP activity, the putative marker of osteoclastic activity. *In vitro* models may be limited by a series of factors like: species and anatomical site specificity, the absence of intermediary or accessory cells, the absence of normal marrow spatial organization and cellular interactions with the extracellular matrix. The present study of GD may constitute an ideal system to obtain additional important insights and to test the validity of conclusions reached *in vitro*. On the basis of the proposed mechanism for T cells involvement on osteoclastic proliferation/differentiation (Horwood et al, 1998), the presence of lower number of T lymphocytes in GD patients presenting bone involvement would result in increased osteoclastic activity. The resultant higher resorptive activity would finally lead to the loss of balance between bone modeling and remodeling homeostasis. Further studies are however necessary to clarify the possible regulatory interactions between these T-cell subsets and the pathophysiology of Gaucher disease. In patients submitted to the alglucerase supplementation therapy, follow-up studies for an about 2 year period

showed that the individual %CD4$^+$/%CD8$^+$ ratio imbalances persisted over time, confirming the existence of a strict homeostatic regulation of the relative numbers of the two major peripheral T lymphocytes sub-populations.

In virtue of these results further studies are necessary in order to establish if the T lymphocytes imbalances may also contribute to the pathogenesis of the disease. The presence of abnormal T lymphocyte major subsets of patients other than the N370S homozygotes may however suggest the involvement of these cells the pathophysiology of GD in the more severely affected patients and thus constitute a new additional hallmark associated with the mild clinical expression and progression of the disease of the N370S homozygous genotype.

Based on the results of the presented investigations a number of follow-up studies should next be undertaken.

The N370S glucocerebrosidase activity seems to be highly dependent on the lysosomal environment, therefore *in vitro* assays do not truly reflect the *in vivo* activity. This finding is likely to account for the lack of correlation between the clinical phenotype and the residual glucocerebrosidase activity. A better insight of the effect of the intralysosomal environment on glucocerebrosidase activity, especially the pH and saposin C concentration, seems to be crucial for the understanding of the pathophysiology of Gaucher disease. It would also be important to establish whether such a correlation exist if the activity is measured in extracts of purified monocytes/macrophages, since it is these cells specially which are involved in the pathology of type 1 Gaucher disease. Moreover, it has been proposed that the hydrolysis of other endogenous substrates than glucocerebroside (e.g. β-xyloside) may also as well be impaired (Aerts, 1995) and it would be of interest to study the extent to which this contributes further to the clinical presentation.

The apparent lack of correlation between clinical GD phenotype and glucocerebrosidase genotype and properties of residual enzyme strongly suggest that epigenetic factors might modulate the outcome of a glucocerebrosidase deficiency. These factors have to be established in the future. Furthermore, attention has to be paid to the role of macrophage-derived factors (cytokines and/or hydrolases) in the pathology of Gaucher disease. For this purpose, the availability of a good animal model of GD or of cultured lipid-loaden macrophages (Gaucher cells) will be essential.

Given the gross abnormality in plasma chitotriosidase in GD patients it is of interest to determine the physiological role of this enzyme to have some insight in the possible consequences of its marked elevation. This will be crucial to the identification of possible effects of the relative common deficiency in enzyme activity in man and to identify the cause and consequences of the strong increase in plasma levels of chitotriosidase in clinically affected Gaucher patients.

Due to the expensive nature of the enzyme supplementation therapy it would be of interest to assess further the feasibility of substrate deprivation as a strategy for the treatment of Gaucher disease. The administration of inhibitors of the enzyme UDPglucose:ceramide glucosyltransferase, the enzyme that catalyses the first step in the glycosphingolipid biosynthesis, has been found to result in extensive glycosphingolipid depletion in cells treated *in vitro*, without causing toxicity. In normal mice fed via the diet for several weeks, glycolipid levels were reduced 50-70% in all tissues examined without resulting pathology (Platt and Butters, 1997). Preliminary results obtained with these inhibitors in the mouse model of another human glycosphingolipidoses, the Tay-Sachs disease, showed that the accumulation of GM2 ganglioside in the brain was prevented by reduction of the number of storage neurones and the quantity of ganglioside per cell (Platt et al, 1997).

Gene therapy of Gaucher disease is also under study (Barranger et al, 1997). One approach involves *ex vivo* retroviral transduction of human CD34 cells. In this FDA approved study, no myeloablation was used and multiple transplants of genetically corrected cells were employed. The results to date revealed the presence of the glucocerebrosidase transgene in peripheral blood leukocytes in three subjects and measurable gene expression. Studies are in progress to determine whether engraftment and persistence of transduced cells can be accomplished in patients. A second approach involves gene transfer and transplantation of retrovirally transduced myoblasts in mice, resulting in engraftment of transduced muscle cells as well as persistent *in vivo* secretion of glucocerebrosidase in the circulation. The secreted enzyme was found to be taken up by resident macrophages in the liver of transplanted animals, showing that transplanted myoblasts may be useful in producing enzyme to correct the deficiency of glucocerebrosidase in tissue macrophages.

Chapter 7
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A

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APPENDIX

Reference values for serum iron level, transferrin saturation (%) and ferritin in the Portuguese population (Porto et al, 1994).

Reference values	males		females	
	mean	range	mean	range
Serum iron (µg/dl)	107	50-181	95	44-160
Transferrin saturation (%)	39	19-62	34	15-59
Serum ferritin (ng/ml) depending on age (years)				
10	32	8-123	32	7-134
20	59	16-218	32	7-134
30	108	29-401	32	7-134
40	155	30-483	38	7-196
50	155	30-483	47	9-242
60	155	30-483	60	12-304
70	155	30-483	75	15-387

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