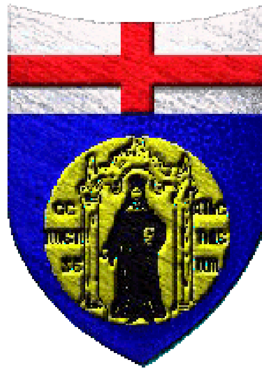

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DEFECTIVE FAS-MEDIATED APOPTOSIS AND IMMUNE DYSREGULATION IN GAUCHER DISEASE

DEFICIT DI APOPTOSI FAS-MEDIATA E DISREGOLAZIONE IMMUNOLOGICA NELLA MALATTIA DI GAUCHER

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1. Introduction

1.1 Gaucher disease

Gaucher disease (GD) is a lysosomal disorder which is inherited as an autosomal recessive condition with an estimated birth frequency of 1 in 57,000. [1]
It is caused by mutations in the GBA1 gene which encodes acid glucocerebrosidase; reduced activity of this enzyme leads to a build-up of glucosylceramide – mainly in the lysosomal compartment of macrophages (giving rise to the so-called 'Gaucher cells').[2]
Accumulation of glucosylceramide and related sphingolipids is associated with multi-system disease and diverse clinical manifestations. GD has been classically divided into three principal types. Type 1 GD (GD1) is mainly characterized by visceral manifestations. Signs and symptoms include splenomegaly, hepatomegaly, thrombocytopenia, anaemia, bone disease and fatigue. In type 2 (GD2) and type 3 (GD3) disease there are neurological manifestations ranging from rapidly progressive neurological deterioration in GD2 leading to death in the first years of life, to a milder neurological phenotype in GD3.[2]
GD1 is the most common type in populations of European ancestry and Ashkenazi Jews in whom it accounts for up to 95% of patients with GD.[2]
The diagnosis of GD can be a challenge not only due to its rare incidence but also due to incomplete penetrance of clinical phenotype, variable onset of the disease (both in childhood and in adulthood), and heterogeneity of clinical symptoms (which can often involve extra-hematologic organs such as bone compartment). Patients are usually referred to hematologists for the presence of cytopenias and/or splenomegaly, and differential diagnosis usually takes into consideration other common hematological disorders. Anemia and/or thrombocytopenia, and rarely also leukopenia, are nearly universal findings among symptomatic patients with GD. Cytopenia may occur at any age, and it usually precedes the appearance of skeletal manifestations.[3]
Disease in different organs may progress at different rates; for example, bone disease may occur without significant hematologic/visceral disease and vice versa, and severity of disease in one organ does not reliably predict disease in other organs or overall disease severity. While some complications of Gaucher disease, such as advanced liver disease and portal hypertension and hepatopulmonary syndrome, always seem to be associated with a high disease severity score index (SSI), there are

other serious Gaucher disease complications, such as pulmonary hypertension, osteonecrosis and fragility fractures that can occur in patients with a relatively low SSI. Similarly, a number of comorbidities are associated with Gaucher disease, such as multiple myeloma and other cancers, Parkinson's disease, peripheral neuropathy, and gallstones, that do not seem to relate to overall disease severity as conventionally defined. Many aspects of Gaucher disease are not fully understood, including an understanding of the pathogenesis and management of neuronopathic disease, the pathogenesis of bone disease, and factors that underlie the variability of Gaucher disease manifestations between patients, even of the same genotype.[4]

Currently, two different therapeutic approaches for the treatment of GD1 are used: enzyme replacement therapy (ERT) and substrate reduction therapy (SRT). Intravenously administered ERT is targeted to macrophages and increases the breakdown of the accumulated glycolipids, while SRT reduces the amount of glucosylceramide by inhibiting its synthesis, representing an oral alternative to ERT. Both treatments have proven to be very effective in the treatment of the visceral and haematological complications of the disease,[5][6][7][8] since decrease in splenic and hepatic size and improvement in cytopenias are usually apparent after 6 months of treatment.[9] Nonetheless, in some patients thrombocytopenia may persist despite treatment and a possible immunological mechanism has been hypothesized. In the last years, a growing interest in immune system involvement in Gaucher disease is developing.

1.2 Autoimmune Lymphoproliferative Syndrome (ALPS)

Autoimmune lymphoproliferative syndrome (ALPS) is a rare congenital disease secondary to a defect of FAS-mediated apoptosis.

ALPS has very low incidence, even if its exact prevalence is still unknown. According to the revised criteria for the diagnosis and classification of ALPS from 2009 International Workshop at the National Institutes of Health (NIH), only approximately 500 patients with ALPS originating from more than 300 families have been investigated all over the world.[10]

It is a genetic disorder caused by germline (62%) or somatic FAS mutations and by mutations in CASP10 and FASLG. The median age at first disease onset is 24 months; however, with increasing awareness of this disease, adult ALPS patients are also now being diagnosed more frequently.[11]

ALPS is often misdiagnosed due to variable phenotypic expressions and the overlap of symptoms with many other haematological disorders.[12]

Lymphoproliferation is the most common clinical manifestation in ALPS, presenting with lymphadenopathy, hepatomegaly or splenomegaly.[13] Lymphoproliferation in ALPS must be chronic (>6 months) and neoplastic and infectious etiology should be ruled out. If an isolated lymphadenopathy is present, it can affect at least two distinct nodal regions. A moderate to massive splenomegaly is detected in more than 85% of patients, and mild to moderate hepatomegaly is also common in ALPS patients.

Autoimmunity is the second most common clinical manifestation in ALPS patients and it frequently requires medical intervention. Autoimmunity usually affects over 70% of patients. Many patients have multiple cytopenias such as Coombs positive autoimmune hemolytic anemia (AIHA) and immune mediated thrombocytopenia. In addition, rashes especially urticarial, immune-mediated pulmonary fibrosis, and SLE are also reported in ALPS. Other autoimmune manifestations including autoimmune nephritis, hepatitis, gastritis, arthritis, and uveitis are infrequently observed.[14]

The elevation of T cell receptor (TCR) αβ+/CD4-/CD8- T cells in peripheral blood and lymphoid tissues is the most significant laboratory characteristic of ALPS patients, but some patients may have normal numbers. The population of TCR αβ+ DNT cells required for the diagnosis is higher than or equal to 1.5 % of total lymphocytes or 2.5% of CD3+ T lymphocytes.[15]

The apoptotic assay of abnormal lymphocytes is previously thought to be the gold standard for the diagnosis of ALPS and documented in the required diagnostic criteria. Apoptotic assay measures the percentage of activated primary lymphocytes undergoing apoptosis after FAS activation (using recombinant Fas ligand/TCR re-stimulation/cytokine starvation).[16]. Approximately 50% or less cell death than the control is considered as abnormal.[15] However, this test is expensive and is available in only few laboratories. Moreover, patients with somatic mutation in FAS and germline FASLG mutations have normal Fas-induced apoptosis assay, so this test is no longer considered as mandatory in the diagnosis of ALPS.[17]

Serum IL-10, IL-18, soluble FAS ligand (FasL), and vitamin B12 are commonly elevated in ALPS patients with FAS mutation and can be useful diagnostic biomarkers.[18] [19]

Recent studies have demonstrated that the presence of elevated TCR αβ+-DNT cells combined with high serum or plasma levels of either IL-10, IL-18, soluble FAS ligand (sFASL), or vitamin B12 can predict the mutation of germline or somatic FAS

gene with an accuracy rate of 85–97%. Due to their high specificity, these biomarkers have been included in the diagnostic criteria.[15]

Since the investigators from NIH have established a triad of criteria for the diagnosis of ALPS in 1999, important research advances have been achieved. In 2009, an international workshop from NIH has revised the criteria for the diagnosis and classification of ALPS and published the revised criteria in 2010.[15]

The presence of both required criteria plus one primary accessory criterion allows a definitive diagnosis of ALPS, while both required criteria plus one secondary accessory criterion suggest a probable diagnosis of ALPS.

<u>Required criteria</u>:

1. Chronic (> 6 months), nonmalignant, noninfectious lymphadenopathy or splenomegaly or both
2. Elevated CD3+TCR+CD4-CD8- DNT cells (≥1.5% of total lymphocytes or 2.5% of CD3+ lymphocytes) in the setting of normal or elevated lymphocyte counts

<u>Accessory criteria</u>:

Primary:

1. Defective lymphocyte apoptosis (in 2 separate assays)
2. Somatic or germline pathogenic mutation in FAS, FASLG, or CASP-10

Secondary:

1. Elevated plasma sFASL levels (>200 pg/mL) OR elevated plasma interleukin-10 levels (>20 pg/mL) OR elevated serum or plasma vitamin B12 levels (>1500 ng/L) OR elevated plasma interleukin-18 levels >500 pg/mL
2. Typical immunohistological findings as reviewed by an experienced hematopathologist
3. Autoimmune cytopenias (hemolytic anemia, thrombocytopenia, or neutropenia) AND elevated immunoglobulin G levels (polyclonal hypergammaglobulinemia)
4. Family history of a nonmalignant/noninfectious lymphoproliferation with or without autoimmunity.[15]

1.3 Study reasons and Objectives

As clinical manifestation of GD can be underhand, in some cases, phenotype can mimic benign lymphoproliferative disorders such as ALPS or other similar disorders whose phenotype does not completely fulfill the ALPS diagnostic criteria that are usually reported as ALPS-like syndromes. The knowledge of these groups of diseases and other primary immune dysregulation disorders (PIRDs) has greatly increased in the last few years, but no patients with such phenotype leading to a diagnosis of GD have been reported so far.

For many years the impairment of monocytes and their transformation to inflammatory subsets of macrophages and dendritic cells has been considered the main pathogenic mechanism of GD; however, in the last few years, the involvement of other players of immune response, such as lymphocytes, including T, B, and natural killer (NK) cells, has been demonstrated, suggesting a more complex scenario.[20][21][22] Nair demonstrated that type II NKT cells with a T follicular helper cell phenotype are increased in an animal model of GD and in GD patients.[22] It is postulated that dendritic cells take up glucosylceramide and glucosylsphingosine release from Gaucher cells, migrate to lymph nodes and present their cargo via CD1d to type II NKT cells. This initial trigger, together with signals from B lymphocytes, drive the activation and differentiation of type II NKT cells, that promote differentiation of B lymphocytes into plasma cells, with associated gammopathy and production of glucosylceramide and glucosylsphingosine specific IgG auto-antibodies.[20] Recently, Pandey has pointed to an unexpected player in GD: the complement system.[23] He showed that glucosylceramide-immunocomplex leads to classical pathway activation, eventually leading to systemic C5 cleavage and C5a generation. In addition, immunocomplexes also bind to activating FcγRs expressed on phagocytes. FcγR aggregation induces local C5 production and non-canonical C5 proteolysis by a cell-specific protease leading to C5a formation. The binding of systemically or locally-generated C5a to C5aR1 enhances the accumulation of glucosylceramide and glucosylsphingosine within phagocytes through increased expression of glucosylceramide synthase, driving a vicious cycle that fuels the autoimmune response directed against these sphingolipids. Furthermore the activation of the C5a/C5aR1 axis in dendritic cells upregulates costimulatory molecules (CD80/CD86/CD40) and drives the activation of NKT effector cells (NKT

Eff) with Th1 (IFN-γ) and Th17 (IL-17A/F) signatures, eventually sparking chronic inflammation and tissue involvement in GD.[23] Pandey also demonstrated that the interruption of this vicious cycle at the level of C5a/C5aR1 interaction is sufficient to massively reduce cellular glucosylceramide accumulation and protect from death in genetic and pharmacologically induced GD models.[23]

In summary, these laboratory results suggest a fundamental role of immunological abnormalities in the pathogenesis of GD.

On the basis of a fortuitous diagnosis of GD in a child previously diagnosed with ALPS and of the frequent phenotypic overlap between these conditions, we decided to investigate the possible presence of an apoptosis defect in GD patients.

The primary objective of the present study was the identification of an ALPS-like immunophenotypic pattern in a wide cohort of GD patients followed in Giannina Gaslini Institute in Genoa.

Secondary objectives were:

- the comparison of immunological alterations between naïve and treated patients
- the correlation of immunological alterations with Gaucher disease activity parameters
- the study of FAS-mediated apoptosis function and caspase activation in a subgroup of patients with indirect signs of apoptosis impairement.

2. Methods

2.1 Patients

All the patients affected by GD evaluated in Rare Diseases Unit of Giannina Gaslini Institute in a period of 18 months were included in the study.

The inclusion criteria were a certain diagnosis of GD (by enzyme assay on leukocytes or lymphocytes or fibroblasts culture and characterization of *GBA1* pathogenic variants) and the informed consent to the participation to the study.

No exclusion criterion was established.

For each patient, the main GD parameters collected at last visit in Gaslini Institute were available: haemoglobin concentration, white blood cell count, platelet count, serum chitotriosidase, spleen and liver volume meausered by MRI, history of splenectomy, bone marrow infiltration and osteonecrosis evaluated through MRI, history of pathological fractures, bone mineralization based on DEXA, diagnosis of respiratory failure, pulmonary hypertension, peripheral neuropathy, Parkinson's disease or parkinsonism. On the basis of these parameters, the Gaucher Disease Severity Score Index - Type I (GauSSI-I) score was calculated for each patient (Table 1).[24]

A flow panel including 4 specific parameters suggestive of ALPS (CD3+CD4-CD8-TCRαβ+, known as "double negative T cells" [DNTs], >1.5%; CD19+CD27+ <10%; B220+DNTs >60%; CD3+CD25+/CD3+HLA-DR+ ratio <1) and serum biomarkers (FAS ligand, IL-18, IL-10, and vitamin B12) evaluation were used as a screening to identify immunological features of ALPS in patients with GD. [25][26][27][28][29]

In those who showed at least 3 ALPS-like immunophenotypic parameters, both FAS-induced apoptosis and caspase activation were assayed in Epstein-Barr virus (EBV)-immortalized B-cell lines.

Furthermore, NK cells, NKT-like cells (CD3+ CD16+CD56+), CD19+ B cells, TCRγδ+, Treg, T naive and T memory cells were studied.

Autoimmune screening included antinuclear, antiextractable nuclear antigen, antithyroid peroxidase, antithyroglobulin, and antitissue transglutaminase immunoglobulin antibodies testing, along with direct and indirect Coombs tests. In the case of thrombocytopenia or neutropenia, antiplatelet or antineutrophil antibodies, respectively, were also tested.

Biological samples were provided by Gaslini Biobank (Target Biobank P00-IGG-GB, request ID 509 and 620, Genoa, Italy); for tests requiring biological material that was not frozen, supplemental blood samples were donated during routine monitoring of the disease. All samples were donated with the patient's or parent/tutor's written informed consent.

2.2 Laboratory methods

2.2.1 Lymphocyte immunophenotype

Peripheral lymphocyte subsets were evaluated from whole blood using an 8-colour immunostaining panel and by *lyse and wash* procedure. Briefly, 50 µl of EDTA anticoagulated whole blood has been incubated with surface fluorochrome-labelled monoclonal antibodies (mAbs) for 20 min at 4°C and lysed with FACS Lysing solution (Becton Dickinson, BD) for10 min at room temperature (RT). Data acquisition and analysis have been performed on a FACS Canto II flow cytometer (BD) equipped with three lasers a Blue (488-nm, air-cooled, 20-mW solid state), a Red (633-nm, 17-mW HeNe), a Violet (405-nm, 30-mW solid state) and FACS Diva™ software (BD). Peripheral lymphocyte subsets were evaluated using the following RUO mAbs: CD3, CD4, CD8, CD16-56, CD19, CD20, CD27, TCRαβ, TCRγδ, HLA-DR, CD25, CD45, CD45RO, CD45RA (all BD).

Fluorochromes differently combined for eight colours antibody panels were: APC, APC-H7, FITC, PE, PE-Cy7, PerCP-Cy5.5, V450, V500.

2.2.2 Interleukin-18, interleukin-10, and FAS ligand

IL-18, IL-10, and FAS ligand were tested using commercially available enzyme-linked immunosorbent assay kits (MBL, Woburn, Mass; Invitrogen, Waltham, Mass; Abnova, Taipei, Taiwan, respectively).

2.2.3 FAS-induced apoptosis and caspase activation

The patients' EBV-immortalized B cells were treated with FAS ligand (10 ng/mL; Enzo Life Science) for 24 hours to induce apoptosis, and cell death was measured by a cytofluorimeter. Apoptosis pathway function was evaluated by the Western-blot analysis of CASP10, CASP8, and PARP proteins using rabbit monoclonal anti-CASP10 antibodies (Abcam), mouse monoclonal anti-CASP8 (1C12), and rabbit polyclonal anti- PARP (Cell Signaling Technology).

2.3 Statistical analysis

Comparison of qualitative variables was performed by applying the Chi-square test or the Fisher exact test. Comparison of quantitative variables between two groups was made by the Mann–Whitney U test.

All correlations were assessed using Spearman's rank order correlation coefficient (r_s). For the purpose of this analysis, correlations >0.8 were considered very high, correlations ranging from 0.6 to 0.79 were considered high, correlations ranging from 0.4 to 0.59 were considered moderate and correlations <0.4 were considered weak.

All statistical tests were two-sided and $p<0.05$ was considered statistically significant.

The statistical package used was Statistica (StatSoft, Tulsa, Oklahoma, USA).

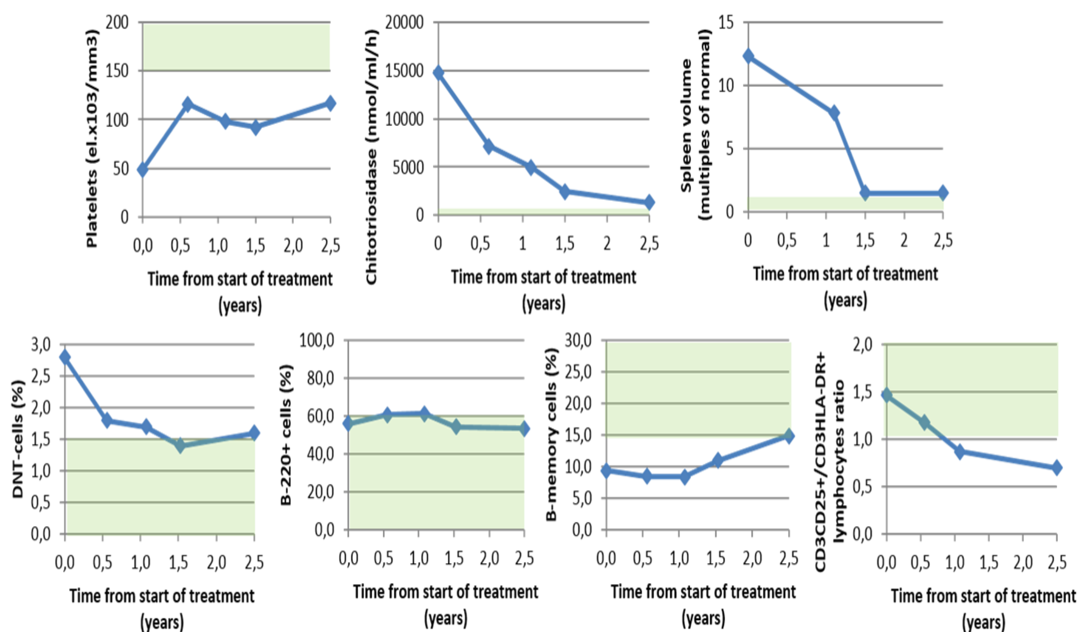
3. Results

3.1 Index case

A 12-year-old child with a history of ALPS diagnosed in another center was referred to our Institution for a second evaluation.

The patient phenotype completely fulfilled the diagnostic criteria of ALPS reported by Oliveira, [15] since he presented with chronic splenomegaly since the age of 22 months, severe persistent thrombocytopenia, increased count of DNTs (2.8%) and defective lymphocyte apoptosis in two different samples (FAS-induced cell death 0% and 13%). No pathogenic mutation was found in *FAS, CASP-10* and *FASLG* genes. Surprisingly, bone marrow biopsy showed the presence of a huge amount of engorged histiocytes PAS+ and CD68+, identified as "Gaucher cells". Chitotriosidase was significantly increased (14760 nmol/ml/h), βglucocerebrosidase activity was markedly reduced (0.9 nmol/mg/h) and *GBA1* gene analysis showed a compound heterozigosity (N370S/G202R), leading to a diagnosis of GD associated to ALPS. MRI showed femoral bone marrow infiltration bilaterally. ERT was started (50 UI/Kg i.v. every other week) and it was able to reverse the clinical signs and symptoms and even the main immunological abnormalities previously noticed (Figure 1).

Figure 1. Changes in platelet count, chitotriosidase, spleen volume and immunological parameters during the 2.5 years following the start of ERT in the first patient diagnosed with ALPS affected by Gaucher disease.



On the basis of this unusual phenotypic overlap, we decided to investigate the ALPS-like immunophenotypic pattern, FAS-mediated apoptosis function, and caspase activation in a wider cohort of patients with GD, with the aim of identifying the presence of apoptosis impairment.

3.2 GD population

In total, 41 patients aged 1.6 to 71.5 years were enrolled. Eight patients were treatment-naïve and 33 were treated with ERT o SRT.

Demographic characteristics and a summary of GD-related parameters (*GBA1* analysis result, age at disease onset, treatment duration, and GauSSI-I score) for each patient are reported in Table 1.

Table 1. Detailed demographic features and Gaucher disease related parameters, including results of molecular analysis of *GBA1* gene and GauSSI-I score (Gaucher Disease Severity Score Index – Type 1), for each patient included in the study. *GauSSI-I score is not validated for children (not applicable, NA). Age at onset of the disease is NA in asymptomatic patients (diagnosed after newborn screening or diagnosis in a sibling).*

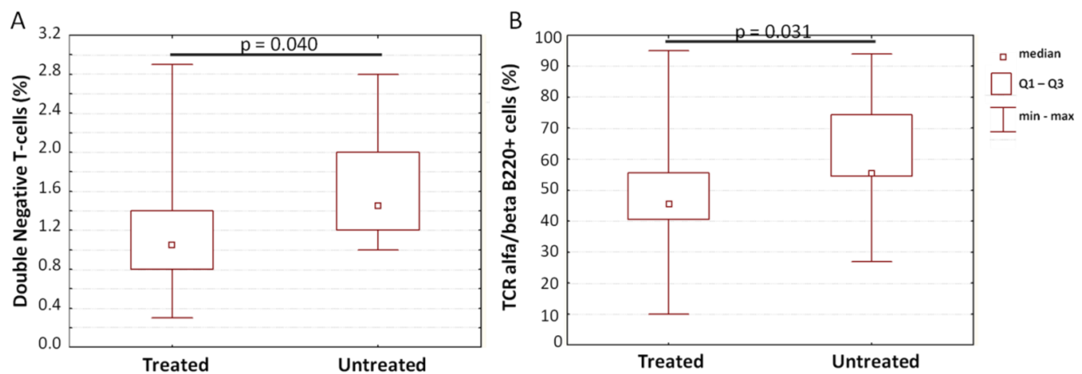
Patient	Sex	Age (years)	Mutation 1	Mutation 2	Age at onset (years)	TRT	TRT duration (years)	GauSSI-I score
1	m	1.6	L444P	N370S	0.5	NO	NA	NA
2	f	6.6	N370S	E388K	NA	NO	NA	NA
3	f	10.5	N370S	E388K	NA	NO	NA	NA
4	f	13.5	N370S	N370S	NA	NO	NA	NA
5	m	12.6	N370S	G202R	1.9	NO	NA	NA
6†	f	14.7	S271G	?	10.0	NO	NA	NA
7	f	37.0	N370S	F213I	23	NO	NA	9
8	m	52.4	L444P	G46E	5.0	NO	NA	8
9	f	65.4	N370S	D409H+H255Q	57.0	NO	NA	4
10	f	65.8	N370S	R120W	49.0	NO	NA	6
11†	f	2.3	E388*	D409H	0.3	YES	1.3	NA
12†	f	5.7	L444P	L444P	4.0	YES	5.0	NA
13	f	5.8	L444P	N370S	3.0	YES	2.5	NA
14	m	7.2	L444P	I402T	4.4	YES	2.4	NA
15†	f	11.2	L444P	L444P	1.0	YES	8.1	NA
16	f	13.1	N370S	IVS2+1G>A	8.0	YES	0.8	NA
17	f	13.9	L444P	W312S	6.0	YES	6.4	NA
18	m	16.0	N370S	N370S	2.6	YES	13.3	NA
19	f	16.5	L444P	W312S	9.0	YES	6.4	NA
20	f	17.2	N370S	RecNciI	9.0	YES	6.8	NA
21	f	19.1	G46E	G202R	5.0	YES	12.7	3
22	m	20.8	N370S	IVS2G>A	5.0	YES	15.4	0

23	m	21.8	N370S	L444P	10.0	YES	11.9	0
24	m	25.3	N370S	IVS2G>A	5.0	YES	18.2	0
25	f	28.7	L444P	W312S	8.0	YES	16.4	1
26†	m	31.5	L444P	F213I	3.0	YES	25.8	0
27	f	32.9	N370S	R131C	11.0	YES	18.7	3
28	m	35.7	N370S	RecNciI	3.0	YES	24.6	0
29	m	36.7	N370S	R131C	2.5	YES	12.3	3
30	f	38.3	N370S	G195R	35.0	YES	2.7	0
31	m	41.6	N370S	R131C	6.0	YES	13.3	3
32	m	49.6	N370S	L444P	15.0	YES	ND	0
33	m	50.0	N370S	L444P	32.0	YES	12.4	2
34	f	52.6	N370S	rec NciI	35.0	YES	12.8	1
35	f	59.0	N370S	L444P	41.0	YES	15.3	1
36	m	59.0	N370S	D409H+H 255Q	40.0	YES	17.5	1
37	f	67.1	N370S	L444P	40.0	YES	21.3	4
38	f	67.2	N370S	N370S	56.0	YES	7.2	0
39	f	69.4	N370S	N370S	50.0	YES	12.8	1
40	f	69.8	N370S	?	54.0	YES	ND	3
41	m	71.5	N370S	S366R	60.0	YES	9.2	3

†GD3 patients. m: male. f: female. NA: not applicable. TRT: treatment. GauSSI-I score: Gaucher Disease Severity Score Index- Type I.[24]

Nine (21%) and 7 (17%) of the patients had high levels of DNTs and B220+DNTs, respectively. Levels of DNTs and B220+DNTs were more frequently raised in naïve subjects (p=0.040 and p=0.031, respectively; Figure 2) and in patients with early onset of the disease (p=0.046 and p=0.011, respectively) compared with treated patients and patients with a late onset phenotype.

Figure 2. Comparison of DNTs and B220+DNTs in treated and untreated GD patients.



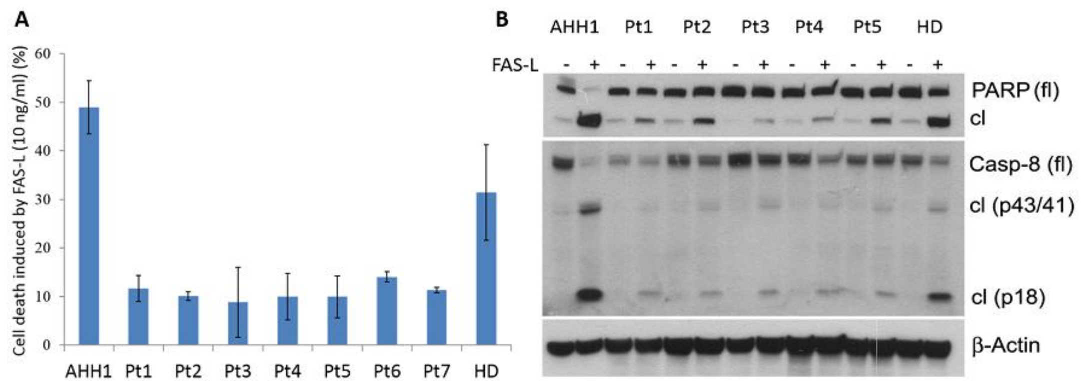
No significant correlation was found between DNTs and B220+DNTs and GauSSI-I score or other Gaucher disease severity parameters.

All patients showed normal levels of IL-10 and serum FAS-L, but 14 of 41 patients (34%) had IL-18 levels of >500 pg/mL, with a trend for higher levels in untreated versus treated patients, although this was not statistically significant (p=0.06).

Overall, 10 of 41 (24%) patients showed an ALPS-like immunological pattern (at least 3 of 4 features of the ALPS panel), that was more frequent in untreated patients (p=0.003) and in patients with an earlier onset of disease (p=0.01).

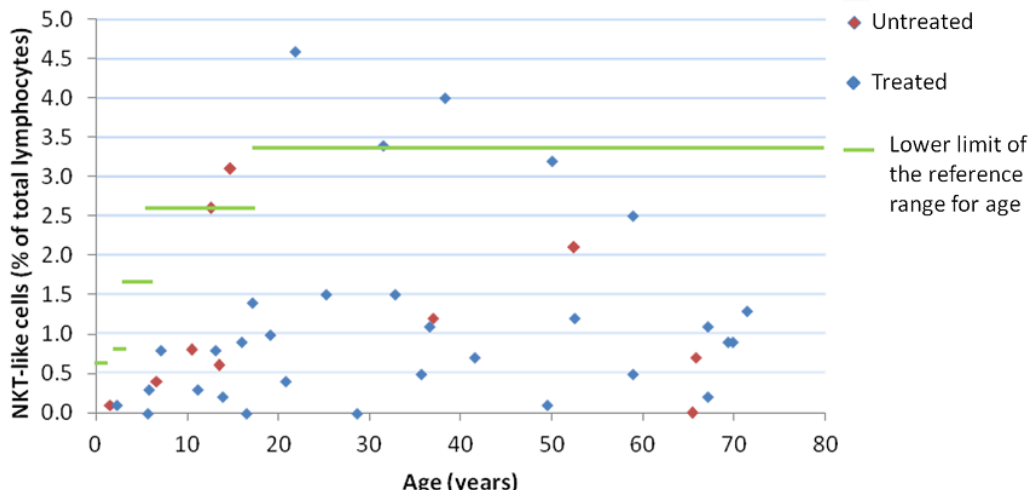
FAS-induced apoptosis and caspase activation were further evaluated in these patients, with 7 out of 10 (70%) shown to be defective (Figure 3).

Figure 3. (A) Cell death and **(B)** apoptosis pathway impairment after FAS ligand-induced stimulation. AHH1 (ATCC CRL-8146), human B-lymphoblastoid wild-type cell line.



Surprisingly, 37 of 41 patients (90%) had CD8+NKT-like cells that were absent or lower than normal values for age (Figure 4).

Figure 4. Reduction of NKT-like cells below the normal values for age in the majority of patients with Gaucher disease, irrespective of treatment.



The only patient evaluated before and after treatment showed complete normalization of lymphocyte subsets after ERT. A comparison of the main clinical and laboratory parameters between treated and untreated patients is shown in Table 2. Lymphocvtes subsets and ALPS immunophenotipic features and biomarkers for each patient included in the study are reported in Table 3 and Table 4, respectively.

Table 2. Summary of the demographic, laboratory and clinical parameters of study population.

	Untreated *(10 patients)*	**Treated** *(31 patients)*	**p**
Age (years)	14.1 (11.0 - 48.6)	30.1 (16.3 - 51.3)	0.286
Age at onset (years)	10.0 (3.5 - 36.0)	8.0 (4.2 - 35.0)	0.971
Age at start of treatment (years)	NA	11.1 (5.7 - 37.6)	NA
Treatment duration (years)	NA	12.7 (6.6 - 17.0)	NA
ERT dosage (U/Kg/2 weeks)	NA	31.0 (29.0 - 38.5)	NA
Chitotriosidase (nmol/ml/h) [n.v. 43.5±23.2]	5120 (1242 - 10660)	480 (320 - 930)	0.223
Platelets (el. x10^3/mm^3) [n.v. 150-400]	188 (93 - 265)	210 (162 - 241)	0.354
Hemoglobin (g/dL) [n.v.:m 13.5-17-5; f 12.0-15.5]	12.8 (11.3 - 13.5)	14.2 (13.5 - 15.2)	0.002*
Ferritin (ng/mL) [n.v. 15-50]	180 (103 - 780)	98 (37 - 170)	0.119
Spleen volume (cc) [n.v.=0.2% of total body weight] [1]	973 (694 - 1124)	247 (216 - 270)	0.009*
Spleen volume (multiples of normal)	1.3 (1.0 - 4.9)	1.5 (1.0 - 2.0)	0.829
GauSSI-I score [n.v. 0] [2]	7.0 (5.5 - 8.3)	1.0 (0.0 - 3.0)	0.002*
NK-T like cells (% total lymphocytes) [3]	0.8 (0.5 - 1.9)	0.9 (0.3 - 1.4)	0.988
Double negative T-cells (% total lymphocytes) [n.v.<1.8]	1.5 (1.2 - 2.0)	1.0 (0.8 - 1.4)	0.040*
B220+ cells (% TCRαβ+ lymphocytes) [n.v.<60]	55.7 (54.5 - 70.0)	44.7 (40.8 - 55.1)	0.031*
B-memory cells (% total B lymphocytes) [n.v.>15]	13.3 (10.0 - 21.4)	24.1 (15.8 - 32.4)	0.063
CD3CD25+/CD3HLA-DR+ lymphocytes ratio [n.v.>1]	1.0 (0.5 - 1.3)	1.2 (0.7 - 1.7)	0.472
IL-18 (pg/mL) [n.v. 36-258]	1350 (1000 -; 1350)	350 (139 -; 469)	0.618
IL-10 (pg/mL) [n.v. <1]	<1 (<1 - <1)	<1 (<1 - 1.5)	0.731
sFASL (pg/mL) [n.v. 0]	0 (0 - 0)	0 (0 - 0)	0.480
Vitamin B12 (pg/mL) [n.v. <663]	429 (322 - 578)	438 (315 - 558)	0.815
Apoptosis defect parameters (n.) [4]	3 (2 - 3)	1 (0 - 2)	0.003*

NA: not applicable. n.v.: normal values. ERT: enzyme replacement therapy.
*GauSSI-I score: Gaucher Disease Severity Score Index – Type I. *p<0.05*
[1]*Spleen volume has been measured by MRI only in adults.*
[2]*GauSSI-I score is not applicable in children and has been calculated only for adults.*
[3]*Normal values for NK-T like cells vary deèending on age.*
[4]*Apoptosis defect parameters include: double negative T-cells ≥1.8%, TCRαβ+B220+ cells ≥60%, B-memory cells <15%, CD3CD25+/CD3HLA-DR+ lymphocytes ratio <1, IL-18 >500 pg/mL, IL-10 >20 pg/mL, sFASL > 200 pg/mL, vitamin B12 > 663 pg/mL, abnormal FAS-apoptosis.*

Table 3. Lymphocvtes subsets for each study patient, expressed as percentage of total lymphocytes .

Patient	CD3+ cells (%) [n.v.60-78]	CD3+CD4+ cells (%) [n.v.33-49]	CD3+CD8+ cells (%) [n.v.20-28]	B cells (%) [n.v.10-22]	NK cells (%) [n.v.10-16]	NK-T like cells (%)*
1	55.8 (L)	32.0 (L)	17.6 (L)	26.3 (H)	15.8	0.1 (L)
2	73.1	35.2	27.3	16.9	10.5	0.4 (L)
3	73.9	34.5	31.0 (H)	17.1	8.8 (L)	0.8 (L)
4	77.8	50.3 (H)	18.3 (L)	14.0	7.9 (L)	0.6 (L)
5	80.1 (H)	34.4	35.4 (H)	15.0	6.4 (L)	2.6 (L)
6†	70.0	30.0 (L)	27.9	13.0	15.4	3.1
7	85.9 (H)	52.2	28.5 (H)	10.5	3.3 (L)	1.2 (L)
8	66.0	33.0	31.0 (H)	21.0	12.6	2.1 (L)
9	71.5	55.0 (H)	12.7 (L)	9.0 (L)	6.0 (L)	0.0 (L)
10	70.6	36.0	33.0 (H)	14.6	15.0	0.7 (L)
11†	64.4	45.0	16.0 (L)	25.3 (H)	8.3 (L)	0.1 (L)
12†	70.4	33.8	23.2	18.9	8.7 (L)	0.0 (L)
13	69.5	31.0 (L)	28.2 (H)	20.7	8.1 (L)	0.3 (L)
14	82.5 (H)	37.7	28.4 (H)	11.1	5.4 (L)	0.8 (L)
15†	63.6	40.2	15.5 (L)	18.7	17.3 (H)	0.3 (L)
16	76.4	49.7 (H)	16.7 (L)	16.3	7.4 (L)	0.8 (L)
17	76.3	46.0	24.0	13.8	10.3	0.2 (L)
18	81.9 (H)	42.5	25.7	11.7	6.0 (L)	0.9 (L)
19	72.8	43.0	22.0	13.3	13.1	0.0 (L)
20	71.3	43.0	24.3	10.0	19.0 (H)	1.4 (L)
21	83.1 (H)	48.6	30.2 (H)	9.8 (L)	6.7 (L)	1.0 (L)
22	70.6	44.0	21.4	-	17.8 (H)	0.4 (L)
23	68.8	40.0	24.5	7.9 (L)	22.6 (H)	4.6
24	80.0 (H)	48.0	23.3	-	10.0	1.5 (L)
25	85.5 (H)	45.0	33.2 (H)	5.7 (L)	8.5 (L)	0.0 (L)
26†	73.9	24.5 (L)	43.4 (H)	10.7	14.6	3.4
27	74.3	45.0	25.0	9.4 (L)	16.2 (H)	1.5 (L)
28	62.4	20.0 (L)	23.0	0.5 (L)	5.9 (L)	0.5 (L)
29	66.6	43.0	21.1	13.0	20.5 (H)	1.1 (L)
30	74.3	48.0	14.5 (L)	7.7 (L)	17.4 (H)	4.0
31	66.2	34.0	30.2 (H)	7.3 (L)	26.0 (H)	0.7 (L)
32	75.3	47.0	27.9	19.4	6.1 (L)	0.1 (L)
33	67.9	30.3 (L)	33.0 (H)	3.3 (L)	5.3 (L)	3.2
34	73.9	44.2	26.0	14.6	15.2	1.2 (L)
35	79.0 (H)	61.0 (H)	14.9 (L)	9.5 (L)	11.6	0.5 (L)
36	63.7	37.7	21.5	15.6	19.9 (H)	2.5 (L)
37	68.0	39.2	25.0	16.3	13.6	0.2 (L)
38	73.8	37.5	34.0 (H)	11.5	13.4	1.1 (L)
39	75.6	48.6	23.5	15.4	9.0 (L)	0.9 (L)
40	61.5	46.0	13.4 (L)	6.4 (L)	32.2 (H)	0.9 (L)
41	53.3 (L)	33.0	17.5 (L)	6.1 (L)	41.4 (H)	1.3 (L)

†GD3 patients. ND: not determined. n.v.: normal values. (H): higher than normal range. (L): lower than normal range.

**NK-T like cells (% of total lymphocytes) n.v. for age: >3.4 (>16 years), >2.6 (5-16 years), >1.7 (2-5 years), >0.8 (1-2 years).*

Table 4. ALPS immunophenotipic features (Table 4A) and biomarkers (Table 4B) for each patient included in the study.

Table 4A.

Patient	FAS-apoptosis test (%)* [n.v.<82]	DNT-cells (% total lymphocytes) [n.v.<1.8]	B220+ cells (% TCRαβ+ lymphocytes) [n.v.<60]	B-memory cells (% total B lymphocytes) [n.v.>15]	CD3CD25+/CD3HLA-DR+ lymphocytes ratio [n.v.>1]
1	64%	2.0 (H)	91.3 (H)	10.0 (L)	0.4 (L)
2	44%	1.9 (H)	56.8	20.6	1.1
3	39%	2.1 (H)	54.5	14.2 (L)	0.4 (L)
4	60%	1.2	74.4 (H)	12.4 (L)	0.9 (L)
5	100% (H)	2.8 (H)	56.0	9.4 (L)	1.5
6†	ND	1.0	52.5	5.6 (L)	0.4 (L)
7	52%	1.5	94.0 (H)	69.0	1.4
8	100% (H)	1.4	55.0	10.0 (L)	2.0
9	ND	1.2	27.0	21.6	1.0
10	43%	1.0	55.3	29.0	1.1
11†	74%	1.4	95.0 (H)	16.5	1.2
12†	52%	2.0 (H)	44.7	11.5 (L)	0.7 (L)
13	69%	1.1	53.7	11.0 (L)	0.3 (L)
14	57%	ND	ND	ND	ND
15†	58%	1.2	40.5	21.6	1.4
16	57%	0.8	41.5	9.7 (L)	2.0
17	ND	1.4	47.5	18.7	1.3
18	60%	0.8	44.0	11.8 (L)	0.5 (L)
19	ND	1.1	42.3	21.6	2.9
20	ND	1.0	50.6	26.5	1.8
21	ND	0.9	60.6 (H)	27.6	1.1
22	61%	1.6	36.3	24.1	2.0
23	56%	0.7	55.6	13.6 (L)	1.2
24	ND	2.2 (H)	32.9	24.8	2.7
25	74%	0.9	58.0	33.0	0.8 (L)
26†	ND	1.6	58.0	42.7	1.0
27	ND	2.0 (H)	43.0	44.0	1.5
28	73%	2.9 (H)	60.0 (H)	0.0 (L)	0.7 (L)
29	28%	0.9	52.5	12.9 (L)	0.7 (L)
30	88% (H)	0.8	33.6	47.0	2.0
31	19%	1.4	64.0 (H)	30.0	0.6 (L)
32	ND	0.3	31.0	63.9	1.4
33	52%	0.8	59.0	56.0	0.5 (L)
34	78%	0.8	10.0	41.5	1.2
35	26%	1.0	30.0	46.5	5.2
36	34%	2.5 (H)	53.0	15.6	1.3
37	ND	1.1	41.6	18.5	1.0
38	43%	1.4	50.0	22.0	0.2 (L)
39	ND	0.6	43.0	30.6	0.4 (L)
40	ND	0.7	34.3	30.7	2.1
41	ND	0.7	46.6	25.0	0.5 (L)

Table 4B.

Patient	IL-18 (pg/mL) [n.v.<500]	IL-10 (pg/mL) [n.v.<20]	sFASL (ng/mL) [n.v.<200]	Vitamin B12 (pg/mL) [n.v.<663]
1	825 (H)	<1	ND	623
2	1000 (H)	<1	0	605
3	275	<1	0	ND
4	900 (H)	<1	ND	498
5	ND	ND	ND	359
6†	2182 (H)	<1	0	ND
7	900 (H)	<1	0	309
8	1350 (H)	<1	0	241
9	550 (H)	3	0	ND
10	1350 (H)	<1	0	ND
11†	600 (H)	2	0	883 (H)
12†	350	3	0	ND
13	2200 (H)	15	ND	941 (H)
14	175	<1	0	636
15†	450	3	0	ND
16	1000 (H)	<1	0	645
17	525 (H)	<1	0	450
18	1200 (H)	<1	ND	508
19	650 (H)	<1	0	379
20	475	<1	0	301
21	132	<1	0.6	ND
22	95	<1	0	ND
23	ND	ND	ND	532
24	100	<1	0	ND
25	250	<1	0	474
26†	135	<1	0	490
27	375	0	ND	ND
28	250	<1	0	446
29	400	<1	0	251
30	15	<1	ND	266
31	400	2	0	212
32	150	<1	0	266
33	450	<1	0	327
34	125	<1	0.2	301
35	500	2	0	437
36	130	2	0	416
37	ND	ND	ND	ND
38	300	<1	0	706 (H)
39	500	<1	0	654
40	500	<1	0	319
41	350	<1	0	438

†GD3 patients. ND: not determined. n.v.: normal values. (H): higher than normal range. (L): lower than normal range.
**Performed on fresh activated T-cells stained by direct immunofluorescence with anti-Fas monoclonal antibody and analysed by cytometry .*

Autoimmunity parameters were negative in most patients, except for 2 cases of antinuclear antibody positivity and 3 cases of antithyroid peroxidase and antithyroglobulin antibodies; antitissue transglutaminase immunoglobulin A antibody and direct and indirect Coombs tests were always negative, as well as antiplatelet antibodies. Antineutrophil antibodies were never tested because no case of neutropenia was detected.

4. Discussion

This study shows that untreated patients with GD have an immune-dysregulation pattern secondary to an FAS-mediated apoptosis defect, adding novel information to the already complex pathogenic scenario of this rare disease. This highlights that hematologists and immunologists should be aware of the possibility of GD among referrals for ALPS. Some common clinical issues of both disorders, such as thrombocytopenia and splenomegaly, should be evaluated not only as an overlapping sign of different diseases, but also as the result of a common pathogenic pathway impaired by different causes.

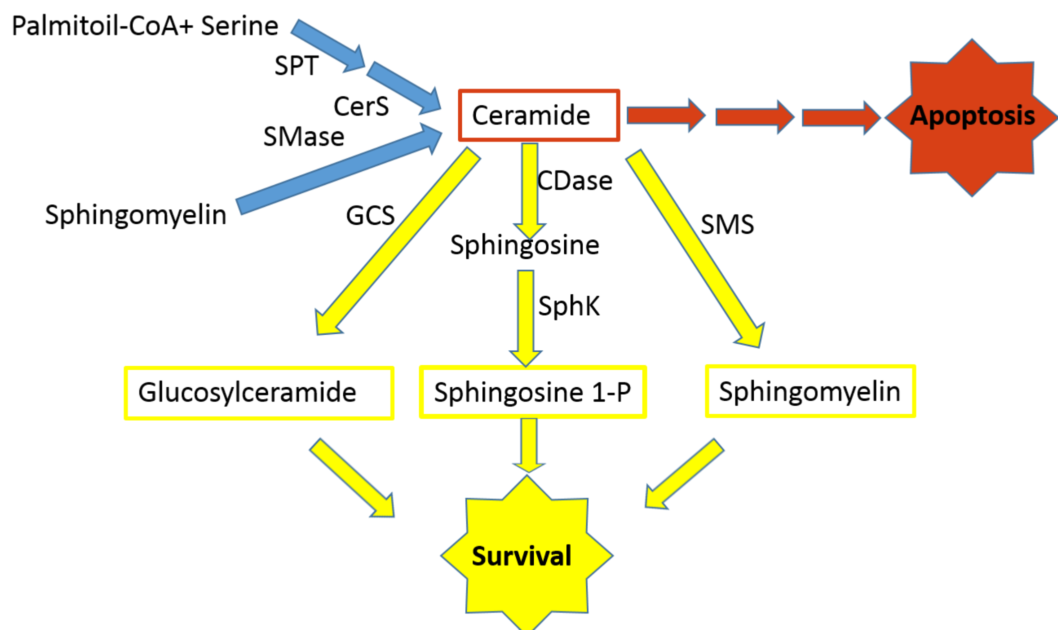
The reasons for a FAS-mediated apoptosis defect in patients with GD are still unclear. We can speculate that the well-known alteration of cell-membrane lipid homeostasis in GD may play a role in such a phenomenon, as already shown in the case of toll-like receptors.[30] Moreover, it is well known that sphingolipids are key players in many major cell biological functions and that ceramide, a central molecule of sphingolipid metabolism, plays a crucial role in programmed cell death, being a mediator of both intrinsic and extrinsic apoptosis pathways.[31] In fact, the increased generation of ceramide in response to cellular stress and apoptosis triggers, such as tumor necrosis factor or FAS ligand, results in cell death by caspase activation. Conversely, glucosylceramide, which is increased in GD because of a GBA defect, is an antiapoptotic sphingolipid (Figure 5).[32] There is growing evidence indicating that, via glucosylceramide synthase (GCS) overexpression, glucosylceramide accumulates in multidrug-resistant tumors and cancer cell line[33][34] and that the consequent abnormal ceramide/glucosylceramide ratio is the cause of drug-induced apoptosis resistance; indeed, the inhibition of GCS reverses drug resistance in cancer cells, thus decreasing the levels of glucosylceramide and enhancing ceramide-induced apoptosis.[35] Although this has not been proved in our patients and needs further study, we may speculate that the abnormal proapoptotic/antiapoptotic lipid ratio, due to defective glucosylceramide degradation, may play an important role in the observed apoptosis defect because it is also known that ERT or inhibition of GCS by eliglustat,[36] leading to restoration of the ceramide/glucosylceramide ratio, induces
apoptosis.

The presence of an apoptosis defect and immune dysregulation in this rare disease may also explain the previously reported cases of patients with GD with immune thrombocytopenic purpura,[37][38] which, in addition to splenomegaly, can

represent a crucial factor for misdiagnosis versus other immunedysregulation syndromes that, in turn, can also be underhand, being secondary to genetic defects known to have variable penetrance and phenotype.[39]

The higher incidence of such defect in naïve patients observed in our study may be explained by the potential role of ERT to improve not the only clinical signs but also immune dysregulation, as demonstrated in our patient with a previous diagnosis of ALPS, who showed complete recovery of immunological impairment after appropriate treatment.

Figure 5. Role of sphingolipids and ceramide in the balance between apoptosis and cell survival.



As already reported in other PIRDs,[40] patients with GD of our cohort showed higher counts of DNTs, thus confirming that this finding cannot be considered a pathognomonic sign of ALPS. Interestingly, the patients of our cohort mostly showed an increase of IL-18 and DNTs but, unlike patients with ALPS, normal levels of IL-10, serum FAS, and vitamin B12, highlighting the important role of such biomarkers in the differential diagnosis of ALPS from other PIRDs or GD and indicating that the already revised diagnostic criteria of ALPS[15] need to be updated. In fact, in the last few years, knowledge of novel genetic defects causing PIRDs has dramatically increased, expanding the category of ALPS-like disorders, but no metabolic diseases have been reported to be associated with this phenotype so far. Our study shows, for the first time, that immune dysregulation can be the sign of a metabolic disorder such as GD and should be taken into consideration by clinicians

when patients presenting with this clinical pattern are referred. For this reason, the GBA1 gene should also be included in next-generation sequencing panels used to screen PIRDs because the identification of a specific underlying genetic defect can redirect care for targeted therapies, as already shown in other disorders such as Evans syndrome.[41][42]

Finally, an incidental finding of our study, not reported before in GD and therefore worthy of mention, was the demonstration of a decrease or absence of CD8+NKT-like cells in 90% of our patients. These cells are CD1d-independent T cells with NK markers, and therefore are distinct from CD1d-dependent invariant NKT cells.[43] CD8+NKT-like cells are especially noted as key players in the progression of chronic obstructive pulmonary disease and bronchiolitis obliterans and in conferring resistance to glucocorticoids.[44] We do not have the data to connect the decrease of CD8+NKT-like cells to sphingolipid dysfunction due to GBA deficiency and/or to lymphocyte apoptosis defect; therefore, this finding needs to be further investigated with specific studies.

In conclusion, the presence of a FAS-mediated apoptosis defect leading to an ALPS-like immune dysregulation in patients with GD suggests that this disorder should be excluded in the diagnostic work-up of ALPS and other PIRDs to administer the right treatment. Further studies are needed to confirm the role of impairment of the cell-membrane lipid structure in the pathogenesis of such a defect and to evaluate prospectively, on a larger cohort of patients, the role of ERT in the normalization of Immune dysregulation.

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