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Original Article

Altered immune phenotypes in subjects with Fabry disease and responses to switching from agalsidase alfa to agalsidase beta

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Abstract: Fabry disease (FD) is a rare X-linked genetic disorder caused by mutations in the *GLA* gene encoding the lysosomal enzyme, α-galactosidase A (α-gal A). The mutations lead to lack of or faulty enzyme causing accumulation of globotriaosylceramide (Gb3) and related glycosphingolipids including globotriaosylsphingosine (lyso-Gb_3). Treatment options for FD include enzyme replacement therapy. There are two different recombinant α-gal A enzymes, where agalsidase beta has been approved by FDA for use in the USA while both agalsidase beta and agalsidase alfa are being prescribed in many other countries. Several FD patients in the USA were switched to agalsidase alfa for a certain period of time due to supply shortage of agalsidase beta but were switched back to agalsidase beta upon availability. Due to the fact that some glycolipids may serve as antigens, various immune abnormalities have been associated with several lysosomal storage disorders (LSDs). In the present clinical study we evaluated alterations in peripheral immune cell subsets in patients with FD (n=27) compared to healthy control group (n=27). Patients with FD showed persistent T cell associated abnormalities, including skewed T helper to cytotoxic T cell ratio and elevated fraction of memory T cells and expression of activation markers on T cell subsets. Further, the study elucidated the effect of switching from agalsidase alfa to agalsidase beta on immune system as well as other clinical markers. While there was relative decrease in plasma lyso-Gb_3 as well as urine lyso-Gb_3 over time, their levels remained above the reference values. The immune abnormalities did not correlate with gender, age or lyso-Gb_3 levels, indicating that these persistent changes were inherent to FD irrespective of the extent of substrate accumulation.

Keywords: Fabry disease, immune cells, enzyme replacement therapy, agalsidase alfa, agalsidase beta, immune alterations, lyso-Gb_3

Introduction

Fabry disease (FD, OMIM 301500) is an X-linked lysosomal storage disorder (LSD) caused by mutations in the *GLA* gene (OMIM#300644). The mutations lead to lack of or faulty lysosomal α-galactosidase A (α-gal A, EC 3.2.1.22) enzyme causing progressive accumulation of substrate glycosphingolipids-globotriaosylceramide (Gb_3) and globotriaosylsphingosine (lyso-Gb_3) within lysosomes in a variety of cell types, including of vascular endothelial cells, smooth muscle cells, renal, cardiac (cardiomyocytes and fibroblasts) and nerve cells [1, 2]. FD is pan-ethnic with reported incidence of 1 in 476,000 to 1 in 117,000 in the general population [2]. FD is a disease with a wide spectrum of heterogeneously progressive clinical phenotypes. A range of clinical manifestations have been described spanning multiple organ systems, prominently featuring renal, neurological, ophthalmological, dermatological gastrointestinal and cardiovascular systems [1]. Irregularities in immune system have been described in LSDs, most prominently in Gaucher disease (GD) where immune dysfunction is seen, most likely as a result of glycosphingolipid accumulation [3, 4]. Immune cell alterations were found to be persistent in GD patients despite long-term treatment, though early initiation of treatment shows beneficial effects on immune alterations [5]. In FD patients, accumu-

lation of glycolipids, Gb3 and lyso-Gb_3 leading to recognition of these molecules as antigens could be resulting in several leukocyte perturbations including increase in total lymphocytes and B cells, and reduced numbers of monocytes, CD8+ T cells and dendritic cells (DCs), while some abnormalities are less marked depending on the treatment status [6, 7]. In addition, glycolipid accumulation was also shown to trigger local and systemic inflammation leading to irreversible organ damage [8, 9].

Enzyme replacement therapy (ERT) with recombinant α-gal A is one of the most acceptable treatment options for FD since it was introduced in 2001 [10]. Currently, two recombinant enzymes, agalsidase alfa (Replagal, Shire human genetic therapies, Cambridge, MA) and agalsidase beta (Fabrazyme, Sanofi, Cambridge, MA) are available for FD patients outside the USA, while only agalsidase beta is approved by the Food and Drug Administration (FDA) for use in the USA. However, there are only a few studies that have directly compared therapeutic responses between the two commercially available enzyme preparations [11].

An extended shortage of agalsidase beta in 2009 as a result of viral contamination in the manufacturing process necessitated the switch to agalsidase alfa for many FD patients in the USA [12]. This inevitable switch in therapies presented a unique opportunity to compare the effects on FD patients of switching from agalsidase beta to agalsidase alfa. Overall, it was shown that the switch was well tolerated and associated with stable clinical conditions [13]. However, once production of agalsidase beta was restored, the patients were switched back to their original treatment regime. The aim of this study was to elaborate the effect of this second switch in treatment, i.e., from agalsidase alfa to agalsidase beta.

In this open label comparative clinical study, three cohorts were included; 1) FD patients who were switching back from agalsidase alfa to agalsidase beta, 2) those that remained on agalsidase beta throughout the study, and 3) FD patients who were untreated at the time of enrollment and were then treated using agalsidase beta. The subjects were evaluated at the time of enrollment and then followed up after 12 months of agalsidase beta. We performed flow cytometry based immunophenotyping, to assess persistent immune cell alterations in FD patients compared to healthy controls. We then compared between the cohorts to study the effect of treatment status on these immune cell alterations. Lyso-Gb_3 in plasma and urine has been shown to be a sensitive surrogate marker to assess treatment efficiency in FD patients [14-16]. We investigated the level of lyso-Gb_3 and its analogs in plasma and urine and any changes in their level after 12 months of agalsidase beta.

Materials and methods

Subjects

Twenty seven patients with confirmed FD (mean age 31±17 years, range 8-59 years; 12 M/15 F) were enrolled into this active comparator study (NCT01745185). Healthy subjects (n=27, mean age 31±17 years, range 5-60 years; 11 M/16 F) with no known FD were enrolled as control group. The handling of tissue samples and patient data was approved by the internal review board (Western IRB) including the procedure whereby all patients gave informed consent to participate in this study. Written informed consent was obtained using IRB approved informed consent form. At enrollment, a medical history was obtained and a detailed physical examination was performed. Medical records were reviewed as a part of the clinical evaluation.

Antidrug antibodies and neutralizing IgG antibodies titration

Samples were first screened for anti-agalsidase alfa antibodies (ADA) using an electro-chemiluminescent (ECL) bridging assay. Samples that screened positive were then confirmed by competition with agalsidase alfa in the ECL bridging format. The titer of confirmed positive samples was determined using the same ECL bridging assay, and all confirmed positive samples were further characterized using an enzyme activity-based neutralizing antibody (NAb) assay. Minimum required dilution for both the ADA and the NAb assay was 1:20.

Immunophenotyping

Direct immunofluorescence with specific antibodies was performed either on peripheral

blood as previously described [5, 17] with some modifications using the following antibodies; anti-IgG1 FITC, anti-CD5-FITC, anti-CD8-FITC, anti-CD14-FITC, anti-CD22-FITC, anti-CD34-FITC, anti-IgG1-PE, anti-CD3-FITC/CD16+CD56-PE, anti-CD11C-PE, anti-CD21-PE, anti-CD27-PE, anti-CD183-PE, anti-CD194-PE, anti-CD20-PerCP and anti-HLA-DR-PerCP (BD Bioscience, San Jose, CA). Anti-CD19-FITC, anti-IgA-FITC, anti-IgD-FITC, anti-CD8-PE, anti-CD19-PE, anti-IgG-PE, anti-IgG1-PerCP, anti-CD3-PerCP, anti-CD4-PerCP, anti-CD8-PerCP and anti-CD3-APC (Invitrogen, Carlsbad, CA). Anti-Lineage-FITC (anti CD3/CD14/CD16/CD19/CD20/CD56), anti-CD196-PerCP, anti-IgM-PerCP and anti-CD-45-APC (Biolegend, San Diego, CA). Anti-CD4-FITC (eBioscience, San Diego, CA), anti-CD-45RO-FITC (Abcam, Cambridge, MA) and anti-BDCA2-APC (Miltenyi Biotech, San Diego, CA).

Briefly, after washing the whole blood with PBS, 100 ul of blood was stained with the relevant cocktail of antibodies at 4°C for 30 min followed by red blood cell lysis using BD FACS lysis solution (BD Bioscience, San Jose, CA). Samples were acquired on Accuri C6 flow cytometer (BD Bioscience, San Jose, CA) and analyzed using FCS express software (De Novo software, Glendale, CA). During acquisition, a lymphocyte gate was assigned and 10,000 events were collected for the T cells and NK cells, and 25,000 events for the B cell analysis. For dendritic cells, a million ungated events were acquired.

Lyso-Gb_3 and related analogs

Plasma and urine samples were collected from the subjects at the time of enrollment (visit 1) and after 12 months of treatment with agalsidase beta (visit 2) and stored at -80°C, till the sample were shipped on dry ice to Université de Sherbrooke for biochemical evaluation. Lyso-Gb_3 and related analogs in plasma and urine were measured using tandem mass spectrometry as described previously [18, 19]. The analogs are lyso-Gb_3 molecules with chemical modifications (-C_2H_4, -C_2H_4+O, -H_2, -H_2+O, +O, +H_2O, +H_2O_2, and +H_2O_3) on their sphingosine moieties [20, 21]. All urinary biomarkers were normalized to creatinine.

Statistical analysis

All statistical analysis was performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). Mean values and their standard deviation were calculated for ages of the subjects in each cohort. Immunophenotyping and antibody results from FD patients (n=27) were compared to reference values calculated from non-FD age and gender matched controls (n=27) and graphs were generated as dot plots. Statistical evaluation of differences were performed using two-tailed unpaired Student's t-test for comparing FD to controls, while paired Student's t-test was used to compare results from visit 1 and 2 from same subjects. *P*-values were indicated where found significant, *: P<0.05; **: P<0.01; ***: P<0.001.

Results

Subjects

Twenty seven patients with confirmed FD were enrolled in an IRB approved study (12 M/15 F). Subjects were then divided into three groups based on their treatment status at the time of enrollment. Fifteen subjects were being treated with agalsidase alfa at the time of enrollment and then switched to agalsidase beta (**Table 1A**). Seven subjects were continued on agalsidase beta, while five subjects were untreated at the time of enrollment and subsequently treated with agalsidase beta (**Table 1B**, **1C**). Basic details with age, sex, treatment status, and causative mutation in *GLA* gene as well as the phenotype are noted. Most of the subjects (n=24) have classic form of FD while 3 subjects have non-classic form of the disease. All the samples were analyzed for ADA and NAb titers at baseline and 12 months. In nine subjects, antibodies against agalsidase were found, while one naïve subject developed measurable titer after administration of ERT. The rest of the subjects tolerated ERT well and did not develop antibodies against α-gal A. NAb were found in three subjects each from cohort A and B, while subject 026 developed NAb after initiation of ERT. Clinical findings at the time of enrolment are summarized in **Table 2**.

T cell abnormalities persist in patients with FD after long term therapy

Flow cytometry based immunophenotyping was performed on peripheral blood cells from 27 FD patients as well as non-FD controls. Overall percentage of T cells did not differ between FD and normal controls. However, when T cells were fractionated into CD4+ T

Table 1. Basic characteristics and antibody status of subjects

ID	Age at enrollment	Age at Dx	Sex	Genotype	Phenotype	ADA status/titer Visit 1-Visit 2	NAb status/titer Visit 1-Visit 2
A.							
001	49	24	M	c.717delAA	Classic	UA-20480	N/A-Negative
004	59	10	F	p.W287C	Classic	Negative-Negative	N/A-N/A
005	58	50	F	p.P205T	Classic	Negative-Negative	N/A-N/A
006	26	14	M	R220X	Classic	160-UA	20-UA
008	18	6	F	p.Q250X	Classic	Negative-Negative	N/A-N/A
009	12	9	F	p.R49P	Classic	Negative-Negative	N/A-N/A
010	9	5	M	p.R49P	Classic	160-2560	Negative-Negative
012	17	14	F	p.R49P	Classic	Negative-Negative	N/A-N/A
013	17	14	F	p.R49P	Classic	Negative-Negative	N/A-N/A
014	55	29	M	large ex 2 del	Classic	40960-40960	500-500
015	58	42	F	c.256delT	Classic	Negative-Negative	N/A-N/A
016	25	7	M	c.256delT	Classic	1280-2560	20-100
021	46	41	M	p.Y207C	Classic	40-Negative	Negative-Negative
022	13	11	F	p.D244N	Classic	Negative-Negative	N/A-N/A
028	35	9	F	IVS5-2, c.3delCA	Classic	Negative-Negative	N/A-N/A
B.							
011	54	38	M	p.Q250X	Classic	Negative-Negative	N/A-N/A
017	26	15	F	p.E79X	Classic	Negative-Negative	N/A-N/A
018	35	31	M	c.668G>A	Classic	640-2560	100-100
020	17	10	M	p.Y134D	Non-classic	Negative-Negative	N/A-N/A
024	15	Infancy	M	p.E103X	Classic	81920-20480	500-500
025	35	6	F	p.E103X	Classic	Negative-Negative	N/A-N/A
029	14	7	M	c.865A>T	Classic	160-1280	20-500
C.							
007	37	32	F	p.R363H	Non-classic	Negative-Negative	N/A-N/A
019	49	47	F	p.Y134D	Non-classic	Negative-Negative	N/A-N/A
023	12	10	F	p.D244N	Classic	Negative-Negative	N/A-N/A
026	8	Infancy	M	p.E103X	Classic	Negative-10240	N/A-100
027	38	34	F	c.668G>A	Classic	Negative-Negative	N/A-N/A

Subjects are classified into three sub-groups, those who have switched from agalsidase alfa at first visit to agalsidase beta at visit 2 (A), those who remained under agalsidase beta throughout the study (B), those who were untreated at visit 1 and subsequently treated with agalsidase beta (C). Dx: Diagnosis, ADA: anti-agalsidase alfa antibody, NAb: anti-agalsidase antibody neutralizing antibody, UA: unavailable, N/A: Not applicable.

helper cells (Th cells) and CD8+ cytotoxic T cells (Tc cells), FD patients showed an increase in the percentage of Tc cells compared to controls (P=0.018) (**Figure 1A**). CD4/CD8 ratio, calculated as ratio of CD4+ T helper cells to CD8+ cytotoxic T cells was found to be significantly lower in FD patients (Data not shown). Memory CD4 and CD8 T cells were assayed by presence of memory cell marker CD45RO. A significantly higher fraction of both memory Th cells (P=0.01) and memory Tc cells (P=0.002) was observed in FD patients compared to controls (**Figure 1B**). CD4 and CD8 T cells were further analyzed for chemokine receptors CCR4, CXCR3 and CCR6. Th cells from FD patients showed significant increase of CCR4 and CCR6 expression (P=0.0002 & 0.02 respectively), while Tc cells showed increase in CCR4, CXCR3 and CCR6 (P=0.0001, 0.017 & 0.02) (**Figure 1C**, **1D**).

B lymphocytes were identified by the expression of CD20 marker and were found to be comparable to controls. Further characterization of B cells showed a lower fraction of memory B cells (CD20+/CD27+) in FD subjects

Table 2. Clinical symptoms of the subjects at the time of enrollment

		Neurological										Cardiovascular						Renal		Pulmonary						Gastro intestinal			General		
	IDs	Depression/ Anxiety	Tinnitus	Acroparesthesia	Strokes	Fatigue	Anhidrosis	Hypohidrosis	Vertigo	Fever pain crisis	TIA	LVH	Bradycardia	ECG abnormalities	Pacemaker	Cardiomyopathy	POTS	Proteinuria	CKD3	Obstruction	Sleep apnea	PFT	Asthma	SOB	Cough	Abdominal pain	Diarrhea	Constipation	Edema	Angiokeratomas	Cornea verticillata
Group A	1	+	+	+	+	+	+		+			+	+		+			+		+						+	+		+	+	+
	4			+		+				+		+						+			+					+	+	+		+	+
	5	+		+		+					+		+					+				+									+
	6		+	+		+							+																	+	+
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	16		+	+		+		+					+													+	+	+	+	+	+
	21	+		+				+	+		+	+						+		+										+	
	22			+		+	+											+								+					
	28																														+	+
Group B	11		+	+	+	+	+				+	+			+											+			+	+	+
	17	+	+					+					+											+		+			+		+
	18	+	+	+	+	+		+			+	+		+										+				+		+	+
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	25	+	+	+		+							+																			
	29			+																							+	+				
Group C	7	+		+									+											+		+					+
	19			+		+												+						+	+							
	23							+																								
	26			+																						+						
	27	+	+			+							+					+														+

Subjects presented with a variety of symptoms which were categorized under; neurological, cardiovascular, renal, pulmonary, gastrointestinal, and other symptoms. TIA: transient ischemic attack, LVH: left ventricular hypertrophy, POTS: postural orthostatic tachycardia, CKD3: Chronic kidney disease stage 3, PFT: pulmonary function test, ECG: electrocardiogram, SOB: shortness of breath.

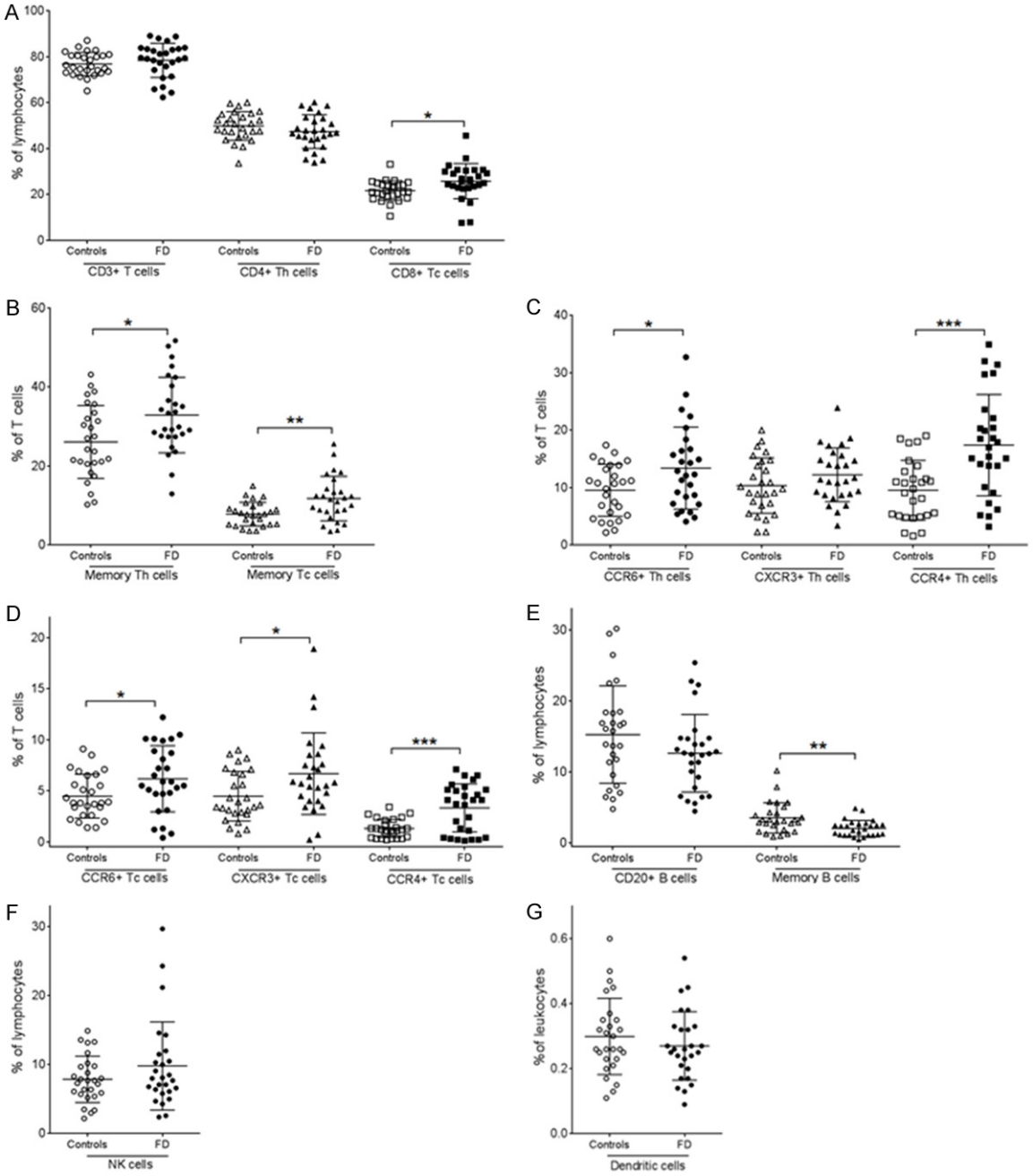

Figure 1. Immune subsets in patients with Fabry disease compared to healthy controls. Percentages of overall T lymphocytes (CD3+), Th cells (CD3+/CD4+) and Tc cells (CD3+/CD8+) in peripheral blood of FD patients and normal controls were assessed using flow cytometry and plotted (A). Similarly, memory subsets of Th (CD3+/CD4+/CD45RO+) and Tc cells (CD3+/CD8+/CD45RO+) were calculated and plotted against normal controls (B). CD4+ Th and CD8+ Tc cells were further analyzed for expression of activation markers (CCR4, CXCR3) and chemokine receptor (CCR6) that have a role in T cell mediated inflammation (C and D). Percentage of B-lymphocytes (CD20+), memory B cells (CD20+/CD27+) and NK cells (CD3-/CD16+ or CD56+) were plotted (E and F). DCs were enumerated as Lin-/CD34-/HLA DR+ cells and plotted as a percentage of total leukocytes (G). Unpaired student's t-test was performed to calculate significance values and included in the plots where significant difference between FD and normal controls was observed. *: P<0.05; **: P<0.01; ***: P<0.001.

(P=0.002) (**Figure 1E**), there were no alterations in individual immunoglobulin producing B cells (data not shown). Natural killer cells (NK cells) are identified as CD45+ lymphocytes that express CD16 or CD56 and lack CD3 expression. No significant difference in NK cell frac-

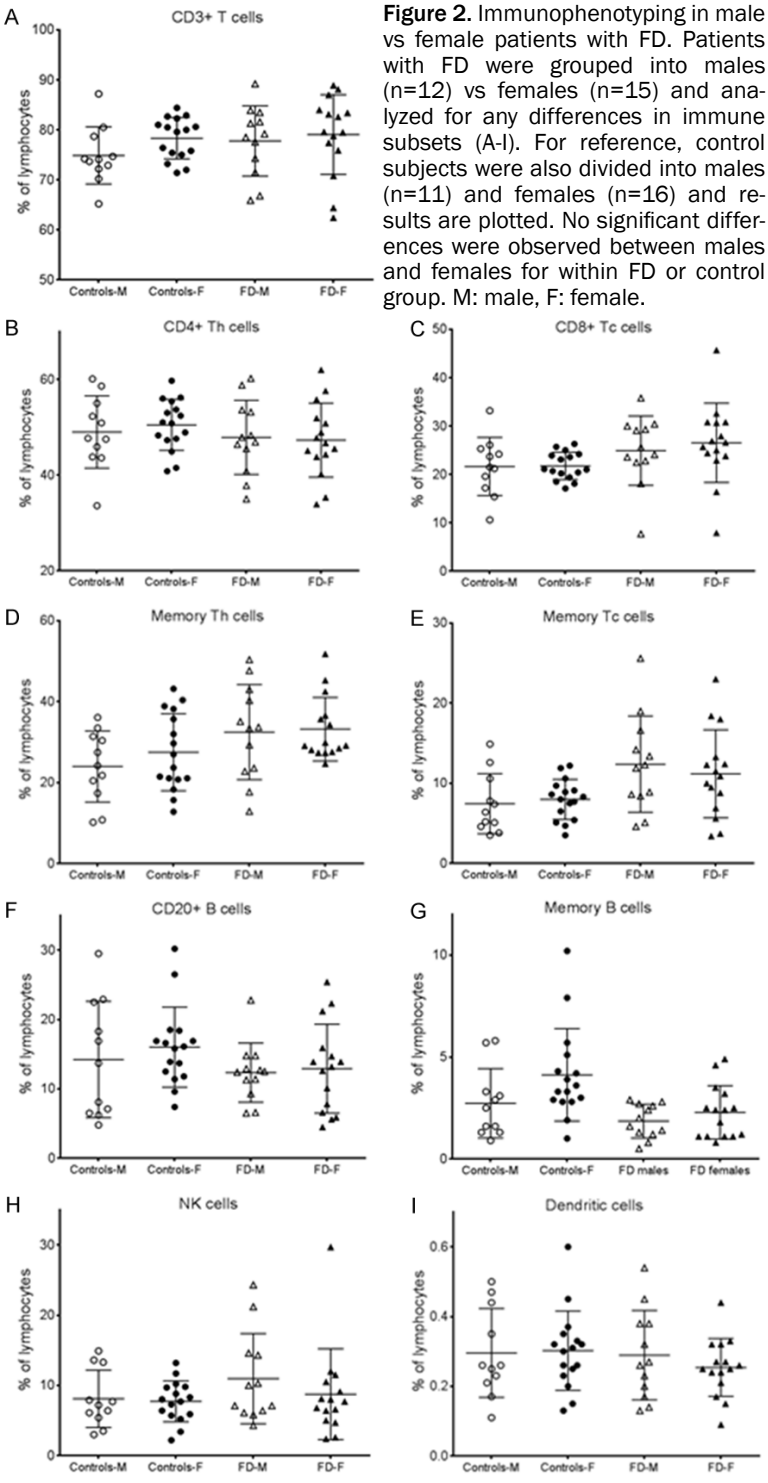

Figure 2. Immunophenotyping in male vs female patients with FD. Patients with FD were grouped into males (n=12) vs females (n=15) and analyzed for any differences in immune subsets (A-I). For reference, control subjects were also divided into males (n=11) and females (n=16) and results are plotted. No significant differences were observed between males and females for within FD or control group. M: male, F: female.

tion was seen between FD and control groups (**Figure 1F**). When DCs were analyzed as those leukocytes which are lineage-1 negative (CD3, CD14, CD16, CD19, CD20 and CD56 negative), CD34 negative and HLA DR positive, no significant difference in overall DC fraction was observed between FD and control group (**Figure 1G**).

Immune cell alterations were not affected by gender or disease duration

FD being an X-linked disorder, severity of symptoms has been shown to vary between males who are hemizygous and females who carry mutated *GLA* gene on only one X chromosome, but nevertheless are affected due to X-inactivation. In order to study if immune cell alterations follow similar pattern and are more prominent in males, FD subjects were divided based on their gender (12 M/15 F) and their immune cell fractions were compared with non FD controls (11 M/16 F). There were no significant differences in any of the immune cell types that were compared, which included T/B cells, DCs and NK cells (**Figure 2A-I**). FD subjects as well as control subjects were then divided based on their age into pediatric (≤ 18 yr.; n=9) and adult (>18 yr.; n=18) and their immune phenotypes were compared. There were no differences in the overall immune cell fractions between both the subgroups of FD subjects (**Figure 3A-C**, **3F-H**). Fraction of memory Th cells were found to be increased significantly in adult FD subjects compared to in pediatric patients (P= 0.0002) as well adult control subjects (P=0.0017). Similarly, fraction of memory Tc cells was also elevated in adult FD subjects compared to control adult subjects (P= 0.014) (**Figure 3D**, **3E**). In contrast, memory B cells were found to be significantly lower in FD adults compared to controls (P=0.002) (**Figure 3I**).

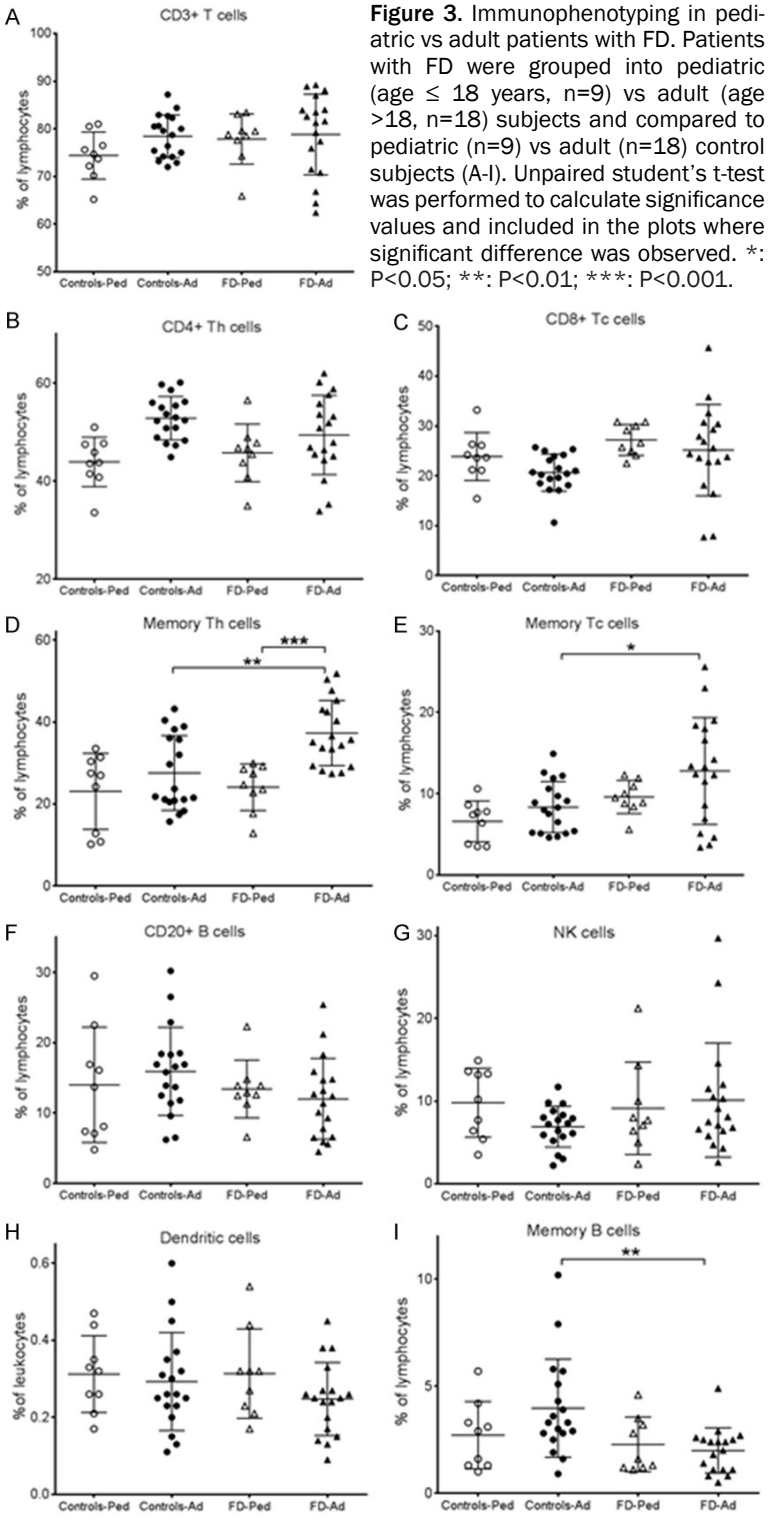

Figure 3. Immunophenotyping in pediatric vs adult patients with FD. Patients with FD were grouped into pediatric (age ≤ 18 years, n=9) vs adult (age >18, n=18) subjects and compared to pediatric (n=9) vs adult (n=18) control subjects (A-I). Unpaired student's t-test was performed to calculate significance values and included in the plots where significant difference was observed. *: P<0.05; **: P<0.01; ***: P<0.001.

Treatment status had no effect on T cell alterations

To investigate if persistent T lymphocyte alterations are affected by FD patients' ERT, subjects were grouped based on their status at baseline, during visit 1: those treated with agalsidase alfa (n=15), agalsidase beta (n=7) and untreated (Naïve, n=5). No significant differences between three cohorts were observed with respect to overall T lymphocytes, Th and Tc cells (**Figure 4A**). Expression of T cell activation markers was found elevated in FD patients, compared to normal controls, however, even though a trend for increase in activation was found in those undergoing ERT (by agalsidase alfa or agalsidase beta) compared to untreated FD patients, these differences were not statistically significant (**Figure 4B**, **4C**).

Treatment status does not correlate with lyso-Gb_3 levels in plasma and urine

Gb3 levels in plasma and urine have been in use as surrogate biomarkers for FD progression. More recently, plasma and urinary lyso-Gb_3 and related analogs have been analyzed as a more sensitive marker [19, 22]. Lyso-Gb_3 and 6 analogs were analyzed from plasma of subjects at baseline (visit 1) and 12 month visit (visit 2), to assess any changes in their concentrations as a result of ERT (**Table 3**). Almost all FD patients showed elevated plasma lyso-Gb_3, except 2 subjects (009 & 007), while majority of them showed elevated concentration of lyso-Gb_3 analogs as well. Overall, there was a decrease in plasma lyso-Gb_3 (mean value 40 to 29 nmol/L) during visit 2 compared to baseline, (**Figure 5A**). However, the reduced levels were still not comparable to normal reference range (**Table 3**). To analyze the changes

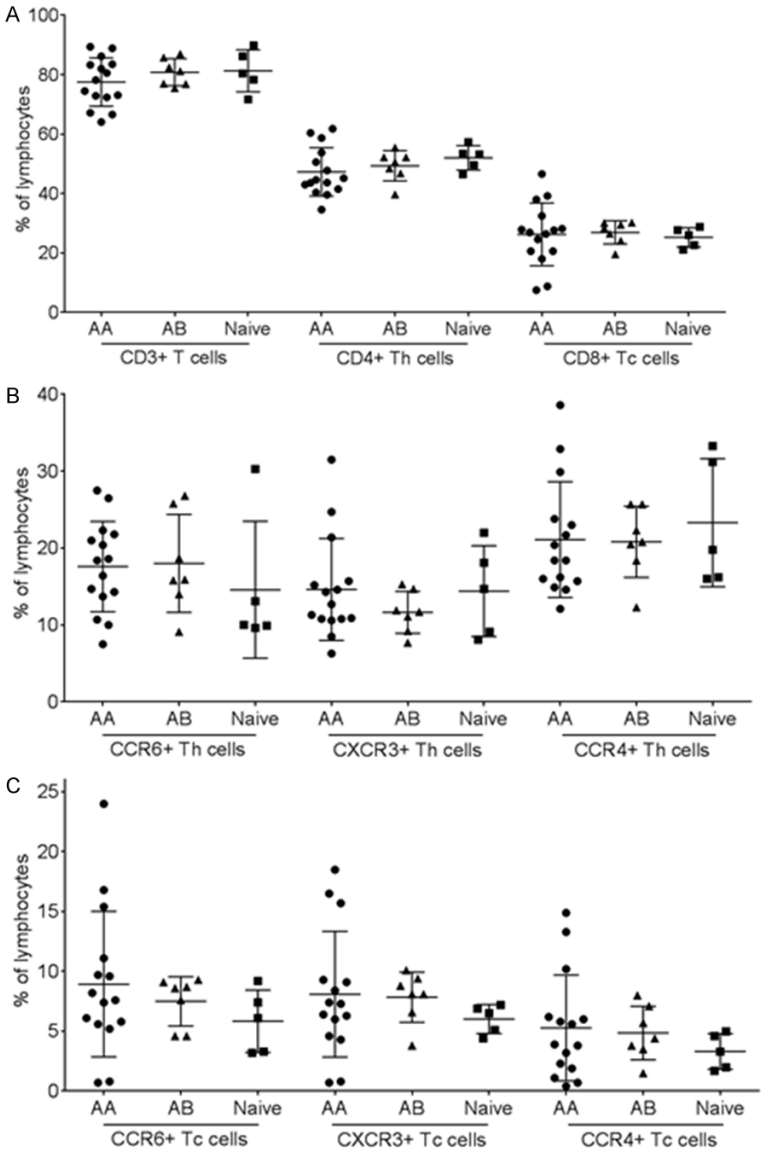

Figure 4. T cell subsets in FD patients with different treatment status. FD subjects were grouped based on their treatment status at initial visit: ERT with agalsidase alfa (AA), agalsidase beta (AB), untreated (naïve) and the T cells and T cell subsets- Th and Tc cells were compared (A). Surface activation markers-CCR6, CXCR3 and CCR4 on Th and Tc cells on individual subjects are plotted (B and C). No significant differences in T cells, subsets or activation markers were observed based on treatment status of the FD patients.

with treatment, plasma lyso-Gb_3 levels were plotted between cohorts for baseline and visit 2 (**Figure 5B-D**). Subject 020 in cohort B showed elevated levels of plasma lyso-Gb_3 and analogs (**Figure 5C**). Similar analysis for urinary lyso-Gb_3 was performed for all subjects at baseline and visit 2 and majority of FD patients showed abnormal levels of lyso-Gb_3 and analogs, compared to normal reference range (**Table 4**). Comparison for urinary lyso-Gb_3 also showed a decrease of mean value (from 27 to 21 pmol/mmol of creatinine) at baseline to concentration at visit 2 (**Figure 5E**). When urinary lyso-Gb_3 was plotted for each cohort, there was a decrease in a majority of FD patients irrespective of their treatment status (**Figure 5F-H**). Similar to the findings from plasma, subject 020 (cohort B) showed an abnormal increase in the urinary lyso-Gb_3 as well (**Figure 5G**), which was not related to an increase of antibody levels. Subject 020 had discontinued ERT in the duration of the study and it is likely that this could have resulted in the elevated lyso-Gb_3 in both plasma and urine.

Discussion

FD is a form of sphingolipidosis which leads to progressive accumulation of glycosphingolipids, gGb3 and lyso-Gb_3 in lysosomes of various cell types leading to a diverse range of symptoms across various tissue systems that range from acroparesthesia, kidney and dermatological manifestations to cardiac complications. Gb3 accumulation also leads to inhibition of CD1d mediated iNKT cell activation by competing with other glycolipid antigens for CD1d binding [23]. While perturbations in other immune cell types including certain leukocytes fractions and monocytes have been demonstrated [6], it is unclear how the two different recombinant enzymes affect normalization of these immune irregularities in FD patients. Agalsidase alfa and agalsidase beta have similar amino acid sequences and functional effects even though certain differences between uptake efficiency have been noted [24-26]. Reverting of ERT from agalsidase alfa to agalsidase beta for FD patients in the USA presented a uni-

Table 3. Plasma lyso-Gb_3 at visit 1 & 2

	Results (nmol/L)													
Ref. values	≤ 2.4		0		≤ 0.9		0		0		≤ 0.3		0	
Subject id	Lyso-Gb_3		Lyso-Gb_3 (-28)		Lyso-Gb_3 (-2)		Lyso-Gb_3 (+16)		Lyso-Gb_3 (+18)		Lyso-Gb_3 (+34)		Lyso-Gb_3 (+50)	
	V1	V2	V1	V2	V1	V2	V1	V2	V1	V2	V1	V2	V1	V2
A.														
004	**11.5**	**10.0**	0	0	**1.9**	**1.5**	0	0	0	0	0	0	0	0
005	**16.4**	**9.9**	0	0	**3.1**	**1.3**	**1.9**	0	**0.6**	0	0	0	0	0
006	**70.0**	**41.0**	**2.3**	**0.5**	**9.0**	**4.1**	**3.0**	**1.2**	**10.9**	**4.7**	**4.3**	**0.9**	0	0
008	**3.9**	1.8	0	0	0	0	0	0	0	0	0	0	0	0
009	2.3	2.1	0	0	0	0	0	0	0	0	0	0	0	0
010	**86.2**		**1.6**		**17.7**		**8.3**		**14.1**		**7.1**		**0.3**	
012	**11.1**	**7.5**	0	0	**1.0**	0.5	0	0	**0.6**	0	0	0	0	0
013	**8.8**	**5.6**	0	0	0.9	0.4	0	0	**0.2**	0	0	0	0	0
014	**105.3**	**109.0**	**3.0**	**2.6**	**12.2**	**12.0**	**4.8**	**4.4**	**13.2**	**10.5**	**7.4**	**6.6**	**0.5**	**0.4**
015	**15.8**	**14.0**	0	0	**2.3**	**1.7**	0	0	**1.1**	**0.6**	0	0	0	0
016	**206.2**		**5.1**		**38.7**		**22.5**		**32.9**		**31.5**		**4.6**	
021	**45.3**	**26.7**	**0.5**	0	**6.4**	**1.9**	**2.4**	0	**4.8**	**1.3**	**2.2**	0	0	0
022	**6.4**	**3.6**	0	0	0.6	0	0	0	0	0	0	0	0	0
028	**9.3**	**7.2**	0	0	0.8	0.5	0	0	0	0	0	0	0	0
B.														
011	**24.5**	**34.1**	0	0	**2.0**	**5.4**	0	**0.8**	**2.1**	**2.3**	0	0	0	0
017	**7.8**	**3.7**	0	0	**1.5**	0.6	0	0	0	0	0	0	0	0
018	**24.2**		0		**4.0**		**7.7**		**1.2**		**2.0**		**0.1**	
020	**28.0**	**159.4**	0	**3.4**	**5.0**	**37.0**	**1.4**	**18.2**	**3.4**	**20.5**	**0.6**	**40.4**	0	**7.2**
024	**78.7**		**1.6**		**21.5**		**6.4**		**10.5**		**4.7**		0	
025	**4.7**	**3.5**	0	0	0	0	0	0	0	0	0	0	0	0
029	**66.5**	**42.7**	**0.6**	0	**12.5**	**6.8**	**5.1**	**3.2**	**6.8**	**5.7**	**4.2**	**1.5**	**0.4**	0
C.														
007	0	0	0	0	0	0	0	0	0	0	0	0	0	0
019	**6.6**	**8.8**	0	0	**1.2**	**1.3**	0	0	0	0	0	0	0	0
023	**3.1**	2.0	0	0	0	0	0	0	0	0	0	0	0	0
026	**58.4**		**1.3**		**8.0**		**3.3**		**12.3**		**5.1**		0	
027	**7.4**	**5.3**	0	0	0.3	0	0	0	0	0	0	0	0	0

Plasma lyso-Gb_3 and their analogs were measured in the plasma samples (nmol/L). Normal reference values are indicated on the top row for each biomarker. Values in bold font indicate values outside normal reference range. V1: Visit 1, V2: Visit 2.

que opportunity to study any effect of two ERTs on immune irregularities in FD patients.

Immune system irregularities have been described in various lysosomal storage disorders including GM2 gangliosidosis, α-mannosidosis, Niemann Pick disease type C, and mucopolysaccharidosis VII [3]. Largest number of irregularities has been found in GD where apart from increased frequency of B cell malignancies, upregulation of several cytokines, alterations in B cell subsets and systemic inflammation have been noted. Results from current study indicate that FD patients present with alterations in T cells and subsets with no notable effect on B cells indicating persistent immune abnormalities mostly in cellular rather than on humoral adaptive immune system. Presence of elevated levels of activation markers on T cells as well as increase in memory T cells could be a result of persistent inflammation as a result of accumulated glycolipids. No significant alterations were seen in innate immune system components, NK cells and DCs. In addition, the extent of T cell abnormalities did not differ based on treatment sta-

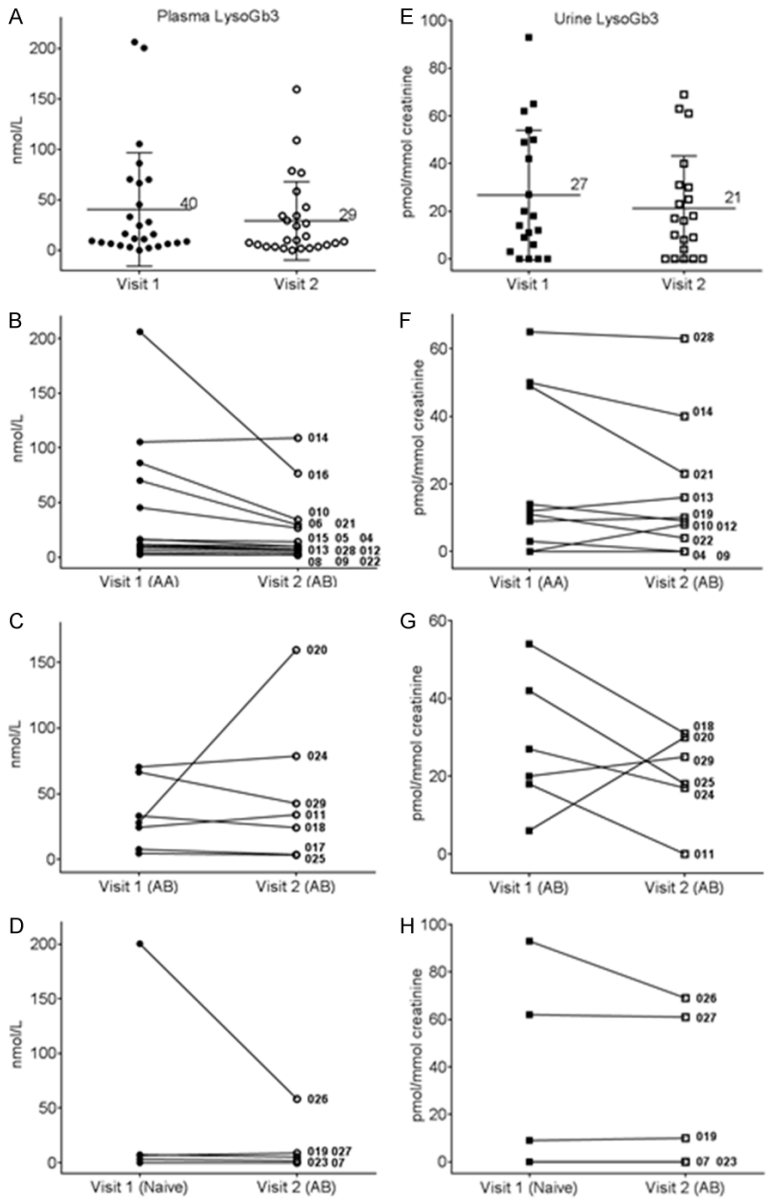

Figure 5. Plasma and urine lyso-Gb_3 in FD patients during visit 1 and visit 2 Lyso-Gb_3 in plasma was measured using mass spectrometry in FD subjects at the time of enrollment into the study (Visit 1) and after 12 months of administering agalsidase beta (Visit 2). Mean lyso-Gb_3 for each visit is indicated (A). Subjects were grouped based on their treatment status and their plasma lyso-Gb_3 levels were plotted. Individual subject IDs are indicated (B-D). Lyso-Gb_3 in urine of FD subjects at Visit 1 and Visit 2 (E). Subjects were grouped based on their treatment status and their lyso-Gb_3 levels in urine were plotted (F-H). Individual subject identification numbers are indicated.

tus or type of ERT administered on the FD subjects.

FD is one of the three X-linked lysosomal storage disorders. Due to X-inactivation leading to random silencing of one of the X-chromosomes, females with mutated *GLA* gene are mosaic for α-gal A expression. Due to this, females usually have delayed and/or milder symptoms compared to males with FD. Interestingly, there were no differences in immune cell alterations between males and females with FD. Similarly, there were no significant differences between pediatric and adult subjects as well. Hence, these long term immune cell abnormalities could be inherent to FD instead of being dependent on dosage or activity of α-gal A present in the system.

Gb3 secreted into plasma or urine has been used as a biomarker to monitor the clinical course and the response to ERT in FD [27-29]. However, more recently lyso-Gb_3 in plasma or urine has been recognized as more sensitive biomarkers to diagnose and monitor FD [15, 30, 31]. Moreover, better biochemical analytical methods have led to the identification of several analogs of both plasma and urine lyso-Gb_3 whose levels correlate with the course of FD [18, 19, 32]. Apart from being valuable as biomarkers, these secreted molecules have been suggested to interact with immune cells and influence immune responses, like antigen presentation, inflammation *etc.* [7-9]. In treatment naïve FD patients no significant difference was seen in urine lyso-Gb_3 upon ERT treatment. Even though plasma lyso-Gb_3 concentrations seemed to decrease upon treatment, the reduced levels still were abnormal compared to normal reference ranges. Similarly in FD patients who have been under long term ERT, concentrations of lyso-Gb_3 and analogs in both plasma and urine either remain unchanged or if they were reduced, they were still found to be in abnormal

Table 4. Urine lyso-Gb_3 at visit 1 & 2

	Results (pmol/mmol creatinine)															
Ref. values	0		0		0		0		0		≤ 25		≤ 20		≤ 85	
Subject id	Lyso-Gb_3		Lyso-Gb_3 (-28)		Lyso-Gb_3 (-12)		Lyso-Gb_3 (-2)		Lyso-Gb_3 (+14)		Lyso-Gb_3 (+16)		Lyso-Gb_3 (+34)		Lyso-Gb_3 (+50)	
	V1	V2	V1	V2	V1	V2	V1	V2	V1	V2	V1	V2	V1	V2	V1	V2
A.																
004	0	0	0	0	**122**	**119**	**17**	**7**	**49**	**46**	**365**	**351**	**148**	**142**	**122**	**124**
009	**3**	0	0	0	**35**	**23**	**6**	**5**	**14**	**6**	**93**	**73**	**61**	**58**	73	51
010	**14**	**9**	**93**	**54**	**614**	**291**	**247**	**119**	**347**	**160**	**2404**	**893**	**1314**	**581**	**1181**	**359**
012	**0**	**8**	**8**	**5**	**61**	**60**	**10**	**9**	**15**	**18**	**203**	**190**	**128**	**114**	**111**	**107**
013	**12**	**16**	**9**	**5**	**57**	**29**	**14**	**9**	**23**	**15**	**179**	**113**	**123**	**61**	64	41
014	**50**	**40**	**57**	**50**	**420**	**366**	**56**	**46**	**101**	**84**	**772**	**590**	**556**	**414**	**640**	**424**
019	**9**	**10**	**6**	0	**44**	**52**	**11**	**2**	**24**	**23**	**161**	**141**	**83**	**70**	51	53
021	**49**	**23**	**25**	0	**296**	**57**	0	**4**	**62**	**12**	**578**	**157**	**433**	**105**	**256**	78
022	**11**	**4**	**13**	**4**	**91**	**37**	**17**	**6**	**26**	**10**	**204**	**105**	**124**	**56**	**77**	43
028	**65**	**63**	**9**	0	**51**	**40**	**24**	**22**	**24**	**18**	**180**	**161**	**82**	**68**	72	55
B.																
011	**18**	0	**16**	**23**	**111**	**161**	**35**	**48**	**41**	**54**	**308**	**445**	**258**	**241**	**156**	**146**
018	**54**	**31**	**13**	**10**	**254**	**137**	**83**	**28**	**172**	**101**	**1084**	579	**611**	**321**	**454**	**227**
020	**6**	**30**	**21**	**79**	**139**	**648**	**48**	**154**	**69**	**452**	**574**	**2852**	**300**	**1225**	**204**	**3138**
024	**27**	**17**	**97**	**51**	**494**	**402**	**240**	**61**	**271**	**203**	**1497**	**1380**	**761**	**650**	**767**	**445**
025	**42**	**18**	0	0	**37**	**45**	0	**3**	0	**21**	**84**	**148**	**69**	**120**	46	64
029	**20**	**25**	**93**	**27**	**537**	**133**	**87**	**46**	**179**	**61**	**956**	**442**	**1094**	**224**	**564**	**152**
C.																
007	0	0	0	0	**10**	0	0	0	0	0	17	8	13	0	**36**	**24**
019	**9**	**10**	**6**	0	**44**	**52**	**11**	**2**	**24**	**23**	**161**	**141**	**83**	**70**	51	53
023	0	0	**17**	0	**145**	**55**	**16**	**0**	**32**	**16**	**257**	**90**	**127**	**36**	**116**	55
026	**93**	**69**	**334**	**65**	**2018**	**300**	**506**	**156**	**1124**	**146**	**4346**	**1112**	**4315**	**633**	**7555**	**400**
027	**62**	**61**	**10**	0	**45**	**11**	**14**	0	**12**	0	**76**	**49**	**69**	**28**	45	32

Urine lyso-Gb_3 and their analogs were measured in urine samples and expressed as a ratio to creatinine (pmol/mmol creatinine). Normal reference values are indicated on the top row for each biomarker. Values in bold font indicate values outside normal reference range. V1: Visit 1, V2: Visit 2.

ranges. Subject 020 in cohort 2 who was being treated with agalsidase beta discontinued ERT during the course of the study. A significant increase (~5 times) of lyso-Gb_3 and analogs in both plasma and urine was observed highlighting that even though lyso-Gb_3 may not be lowered to normal reference range, ERT still plays a role in reducing the lyso-Gb_3 concentration. Even though this observation is from a single subject, it indicates the importance of consistent ERT in FD patients.

Overall, current study details the persistent immune alterations in T cells, subsets and activation markers in FD subjects compared to controls. There are no long term adverse effects of switching from agalsidase alfa to agalsidase beta and continued ERT leads to reduction in plasma and urinary lyso-Gb_3 in majority of FD subjects.

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Disclosure of conflict of interest

None.

Abbreviations

FD, Fabry disease; α-gal A, α-Galactosidase A; GLA, α-Galactosidase A; Gb3, Globotriaosylceramide; Lyso-Gb_3, Globotriaosylsphingosine; FDA, Food and drug administration; LSD, Lysosomal storage disorder; GD, Gaucher disease; DCs, Dendritic cells; ERT, Enzyme replacement therapy; M, Male; F, Female; IRB, Internal review board; ADA, Anti-agalsidase alfa antibodies (ADA); ECL, Electro-chemiluminescent; Nab, Neutralizing antibody; NK cells, Natural killer cells; Th, T helper cells; Tc, Cytotoxic T cells; AA, Agalsidase alfa; AB, Agalsidase beta.

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