Involvement of hepcidin in iron metabolism dysregulation in Gaucher disease

Thibaud Lefebvre,[1,2*] Niloofar Reihani,[3*] Raed Daher,[1] Thierry Billette de Villemeur,[4] Nadia Belmatoug,[5] Christian Rose,[6] Yves Colin-Aronovicz,[3] Hervé Puy,[1,2] Caroline Le Van Kim,[3] Mélanie Franco[3] and Zoubida Karim[1**]**

[1]University Sorbonne Paris Cité, Paris Diderot University, Inserm U1149 / ERL 8252, Inflammation Research Center (CRI), Laboratory of Excellence GR-Ex, Paris; [2]AP-HP, Centre Français des Porphyries, Hôpital Louis Mourier, Colombes; [3]University Sorbonne Paris Cité, Paris Diderot University, Inserm, INTS, "Biologie Intégrée du Globule Rouge" Department, Laboratory of Excellence GR-Ex, Paris; [4]Sorbonne Universités, UPMC, GRC ConCer-LD and AP-HP, Hôpital Trousseau, Service de Neuropédiatrie, Centre de Référence des Maladies Lysosomales, Paris; [5]Hôpitaux Universitaires Paris Nord Val de Seine, Assistance Publique-Hôpitaux de Paris, Hôpital Beaujon, Service de Médecine Interne, Centre de Référence des Maladies Lysosomales, Clichy and [6]Université Catholique de Lille, Hôpital Saint Vincent de Paul, Service d'Hématologie, France

**TL and NR contributed equally to this work. **MF and ZK contributed equally to this work.*

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Correspondence: zoubida.karim@inserm.fr

Supplementary Data

Materials and Methods:

Glucocerebrosidase activity measurement

Glucocerebrosidase activity was measured in J774 cells, pre-incubated or not for 96 h with 1 mM conduritol B epoxide, a glucocerebrosidase inhibitor (CBE, Sigma-Aldrich). Enzyme activity was measured by using the fluorogenic glucocerebrosidase substrate PFB-FDGlu (5-Pentafluorobenzoylamino Fluorescein Di-β-D-Glucopyranoside) (Life Technologies). J774 cells were incubated with 1 mM of PFB-FDGlu or DMSO as control for 20 minutes at 37°C in 5% CO2. The reaction was stopped with ice-cold PBS, and the fluorescence intensity was measured within 15 minutes by flow cytometry. Acquisition and analysis were performed with the FACS Canto II flow cytometer (BD Biosciences) and with FlowJo software (Version 7.6.5).

Quantitative real-time PCR

Total RNA was isolated from J774 cell pellets using RNA-PLUS reagent (MP Biomedicals, Santa Ana, CA-USA) according to the manufacturer's recommendations. cDNA was then synthesized using SuperScript II Reverse Transcriptase (ThermoFisher Scientific, Villebon-sur-Yvette, France) per the manufacturer's instructions using 1 µg of total RNA template per sample. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed with specific sets of primers and LC 480 SybrGreen I Master, and the samples were amplified on a LightCycler 480 Instrument (Roche Diagnostics, Meylan, France). In parallel, the housekeeping Hprt1 transcript was amplified under similar conditions to serve as an internal control. Standard curves for target genes were generated from accurately determined dilutions of cDNA. Samples were analyzed in duplicate for each experiment, and the results were reported as the ratio of mean values for each gene to HPRT1. The target gene primers were as follows:

Hamp: For. CGATACCAATGCAGAAGAGAAGG; Rev. TTTGCAACAGATACCACACTGGG

FPN: For. CCCATAGTCTCTGTCAGCCTGC; Rev. CCGTCAAATCAAAGGACCAAA

HPRT1: For. AGCTACTGTAATGATCAGTCAACG; Rev. AGAGGTCCTTTTCACCAGCA

Cytokines and ferritin assays on J774 cells

Measurement of cytokines and chemokines levels in J774 cells supernatant was performed using MILLIPLEX MAP Mouse Cytokine (EMD Millipore, Saint-Quentin-en-Yvelines, France) according to the manufacturer's instructions.

For the quantification of intracellular ferritin, cells were lysed using RIPA lysis buffer supplemented with 1x protease inhibitor cocktail (EDTA Complete; ThermoFisher Scientific, Paris France)). The homogenate was centrifuged and ferritin level was measured using an AU400 automate (Olympus, Tokyo, Japan).

Tables :

Supplementary Table 1:

Clinical and biological parameters of untreated Gaucher disease patients (34 individuals). Patients with iron overload, defined by hyperferritinemia associated with a transferrin saturation exceeding 45% were excluded from the study. Soluble transferrin receptor sTFR >1.76 mg/L and GDF15>254 pg/ml indicate dyserythropoiesis.

Group	Age (year)	Hepatomegaly	Splenomegaly	Bone lesions	Serum Ferritin (µg/L)	TSAT(%)	sTFR (mg/L)	serum GDF15 pg/mL (see reference 7)
M	20	-	-	-	65	11	1.74	3018
M	23	no	yes	no	459	36	2.1	4376
M	26	yes	yes	yes	655	22	2.84	1975
M	30	yes	yes	yes	868	24	1.98	7050
M	33	-	-	-	1443	30	-	-
M	45	yes	yes	yes	188	23	-	-
M	50	no	yes	no	53	10	1.37	1290
M	58	no	yes	no	761	32	-	-
M	65	no	yes	yes	578	-	-	-
F	18	no	yes	no	248	-	2.76	4991
F	19	no	yes	no	395	20	2.66	5861
F	20	-	-	-	126	22	1.49	2834
F	24	yes	yes	yes	125	10	-	-
F	28	-	-	-	594	22	2.05	-
F	31	yes	yes	yes	1407	24	2.39	5680
F	41	yes	yes	yes	363	34	-	-
F	41	yes	yes	-	955	10	-	-
F	44	no	yes	no	99	-	0.819	-
F	47	no	yes	no	136	29	-	351
F	48	no	yes	no	27	-		-
F	51	no	yes	no	111	25	-	-
F	52	yes	yes	yes	526	22	2.23	-
F	63	yes	yes	no	2141	-		-
C	3	-	-	-	227	-	3,0	-
C	3	-	-	-	779	-	6.52	-
C	3.8	yes	yes	no	132	12	2.53	-
C	5	-	-	-	97	-	1.83	-
C	6	-	-	no	128	21	1.87	-
C	6	no	yes	no	215	20	3.01	1503
C	6.76	no	yes	no	151	11	2.47	-
C	9	yes	yes	no	278	13	-	3773
C	11	ouo	yes	no	418	14	2.31	-
C	11	yes	yes	no	498	14	0.758	-
C	13	yes	yes	no	231	-	2.02	-
Normal Values	*Men (M)*				*30-300*	*20-40*	<1.76	*254 in 8 healhty controls*
	Women(F)				*15-150*	*15-35*		
	Children(C)				*15-150*	*15-35*		
(-)	*not available*							

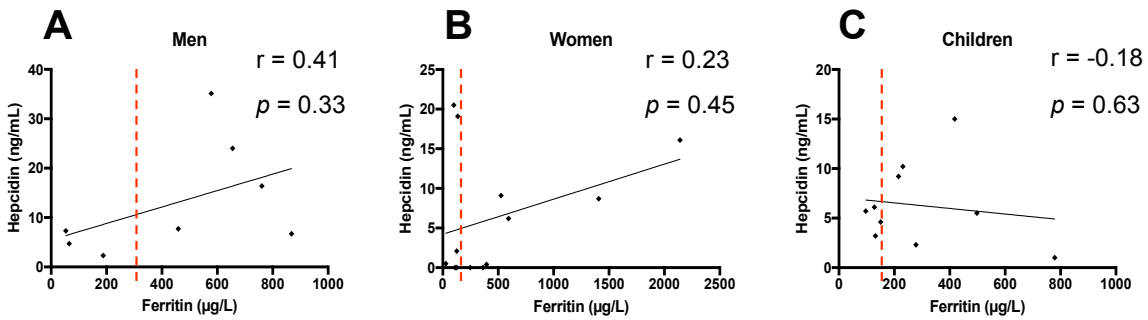
Supplementary Table 2: Contingency of patients with normal serum ferritin and hyperferritinemia according to treatment (Chi-squared test) and taking account of specific cutoff in each subgroup. Specific ferritin cutoff of each subgroup was considering: 300 µg/mL for men, 150 µg/mL for women and children.

Subgroups	Untreated		Treated		*p*
	n/total	%	n/total	%	
Men	**6/9**	**67**	**14/30**	**47**	**0.15**
Women	**8/14**	**57**	**13/29**	**45**	**0.22**
Children	**8/11**	**73**	**1/7**	**14**	**0.008****
Total	**22/34**	**65**	**28/66**	**42**	**0.02***

Supplementary Table 3: Contingency of patients with normal and low hemoglobin values according to treatment (Chi-squared test). Specific hemoglobin cutoff of each subgroup was considering: 13 g/dL for men, 12 g/dL for women and 11.5 g/dL for children.

Subgroups	Untreated		Treated		*p*
	n/total	%	n/total	%	
Men	1/9	11	0/30	0	0.03*
Women	6/14	43	0/29	0	<0.0001****
Children	4/10	40	0/7	0	0.03*
Total	11/33	33	0/66	0	<0.0001****

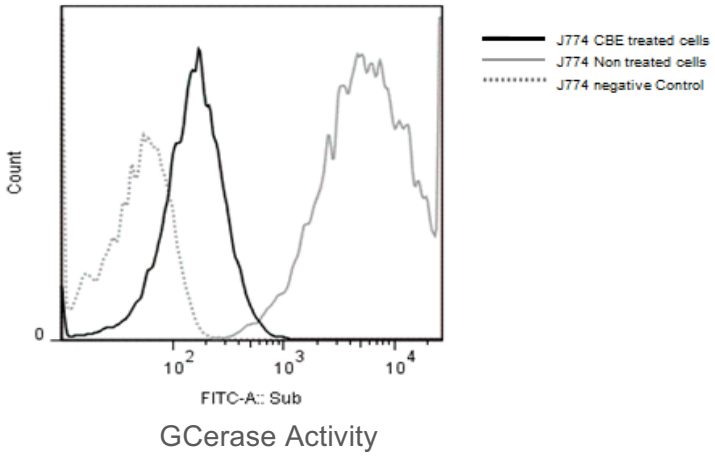
Figures:



Supplementary Figure 1

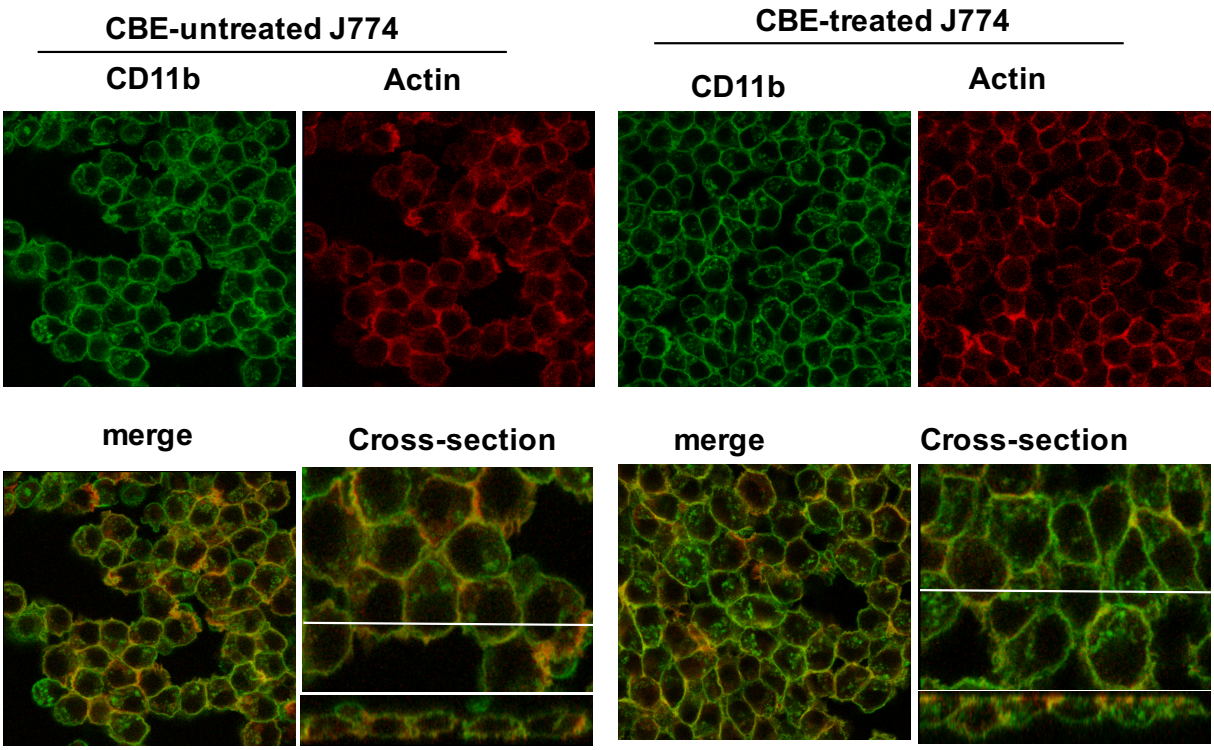
Correlation between serum ferritin and hepcidin in untreated patients. men, women and chidren. According to the Spearman test, no significant correlation was found (p>0.05).

The red dotted lines represent the high normal value of ferritin on the x-axis.

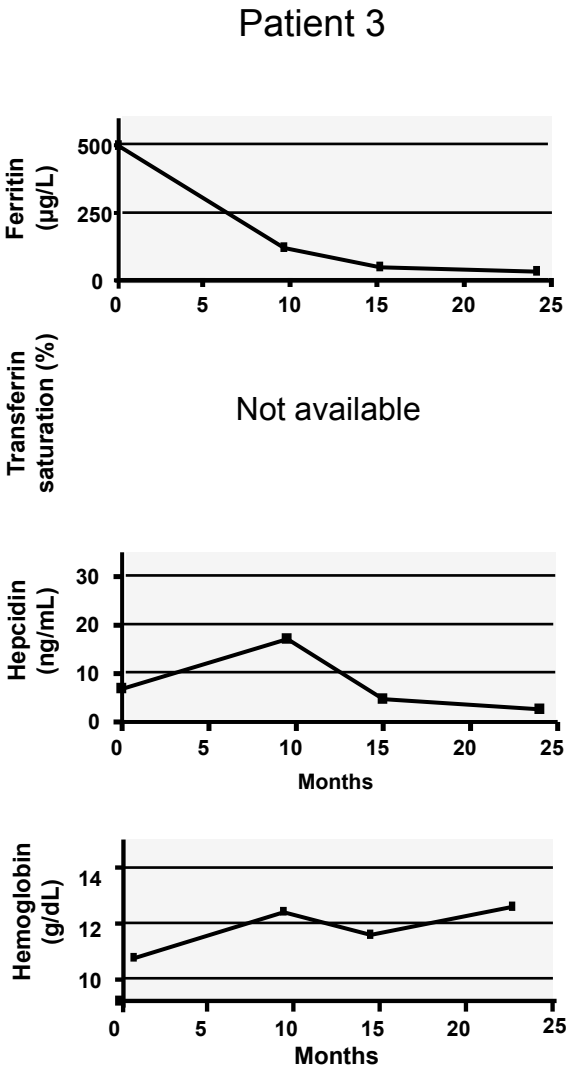


Supplementary Figure 2: Glucocerebrosidase activity inhibition in J774 cells.

The inhibition of glucocerebrosidase activity was confirmed after 96 h of CBE treatment (J774 negative CTL - dash line, J774 untreated cells - gray line, J774 cells treated by CBE for 96 h - black line).



Supplementary Figure 3: Staining of CD11B and actin were performed as described in methods. CD11b is labeled in green and actin in red. In the absence of CBE, CD11b is stained at the plasma membrane. The focal plan and the cross-section images showed no significant differences of the CD11b staining in the presence of CBE (CBE-treated J774) compared to the absence of CBE (CBE-untreated J774). Images are taken by confocal microscopy (60x).



Supplementary Figure 4: Follow-up study in other one GD treated patient.

Time course of iron-related parameters in treated patient 3 (11-year-old girl) from ERT initiation until 18-25 months later.