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Juilette Berger, Séverine Lecourt, Valérie Vanneaux, Chantal Rapatel, Stéphane Boisgard, et al.. Glucocerebrosidase deficiency dramatically impairs human bone marrow haematopoiesis in an in vitro model of Gaucher disease.. *British Journal of Haematology*, 2010, 10.1111/j.1365-2141.2010.08214.x . hal-00552595

HAL Id: hal-00552595

<https://hal.science/hal-00552595>

Submitted on 6 Jan 2011

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**Glucocerebrosidase deficiency dramatically impairs human
bone marrow haematopoiesis in an in vitro model of
Gaucher disease.**

Journal:	<i>British Journal of Haematology</i>
Manuscript ID:	BJH-2009-01969.R1
Manuscript Type:	Ordinary Papers
Date Submitted by the Author:	03-Mar-2010
Complete List of Authors:	Berger, Juliette; CHU- Hotel-Dieu, Hematologie (Biologie) Lecourt, Séverine; Hopital St Louis, Unité de Thérapie Cellulaire Vanneaux, Valérie; Hopital St Louis, Unité de Thérapie Cellulaire Rapatel, Chantal; CHU- Hotel-Dieu, Hematologie (Biologie) Boisgard, Stéphane; CHU, Hop G. Montpied, Orthopédie, Traumatologie et Chirurgie Reconstructive Caillaud, Catherine; Hopital Cochin, Laboratoire de Génétique Métabolique Boiret-Dupré, Nathalie; CHU- Hotel-Dieu, Hematologie (Biologie) marolleau, jean-pierre; Hopital d'Amiens, Service clinique des Maladies du Sang Larghero, Jerome; Hopital St Louis, Unité de Thérapie Cellulaire Chomienne, Christine; Hopital St Louis, INSERM UMR940, Institut Universitaire d'Hématologie Berger, Marc; CHU- Hotel-Dieu, Hematologie (Biologie)
Key Words:	CYTOPENIA, GAUCHERS DISEASE, HAEMATOPOIESIS

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3 Title: **Glucocerebrosidase deficiency dramatically impairs human bone**
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6 **marrow haematopoiesis in an *in vitro* model of Gaucher disease.**
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3 Running short title: (60 caractères): Glucocerebrosidase directly impairs haematopoiesis.
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5 **Summary (200 mots)**
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7 One of the cardinal symptoms of type 1 Gaucher Disease (GD) is cytopenia, usually explained
8 by bone marrow (BM) infiltration by Gaucher cells and hypersplenism. However, some cases
9 of cytopenia in splenectomised or treated patients suggest possible other mechanisms. To
10 evaluate intra-cellular glucocerebrosidase (GlcC) activity in immature progenitors and to
11 prove the conduritol B epoxide (CBE)-induced inhibition of the enzyme, we used an adapted
12 flow cytometry technique before assessing the direct effect of GlcC deficiency in functional
13 assays. Among haematopoietic cells from healthy donors, monocytes showed the highest
14 GlcC activity but we demonstrated that immature CD34⁺ and mesenchymal cells have
15 significant GlcC activity. CBE greatly inhibited the enzyme activity of all cell categories.
16 GlcC-deficient CD34⁺ cells showed impaired ability to proliferate and differentiate in the
17 expansion assay and had lower frequency of BFU-E, CFU-G and CFU-M progenitors, but the
18 effect of **GlcC** deficiency on CFU-Mk lineage was not significant.
19

20 GlcC deficiency strongly impaired primitive haematopoiesis in long-term culture.
21

22 Furthermore, GlcC deficiency progressively impaired proliferation of mesenchymal
23 progenitors. These data suggest an intrinsic effect of GlcC deficiency on BM immature cells
24 that supplements the pathophysiology of GD and opens new perspectives of therapeutic
25 approach.
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49 **Keywords: Gaucher disease, glucocerebrosidase activity, haematopoiesis, cytopenia,**

50 **LTC-IC, mesenchymal stem cell**
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Introduction

Gaucher disease (GD) is a genetic lysosomal disease characterised by deficiency in glucocerebrosidase (Acid β glucosidase), a lysosomal enzyme involved in the first step of glucocerebroside breakdown into glucose and ceramide. This abnormality affects all body cells but is particularly expressed in macrophage-like cells where glucocerebroside accumulates in the lysosomes that induce a typical morphology. These cells - Gaucher cells - infiltrate **bone marrow** (BM), spleen, liver and brain and are considered to be mainly responsible for the clinical manifestations of GD. Three clinical types of GD have been identified: a perinatal lethal form (type 2); a form exhibiting neurological symptoms (type 3) and the most frequent (type 1) presenting haematologic and skeletal complications (Grabowski 2008).

The haematologic consequences of enzyme deficiency consist principally in cytopenias. Sixty percent of GD patients present with thrombocytopenia and 37% with anaemia (Pastores, *et al* 2004, Weinreb, *et al* 2002), whilst leucopenia is rare, occurs later in life and is rarely severe. Two main hypotheses have been proposed to explain cytopenia: hypersplenism and haematopoietic impairment due to infiltration of BM by Gaucher cells. However, hypersplenism usually leads to a decrease in platelets and leucocytes but hardly at all in erythrocytes, and some cases remain difficult to explain, particularly those where cytopenia continues after splenectomy and those where cytopenia persists during enzyme replacement therapy (ERT) even though spleen size has normalised. In addition, there is no correlation between the number of Gaucher cells in BM and severity of cytopenia. Several studies confirmed that Gaucher cells are macrophages and have the ability to produce IL-10, CCL-18, and chitotriosidase, (Allen, *et al* 1997, Deegan, *et al* 2005, van Breemen, *et al* 2007). Furthermore, there are high levels of numerous cytokines in plasma, both inflammatory and

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3 anti-inflammatory (Yoshino, *et al* 2007). We can suppose that all these modifications could
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5 affect haematopoiesis.
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8 It should be noted that thrombocytopenia and anaemia can be corrected with ERT but
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10 cellular kinetics are slow, and in a significant number of cases remain under or around the
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12 lower limit of normal values despite improving biological biomarkers and normalizing
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14 splenomegaly. Furthermore, severe thrombocytopenia is more difficult to correct (Pastores, *et*
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16 *al* 2004, Weinreb, *et al* 2002). All these observations raise the question of the exact role of
17
18 glucocerebrosidase deficiency in the pathophysiology of cytopenia which might be triggered
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20 by more complex mechanisms. However, we know little about other modifications possibly
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22 related to the intrinsic effect of enzyme deficiency on haematopoietic and mesenchymal
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24 progenitors. A recent study reported for the first time intrinsic impairment of BM
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26 Mesenchymal Stem Cells (MSC) (Campeau, *et al* 2009), but the consequences of
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28 glucocerebrosidase (GlcC) deficiency on haematopoietic progenitors/stem cells remain
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30 unknown. Further understanding of the pathophysiology of BM in these forms of cytopenia
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32 would make it possible to adapt treatment and/or to propose adapted complementary
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34 treatment.
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Materials and Methods

Cells and tissue culture

THP-1 human monocytic cell line (DSMZ, Braunschweig, Germany) was maintained at a density of 2×10^5 cells/ml in RPMI 1640 (Lonza, Verviers, Belgium) supplemented with 10% foetal calf serum (FCS) (Biowest, Nuaille, France) with 2 mmol/L L-glutamine (Lonza, Verviers, Belgium). Peripheral blood samples were obtained from the left over part of blood collected for routine analyses for benign non-inflammatory disease (control donors; n = 15) or from Gaucher patients (n = 4). The left over part of biological samples collected for routine analysis could be used for research because patients had been informed and did not verbally express any disagreement as stipulated in French law. (*Decree No. 2007-1220 dated August 10, 2007, Order dated August 16, 2007, French Public Health Code (Articles L.1232-6 and L.1243-9)*). Nucleated cells were isolated by collecting buffy coat and red cells were lysed in ammonium chloride (Stemcell Technologies Inc., Vancouver, Canada) for 10 min. on ice and immediately processed for substrate loading. BM cells were isolated from spongy bone (SB) collected from the femoral head during hip arthroplasty with the patient's informed written consent, according to the Declaration of Helsinki, the protocol being approved by the Regional Ethics Committee as already reported (Veyrat-Masson, *et al* 2007). Briefly, SB was fragmented and adherent cells were collected after 10 min. incubation with collagenase (Sigma Aldrich, St Quentin Fallavier, France) at 37°C. BM tissue was washed and filtered through a 100 µm nylon mesh (Falcon™, BD Biosciences, Le Pont de Claie, France), followed by Ficoll sedimentation (Histopaque 1077, Hybrimax (Sigma Aldrich, St Quentin Fallavier, France)); MSC were isolated in culture conditions validated in a series of more than 200 BM (Veyrat-Masson, *et al* 2007). In parallel, CD34⁺ cells were selected by using MACS Separation Columns (Miltenyi Biotec, Paris, France) according to the manufacturer's recommendations.

Flow cytometry analysis of glucocerebrosidase activity

Flow cytometry analysis of intra-cellular GC activity was conducted using the fluorogenic GlcC substrate 5'pentafluorobenzoylamino fluorescein-di- β -D-glucoside PFBFDGlu (Invitrogen, Cergy Pontoise, France) according to the protocol of Lorincz *et al.* (Lorincz, *et al* 1997); PFBFDGlu stock was maintained in the dark at 50 mmol/L (in 100% dimethyl sulfoxide DMSO) and was stable for several month at -20°C. Briefly, for GlcC activity and inhibition studies, cells were mixed with an equal volume of PFBFDGlu (2mM). In a first series of experiments using monocytic THP-1 cells, we evaluated the influence of incubation temperature and time (10, 30, 60 and 120 min.) on generating fluorescence. We retained incubation conditions of 37°C for one hour as more reproductive and reliable. In these conditions, the mean fluorescence of the isotypic control was highly reproducible and the fluorescence intensity was directly correlated with glucocerebrosidase functional activity, thus we considered additional fluorescence of cells of interest expressed in arbitrary units under control. For GlcC inhibition studies, THP-1 cells and haematopoietic cells were suspended at a concentration of 10^5 - 10^6 cells/mL and incubated at 37°C for 24 hours with the optimal 500 μ M dose of CBE (Sigma Aldrich, St Quentin Fallavier, France) (see results, figure 1), a specific chemical inhibitor of glucocerebrosidase (Das, *et al* 1986), before adding substrate. Flow cytometry analysis was performed on a Coulter Epics flow cytometer with a single argon laser at 488 nm. In a limited series, glucocerebrosidase activity was also analysed by a standard method (Peters, *et al* 1976).

In vitro Expansion, Clonogenic assays and Conduritol B Epoxide (CBE) inhibition studies

The CD34⁺ cells were purified using the direct CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) following the manufacturer's protocol.

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3 CD34⁺ cells were expanded in complete medium consisting in IMDM supplemented with
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5 10% FBS, recombinant human (rh) Kit Ligand (KL) (50ng/mL), TPO (50ng/mL), rh IL-3
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7 (20ng/mL) and rh Flt3-L (20ng/mL) (Tebu-bio, Le Perray en Yvelines Cedex, France) at
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9 37°C in 5% CO₂ for 14 days and fed, after 7 days, by the addition of fresh 'complete
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11 medium'. On day 14 of culture, cells were sampled for count and cell viability measurement
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13 using 7-aminoactinomycin D (7-AAD) staining, CD34⁺ cell count (ISHAGE protocol) and
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15 CFU-GM assays. Expanded cells on day 14 of culture were then cultured in Methocult GF
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17 H4534 (Stemcell Technologies Inc., Vancouver, BC, Canada) and CFU-GM were identified
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19 and counted using standard criteria at D14. The expansion values represent the fold increase
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21 over the number of CD34⁺ cells initially purified. All the reagents were purchased from
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23 Becton Dickinson.
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29 To determine the effect of glucocerebrosidase on native colony-forming cells (CFCs), and to
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31 avoid any influence from accessory cells, with or without CBE, 2000-500 cells/mL fresh
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33 immunoselected CD34⁺ cells (purity > 90 %) were seeded in Methocult H4434 (Stemcells
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35 Technologies Inc., Vancouver, Canada). At day 14, we distinguished burst forming unit-
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37 erythroid (BFU-E), colony forming unit-granulocytes and/or macrophages (CFU-G, CFU-M)
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39 scored by direct visualisation using an inverted microscope 14 days later using standard
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41 criteria (Boiret, *et al* 2003). CFU-MK assays were performed in a collagen-based, serum-free
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43 medium supplemented with a cytokine cocktail affording 20 ng/mL rh TPO, 10 ng/mL rh IL6,
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45 20 ng/ml rh KL (AbCys, Paris, France) at the final concentration. Cells were seeded at two
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47 different concentrations. After 10 days of incubation, cultures were dehydrated and fixed with
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49 methanol-ethanol and revealed after May-Grünwald-Giemsa (MGG) staining.
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53 The frequency of long-term culture-initiating cells (LTC-IC) was determined in limiting
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55 dilution assay (LDA) (Sutherland, *et al* 1990). Briefly, CD34⁺ cells were suspended in LTC
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57 media (IMDM supplemented with 12.5% horse serum (Stemcell Technologies Inc.,
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3 Vancouver, Canada), 12.5% FCS and 10^{-4} mol/L hydrocortisone (Sigma Aldrich, St Quentin
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5 Fallavier, France), 1% glutamine) which was then plated in limiting dilution in a 96-well plate
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7 on pre-established, irradiated normal MSC as marrow feeders. Half the medium was replaced
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9 weekly and after 5 weeks, all non-adherent cells and adherent cells were mixed and assayed
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11 for clonogenic assays. The number of wells containing secondary clonogenic cells at 5 weeks
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13 provided an indirect measure of the frequency of LTC-IC present at day 0. The dishes
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15 supplemented with 500 μ M CBE received a new dose every 3-4 days to maintain enzyme
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17 inhibition.
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22 23 24 ***CFU-F assay and mesenchymal cell in vitro expansion***

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26 Mononuclear cells from SB BM were seeded in 25 cm² culture flasks (Falcon™, BD
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28 Biosciences, Le Pont de Claie, France) at 2×10^4 /cm² in basic mesenchymal medium,
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30 consisting of IMDM, 10% FCS, 1% glutamine, 1 ng/mL bFGF (AbCys, Paris, France), with
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32 or without CBE. Progenitor cells -colony forming unit-fibroblasts (CFU-F)- were quantified
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34 directly by microscopic examination of flasks at day 10 and identified by their size (<25, 25-
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36 50 and >50 cells). At the end of primary culture, MSC were trypsinised (Sigma Aldrich, St
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38 Quentin Fallavier, France) then cultured at 500 cells/mL for a further expansion passage (P1)
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40 to evaluate the effect of extended inhibition on the ability of cell progeny to proliferate and to
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42 produce secondary CFU-F.
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50 51 ***Statistical analysis***

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53 Results are expressed as mean \pm SEM. Differences between mean values were evaluated by
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55 paired or bilateral Student's t-tests. Limiting dilution assays for determining the frequency of
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57 LTC-IC were performed according to the Poisson statistical model (L-Calc™, StemCell
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59 Technologies Inc., Canada).
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Results

Assesment of glucocerebrosidase activity and effect of CBE in blood and BM cells

With the aim of evaluating intra-cellular GlcC activity of defined cell subsets and to prove the inhibiting effect of CBE, we chose to adapt a flow cytometry technique (Lorincz, *et al* 1997) with the monocytic THP-1 cell line and we adopted one hour substrate incubation at 37°C as the most reproducible and reliable conditions (data not shown).

We then analysed the intra-cellular enzyme activity of circulating blood cells from healthy donors (**Fig 1A**). We observed a high variability of GlcC activity according to blood nuclear cell subsets (**Fig 1A, table1**). Monocytes had a 48 fold higher activity than lymphocytes and polymorphonuclear cells. This observation provides further explanation as to why enzyme deficiency is particularly expressed in a monocyte subset - Gaucher cells - in patients.

In parallel, we checked that CBE was able to inhibit GlcC activity. This activity was proportionally inhibited by CBE as shown by the dose response curve, validating the already published 500 µM optimal dose (**Fig 1B**). At this dose, we observed inhibition of at least **99%** GlcC activity in all cell categories. By analysing GlcC activity by a standard method (after cell lysis) in parallel (Peters, *et al* 1976), we obtained satisfactory correlation between the two methods (**Fig 1E**) except for lower **GlcC** values; flow cytometry **being** more sensitive, it showed differences that were not detected with the **standard** technique. Furthermore, we confirmed that **the effect of CBE** was transient (Yatziv, *et al* 1988) and that maintaining its effect required sequential adding of CBE (data not shown).

We then compared GlcC activity in the two main cell **categories involved in** haematopoiesis, i.e. CD34⁺ immature haematopoietic cells and MSC. Surprisingly, we detected significant activity in these cell categories (**Fig 1C, D; table 1**), slightly higher than that of lymphocytes or polymorphonuclear cells. Lastly, we validated the method by analysing blood monocytes from **four** untreated type 1 GD patients. Intra-monocyte GlcCA was dramatically decreased

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3 compared with that of healthy subjects (**table 1**). Thus, circulating blood monocytes might be
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5 an interesting satisfactory cell surrogate for BM Gaucher cells.
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10 *Glucocerebrosidase deficiency dramatically impaired mature and primitive haematopoiesis*

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12 To evaluate the consequences of GlcC deficiency on haematopoiesis, we analysed the effect
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14 of adding CBE in functional assays. CD34⁺ cell culture for 14 days led to a significant
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16 decrease in nucleated cell expansion in the presence of CBE (cumulative population
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18 doublings 4.1 ± 0.9 vs 3.4 ± 1.1 , $p=0.007$) (Figure 2A). Moreover, a decrease in the
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20 percentage of CD34⁺ during expansion was also observed ($4.4\% \pm 2.2$ vs $2\% \pm 1.9$, $p=0.04$).
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22 The number of CFU-GM, assessed after 14 days expansion, was dramatically reduced in the
23
24 CBE-treated group as compared to untreated cells (7 ± 4 and $39 \pm 9.0 / 500$ expanded cells
25
26 respectively, $p=0.04$) (Figure 2A). Furthermore, inhibition of GlcC activity in short-term
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28 culture of fresh CD34⁺ cells showed a significant decrease in the frequency of CFU-G (16 ± 3
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30 vs $30 \pm 4 / 10^4$ CD34⁺ cells; $p= 0.0036$), CFU-M (3 ± 1 vs $5 \pm 1 / 10^4$ CD34⁺ cells; $p=0.0297$)
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32 and of BFU-E (21 ± 5 vs $32 \pm 8 / 10^4$ CD34⁺ cells; $p=0.0143$) ($n=9$) and a non-significant
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34 trend to lower frequency of CFU-MK (Figure 2B). Finally, we evaluated the effect of CBE in
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36 long-term culture. GlcC deficiency dramatically impaired primitive haematopoiesis making
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38 LTC-ICs almost undetectable when CBE was added twice weekly (1 LTC-IC / 3218 ± 1650
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40 vs 1 LTC-IC / 58 ± 20 CD34⁺ cells; $n=3$; $p= 0.001$) (Figure 2C). Due to the impossibility of
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42 distinguishing the effect of CBE on CD34⁺ cells from that on MSC, we evaluated the
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44 consequences on viability and proliferation of MSC ($n=4$) (Figure 2D). We observed no
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46 change in native mesenchymal progenitor frequency, but their number (100 ± 9 vs 154 ± 13
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48 CFU-F / 10^3 cells) and their proliferative capacity, shown by colony size distribution, were
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50 impaired from the end of primoculture. The number of expanded mesenchymal cells then
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52 progressively decreased, the difference with control cells being more evident at the end of P1.
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Thus this observation suggests that enzyme deficiency must be present a certain length of time before effects are observed.

For Peer Review

Discussion

The main goal of this study was to research a possible intrinsic effect of glucocerebrosidase deficiency on BM immature cells which would shed new light on the pathophysiology of cytopenias in Gaucher Disease. Several steps were defined (table 2).

The first step of this study was to develop tools to validate the *in vitro* model. In order to assess glucocerebrosidase activity and its inhibition by CBE as accurately as possible, we adapted the flow cytometry technique of Lorincz *et al.* (Lorincz, *et al* 1997). Substrate incubation and analysis after erythrocyte lysis, instead of Ficoll density centrifugation, enabled us to compare the activity of cell subsets in different samples at different times. The correlation between results of flow cytometry and of the standard technique (Beutler and Kuhl 1990) was satisfactory, but flow cytometry is more sensitive and glucocerebrosidase activity in cell subsets **could be evaluated.**

The second step was to describe natural GlcC activity in blood leucocytes and BM haematopoietic and mesenchymal immature cells. The enzyme activity of circulating monocytes from healthy donors was greater than that of PMN or lymphocytes. Under our test conditions, the intra-monocyte activity of the **four** type 1 GD patients in this study was proportionally lower than reported previously (Lorincz, *et al* 1997). The heterogeneity of patient phenotype and the different analytical method used may explain these findings (Grabowski, *et al* 2006, Weinreb, *et al* 2002). **The first result confirmed that circulating monocytes represent an interesting leucocyte subset to analyse for Gaucher Disease. The second interesting observation concerned enzyme activity of bone marrow immature cells.** We have assessed, for the first time, the intra-cellular activity in normal CD34⁺ cells themselves and not in their progeny (Nimgaonkar, *et al* 1995) nor in glucocerebrosidase gene transduced CD34⁺ cells (Nimgaonkar, *et al* 1994). Surprisingly, GlcC activity in CD34⁺ cells

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3 was higher than in PMN or lymphocytes, suggesting a potentially unknown role for primitive
4 haematopoietic cells. Similarly, the enzyme activity of expanded mesenchymal stem cells was
5 also significant. **The GlcC activity present in immature BM cells that can be inhibited by
6 CBE suggested that the enzyme deficiency seen in GD patients may have intrinsic
7 and unknown effects on cells indispensable for haematopoiesis.**

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10 **The third step was to validate the *in vitro* model of glucocerebrosidase deficiency. The *in*
11 *vitro* use of CBE induced a collapse of intra-cellular GlcC activity in all tested cell
12 subsets, by inhibiting at least 99 % of the initial activity.**

13
14
15 **The fourth step was assessment of the effect of enzyme deficiency on mature**
16 **haematopoiesis.** Functional assays with CBE revealed a marked impairment of
17 haematopoiesis. During 14-day *in vitro* expansion, we saw a decreased proliferative capacity
18 of CD34⁺ cells and CFU-GM production. In short-term culture, inhibition of
19 glucocerebrosidase caused a significant decrease in the frequency of native CFU-G, CFU-M
20 and BFU-E, but in this small series we did not see a significant effect on the frequency of
21 CFU-Mk, suggesting that thrombocytopenia in patients could result from more complex and
22 extrinsic mechanisms. We thus observed the direct effect of enzyme deficiency on CD34⁺
23 cells, since their selection eliminated the possible role of accessory cells. Moreover, it is
24 unlikely that the observed effect was related to CBE toxicity, since CBE is not known to
25 cause cell apoptosis (Das, *et al* 1987), and cell viability curves could be superimposed onto
26 those of control cells (data not shown).

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29 **The fifth step was the evaluation of the consequences of GlcC deficiency on primitive**
30 **haematopoiesis in long-term culture.** Primitive haematopoiesis was greatly impaired when
31 glucocerebrosidase was inhibited, as seen by the collapse of LTC-ICs. However, in this
32 system, the effect of CBE on CD34⁺ cells could not be differentiated from its effect on the
33 mesenchymal cells used as a feeder layer, and we confirmed that glucocerebrosidase
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3 deficiency impaired mesenchymal cells as recently reported (Campeau, *et al* 2009). The
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5 mechanisms of this impairment of proliferation and differentiation remain to be specified.
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7 However, the impairment of intra-cellular signalling pathways (Fredman 1998) or modified
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9 calcium mobilisation (Lloyd-Evans, *et al* 2003) may be involved in, and have an impact on,
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11 haematopoiesis (Paredes-Gamero, *et al* 2008). Similarly the gradual accumulation of
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13 ceramide (Luberto, *et al* 2002) and its effect on the lipid composition of microdomains may
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15 be involved (Hein, *et al* 2008). Overall, these results suggest that glucocerebrosidase
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17 deficiency may have a directly harmful effect on immature haematopoietic cells and/or cells
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19 of the stem cell niche and that cytopenias are not just the results of hypersplenism, Gaucher
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21 cells or modification in cytokine secretion (Barak, *et al* 1999, Yoshino, *et al* 2007).
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26 The results with mesenchymal cells are particularly interesting for one point: the initial
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28 frequency of native CFU-F remained unmodified and it would appear that minimal exposure
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30 to enzyme deficiency is necessary to induce damage. This notion corresponds to the clinical
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32 signs of type 1 GD, where diagnosis is often made several years after its onset (Grabowski
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34 2008).
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37 However, any correlation with clinical signs is slight since the most visible effect observed
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39 here concerned erythroid and granulo-monocyte progenitors whereas patients usually have
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41 thrombocytopenia (Weinreb, *et al* 2002). Little or no BM aplasia was observed, as might be
42
43 expected with major impairment of primitive haematopoiesis. But we did notice that residual
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45 enzyme activity after CBE represented less than **1%** of initial activity, whilst it ranged from
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47 10-30% in type 1 GD patients (Beutler and Kuhl 1970a, Beutler and Kuhl 1970b, Jmoudiak
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49 and Futerman 2005, Van Weely, *et al* 1991). Consequently, the model presented here may
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51 correspond to a major deficiency, as in type 3 or type 2 GD. Major impairment of mature and
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53 primitive haematopoiesis may contribute to **poor** foetal prognosis and early death that occurs
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60 in these clinical forms. **However, in our small series of GD patients, even if we observed**

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3 **higher values with Gaucher patient monocytes than with CBE-inhibited normal**
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5 **monocytes, the residual intra-cellular GlcC activity was of between 3-8% of the normal**
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7 **value. However tests should be performed with a larger sample size.**

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10 In conclusion, **the major result of this study is proof of the harmful intrinsic effect of**
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12 **glucocerebrosidase deficiency on the proliferation and differentiation of BM immature cells**
13
14 **resulting in mature and primitive haematopoiesis impairment.** The dramatic *in vitro*
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16 **impairment of haematopoiesis, in particular of primitive progenitors, when enzyme activity is**
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18 **lower than 1%, suggests that intrinsic consequences might depend on the level of residual**
19
20 **enzyme activity, cell type and perhaps disease type, thus explaining to some degree why**
21
22 **cytopenias can sometimes be corrected partially by imiglucerase. However, no correlation**
23
24 **has yet been found between phenotype and residual enzyme levels. A more accurate**
25
26 **assessment of enzyme activity in target cells would confirm whether or not there is any**
27
28 **such relationship. Furthermore, since we observed no Gaucher-type cells (large size,**
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30 **wrinkled tissue paper appearance of cytoplasm and eccentric nucleus) in this model, we**
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32 **postulate that the functional effects of deficiency are present before the accumulation of**
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34 **glucosylceramide has modified cell morphology, this aspect remaining quite specific to**
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36 **the monocyte cell lineage.** A better understanding of these cellular mechanisms would help
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us to develop treatments complementary to enzyme replacement therapy.

Acknowledgements

50
51 This work was supported by grants from Genzyme Corporation. S.L. was sponsored as a
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doctoral student by Genzyme SAS (CIFRE scholarship). The authors would like to thank
Dominique Chadeyron **and Philippe Bonnet** for manuscript preparation

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3 **Table 1: Evaluation of intra-leucocyte glucocerebrosidase activity in healthy donors and**
4
5 **Gaucher patients.**
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7
8 Activity of glucocerebrosidase was evaluated by flow cytometry (see Fig. 1) in blood
9
10 circulating leucocyte subsets from healthy donors (n=15) and Gaucher patients (n=4),
11
12 and in CD34+ cells (n=3) and MSC collected at the end of primary culture (n=5). Results
13
14 are expressed as mean \pm SEM of fluorescence arbitrary units (fluorescence mean). Mo:
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16 Monocytes; PMN: polymorphonuclear cells; Ly: Lymphocytes.
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24 **Table 2: Summary of objectives and main results.**
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27 This table summaries the main steps of the study and the major results obtained from
28
29 the study of haematopoiesis using an *in vitro* model of Gaucher disease.
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3 **Figure 1: *In vitro* inhibition of glucocerebrosidase in different categories of normal cells.**

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5 Flow cytometry functional assay was used for determining the activity of GlcC in normal
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7 blood cell subsets (A). Representative profiles show GlcC-generated fluorescence in
8
9 polymorphonuclear (PMN) cells, monocytes (Mo) and lymphocytes (Ly). Unspecific isotypic
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11 control (IC) fluorescence, GlcC-dependant and CBE-reduced (CBE) fluorescences are shown
12
13 underlining the clear decrease (> 95%) in enzyme activity in all tested cells. Intra-cellular
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15 GlcC activity was particularly expressed in blood monocytes. Inhibition of intra-cellular
16
17 GlcC activity was particularly expressed in blood monocytes. Inhibition of intra-cellular
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19 activity by CBE was dose-dependent (B; n=3) and we chose the optimal 500 µM dose.
20
21 Significant activity sensitive to CBE effect was detected in **bone marrow immature cells i.e.**
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23 **CD34⁺ cells (C) and MSC (D)** as shown by representative profiles. **A satisfactory**
24
25 **correlation was observed between flow cytometry and a standard technique (D).**
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32 **Figure 2: Glucocerebrosidase deficiency dramatically impaired *in vitro* mature and**
33
34 **primitive haematopoiesis.**

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36 The effect of CBE-inhibited GlcC activity was evaluated on mature and primitive
37
38 haematopoiesis. The consequences of enzyme deficiency on proliferative ability of CD34⁺
39
40 were assessed after *in vitro* expansion (A; n=8). The number of doubling populations was
41
42 significantly lower and we collected lower numbers of CD34⁺ cells or CFU-GM at D14. In
43
44 fresh CD34⁺ cells, native BFU-E, CFU-G and CFU-M were significantly impaired in CFC
45
46 functional assay (B; n=9), but the consequence on Mk lineage was not significant. In long-
47
48 term culture, addition of CBE dramatically impaired proliferation and differentiation of LTC-
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50 ICs (C; n=3), the representative cells of primitive haematopoiesis. From this data, we
51
52 evaluated the effect of CBE on BM MSC (D; n= 4). The frequency of native CFU-F was not
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54 modified, but the frequency of expanded CFU-F from the end of primary culture (P0) was
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56 decreased and their ability to produce mesenchymal cell progeny was clearly impaired as
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3 demonstrated by their colony size distribution (<25 cells, 25-50 cells and > 50cells). The
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5 number of expanded mesenchymal cells decreased progressively during passage 1. Ctr:
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7 control refers to culture without CBE, CBE: culture in the presence of conduritol B epoxide.
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10 Results are expressed as mean \pm SEM; *: P<0.05 when not specified.
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For Peer Review

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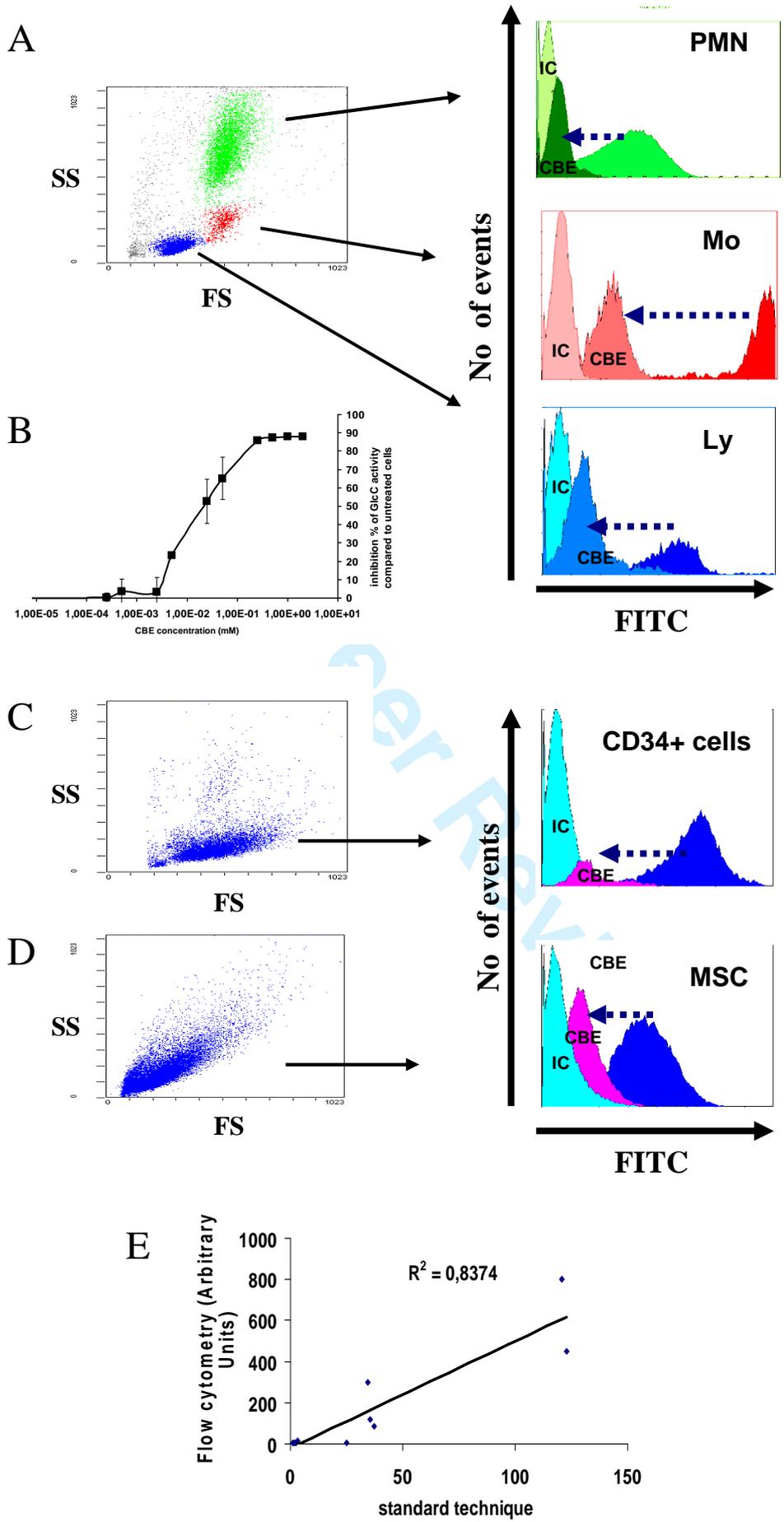


Figure 1

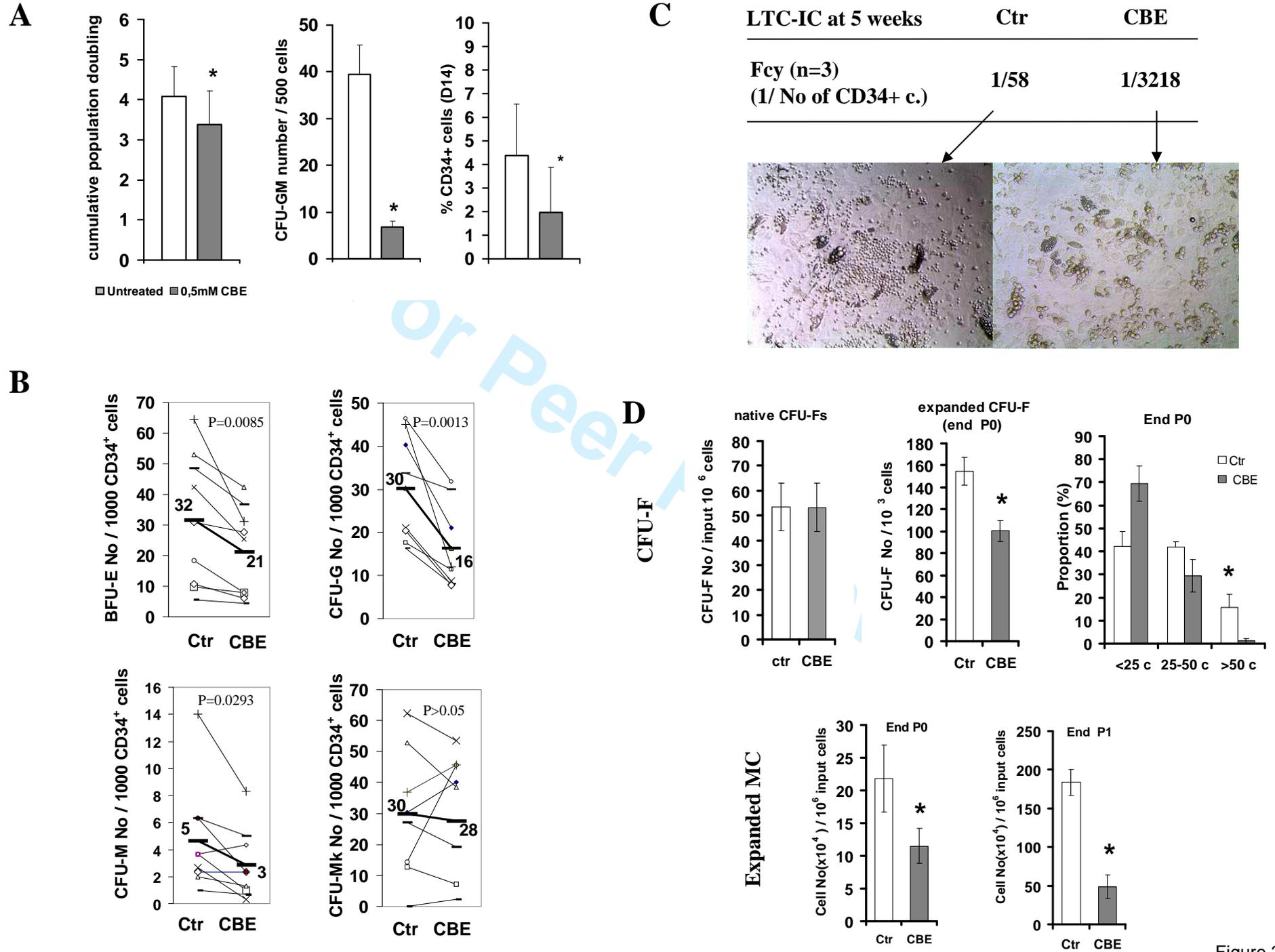


Figure 2

Table 1

	Healthy donors					Gaucher patients		
	Mo	PMN	Ly	CD34+	MSC	Mo	PMN	Ly
Fluorescence (Arbit. Units)	300.3 ± 28.1	6.2 ± 1.8	6.1 ± 0.7	32.2 ± 2.3	21.2 ± 9.1	10.3 ± 4.7	0.4 ± 0.1	0.6 ± 0.2
Exp No (n)	15	15	15	3	5	4	4	4

Table 2

Objectives / Questions	Results
# 1 Developing a reliable tool to assess intra-cellular GlcC	Optimisation of flow cytometry technique
# 2 Descriptive study of natural GlcC activity in haematopoietic cells and immature BM cells.	Blood monocytes have the highest GlcC activity. Bone marrow D34+ cells and mesenchymal cells have significant GlcC activity.
# 3 Validation of <i>in vitro</i> model	Intra-cellular GlcC activity in all cells subsets can be inhibited by CBE.
# 4 Consequences on mature haematopoiesis	Impairment of CD34+ cell proliferation and differentiation towards GM- and E- lineages.
# 5 Consequences on primitive haematopoiesis	Dramatic impairment of primitive haematopoiesis in long-term culture.