TREATING NEUTROPENIA AND NEUTROPHIL DYSFUNCTION IN GLYCOGEN STORAGE DISEASE IB WITH AN SGLT2 INHIBITOR

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SUPPLEMENTARY METHODS

Ethical considerations and study medication

Phase 1 studies in 27 or 33 adolescents, respectively on empagliflozin[1] or dapagliflozin[2] did not indicate relevant pharmacokinetic differences nor safety concerns in comparison to adults. Long-term treatment data was described for 27 diabetics including adolescents under canagliflozin that were followed-up in mean 29 days.[3] Further, the product characteristics (https://www.ema.europa.eu/en/documents/product-information/jardiance-epar-product-information_en.pdf) state: a paediatric Phase 1 study examined the pharmacokinetics and pharmacodynamics of empagliflozin (5 mg, 10 mg and 25 mg) in children and adolescents ≥10 to <18 years of age with type II diabetes mellitus. The observed pharmacokinetic and pharmacodynamic responses were consistent with those found in adult subjects.

Institutional Review Board approval is not required, the patients underwent an individualized treatment. In the Netherlands (PT3) off-label experimental prescription of medication is part of the Medicines Act and concretely worked out in the guideline of the Royal Dutch Medical Association (RDMA). The Medical Ethical Committee of the University Medical Center in Groningen confirmed that the Medical Research Involving Human Subjects Act does not apply for retrospective, observational studies and that official approval of this study by the Medical Ethical Committee was not required (METc code 2016/470). Obviously, parents/patients have to give written consent. The same regulation holds for Austria (PT1, PT2). No ethics approval is required for an individual treatment attempt and it was therefore not requested. For PT 4, after review and consent of the parents, the medication was initiated as an off-label use weighing risks and benefits. Clinical review of this single individual local

case, and study procedures that were not clinically indicated were performed after obtaining informed consent on an IRB-approved study (COMIRB# 16-0146).

Detection and quantification of 1,5-anhydroglucitol (1,5AG) in plasma

1,5AG in plasma was quantified by LC-MS as previously described[4]. Plasma was isolated after centrifugation (5 min at 500 x g; 22°C) of 0.4 ml of freshly collected EDTA blood and kept at -80°C until analysis. Practically, we added 4 µl of plasma to 196 µl of extraction solution (10% H_2O/90% of a solution containing 90% methanol and 10% chloroform) and used the supernatant of a 10 min centrifugation at 16000 x g to quantify 1,5AG in the sample. Absolute concentrations were determined by comparing the integrated extracted ion chromatograms corresponding to 1,5AG (m/z = 163.0612) with those of the standard 2-[D]-1,5AG (m/z = 164.0674) prepared by adding either 5 or 10 µl of 30 µM 2-[D]-1,5AG (0.75 – 1.5 µM final) to 196 µl of extraction solution.

Detection and quantification of granulocyte 1,5-anhydroglucitol-6-phosphate (1,5AG6P)

To get an estimate of the 1,5AG6P concentration in granulocytes, this metabolite was quantified by LC-MS in three types of samples that are described below. Data shown in **Fig. 2C** was collected by using the same 0.4 ml blood sample that was centrifuged for the analysis of 1,5-AG in plasma (see above). Practically, subsequent to removing the plasma (without disturbing the leukocytes - buffy coat - and red blood cell pellet) 1,5AG6P was extracted from the pellet by adding 1 ml of extraction solution (10% H_2O/90% of a solution containing 90% methanol and 10% chloroform), vortexing for 1 min and centrifuging (10 min at 16 000 x g; 4°C) twice to completely clarify the supernatant. This could be stored at -80°C and quickly centrifuged before LC-MS analysis. The values for 1,5AG6P shown correspond to extracted-ion chromatograms of the [M-H]- form and the areas under the curve (m/z = 243.0273) were normalized to (1) total ion current (TIC)[4], which takes into account any variability in the sensitivity of the LC-MS measurements, and (2) the ANC present in the respective blood sample, since most of the 1,5AG6P is present in the neutrophils[4]. The values obtained in this way are proportional to the intracellular concentration of 1,5AG6P in neutrophils. Though the method may overestimate the 1,5AG6P content of neutrophils due to the presence of small

amount of this compound in other white blood cells (Fig. 2C), we observed the same relative decrease of 1,5AG6P upon empagliflozin treatment in isolated granulocytes (Fig. 2D and Fig. S1). This means that any misinterpretation we might make by assuming that 1,5AG6P is essentially present in neutrophils does not affect the validity of our conclusion.

To quantify 1,5AG6P in leukocytes of PT3 (see **Fig. S1**), we added 0.4 ml of EDTA blood to 10 ml of red blood cell lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA, pH 7.2). After lysis (≈ 15 min), the tubes were centrifuged (10 min at 500 x g) and the pellet containing the leukocytes washed with 10 ml 0.9% NaCl and centrifuged as above. The supernatant was removed, the pellet resuspended in 1 ml of extraction solution, vortexed for 1 min and the supernatant finally recovered after centrifugation (10 min at 16 000 x g; 4°C) and stored at -80°C before LC-MS analysis. The values shown for 1,5-AG6P in leukocytes were normalized to TIC and ANC as described above.

Isolation and quantification of 1,5-AG6P in PMNs and PBMCs (**Fig. 2D**) isolated from 5 ml of EDTA blood from PT1 and a healthy control was as previously described[4].

Detection and quantification of glycosylated LAMP2 in isolated granulocytes (PMN) by western blot

Protein glycosylation was estimated in neutrophils by western blot analysis of LAMP2[5]. For that PMNs for PT1, PT3 and a healthy control were isolated from 5 ml of EDTA blood as described[4] for quantification of 1,5AG6P, with the difference that after the last centrifugation step cells were resuspended in 150 µl of RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% Na deoxycholate, 1% NP40) supplemented immediately before use with a protease inhibitor cocktail (Roche Diagnostics, Germany) and 2 mM of a 200 mM stock solution of phenylmethylsulfonyl fluoride (PMSF) made in isopropanol. The lysate was frozen and the supernatant containing the solubilized proteins was recovered after defrosting and centrifugation (10 min at 16 000 x g; 4°C). Protein in the supernatant was assayed using a Bradford[6] microplate protein assay (Bio-Rad, USA), with BSA as a standard. To estimate protein glycosylation, 10 µg of protein were separated in NuPAGE 4-12% gradient Bis-Tris precast protein gels (Thermofisher, USA) run in MOPS buffer and transferred to a nitrocellulose membrane Hybond ECL (GE Healthcare, UK). The membrane was blocked in PBS with 5% milk powder for 1 h at room temperature and further incubated overnight at 4°C in PBS with 0.1% Tween 20 (PBS-T) and 5% milk powder with 500-fold diluted anti-LAMP2

antibody (H4B4; sc-18822; Santa Cruz Biotechnology, USA). After washing 3 times for 5 min in PBS-T, the membrane was incubated for 1h at room temperature in PBS-T and 5% milk powder with 5000-fold diluted goat anti-mouse IgG HRP-conjugated antibody (A5278; Sigma, USA). Before developing, the membrane was washed twice for 5 min in PBS-T and once in PBS and signals were detected with the Immobilon Western Chemiluminescent HRP Substrate reagent (WBKLS0500; Millipore, USA) on imaging film. The proportion of the partially glycosylated LAMP2 in the samples was estimated with the help of ImageJ (https://imagej.nih.gov/ij) by quantifying the intensity of the signal corresponding to the partially glycosylated LAMP2 and comparing it to the total LAMP2 signal.

Neutrophil function assays of oxidative burst, chemotaxis and bactericidal activity.

Oxidative burst was assayed as previously described[7] in two different laboratories. For PT4 (**Fig. 3C**) whole blood was loaded with dihydrorhodamine 123 (DHR-123), stimulated with phorbol 12-myristate 13-acetate (PMA) for 15 minutes and NADPH oxidase activity assessed by increase in DHR fluorescence using a Navios flow cytometer and Kaluza analysis software (Beckman Coulter, Indianapolis, IN). Data was expressed as the stimulation index (SI, ratio of fluorescence of stimulated cells to unstimulated cells)[7]. For PT1, PT2 and the respective controls (**Fig. S2**), 100 µl of whole blood was incubated or not with DHR-123 (Thermo Fisher Scientific, D23806) ± PMA. Following red blood cell lysis, the leukocytes were fixed using BD Phosflow™ Lyse/Fix Buffer (BD Biosciences) according to the manufacturer's instructions. Leukocytes were evaluated for size/granules and granulocytes (PMNs) were analyzed for rhodamine 123 fluorescence by flow cytometer analysis (FC500 and Kaluza 2.1 analyzing software, Beckman Coulter).

Neutrophil chemotaxis was assessed using the modified Boyden chamber method in which purified neutrophils migrate from an upper chamber through a 4.5 micron cellulose nitrate filter towards a chemotactic stimulus placed in the lower chamber[8]. Migrating neutrophils, arrested on a 3.0 micron filter, were visualized by hematoxylin staining and under light microscopy with 25x magnification. Chemotaxis was measured as the distance (in microns) of the leading edge of the neutrophils in response to either buffer or activated serum[8]. Bactericidal activity was assessed by incubating neutrophils with opsonized *S. aureus* in a 1:1 ratio and followed by measurement of the number of viable bacteria remaining over time after phagocytosis had occurred[9]. Viable bacteria were measured by standard colony

counts. The data was expressed as the percentage of viable bacteria at each time point relative to time point zero (100%).

SUPPLEMENTARY FIGURES

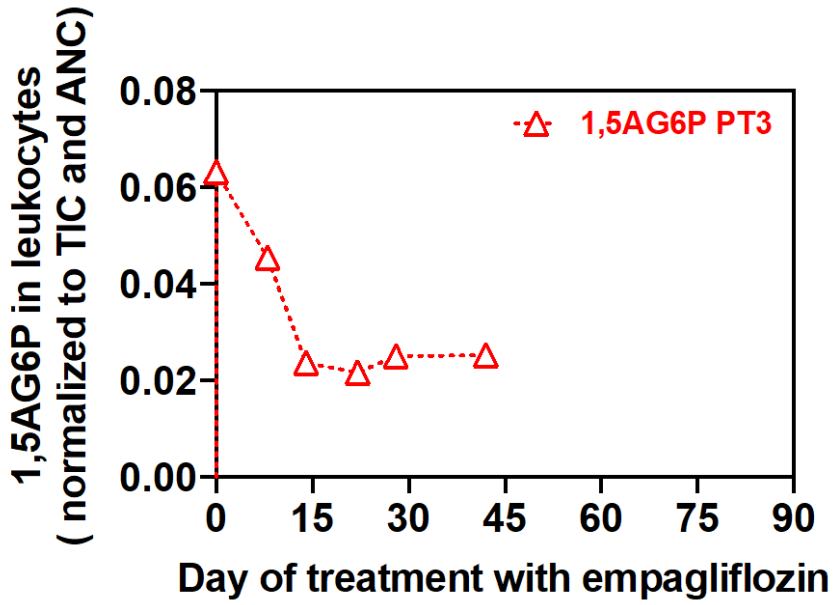
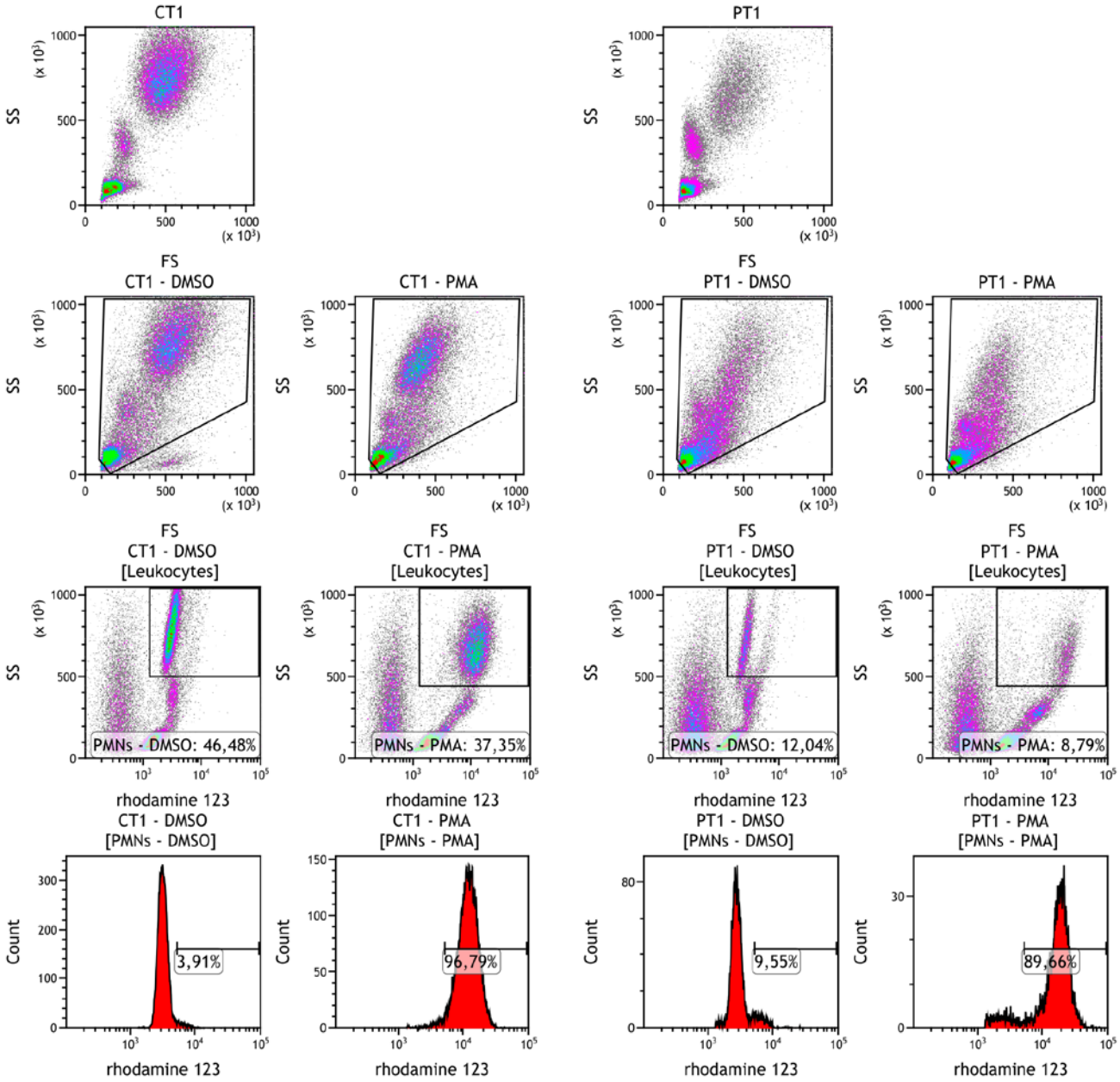


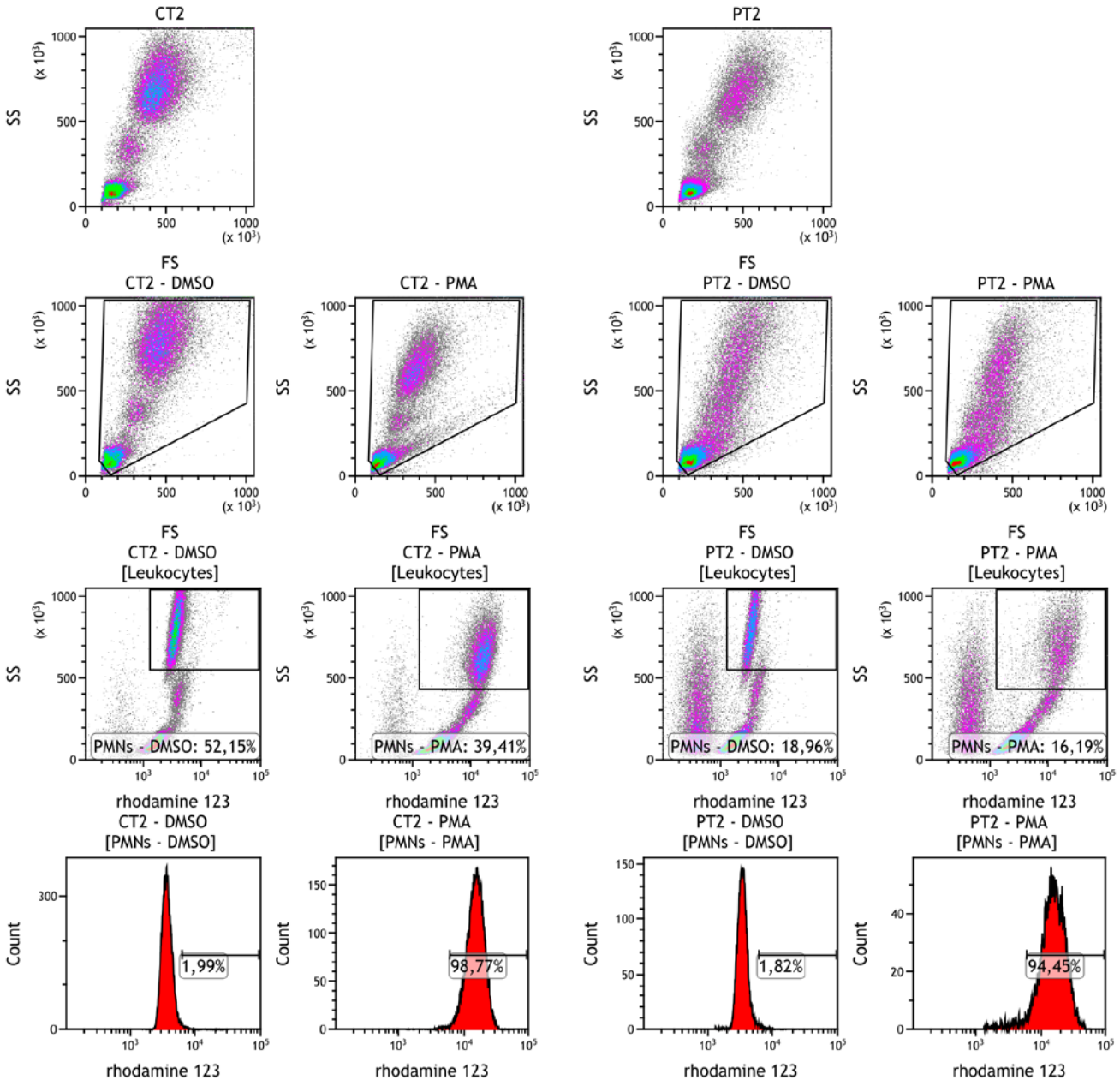
Figure S1. Empagliflozin lowers 1,5-anhydroglucitol-6-phosphate in leukocytes from GSD 1b patients.

The impact of Empagliflozin on the intracellular 1,5-anhydroglucitol-6-phosphate (1,5AG6P) determined by LC-MS in leucocytes from PT3 extracted after red blood cell lysis. As for quantification in whole blood (see **Fig. 1C**) the values for 1,5AG6P shown correspond to extracted-ion chromatograms of the [M-H]- forms and the areas under the curve (m/z = 243.0273) normalized to total ion current (TIC)[4] and the absolute neutrophil counts (ANC) present in respective blood sample.

A


CT1
PT1
SS
(x 10³)
FS
CT1 - DMSO
CT1 - PMA
PT1 - DMSO
PT1 - PMA
[Leukocytes]
PMNs - DMSO: 46,48%
PMNs - PMA: 37,35%
PMNs - DMSO: 12,04%
PMNs - PMA: 8,79%
rhodamine 123
[PMNs - DMSO]
[PMNs - PMA]
Count
3,91%
96,79%
9,55%
89,66%

B


CT2
PT2
SS
(x 10³)
FS
CT2 - DMSO
CT2 - PMA
PT2 - DMSO
PT2 - PMA
[Leukocytes]
PMNs - DMSO: 52,15%
PMNs - PMA: 39,41%
PMNs - DMSO: 18,96%
PMNs - PMA: 16,19%
rhodamine 123
[PMNs - DMSO]
[PMNs - PMA]
Count
1,99%
98,77%
1,82%
94,45%

C

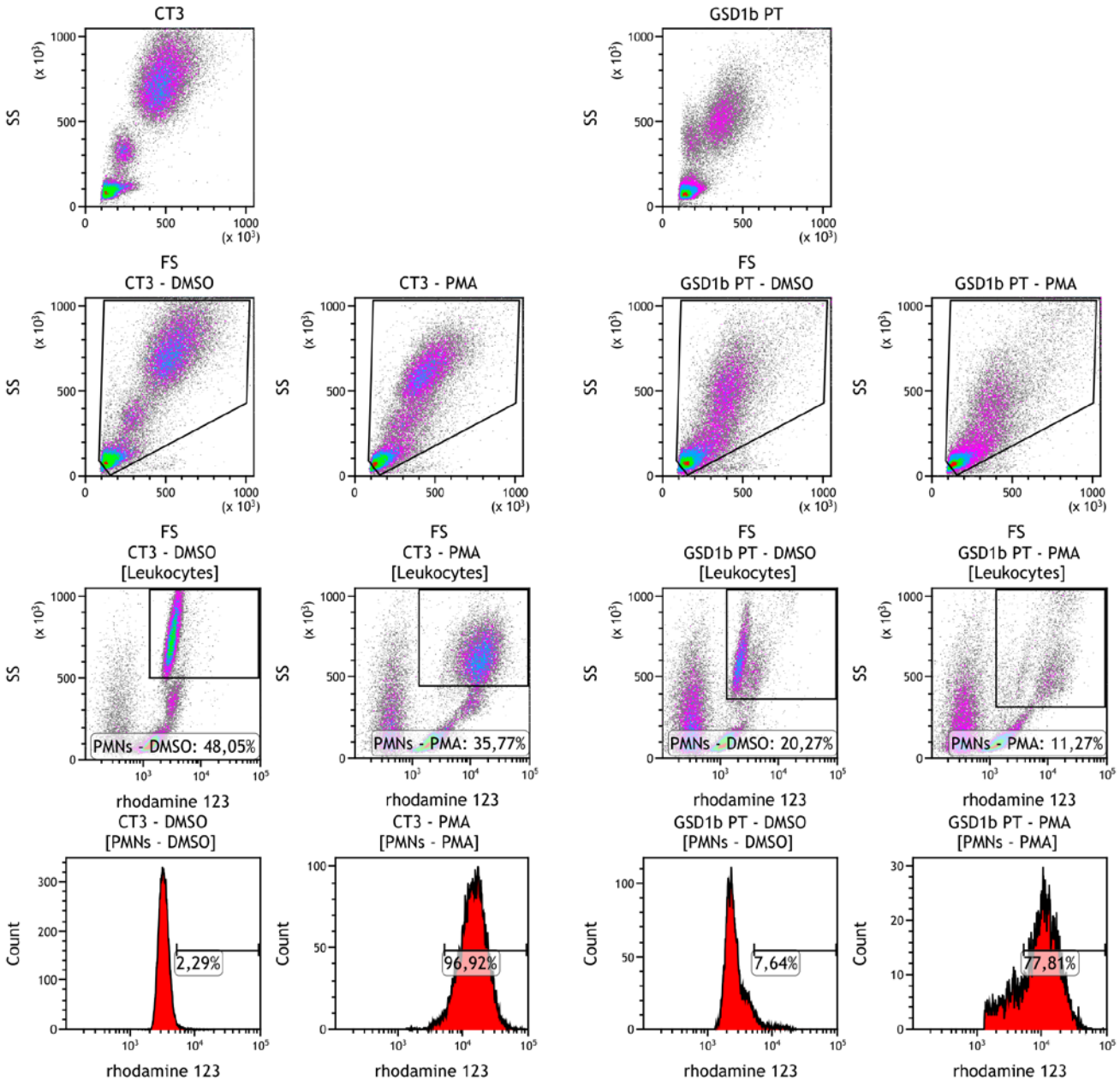

CT3
GSD1b PT
SS
FS
CT3 - DMSO
CT3 - PMA
GSD1b PT - DMSO
GSD1b PT - PMA
[Leukocytes]
PMNs - DMSO: 48,05%
PMNs - PMA: 35,77%
PMNs - DMSO: 20,27%
PMNs - PMA: 11,27%
rhodamine 123
[PMNs - DMSO]
[PMNs - PMA]
Count
2,29%
96,92%
7,64%
77,81%

Figure S2. Normalization of the respiratory burst in empagliflozin-treated PT1 and PT2. Oxidative burst assay was performed as described[7] in whole blood samples (100 µl) and analyzed by flow cytometry. Whole blood (100 µl) was stimulated with PMA at 37°C for 15 minutes. After PMA stimulation (100 ng/ml; 15 min at 37°C), the red blood cells were lysed and the sample was analyzed immediately by flow cytometry with gating set on PMNs. The gate used to construct the histograms, corresponds to the percentage of PMNs showing increased rhodamine-123 fluorescence compared with background fluorescence in unstimulated cells and equivalent rhodamine-123 intensity to the date-matched controls (CT1, CT2 and CT3). **Panels A and B**: analysis of the oxidative burst in PMNs from PT1 (A) and PT2 (B) both during empagliflozin treatment and the respective untreated date-matched healthy controls CT1 (A) and CT2 (B). **Panel C**: analysis of the oxidative burst in PMNs from an *untreated* GSD-1b patient (GSD1b PT) and the respective date-matched healthy control (CT3).

SUPPLEMENTARY REFERENCES

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