blood advances

American Society of Hematology
2021 L Street NW, Suite 900,
Washington, DC 20036
Phone: 202-776-0544 | Fax 202-776-0545
bloodadvances@hematology.org

Neutrophil functions in patients with neutropenia due to glycogen storage disease type 1b treated with empagliflozin

Tracking no: ADV-2023-012403R1

Magdalena Kaczor (Children's Memorial Health Institute, Poland) Stanislaw Malicki (Jagiellonian University, Poland) Justyna Folkert (Jagiellonian University, Poland) Ewelina Dobosz (Jagiellonian University, Poland) Danuta Bryzek (Jagiellonian University, Poland) Barbara Chruścicka-Smaga (Jagiellonian University, Poland) Milena Greczan (Children's Memorial Health Institute, Poland) Dorota Wesół- Kucharska (Children's Memorial Health Institute, Poland) Barbara Piatosa (Children's Memorial Health Institute, Poland) Emilia Samborowska (Children's Memorial Health Institute, Poland) Joanna Madzio (Medical University of Lodz, Poland) Janusz Książyk (Children's Memorial Health Institute, Poland) Ewa Ehmke vel Emczyńska (Children's Memorial Health Institute, Poland) Małgorzata Hajdacka (Children's Memorial Health Institute, Poland) Jan Potempa (Dental School, University of Louisville, United States) Wojciech Młynarski (Medical University of Lodz, Poland) Dariusz Rokicki (Children's Memorial Health Institute, Poland) Florian Veillard (Jagiellonian University, Poland)

Abstract:
Neutropenia and neutrophil dysfunction in glycogen storage disease type 1b (GSD1b) are caused by the accumulation of 1,5-anhydroglucitol-6-phosphate (1,5-AG6P) in granulocytes. The antidiabetic drug empagliflozin reduces the concentration of 1,5-anhydroglucitol (1,5 AG), thus restoring neutrophil counts and functions, leading to promising results in previous case reports. Here, we present a comprehensive analysis of neutrophil function in seven GSD1b patients and 11 healthy donors, aiming to evaluate the immediate (after 3 months) and long-term (after 12 months) efficacy of empagliflozin compared to the reference treatment with granulocyte-colony stimulating factor (G-CSF). We found that most patients receiving G-CSF remained neutropenic with dysfunctional granulocytes, whereas treatment with empagliflozin increased neutrophil counts and improved functionality by inhibiting apoptosis, restoring phagocytosis and the chemotactic response, normalizing the oxidative burst, and stabilizing cellular and plasma levels of defensins and lactotransferrin. These improvements correlated with the decrease in serum 1,5-AG levels. However, neither G-CSF nor empagliflozin overcame deficiencies in the production of cathelicidin/LL-37 and neutrophil extracellular traps. Given the general improvement promoted by empagliflozin treatment, patients were less susceptible to severe infections. G-CSF injections were therefore discontinued in six patients (and the dose was reduced in the seventh) without adverse effects. Our systematic analysis, the most extensive reported thus far, has demonstrated the superior efficacy of empagliflozin compared to G-CSF, restoring the neutrophil population and normal immune functions. EudraCT 2021-000580-78

Conflict of interest: No COI declared

COI notes:

Preprint server: No;

Author contributions and disclosures: All authors reviewed and approved the article and participated in the discussion and interpretation of data. Dariusz Rokicki, Florian Veillard, Wojciech Młynarski, Jan Potempa, and Magdalena Kaczor conceptualized and designed the study. Magdalena Kaczor, Milena Greczan, Dorota Wesół-Kucharska, Janusz Książyk and Dariusz Rokicki provided direct patient care and collect patient data and Wojciech Młynarski provided access to control donors. Magdalena Kaczor, Stanislaw Malicki, Justyna Folkert, Ewelina Dobosz, Danuta Bryzek, Barbara Chruscicka-Smaga, Barbara Piątosa, Emilia Samborowsk, Joanna Madzio, Ewa Ehmke vel Emczyńska, Małgorzata Hajdacka carried out experiments, data analysis and figures preparation. Florian Veillard and Magdalena Kaczor wrote the manuscript with critical evaluations and revisions from Dariusz Rokicki, Wojciech Młynarski, Jan Potempa and Janusz Książyk.

Non-author contributions and disclosures: No;

Agreement to Share Publication-Related Data and Data Sharing Statement: The data that support the findings of this study are available upon reasonable request to the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Clinical trial registration information (if any): EudraCT 2021-000580-78

Neutrophil functions in patients with neutropenia due to glycogen storage disease type 1b treated with empagliflozin

Magdalena Kaczor[1]*, Stanislaw Malicki [2]*, Justyna Folkert[2], Ewelina Dobosz[2], Danuta Bryzek[2], Barbara Chruscicka-Smaga[2], Milena Greczan[1], Dorota Wesół- Kucharska[1], Barbara Piątosa[3], Emilia Samborowska[4], Joanna Madzio[5], Janusz Książyk[1], Ewa Ehmke vel Emczyńska[1], Małgorzata Hajdacka[1], Jan Potempa[2,6], Wojciech Młynarski[5], Dariusz Rokicki[1], Florian Veillard[2]

*Equally co-author.

1) Department of Pediatrics, Nutrition and Metabolic Diseases, Children's Memorial Health Institute, Warsaw, Poland
2) Department of Microbiology, Faculty of Biochemistry, Biophysics and Biotechnologies, Jagiellonian University, Krakow, Poland
3) Histocompatibility Laboratory, Children's Memorial Health Institute, Warsaw, Poland
4) Inborn Errors of Metabolism Laboratory, Children's Memorial Health Institute, Warsaw, Poland
5) Department of Pediatrics, Oncology & Hematology, Medical University of Łódź, Łódź, Poland
6) Department of Oral Immunology and Infectious Diseases, University of Louisville School of Dentistry, KY, USA

Corresponding Author

Florian Veillard

Jagiellonian University

Krakow, Poland

florian.veillard@uj.edu.pl

The data that support the findings of this study are available upon reasonable request to the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Abstract

Neutropenia and neutrophil dysfunction in glycogen storage disease type 1b (GSD1b) are caused by the accumulation of 1,5-anhydroglucitol-6-phosphate (1,5-AG6P) in granulocytes. The antidiabetic drug empagliflozin reduces the concentration of 1,5-anhydroglucitol (1,5-AG), thus restoring neutrophil counts and functions, leading to promising results in previous case reports. Here, we present a comprehensive analysis of neutrophil function in seven GSD1b patients and 11 healthy donors, aiming to evaluate the immediate (after 3 months) and long-term (after 12 months) efficacy of empagliflozin compared to the reference treatment with granulocyte-colony stimulating factor (G-CSF). We found that most patients receiving G-CSF remained neutropenic with dysfunctional granulocytes, whereas treatment with empagliflozin increased neutrophil counts and improved functionality by inhibiting apoptosis, restoring phagocytosis and the chemotactic response, normalizing the oxidative burst, and stabilizing cellular and plasma levels of defensins and lactotransferrin. These improvements correlated with the decrease in serum 1,5-AG levels. However, neither G-CSF nor empagliflozin overcame deficiencies in the production of cathelicidin/LL-37 and neutrophil extracellular traps. Given the general improvement promoted by empagliflozin treatment, patients were less susceptible to severe infections. G-CSF injections were therefore discontinued in six patients (and the dose was reduced in the seventh) without adverse effects. Our systematic analysis, the most extensive reported thus far, has demonstrated the superior efficacy of empagliflozin compared to G-CSF, restoring the neutrophil population and normal immune functions. EudraCT 2021-000580-78

Key words: glycogen storage disease type 1b, neutropenia, *SLC37A4,* SGLT2 inhibitor, empagliflozin

Key points:

- Empagliflozin decreases the susceptibility to infection in Glycogen storage disease type 1b
- Empagliflozin significantly improves neutrophil population and functions in GSD1b, surpassing G-CSF limited efficacy

Introduction

Glycogen storage disease type 1b (GSD1b, OMIM: #232220) is a rare metabolic defect with a prevalence of 1:500,000 that predominantly affects carbohydrate metabolism[1]. It is caused by biallelic mutations in the *SLC37A4* gene, resulting in a deficient glucose-6-phosphate transporter (translocase G6P) in the endoplasmic reticulum (ER) membrane[2]. The final step in gluconeogenesis is G6P hydrolysis to glucose, which only takes place in the ER[3]. Translocase G6P deficiency therefore leads to episodes of hypoglycemia and elevated levels of G6P in the cytoplasm[4]. The accumulation of G6P triggers abnormal metabolic processes that typify GSD1b, including hyperlactatemia, hypertriglyceridemia, hyperuricemia and hypercholesterolemia[4,5]. Furthermore, the storage of excess glycogen and fats causes the enlargement and dysfunction of the liver and kidneys.

The primary treatment for severe hypoglycemia accompanied by metabolic disruption is the frequent consumption of protein-rich meals with minimal levels of simple sugars, including feeding infants at 2-h intervals day and night[4]. However, GSD1b also causes neutrophil deficiency and dysfunction, which has a severe impact on quality of life from the neonatal period onward by making patients susceptible to frequent, life-threatening infection[4,6]. Later in life, neutropenia causes other comorbidities such as inflammatory bowel disease (IBD) and recurrent aphthous stomatitis. Until recently, granulocyte-colony stimulating factor (G-CSF) was the only approved treatment for congenital neutropenia[4,7]. However, although G-CSF can restore the neutrophil count, its ability to address neutrophil dysfunction is more limited and treatment may increase long-term susceptibility to acute myeloid leukemia, myelodysplasia and other forms of cancer[8–11].

The accumulation of 1,5 anhydroglucitol-6-phosphate (1,5-AG6P) in granulocytes was recently identified as the cause of neutropenia in GSD1b[2]. This compound is formed when 1,5-anhydroglucitol (1,5-AG), a glucose analog found in the diet, is phosphorylated by hexokinases. Normally, 1,5-AG6P is transported from the cytosol of neutrophils to the ER, where it is dephosphorylated by glucose-6-phosphatase (G6PC3) and then eliminated. But a deficiency for translocase G6P causes 1,5-AG6P to accumulate in the cytosol, where it inhibits glucose phosphorylation by hexokinases, thereby preventing glycolysis, the primary energy source for mature neutrophils in the absence of mitochondria[3,12]. These deficiencies impair the bactericidal activity of neutrophils, and cause them to undergo premature apoptosis[13–15].

The discovery of the underlying cause of this congenital form of neutropenia has facilitated the pursuit of more targeted treatment options. Inhibitors of renal sodium-glucose cotransporter type 2 (SGLT2), such as empagliflozin, appear to be the most promising options[3,8]. Empagliflozin is used to prevent the reabsorption of glucose and its derivatives in the proximal tubule of the nephron in diabetes patients[16,17]. In GSD1b, empagliflozin also inhibits the feedback of 1,5-AG and reduces 1,5-AG6P levels in the blood and granulocytes[8]. Empagliflozin has been shown to increase the population of viable and functional neutrophils in a limited number of patients[3,8]. Furthermore, retrospective analysis have shown that empagliflozin have positive effects on neutrophil dysfunction-related symptoms including inflammatory bowel disease, oral and urogenital mucosal lesions, infections or skin abscesses[18–20]. Here, we present the first prospective clinical trial evaluating the effectiveness of SGLT2 inhibition in GSD1b with the most extensive and comprehensive analysis of neutrophil dysfunction in patients thus far, in order to investigate both the immediate and long-term efficacy of empagliflozin. We evaluated the effect of empagliflozin on neutrophil apoptosis, phagocytosis, chemotaxis, the oxidative burst, cellular and plasma levels of antimicrobial peptides (AMPs) and LL-37, and the formation of neutrophil extracellular traps (NETs) compared to the control treatment with G-CSF.

Materials and methods

EMPAtia clinical trial

The EMPAtia clinical trial "Evaluation of the effectiveness and safety of empagliflozin in treating neutropenia in patients with GSD1b" (https://www.clinicaltrialsregister.eu/ctr-search/trial/2021-000580-78/PL) has been running in Poland since March 2022. This trial, funded by the Medical Research Agency (ABM), was designed by researchers from the Children's Memorial Health Institute in Warsaw and has enrolled 92% of identified pediatric patients with GSD1b in Poland (12 patients in total). Informed written consent to participate in the EMPAtia clinical trial was obtained from the patients and/or their parents. In collaboration with the FixNet project (Fix Neutropenia: Focusing on neutrophil proteases defect which serve as novel diagnostic and therapeutic options), seven of these patients (58%) qualified for the additional assessment of neutrophil functions according to the EMPAtia/FixNet substudy protocol (Suppl. Figure S1). Given the use of empagliflozin for an average of 1 year, four patients were not included because neutrophils could not be collected at baseline before the initiation of treatment, and one additional patient was excluded because the parents did not provide consent. The EMPAtia substudy was reviewed and approved by the bioethics commission at the Children's Memorial Health Institute in Warsaw (16/KBE/2021). It was conducted in accordance with the Declaration of Helsinki and its subsequent amendments. No personal or identifiable data relating to the substudy are included in this article. Healthy controls were originally enrolled in the FixNet project from the Foundation for Polish Science with informed written consent (approved by the Human Research Ethics Committee, Medical University of Łódź – RNN/211/22/KE and RNN/353/19/KE).

Before the trial started, all seven patients were treated daily with G-CSF. This was initially maintained when daily treatment with empagliflozin commenced (5 mg at weights below 20 kg, 10 mg at weights of 20–39 kg, and 20 mg at weights above 39 kg). The administration of G-CSF was stopped when the neutrophil count exceeded 1×10^3 cells/L at follow-up visits scheduled in the protocol (every 3 months on average). However, G-CSF treatment was reinstated if neutrophil numbers dipped below this threshold. Inclusion and exclusion criteria, as well as the protocol used to address the potential side effects of empagliflozin, are provided in the supplementary materials and methods. Neutrophils were isolated from the peripheral blood of patients and healthy donors as previously described[21]. The complete protocol is provided in the supplementary materials. Blood was collected

before the trial as well as 3 and 12 months after the initiation of empagliflozin treatment. Complete blood counts were determined at the same times.

Apoptosis assay

Freshly-isolated neutrophils were labeled with annexin V (BD Pharmingen) and apoptotic cells were detected by flow cytometry using a BD FACSLyric system.

Migration and chemotactic response assay

Neutrophil migration and chemotaxis in response to 10% human serum (Sigma-Aldrich) were determined using a Boyden's chamber model and the fluorogenic CytoSelect 96-well cell migration assay with 3-µm ports (Cell Biolabs) according to the manufacturer's instructions.

Induction and quantification of NETs

Freshly isolated neutrophils were stimulated with 25 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and extracellular DNA was recovered after 3 h and digested with micrococcal nuclease (Thermo Fisher Scientific). The quantity of DNA was determined using SYTOX Green Nucleic Acid Stain (Thermo Fisher Scientific) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. NETs were also identified by confocal microscopy as previously described[21]. Cells were stained with a rabbit anti-human neutrophil elastase antibody (#01-14-051200, Athens Research and Technology) diluted at 1:500 in phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA) and 0.1% saponin for 1 h, followed by APC-conjugated goat anti-rabbit IgG $F(ab')_2$ (#111-136-144, Jackson ImmunoResearch Laboratories) diluted at 1:1000 for 45 min. Nuclei were counterstained with 1 µg/mL Hoechst 33342 (Invitrogen).

NADPH oxidase assay

The ability of neutrophils to generate reactive oxygen species via NADPH oxidase was determined using a dihydrorhodamine 123 (DHR) assay followed by flow cytometry as previously described[22].

Plasma concentration of 1,5-AG ([plasma 1,5-AG])

50 µL of serum were spiked with 10 µL of the internal standard (100 µg/mL 1,5-AG-$^{13}C_6$) and 150 µL of acetonitrile to precipitate proteins. Samples were vortexed for 2 min and centrifuged before [plasma 1,5-AG] was measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a Waters ACQUITY UPLC glycan BEH amide column (2.1 × 150 mm, 1.7 µm particle size) with isocratic elution and a total runtime of 10 min.

The mobile phase consisted of solvent A (water with 0.1% (v/v) ammonium hydroxide) and solvent B (acetonitrile with 0.1% (v/v) ammonium hydroxide). Eluted fractions were injected into the mass spectrometer followed by negative electrospray ionization (ESI) and analysis by multiple-reaction monitoring (MRM) with the quantification range 1–100 µg/mL.

Enzyme-linked immunosorbent assay (ELISA)

The following commercially available ELISA assay kits were used to quantify various AMPs in cell lysates and plasma samples according to the manufacturers' instructions: Human G-CSF Instant ELISA kit (Invitrogen, BMS2001NST), EnzCheck Myeloperoxidase (MPO) Activity Assay kit (Invitrogen, E33856), Human LTF/LF (lactoferrin) ELISA kit (Elabscience, E-EL-H5200), Human CAMP/Cathelicidin antimicrobial ELISA kit (EIAab, E1046h) and Human HNP1-3 (neutrophil peptide 1-3) ELISA kit (Elabscience, E-EL-H2299).

Statistical analysis

Data were analyzed and visualized using Graphpad Prism (Graphpad Software). Statistical differences between patients and controls were determined by ordinary one-way analysis of variance (ANOVA) for unpaired data. Statistical differences between datasets collected before and during empagliflozin treatment (0, 3 and 12 months) were determined by mixed-effect analysis. Two-tailed nonparametric Spearman correlation assay was used to determine potential correlations with [plasma 1,5-AG] or [plasma G-CSF]. Statistical significance was defined as $p < 0.05$.

The EMPAtia study was approved by bioethics commission at the Children's Memorial Health Institute in Warsaw no 16/KBE/2021 and FixNet project was approved by the Human Research Ethics Committee, Medical University of Łódź (RNN/211/22/KE and RNN/353/19/KE).

Results

Clinical course during empagliflozin treatment

Seven pediatric patients (three girls and four boys, median age 11 years, age range 3–19 years) with various biallelic mutations in the *SLC37A4* gene were enrolled for neutrophil functional tests (Tables I and II and Figure 1). Before empagliflozin treatment commenced, all seven were treated with G-CSF (median dose 2 μg/kg/day, dose range 1–6.5 μg/kg/day). The median dose of empagliflozin as the trial began was 0.42 mg/kg/day (range 0.3–0.47 mg/kg/day).

During G-CSF treatment, in the year before the start of the trial, the median number of infections requiring antibiotics was three (Q1 = 1.5; Q3 = 5) (Tables 1 and 2). The year following the start of empagliflozin treatment, the median number of infections decreased to one (Q1 = 0.5; Q3 = 2.5) and only *patient 4* experienced an increase in the number of infections, from 0 to 1 the following year (Table 1). One patient was diagnosed with IBD before the study. During the first year of empagliflozin treatment, IBD symptoms did not become any more severe in this patient and were not observed in the other six. There was also no significant increase in the frequency of hypoglycemic episodes or side effects during the first year of empagliflozin treatment in any of the patients.

Measurement of [plasma 1,5-AG]

Before empagliflozin treatment, the median concentration of 1,5-AG in the plasma of the seven patients was 47.2 μg/mL (Q1 = 43.35 μg/mL; Q3 = 52.95 μg/mL) (Table 2 and Figure 1A). This decreased significantly ($P < 0.0001$) to 6.2 μg/mL (Q1 = 2.85 μg/mL; Q3 = 11.2 μg/mL) after 3 months and to 5.3 μg/mL (Q1 = 4.6 μg/mL; Q3 = 9.45 μg/mL) after 12 months (Table 2 and Figure 1A). Substantially lower 1,5-AG levels were observed in all seven patients (Figure 1B).

Leukocyte and neutrophil counts

Under G-CSF treatment, the median neutrophil count in the seven patients was 0.86×10^3 cells/μL (Table 2 and Figure 1C). This was significantly lower than the median count of 3.1×10^3 cells/μL in the 11 control donors ($P = 0.001$). During empagliflozin treatment, the median neutrophil count of the seven patients increased to 1.95×10^3 cells/μL after 3 months and 2.24×10^3 cells/μL after 12 months (Table 2 and Figure 1C). Only *patient 2* showed a significant decline in the neutrophil count between 3 and 12 months of empagliflozin

treatment (Figure 1D). G-CSF was therefore discontinued in six of the seven patients, and the dosage was reduced in *patient 2* (Table 1). Spearman analysis revealed a correlation between the neutrophil count and [plasma 1,5-AG] in the patients (P = 0.018) (Figure 1E). G-CSF was therefore discontinued in five of the seven patients during the first 3 months, and only one patient still required G-CSF after 1 year, at a reduced dosage (Table 1). This increase in neutrophil count also affected the leukocyte count, which similarly increased after empagliflozin treatment (Table 2 and Figure 1F-H). However, there was no significant effect on the lymphocyte or monocyte populations (Table 2 and Figure 1I-N).

Neutrophil viability

The proportion of viable neutrophils (negative for annexin V) varied widely among the seven patients during G-CSF treatment, with a median of 34.8% (Q1 = 30.9%; Q3 = 76.3%) (Table 2 and Figure 2A). After 3 and 12 months on empagliflozin, the proportion increased to 61.7% (Q1 = 44.9%; Q3 = 71.3%) and 67.8% (Q1 = 57.1%; Q3 = 74.6%), respectively (Table 2 and Figure 2A). However, the effect varied greatly, improving cell viability in patients with the lowest initial viability under G-CSF treatment but achieving limited or zero improvement in patients with a higher initial proportion of viable neutrophils (Figure 2B). Accordingly, there was no clear correlation between cell viability and [plasma 1,5-AG] (P = 0.1979) (Figure 2C). Given the inverse relationship between the proportions of viable and apoptotic cells, empagliflozin reduced the frequency of apoptosis in neutrophils when it was high under G-CSF treatment (Table 2 and Figure 2D–F).

Neutrophil mobility and chemotactic response

The mobility and chemotactic response of freshly isolated neutrophils were examined using a Boyden's chamber model. In the absence of a chemoattractant, the mobility of neutrophils from patients receiving G-CSG was deficient compared to neutrophils from healthy donors (median of 192 vs 1010 migrating cells, P < 0.001) (Table 2 and Figure 2G). After 12 months of empagliflozin treatment, median neutrophil mobility had increased in the seven patients but still lagged behind the healthy donors. Furthermore, neutrophil mobility decreased in some patients treated with empagliflozin and we observed no clear correlation between neutrophil mobility and [plasma 1,5-AG] (Figure 2H,I). The chemotactic response of neutrophils to human serum was deficient in patients receiving G-CSG compared to neutrophils from healthy donors (1421 vs 2487 migrating cells, P = 0.345) (Table 2 and Figure 2J). Following empagliflozin treatment, the median neutrophil chemotactic response

in the seven patients was restored to the level seen in healthy donors (Figure 2J). Chemotaxis increased in all patients, and was inversely correlated with [plasma 1,5-AG] (P = 0.0457) (Figure 2K,L).

Oxidative burst

We also analyzed the PMA-induced oxidative burst in neutrophils using a DHR test. Only *patient 2* showed a defective oxidative system, with a low proportion of positive neutrophils under G-CSF treatment (Figure 3A,B). This deficiency was corrected by empagliflozin, and no differences were observed in the other patients when G-CSF treatment was discontinued (Figure 3B). Therefore, there was no significant correlation between the DHR index and [plasma 1,5-AG] (P = 0.1905) (Figure 3C). There was also no significant difference in signal intensity between G-CSF and empagliflozin treatment (Figure 3D–F). The median intracellular concentration of MPO in neutrophils from patients treated with G-CSF and empagliflozin treatment was similar to that of healthy donors (Table 2 and Figure 3H). Individual variations were observed, but there was no overall increase in MPO levels in patients treated with empagliflozin (Figure 3I). Similarly, there was no correlation between [intracellular MPO] and [plasma 1,5-AG] (Figure 3J).

Phagocytosis

Neutrophil-mediated phagocytosis was assessed using fluorescent and pH-sensitive particles of *Staphylococcus aureus*. The median phagocytic activity of neutrophils from patients treated with G-CSF was significantly lower compared to that of healthy donors (1546 vs 3735 fluorescence units; P < 0.0001) (Table 2 and Figure 4A). However, after 12 months of empagliflozin treatment, the median phagocytic activity increased significantly (P = 0.028). All individual patients showed an increase in phagocytosis (Figure 4B) and there was a negative correlation between phagocytic activity and [plasma 1,5-AG] (P = 0.0328).

NETosis

Patients treated with G-CSF were deficient for NETosis following stimulation with PMA compared to healthy donors (P < 0.0001) (Table 2 and Figure 4D). Treatment with empagliflozin caused a significant increase in NETosis (P < 0.05), however not to the level observed in healthy donor neutrophils (Figure 4D). This slight increase was apparent in all seven patients (Figure 4E) and we observed a negative correlation between the induction of NETosis and [plasma 1,5-AG] (P = 0.0007) (Figure 4F). Furthermore, confocal microscopy demonstrated that neutrophils from patients, with G-CSF or empagliflozin, failed to produce

visible NETs structures following PMA stimulation, in contrast to neutrophils from the control donor (Figure 4G).

Production of AMPs

Cellular and secreted AMPs such as lactoferrin (LTF), defensins and LL-37 play a significant role in the antibacterial response of neutrophils. Median intracellular LTF concentrations were similar in patients treated with G-CSF or empagliflozin and in healthy donors and there was no consistent LTF profile at the individual level once empagliflozin treatment had commenced (Table 2 and Figure 5A–C). However, the median concentrations of intracellular defensins, plasma LTF and plasma defensins were significantly higher in patients treated with G-CSF compared to healthy controls (Table 2 and Figure 5E,I,M). Empagliflozin treatment resulted in a significant decrease in the concentration of these AMPs in all patients (Figure 5E,J,N). It is unclear whether the concentrations of these AMPs were higher in patients than healthy controls due to the disease or the high concentration of plasma G-CSF (Figure S2). The median levels of intracellular defensins, plasma LTF and plasma defensins were negatively correlated with [plasma 1.5-AG] and positively correlated with [plasma G-CSF] in all seven patients (Table 2 and Figure 5). The median [plasma CAMP/LL-37] in all seven patients was significantly lower ($P < 0.0001$) during treatment with G-CSF or empagliflozin compared to healthy donors (Table 2 and Figure 5Q). At the individual level, [plasma CAMP/LL-37] decreased in all patients following the start of empagliflozin treatment, but there was no correlation between [plasma CAMP/LL-37] and [plasma 1,5-AG], or between [plasma CAMP/LL-37] and [plasma G-CSF] (Figure 5R–T).

Discussion

The accumulation of 1,5-AG6P within the neutrophil cytosol has recently been identified as the cause of neutropenia in GSD1b. Lowering the plasma concentration of 1,5-AG using antidiabetic SGLT2 inhibitors such as empagliflozin has shown promising results[2,3]. Several case reports have indicated that empagliflozin normalizes the neutrophil count and partially restores neutrophil function in some patients[8,23–28]. In this setting, the EMPAtia clinical trial was initiated to evaluate the efficacy and safety of empagliflozin for the treatment of neutropenia in GSD1b patients on a larger scale. Twelve children, representing 92% of identified pediatric GSD1b patients in Poland, have been enrolled thus far. Seven were qualified for the additional assessment of neutrophil function under G-CSF treatment as well as 3 and 12 months after the initiation of empagliflozin therapy. We observed no side effects such as changes in the frequency of hypoglycemic episodes. Moreover, one patient already diagnosed with IBD did not experience any deterioration of the condition.

The ability of G-CSF to restore the immune functions of neutrophils in GSD1b patients has not been addressed adequately because there is only a small pool of patients and many of them do not produce enough neutrophils for testing[9]. During G-CSF treatment, before the administration of empagliflozin, six of the seven patients remained neutropenic with a neutrophil count below 1×10^3 cells/μL (Table 2 and Figure 2). The neutrophils from these patients showed various deficiencies despite the G-CSF treatment, including an inconsistent rate of apoptosis, lower mobility, a weaker chemotactic response, ineffective phagocytic activity, and impaired production of NETs following stimulation. As a result, these patients experienced frequent serious infections during the year preceding the start of the trial (median of three infections per year). However, with the exception of one patient, there was no serious deficiency in the oxidative burst, which suggests that G-CSF can at least restore this function. This last observation agrees with a previous report showing that most GSD1b patients treated with G-CSF have a normal oxidative system[8]

The dose of empagliflozin for pediatric GSD1b patients was based on the recommendations for adults with diabetes. We used a dose of 0.43 mg/kg/day, which is lower than that used in previous studies (0.7 mg/mL) [8]. Even so, this lower dose was sufficient to reduce the median [plasma 1,5-AG] in the patients from 47.2 to 6 μg/mL (Figure 1). We also observed higher neutrophil counts in six of the seven patients, with median values of 1.95×10^3 cells/μL after 3 months and 2.24×10^3 cells/μL after 12 months of empagliflozin treatment. The neutrophil count declined in one patient between months 3 and 12 despite an

initial rise at the beginning of the trial. G-CSF treatment was therefore discontinued in the six patients with higher neutrophil counts and reduced in the other one. Empagliflozin did not significantly affect the monocyte or lymphocyte populations in any of the patients.

Empagliflozin reduced the inconsistent viability of neutrophils in the seven patients, indicating its ability to inhibit apoptosis compared to the limited efficacy of G-CSF. However, it did not fully restore the viability observed in healthy donors. Empagliflozin had only a mild impact on neutrophil mobility, but it increased the chemotactic response to human serum in all patients. This is likely to facilitate the recruitment of neutrophils to infection sites. Similarly, empagliflozin partially restored the phagocytic activity of neutrophils, which is necessary to eliminate pathogens. Despite an overall increase in NETosis following the administration of empagliflozin, the response still lagged behind that observed in healthy donors. Furthermore, we observed inverse correlations between [plasma 1,5-AG] and the neutrophil count, chemotactic response, phagocytosis, and NETosis. This highlights the central role of 1,5-AG and its derivative 1,5-AG6P in GSD1b patients, and confirms the mode of action of empagliflozin. Neutrophils from all patients treated with empagliflozin retained their normal oxidative burst following stimulation, as observed in previous studies. The lower number of infections probably reflects the enhancement of neutrophil antimicrobial defense mechanisms, improving the quality of life for patients by reducing the number and duration of hospital stays, and allowing the treatment of infections in an outpatient setting.

To the best of our knowledge, the production of AMPs by neutrophils in GSD1b patients has not been studied before. During treatment with G-CSF, the concentration of intracellular and plasma defensins and plasma LTF increased in all seven patients, which may reflect the dysfunctional neutrophils or the administration of G-CSF. Empagliflozin treatment reduced the levels of these AMPs, but the absence of untreated patients in the trial made it impossible to determine whether these AMP concentrations correlated with [plasma 1,5-AG] or [plasma G-CSF]. Interestingly, neither G-CSF nor empagliflozin were able to restore the production of CAMP/LL-37 and effective NETosis, despite the inverse correlation between NETosis and [plasma 1,5AG]. Accordingly, additional pathways that do not involve 1,5-AG or 1,5-AG6P must also be deficient in GSD1b patients.

This study included the most extensive and comprehensive examination of neutrophil functions in GSD1b patients reported thus far, and demonstrated the inability of G-CSF to reverse neutropenia and restore normal neutrophil antimicrobial functions. In contrast, our

analysis of the immediate and long-term effects of empagliflozin confirms the efficacy and safety of this antidiabetic drug for the treatment of GSD1b.

Acknowledgment

The Foundation for Polish Science (FNP) TEAM NET Programme, POIR.04.04.00–00–1603/18. Project title: Fix Neutropenia (FIXNET): focusing on neutrophil proteases defects which serve as novel diagnostic and therapeutic options and the EMPAtia project financed by the Medical Research Agency (MRA) grant No 2020/ABM/01/00047-00. The research by FV, JP, BCS, DB, ED, JF and SM has also been supported by a grant from the Priority Research Area BioS under the Strategic Programme Excellence Initiative at the Jagiellonian University.

Author contributions

All authors reviewed and approved the article and participated in the discussion and interpretation of data. Dariusz Rokicki, Florian Veillard, Wojciech Młynarski, Jan Potempa, and Magdalena Kaczor conceptualized and designed the study. Magdalena Kaczor, Milena Greczan, Dorota Wesół-Kucharska, Janusz Książyk and Dariusz Rokicki provided direct patient care and collect patient data and Wojciech Młynarski provided access to control donors. Magdalena Kaczor, Stanislaw Malicki, Justyna Folkert, Ewelina Dobosz, Danuta Bryzek, Barbara Chruscicka-Smaga, Barbara Piątosa, Emilia Samborowsk, Joanna Madzio, Ewa Ehmke vel Emczyńska, Małgorzata Hajdacka carried out experiments, data analysis and figures preparation. Florian Veillard and Magdalena Kaczor wrote the manuscript with critical evaluations and revisions from Dariusz Rokicki, Wojciech Młynarski, Jan Potempa and Janusz Książyk.

Conflict of interest

The authors declare no conflicts of interest.

Bibliography

1. Chou JY, Jun HS, Mansfield BC. Type I glycogen storage diseases: disorders of the glucose-6-phosphatase/glucose-6-phosphate transporter complexes. *J. Inherit. Metab. Dis.* 2015;38(3):511–519.

2. Veiga-da-Cunha M, Gerin I, Chen YT, et al. A gene on chromosome 11q23 coding for a putative glucose- 6-phosphate translocase is mutated in glycogen-storage disease types Ib and Ic. *Am. J. Hum. Genet.* 1998;63(4):976–983.
3. Maiorana A, Tagliaferri F, Dionisi-Vici C. Current understanding on pathogenesis and effective treatment of glycogen storage disease type Ib with empagliflozin: new insights coming from diabetes for its potential implications in other metabolic disorders. *Front. Endocrinol.* 2023;14:1145111.
4. Kishnani PS, Austin SL, Abdenur JE, et al. Diagnosis and management of glycogen storage disease type I: a practice guideline of the American College of Medical Genetics and Genomics. *Genet. Med. Off. J. Am. Coll. Med. Genet.* 2014;16(11):e1.
5. Sentner CP, Hoogeveen IJ, Weinstein DA, et al. Glycogen storage disease type III: diagnosis, genotype, management, clinical course and outcome. *J. Inherit. Metab. Dis.* 2016;39(5):697–704.
6. Visser G, Rake JP, Fernandes J, et al. Neutropenia, neutrophil dysfunction, and inflammatory bowel disease in glycogen storage disease type Ib: results of the European Study on Glycogen Storage Disease type I. *J. Pediatr.* 2000;137(2):187–191.
7. Visser G, Rake JP, Labrune P, et al. Consensus guidelines for management of glycogen storage disease type 1b - European Study on Glycogen Storage Disease Type 1. *Eur. J. Pediatr.* 2002;161 Suppl 1:S120-123.
8. Wortmann SB, Van Hove JLK, Derks TGJ, et al. Treating neutropenia and neutrophil dysfunction in glycogen storage disease type Ib with an SGLT2 inhibitor. *Blood*. 2020;136(9):1033–1043.
9. Roe TF, Coates TD, Thomas DW, Miller JH, Gilsanz V. Brief report: treatment of chronic inflammatory bowel disease in glycogen storage disease type Ib with colony-stimulating factors. *N. Engl. J. Med.* 1992;326(25):1666–1669.
10. Dale DC, Bolyard AA, Marrero T, et al. Neutropenia in glycogen storage disease Ib: outcomes for patients treated with granulocyte colony-stimulating factor. *Curr. Opin. Hematol.* 2019;26(1):16–21.
11. Li AM, Thyagu S, Maze D, et al. Prolonged granulocyte colony stimulating factor use in glycogen storage disease type 1b associated with acute myeloid leukemia and with shortened telomere length. *Pediatr. Hematol. Oncol.* 2018;35(1):45–51.
12. Borregaard N, Herlin T. Energy metabolism of human neutrophils during phagocytosis. *J. Clin. Invest.* 1982;70(3):550–557.
13. Kuijpers TW, Maianski NA, Tool ATJ, et al. Apoptotic neutrophils in the circulation of patients with glycogen storage disease type 1b (GSD1b). *Blood*. 2003;101(12):5021–5024.
14. Jun HS, Weinstein DA, Lee YM, Mansfield BC, Chou JY. Molecular mechanisms of neutrophil dysfunction in glycogen storage disease type Ib. *Blood*. 2014;123(18):2843–2853.
15. Ambruso DR, McCabe ER, Anderson D, et al. Infectious and bleeding complications in patients with glycogenosis Ib. *Am. J. Dis. Child. 1960*. 1985;139(7):691–697.
16. DeFronzo RA, Hompesch M, Kasichayanula S, et al. Characterization of renal glucose reabsorption in response to dapagliflozin in healthy subjects and subjects with type 2 diabetes. *Diabetes Care*. 2013;36(10):3169–3176.
17. Al-Jobori H, Daniele G, Cersosimo E, et al. Empagliflozin and Kinetics of Renal Glucose Transport in Healthy Individuals and Individuals With Type 2 Diabetes. *Diabetes*. 2017;66(7):1999–2006.
18. Grünert SC, Derks TGJ, Adrian K, et al. Efficacy and safety of empagliflozin in glycogen storage disease type Ib: Data from an international questionnaire. *Genet. Med. Off. J. Am. Coll. Med. Genet.* 2022;24(8):1781–1788.

19. Collen LV, Newburger PE, Snapper SB. Clinical Remission of Severe Crohn's Disease with Empagliflozin Monotherapy in a Pediatric Patient with Glycogen Storage Disease Type 1b. *JPGN Rep.* 2023;4(4):e356.
20. Calia M, Arosio AML, Crescitelli V, et al. Crohn-like disease long remission in a pediatric patient with glycogen storage disease type Ib treated with empagliflozin: a case report. *Ther. Adv. Gastroenterol.* 2023;16:17562848231202138.
21. Bryzek D, Ciaston I, Dobosz E, et al. Triggering NETosis via protease-activated receptor (PAR)-2 signaling as a mechanism of hijacking neutrophils function for pathogen benefits. *PLoS Pathog.* 2019;15(5):e1007773.
22. O'Gorman MR, Corrochano V. Rapid whole-blood flow cytometry assay for diagnosis of chronic granulomatous disease. *Clin. Diagn. Lab. Immunol.* 1995;2(2):227–232.
23. Makrilakis K, Barmpagianni A, Veiga-da-Cunha M. Repurposing of Empagliflozin as a Possible Treatment for Neutropenia and Inflammatory Bowel Disease in Glycogen Storage Disease Type Ib: A Case Report. *Cureus*. 2022;14(7):e27264.
24. Grünert SC, Elling R, Maag B, et al. Improved inflammatory bowel disease, wound healing and normal oxidative burst under treatment with empagliflozin in glycogen storage disease type Ib. *Orphanet J. Rare Dis.* 2020;15(1):218.
25. Rossi A, Miele E, Fecarotta S, et al. Crohn disease-like enterocolitis remission after empagliflozin treatment in a child with glycogen storage disease type Ib: a case report. *Ital. J. Pediatr.* 2021;47(1):149.
26. Mikami M, Arai A, Mizumoto H. Empagliflozin ameliorated neutropenia in a girl with glycogen storage disease Ib. *Pediatr. Int. Off. J. Jpn. Pediatr. Soc.* 2021;63(11):1394–1396.
27. Halligan RK, Dalton RN, Turner C, Lewis KA, Mundy HR. Understanding the role of SGLT2 inhibitors in glycogen storage disease type Ib: the experience of one UK centre. *Orphanet J. Rare Dis.* 2022;17(1):195.
28. Hexner-Erlichman Z, Veiga-da-Cunha M, Zehavi Y, et al. Favorable outcome of empagliflozin treatment in two pediatric glycogen storage disease type 1b patients. *Front. Pediatr.* 2022;10:1071464.

Table 1: Pediatric patients with GSD1b recruited for the EMPAtia/Fixnet observational substudy.

	Sex	Age *(Years)*	SLC374A mutation NM_001164278.1	Protein Mutation NP_001157750	G-CSF (µg/kg/day)			Empagliflozin (mg/kg/day)			Antibiotics requiring infections	
					0	3M	12M	0	3M	12M	1 year before	1 year after
P1	M	17	c.[1042_1043del];[1042_1043del]	p.[(Leu370Valfs)];[(p.Leu370Valfs)]	2	1	0	0.45	0.45	0.44	3	1
P2	M	3	c.[1042_1043del];[1042_1043del]	p.[(Leu370Valfs)];[(p.Leu370Valfs)]	5.4	2.5	2.5	0.3	0.3	0.27	6	4
P3	M	11	c.[898C>T];[1042_1043del]	p.[(Arg300Cys)];[(p.Leu370Valfs)]	2	0	0	0.47	0.47	0.46	7	6
P4	F	7	c.[1042_1043del];[1042_1043del]	p.[(Leu370Valfs)];[(p.Leu370Valfs)]	1	0	0	0.44	0.44	0.42	0	1
P5	M	14	c.[1015G>T];[1042_1043del]	p.[(Gly361Cys)];[(p.Leu370Valfs)]	6	0	0	0.43	0.42	0.4	1	1
P6	F	19	c.[1042_1043del];[1042_1043del]	p.[(Leu370Valfs)];[(p.Leu370Valfs)]	4	0	0	0.3	0.3	0.27	4	0
P7	F	8	c.[1015G>T];[1042_1043del]	p.[(Gly361Cys)];[(p.Leu370Valfs)]	2	1	0	0.3	0.3	0.27	2	0

Table 2: Effect of G-CSF and empagliflozin on neutrophils.

	Control donors	Patients			Patients *vs* Control donors (P values)			Empagliflozin *vs* G-CSF (P values)		Correlation with [plasma 1,5-AG] (P values)
		Under G-CSF (0)	3 months (3M)	12 months (12 M)	G-CSF *vs* control	3M *vs* control	12M *vs* control	3M *vs* G-CSF	12M *vs* G-CSF	
Number of samples	11	7	7	6						
Sex	6M / 5F	4M / 3F	4M / 3F	3M / 3F						
Age (Years)	14 (9-17)	11.5 (7.5-15.5)								
Severe infections / years	n.d.	3 (1.5-5)		1 (0.5-2.5)			*n.d.*		*0.0582*	
[plasma 1,5-AG] (µg/ml)	n.d.	47.2 (43.35-52.95)	6.2 (2.85-11.2)	5.3 (4.6-9.45)	*n.d.*	*n.d.*	*n.d.*	***<0.0001***	***<0.0001***	
[plasma G-CSF] (ng/ml)	17.9 (15.8-20.8)	135.0 (46.9-147.9)	43.1 (23.8-72.3)	21.4 (18.5-29.9)	***<0.0001***	*0.2546*	*0.9669*	*0.2467*	***0.09***	
Leukocytes count (× 1000 cells/µL)	6.21 (5.83-7.46)	2.55 (2.24-4.55)	4.4 (3.76-6.92)	3.98 (3.62-4.34)	***0.0021***	*0.38*	*0.0875*	*0.0352*	*0.3783*	***0.0473***
Neutrophils count (× 1000 cells/µL)	3.10 (2.69-3,61)	0.86 (0.58-1.18)	1.95 (1.44-2.78)	2.24 (2.13-2.50)	***0.001***	*0.2347*	*0.3247*	*0.0699*	*0.0945*	***0.018***
Lymphocytes count (× 1000 cells/µL)	2.24 (1.8-3.10)	1.59 (1.18-2.52)	1.83 (1.56-3.15)	1.26 (1.12-1.83)	*0.2934*	*0.8721*	*0.2044*	*0.1034*	*0.7548*	*0.3620*
Monocytes count (× 1000 cells/µL)	0.56 (0.51-0.64)	0.26 (0.21-0.44)	0.30 (0.25-0.49)	0.32 (0.30-0.39)	*0.0875*	*0.4193*	*0.6111*	*0.1354*	*0.4787*	*0.1945*
Healthy - Annexin V negative (neutrophils %)	77.0 (73.2-78.2)	34.8 (30.9-76.3)	61.7 (44.9-71.3)	67.8 (57.1.1-74.6)	***0.0183***	*0.0605*	*0.5127*	*0.9597*	*0.5335*	*0.1979*
Apoptotic - Annexin V positive (neutrophils %)	23.0 (19.7-27.5)	65.1 (23.5-68.5)	38.3 (28.7-55.1)	32.2 (25.2-42.8)	***0.0181***	*0.0567*	*0.4961*	*0.9643*	*0.5381*	*0.1915*
Migration (cells)	1010 (936-1201)	192 (124-420)	350 (196-450)	616 (558-653)	***<0.0001***	***<0.0001***	***0.0132***	*0.9083*	*0.1676*	*0.4290*
Chemotaxis response to 10 % HS (Cells)	3735 (3071-4828)	1546 (1343-2298)	3110 (2837-4462)	3513 (2924-4065)	***0.0345***	*0.9977*	*0.9971*	*0.0844*	***0.0293***	***0.0457***
NETosis (PMA induction fold)	4.33 (2.84-4.77)	1.03 (0.98-1.07)	1.16 (1.05-1.27)	1.58 (1.41-1.80)	***<0.0001***	***<0.0001***	***<0.0001***	*0.1826*	***0.02***	***0.0007***
Phagocytosis of *S. aureus* bioparticles (FU)	3352 (2471-4440)	1365 (774-1424)	1809 (1342-2046)	2470 (1904-2920)	***<0.0001***	***0.0143***	*0.1231*	*0.1656*	***0.028***	***0.0328***
Oxydative burst - Index DHR (%)	*n.d.*	0.89 (0.845-0.955)	*n.d.*	0.96 (0.93-1.015)	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*0.1292*	*0.1905*
Oxydative burst - Index DHR (FMI)	*n.d.*	0.97 (0.955-0.985)	*n.d.*	0.94 (0.925-1.01)	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*0.9444*	*0.5977*
[cell. MPO] (ng/µg)	55.3 (40.5-63.0)	47.3 (37.2-53.0)	43.9 (37.4-62.0)	58.7 (46.9-61.6)	*0.9485*	*0.8253*	*0.9760*	*0.9833*	*0.8152*	*0.2521*
[cell. LTF] (ng/µg)	11.84 (10.96-13.51)	9.52 (8.24-10.82)	8.59 (6.06-8.92)	8.96 (7.72-11.72)	*0.1982*	***0.0073***	*0.2876*	*0.3453*	*0.9954*	*0.7215*
[cell. Defensins] (ng/µg)	44.2 (35.0-46.2)	56.9 (42.2-72.4)	17.15 (15.0-44.9)	22.36 (20.72-38.69)	*0.2893*	*0.3701*	*0.2920*	***0.0451***	***0.0419***	***0.0316***
[plasma LTF] (ng/ml)	0.32 (0.27-0.36)	0.74 (0.61-0.83)	0.19 (0.15-0.21)	0.18 (0.10-0.34)	***<0.0001***	*0.1757*	*0.4234*	***0.0061***	***0.0045***	***0.0005***
[plasma Defensins] (ng/ml)	0.53 (0.33-0.82)	3.06 (2.24-3.21)	2.25 (1.46-2.31)	1.17 (1.01-1.60)	***<0.0001***	***0.0003***	*0.0759*	***0.0098***	***0.0121***	*0.2356*
[plasma CAMP/LL37] (ng/ml)	7.16 (5.38-8.03)	1.36 (0.48-3.83)	1.04 (0.165-2.12)	0.29 (0-1.36)	***0.0005***	***<0.0001***	***<0.0001***	*0.5726*	*0.4034*	*0.8343*

Figures and tables legends

Table 1: Pediatric patients with GSD1b recruited for the EMPAtia/Fixnet observational substudy.

Table 2: Effect of G-CSF and empagliflozin on neutrophils.

Results are presented as median values with Q1 and Q3. Statistically significant differences between patients and healthy controls were determined by ordinary one-way ANOVA for unpaired data, whereas mixed-effect analysis for paired data was used to compare values 3 and 12 months after the start of empagliflozin treatment to values obtained before the start of treatment. The potential correlation between each parameter and [plasma 1,5-AG] were analyzed by two-tailed Spearman correlation assay.

Figure 1: Empagliflozin lowers [plasma 1,5-AG] and increases the neutrophil count.

(A, B) The plasma concentration of 1,5-AG was determined before and 3 and 12 months after the start of empagliflozin treatment in seven GSD1b patients and 11 healthy controls: (A) median ± quartiles for the patients and controls, and (B) individual patient profiles. (C, D) Neutrophil counts: (C) median ± quartiles for the patients and controls, and (D) individual profiles. (E) Spearman correlation analysis between [plasma 1,5-AG] and neutrophil count. (F) median ± quartiles of leukocyte count for the patients and controls, and (G) individual patient profiles. (H) Spearman correlation analysis between [plasma 1,5-AG] and leukocyte count. (I) median ± quartiles of lymphocyte count for the patients and controls, and (J) individual profiles. (K) Spearman correlation analysis between [plasma 1,5-AG] and lymphocyte count. (L) median ± quartiles of monocytes count for the patients and controls, and (M) individual profiles. (N) Spearman correlation analysis between [plasma 1,5-AG] and monocyte count. Statistically significant differences between patients and healthy controls (black) were determined by ordinary one-way ANOVA for unpaired data, whereas mixed-effect analysis for paired data was used to compare values 3 and 12 months after the start of empagliflozin treatment to values obtained before the start of treatment (blue). For clarity, only P values < 0.5 are shown.

Figure 2: Effect of empagliflozin on neutrophil viability and the chemotactic response.

(A-F) Cell viability and the frequency of apoptosis in freshly isolated neutrophils were determined in seven GSD1b patients and 11 healthy controls at three time points by labeling with annexin V. (A) Median ± quartiles of neutrophil viability for the patients and controls, and (B) individual profiles. (D) Median ± quartiles of apoptotic frequency for the patients and controls, and (E) individual profiles. Spearman correlation analysis between [plasma 1,5-AG] and neutrophil viability (C) and apoptosis (F). (G-L) The mobility and chemotactic response of neutrophils determined in a Boyden's chamber model with or without human serum. (G) Median ± quartiles of the number of migrating neutrophils in the absence of human serum for the patients and controls, and (H) individual profiles. (J) Median ± quartiles of the number of migrating neutrophils in in response to human serum for the patients and controls, and (K) the individual profiles. Spearman correlation analysis between [plasma 1,5-AG] and (I) neutrophil mobility and (L) chemotactic response. Statistically significant differences between patients and healthy controls (black) were determined by ordinary one-way ANOVA for unpaired data, whereas mixed-effect analysis for paired data was used to compare values 3 and 12 months after the start of empagliflozin treatment to values obtained before the start of treatment (blue). For clarity, only P values < 0.5 are shown.

Figure 3: Effect of empagliflozin on the oxidative burst response and intracellular MPO concentration in neutrophils.

(A-F) The neutrophil oxidative burst response to PMA stimulation in the seven GSD1b patients and 11 healthy controls determined using a DHR assay. (A) Median ± quartiles of the percentage of responsive cells (DHR index) for the patients and controls, and (B) the individual profiles. (D) Median ± quartiles of fluorescence medium intensity (DHR index FMI) for the same neutrophils, and (E) the individual profiles. Spearman correlation analysis between [plasma 1,5-AG] and (C) DHR index and (F) DHR index FMI. (G) Representative flow cytometry analysis for a control donor and *patient 2*. (H,I) Intracellular MPO concentrations determined by ELISA: median ± quartiles of (H) [MPO] for the patients and controls, and (I) the individual profiles. (J) Spearman correlation analysis between [plasma 1,5-AG] and [intracellular MPO]. Statistically significant differences between patients and healthy controls (black) were determined by ordinary one-way ANOVA for unpaired data, whereas mixed-effect analysis for paired data was used to compare values 3 and 12 months

after the start of empagliflozin treatment to values obtained before the start of treatment (blue). For clarity, only P values < 0.5 are shown.

Figure 4: Effect of empagliflozin on phagocytosis and NETosis.

(A-C) The phagocytic activity of freshly isolated neutrophils was determined in the seven GSD1b patients and 11 healthy donors at three time points using pH-sensitive particles of *Staphylococcus aureus*: (A) median ± quartiles of phagocytosis (fluorescence units) for the patients and controls, and (B) the individual profiles. (C) Spearman correlation analysis between [plasma 1,5-AG] and neutrophil phagocytosis. (D-F) NET production determined by the quantification of extracellular DNA before and after PMA stimulation: (D) median ± quartiles of NETosis induction for the patients and controls, and (E) the individual profiles. (F) Spearman correlation analysis between [plasma 1,5-AG] and neutrophil NETosis. (G) Representative confocal images of NETosis based on the labeling of DNA (blue) and neutrophil elastase (pink). The scale bar represents 20 μM and white arrows indicate NETs structures. Statistically significant differences between patients and healthy controls (black) were determined by ordinary one-way ANOVA for unpaired data, whereas mixed-effect analysis for paired data was used to compare values 3 and 12 months after the start of empagliflozin treatment to values obtained before the start of treatment (blue). For clarity, only P values < 0.5 are shown.

Figure 5: Effect of empagliflozin on intracellular and plasma AMPs.

(A) Median ± quartiles of [intracellular LTF] for the seven GSD1b patients and 11 healthy controls, and (B) the individual profiles. (E) Median ± quartiles of [intracellular defensins] for the patients and controls, and (F) the individual profiles. (I) Median ± quartiles of [plasma LTF] for the patients and controls, and (J) the individual profiles. (M) Median ± quartiles of [plasma defensins] for the patients and controls, and (N) the individual profiles. (Q) Median ± quartiles of [plasma LL-37] for the patients and controls, and (R) the individual profiles. Spearman correlation analysis between [plasma 1,5-AG] or [plasma G-CSF] and [intracellular LTF] (C and D), [intracellular defensins] (G and H), [plasma LTF] (K and L), [plasma defensins] (O and P) and [plasma LL-37] (S and T). Statistically significant differences between patients and healthy controls (black) were determined by ordinary one-way ANOVA for unpaired data, whereas mixed-effect analysis for paired data was used to compare values 3 and 12 months after the start of empagliflozin treatment to values obtained before the start of treatment (blue). For clarity, only P values < 0.5 are shown.

Figure 1

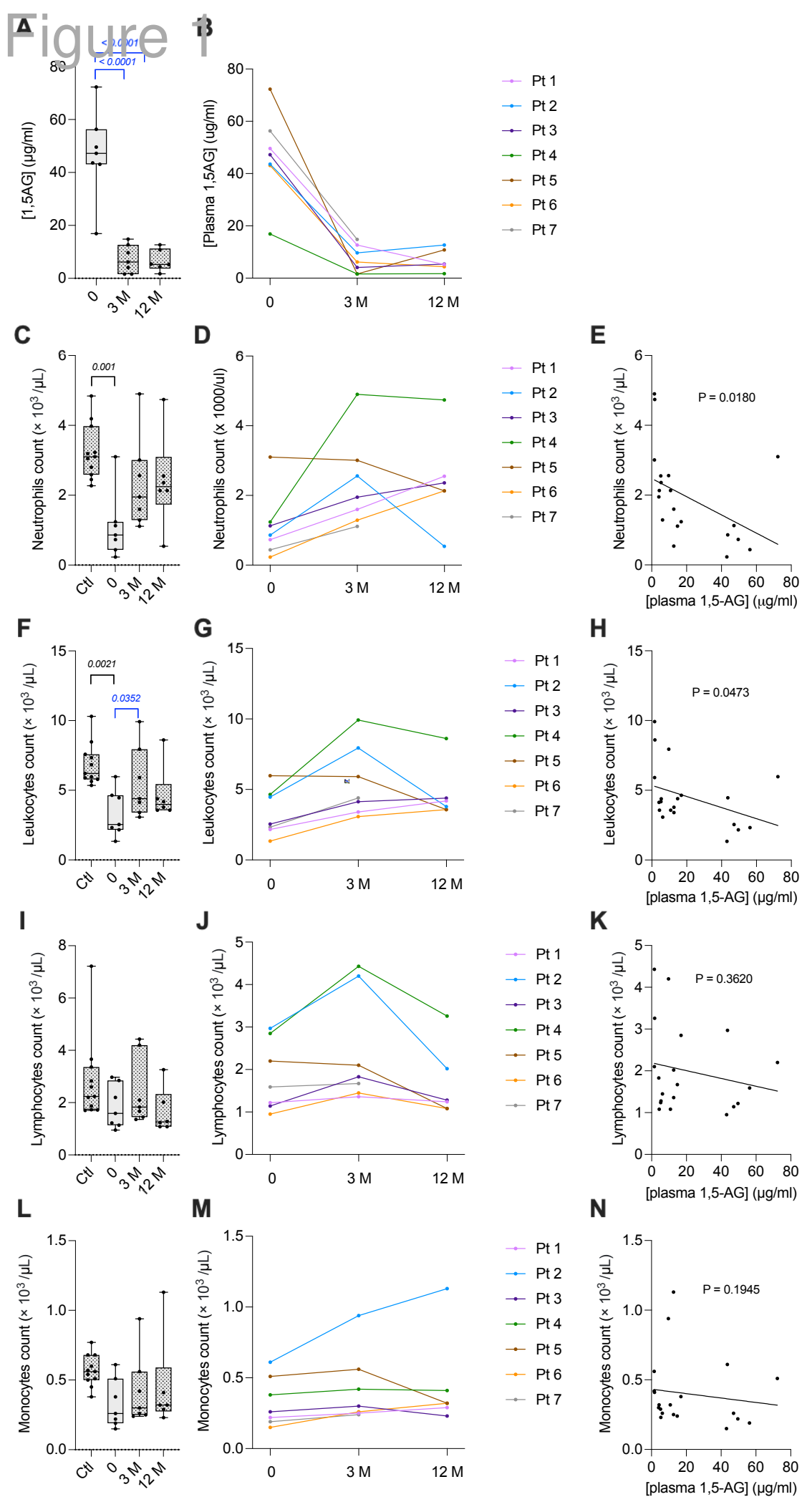


Figure 2

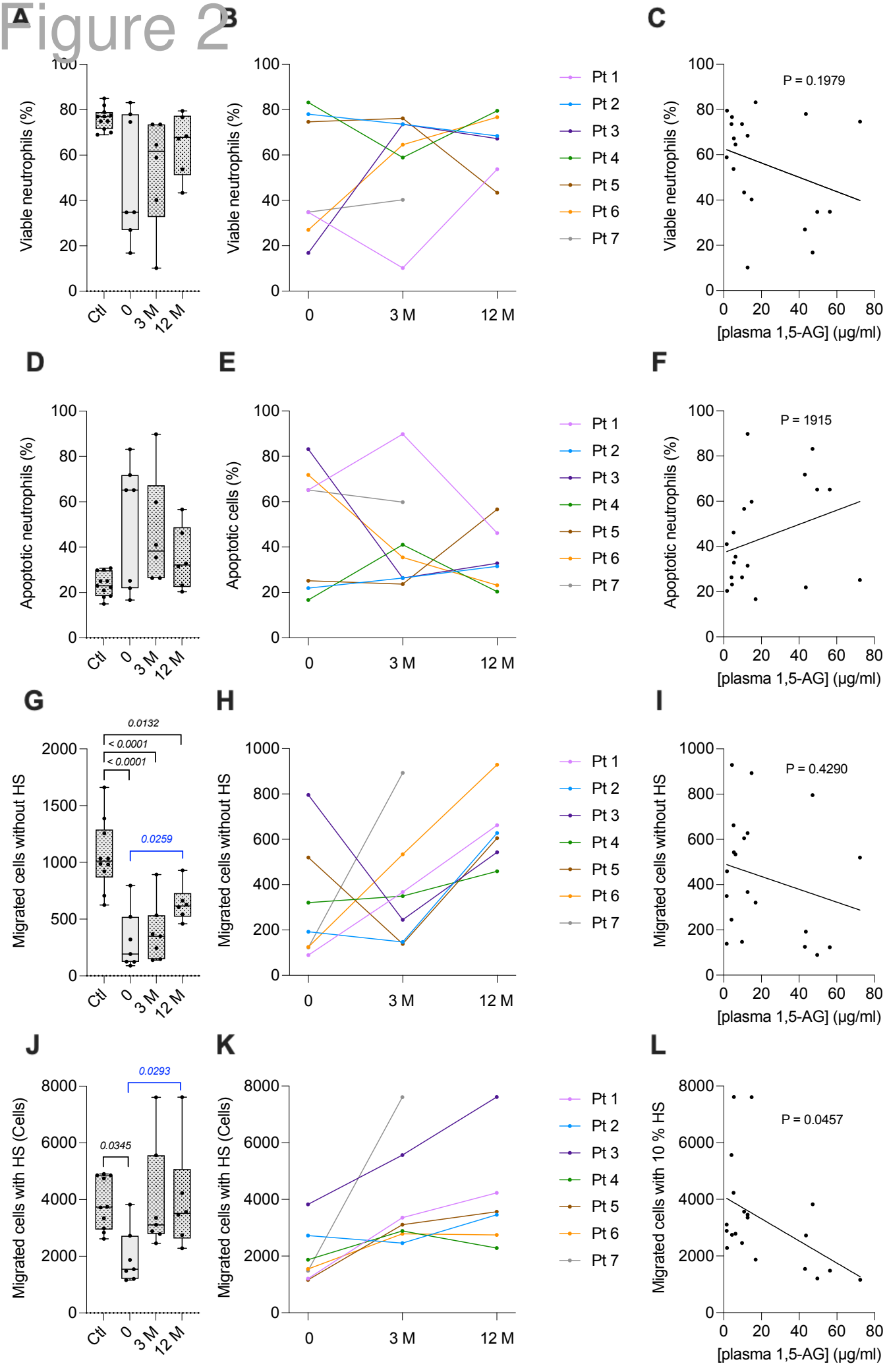


Figure 3

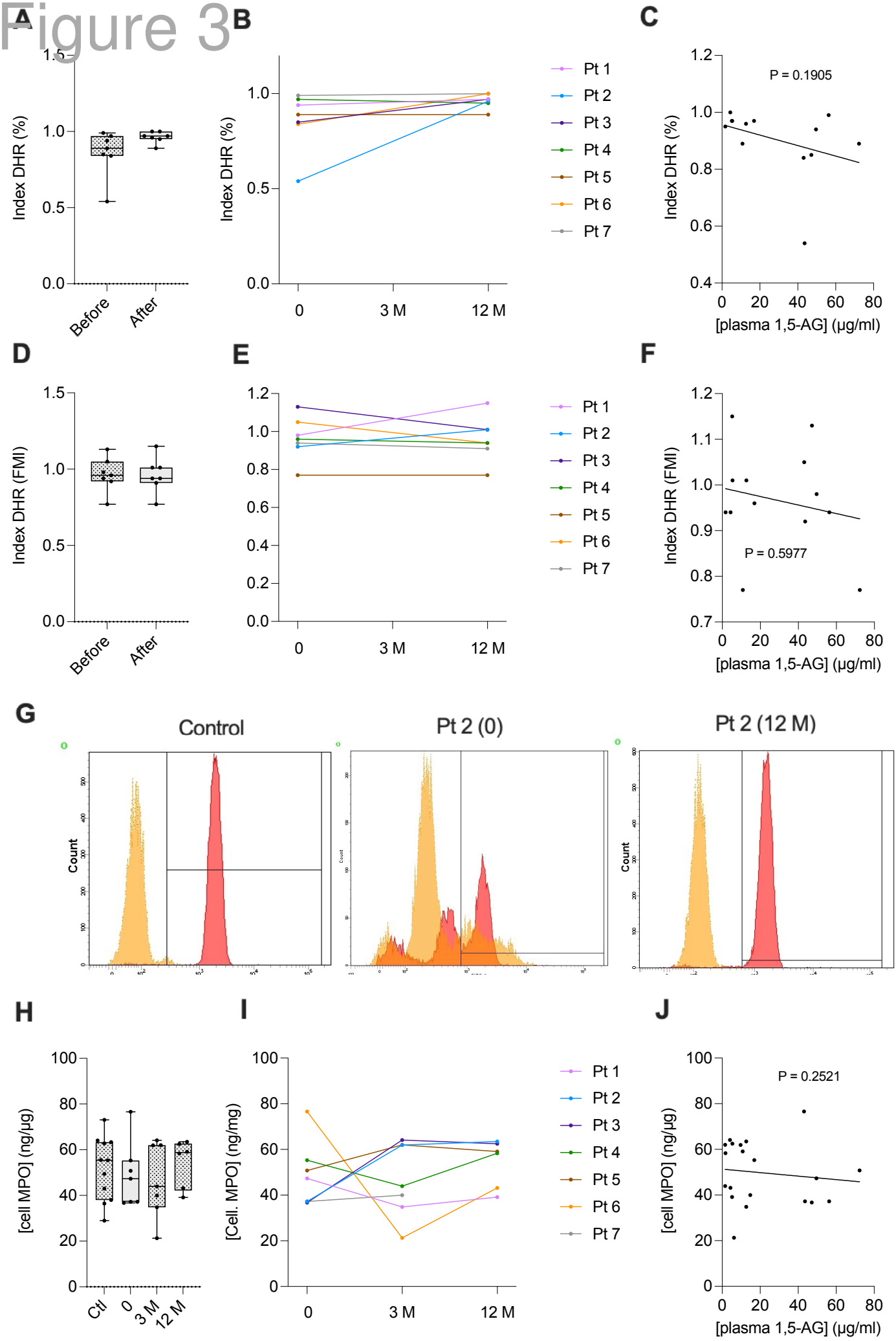
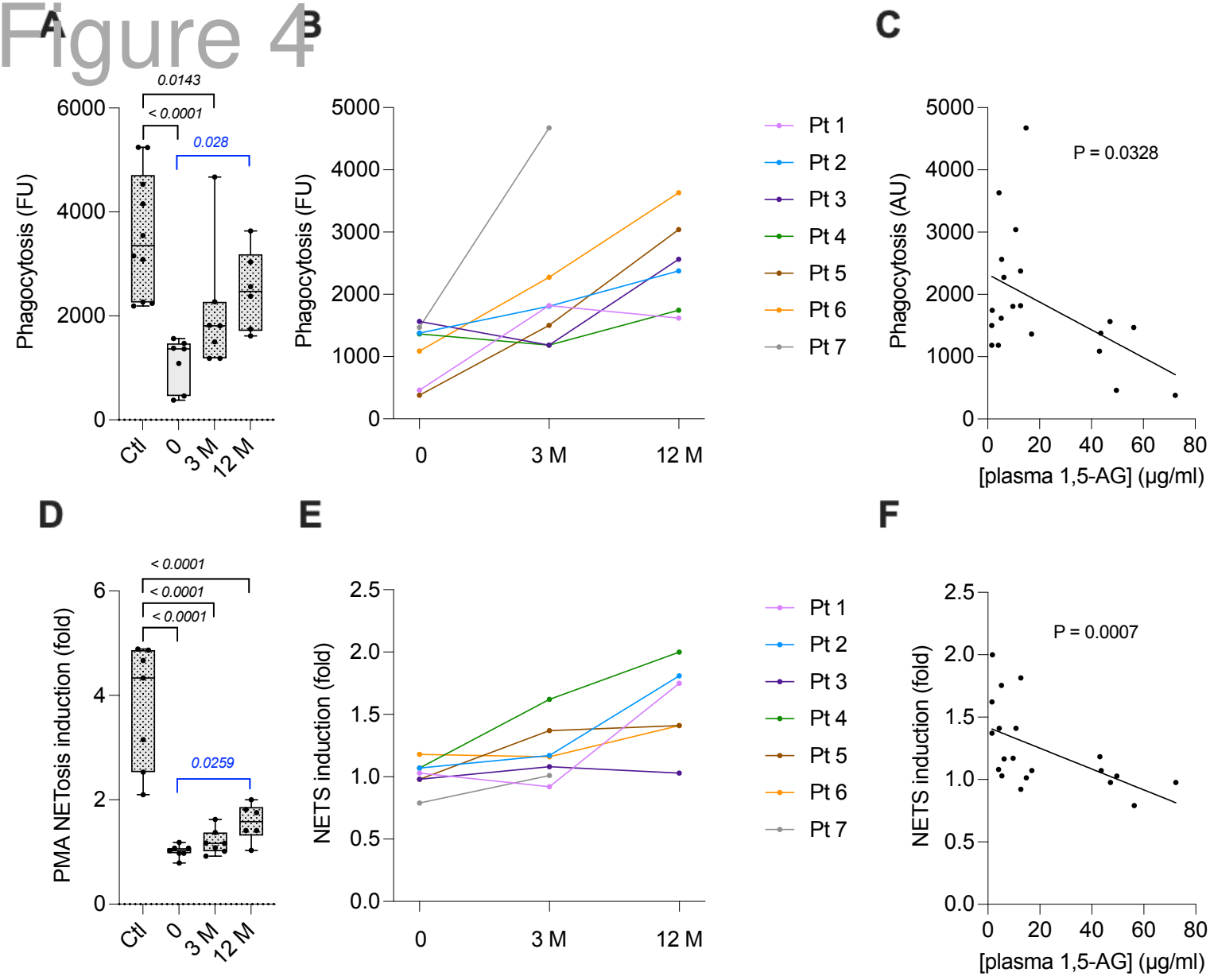


Figure 4



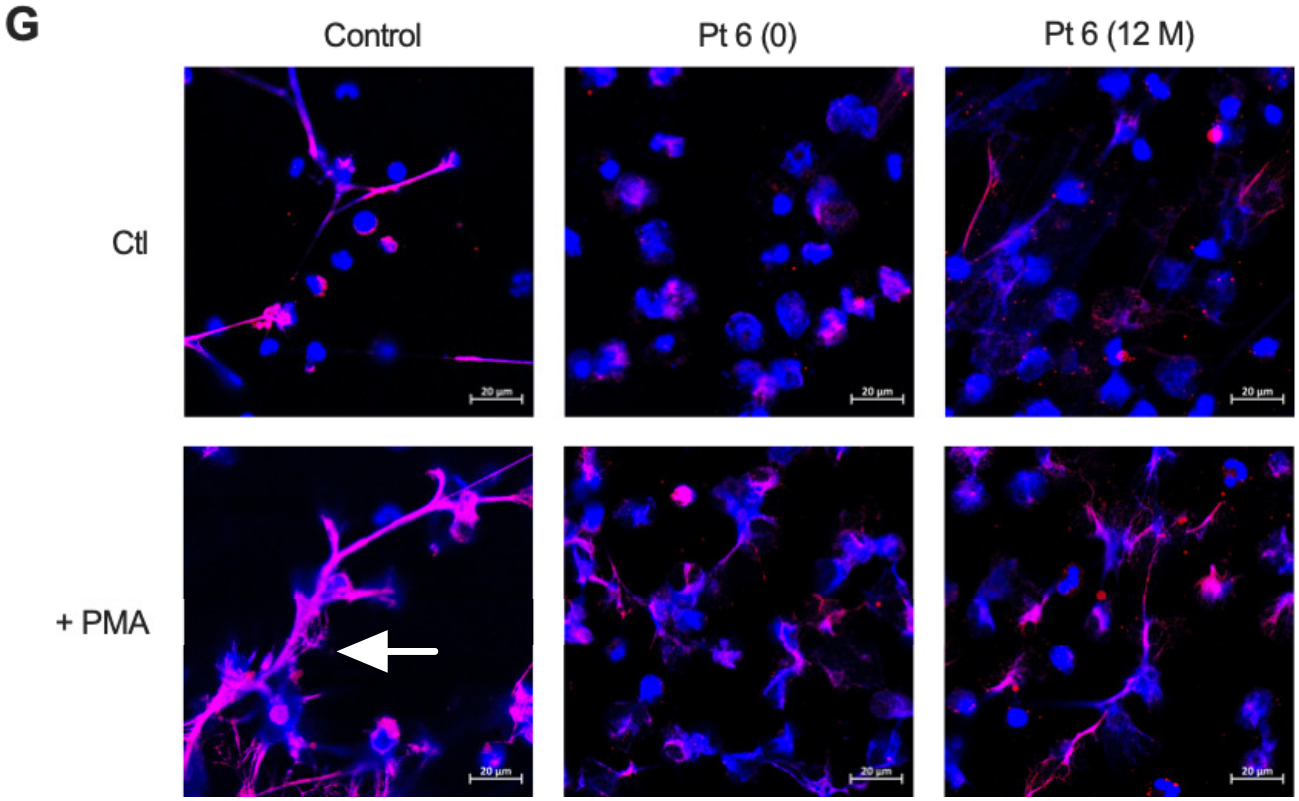


Figure 5