PIEZO1 gain-of-function mutations delay reticulocyte maturation in hereditary xerocytosis

Pedro L. Moura,[1,2] Bethan R. Hawley,[1,3] Johannes G.G. Dobbe,[4] Geert J. Streekstra,[4] Minke A.E. Rab,[5,6] Paola Bianchi,[7] Richard van Wijk,[5] Ashley M. Toye[1,2,8#] and Timothy J. Satchwell[1,2,8#]

[#]AMT and TJS contributed equally as co-senior authors.

[1]School of Biochemistry, University of Bristol, Bristol, UK; [2]NIHR Blood and Transplant Research Unit in Red Cell Products, University of Bristol, Bristol, UK; [3]School of Physiology, Pharmacology and Neuroscience, University of Bristol,Britsol, UK; [4]Amsterdam UMC, University of Amsterdam, Department of Biomedical Engineering and Physics, Amsterdam, the Netherlands; [5]Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht University, Utrecht, the Netherlands; [6]Van Creveldkliniek, University Medical Center Utrecht, Utrecht University, Utrecht, the Netherlands; [7]UOC Ematologia, UOS Fisiopatologia delle Anemie, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milano, Italy and [8]Bristol Institute for Transfusion Sciences, National Health Service Blood and Transplant (NHSBT), Bristol, UK

Correspondence:

TIMOTHY J. SATCHWELL - t.satchwell@bristol.ac.uk

ASHLEY M. TOYE - ash.m.toye@bristol.ac.uk

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SUPPLEMENTAL PATIENT CASE HISTORIES

Patient 1 is a 33-year-old male. He was diagnosed with congenital non-spherocytic hemolytic anemia at 3 years of age. At the time, he suffered from progressive fatigue and jaundice. There is no documented history of perinatal ascites. He suffers from severe hyperbilirubinemia (in part due by co-inheritance for Gilbert's syndrome) and hepatosplenomegaly. He underwent cholecystectomy at an unknown date. At 17 years of age, he was diagnosed with secondary hemochromatosis, for which he undergoes monthly phlebotomies. The diagnosis of hereditary xerocytosis (HX) was first established through osmotic gradient ektacytometry (which displayed the characteristic left shift) and increased osmotic resistance of the red blood cells. This diagnosis was later confirmed by DNA sequence analysis of *PIEZO1* (displaying heterozygosity for c.6262C>G, p.Arg2088Gly[1]). Regarding information on the patient's family history, both the brother and the father of the patient also suffer from HX (not studied). The patient's mother is normal at both the clinical and hematological levels.

Patient 2 is a 23-year-old female. She presented with fatigue, abdominal pain, pallor and jaundice at 6 years of age. At the time, her hemoglobin levels were low to normal and she displayed signs of mild Coombs-negative hemolysis (reticulocytosis, increased bilirubin levels and increased osmotic resistance), mild hepatomegaly and no splenomegaly. There is no documented history of perinatal ascites. She underwent cholecystectomy at 8 years of age and was splenectomized at 12 years of age due to hemolytic anemia. Her clinical parameters improved at the time, but she developed deep venous thrombosis at 15 years of age and again at 18 years of age. She is currently clinically well and displays compensated hemolysis, without anemia. The diagnosis of HX was established when she was 16 years old through osmotic gradient ektacytometry (which displayed the characteristic left shift) and later confirmed by DNA sequence analysis of *PIEZO1* (displaying heterozygosity for c.7367G>A, p.Arg2456His[2]). Regarding information on the patient's family history, she comes from a large family with many affected family members over the course of 3 generations. These family members include **Patient 3** (aged 47) and **Patient 10** (aged 18). The former is an uncle of **Patient 2**, whilst the latter is her nephew. **Patient 3** and **Patient 10** were diagnosed with HX only as a result of the diagnosis of **Patient 2**. Both osmotic gradient ektacytometry measurements displayed the typical left shifted curve. Until then, they had not been diagnosed with hemolytic anemia; however, **Patient 10** was known to suffer from severe iron overload and consequent organ damage (liver), for which he was phlebotomized. No documented history of perinatal ascites was reported for either **Patient 3** or **Patient 10**. Upon molecular

diagnosis, both **Patient 3** and **Patient 10** displayed the same *PIEZO1* pathological variant, c.7367G>A (p.Arg2456His[2]).

Patient 4 is a 19-year-old male. Unfortunately, comparatively little information is known about this patient's clinical history. He was diagnosed with HS-like hemolytic anemia at 12 years of age and underwent splenectomy at 16 years of age (presumably due to his HS-like hemolytic anemia). Osmotic gradient ektacytometry displayed a slightly left-shifted curve, and the same feature was observed in his clinically unaffected father. DNA sequence analysis of *PIEZO1* displayed heterozygosity for a c.1792G>A (p.Val598Met[3]) mutation in both the patient and his father. To date, the patient has not experienced any thrombotic events.

Patient 5 (and **Patient 6,** which corresponds to the second visit of **Patient 5** to the clinic) is a 47-year-old female. A detailed clinical history of **Patient 5** has previously been reported by Fermo E et al.[4]

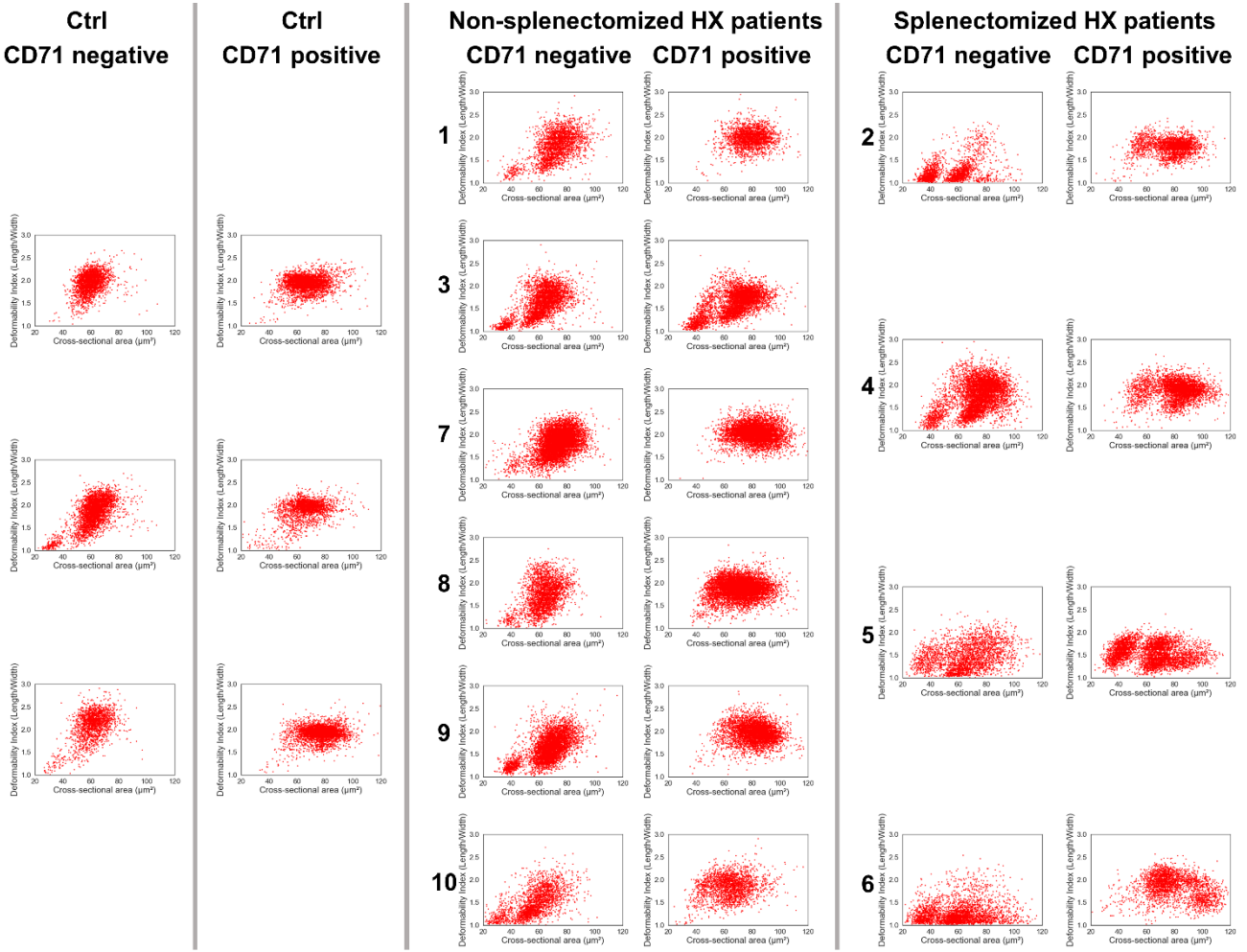
Patient 7 is a 55-year old female. The patient was asymptomatic until 24 years of age, when fatigue and abdominal pain developed accompanied by mild chronic macrocytic hemolytic anemia with reticulocytosis and splenomegaly. At the time, the patient was diagnosed with hereditary spherocytosis. There is no documented history of perinatal ascites. The patient was re-evaluated at 53 years of age due to exacerbation of the anemia and fatigue. At that point, the patient displayed the following hematological parameters: hemoglobin 9.1 g/dL, mean cell volume (MCV) 106.5 fL, absolute reticulocyte number $104x10^9$/L, unconjugated bilirubin 3.01 mg/dL, consumed haptoglobin and increased serum ferritin levels 1464 ng/mL. The EMA binding test results, red cell membrane protein content, and red cell enzyme activities were normal, thus excluding a cytoskeletal or metabolic defect. Bone marrow evaluation showed mild signs of dyserythropoiesis. Finally, osmotic gradient ektacytometry displayed the characteristic left shift suggestive of HX, which was later confirmed by an NGS targeted sequencing panel which displayed the presence of heterozygosity for c.1792G>A, p.(Val598Met[3]) in the *PIEZO1* gene.

Patient 8 is a 46-year-old male. He suffered from neonatal jaundice at birth, with no signs of hemolysis until 18 years of age. He underwent cholecystectomy at 15 years of age due to the presence of gallstones, at which point splenomegaly was also detected. An extensive hematological investigation for chronic hemolytic anemia was performed when the patient was 28 years old, displaying the following hematological parameters: hemoglobin 14.2 g/dL, MCV 100 fL, absolute reticulocyte number $899x10^9$/L, consumed haptoglobin and increased unconjugated bilirubin 22.4 mg/dL (which was later justified by a diagnosis of concomitant

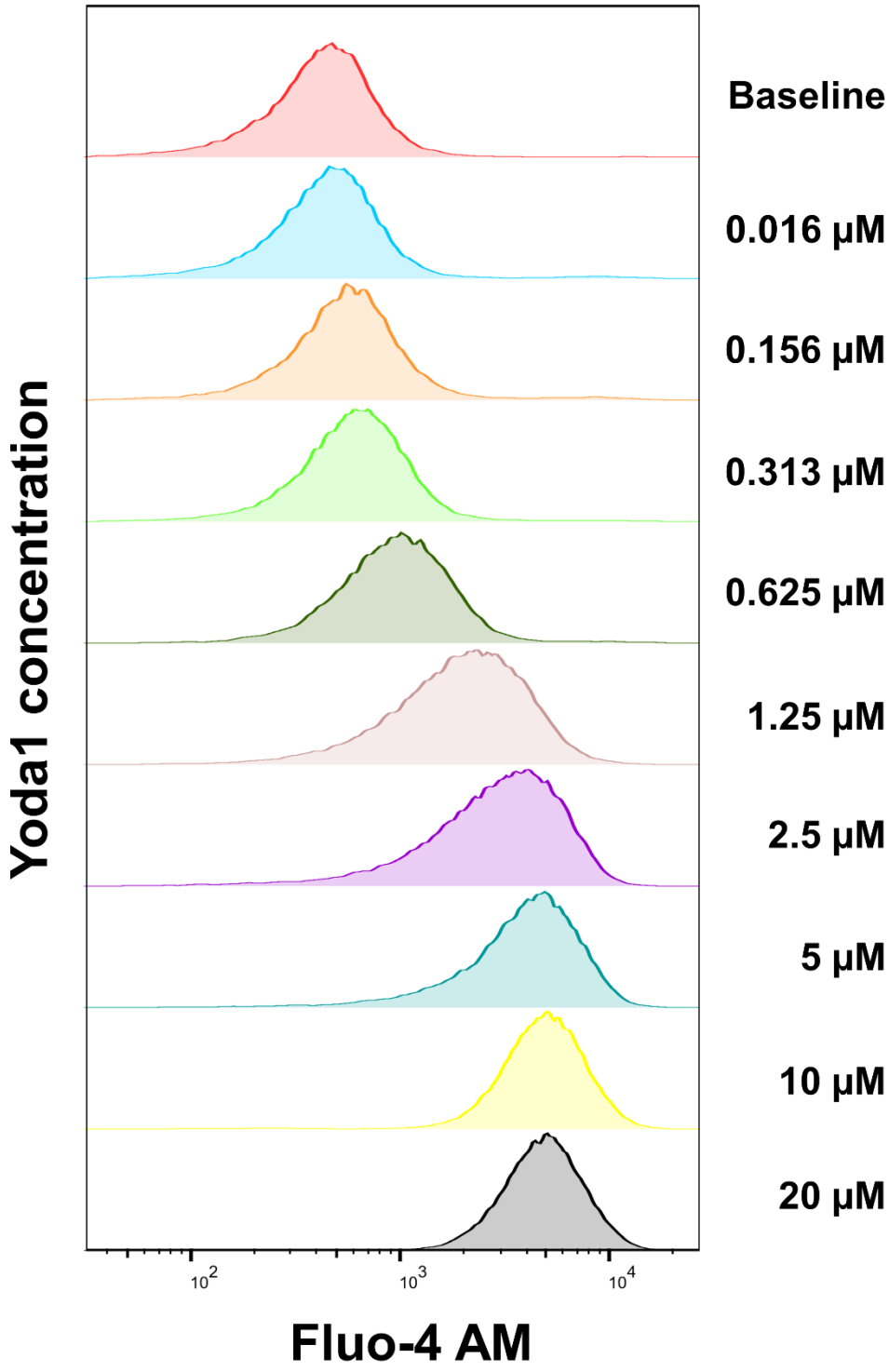
Gilbert's syndrome). Osmotic fragility tests, red blood cell membrane protein content and enzyme activity displayed normal results, thus excluding a cytoskeletal or metabolic defect. The patient was diagnosed with HX more recently, following osmotic gradient ektacytometry (which displayed the characteristic left shift) and molecular investigation that showed the presence of a known pathogenic variant, c.7367G>A (p.Arg2456His[2]), in *PIEZO1*. Neither parent displayed anemia; however, the father (not studied) suffered from jaundice, increased bilirubin levels and reticulocytosis.

Patient 9 is a 43-year-old female and is the sister of **Patient 8**. She underwent clinical investigation for the first time at the age of 25 due to being subjected to cholecystectomy (performed due to the presence of gallstones). Mild macrocytic hemolytic anemia was observed at the time, with the following hematological parameters: hemoglobin 10.7g/dL, MCV 112.8 fL, absolute reticulocyte number $371x10^9$/L, unconjugated bilirubin 9.2 mg/dL (concomitant Gilbert's syndrome), consumed haptoglobin and normal serum ferritin levels. Osmotic fragility test results, red cell membrane protein content and RBC enzyme activities were normal, thus excluding a cytoskeletal or metabolic defect. Similarly to **Patient 8**, the diagnosis of HX was performed more recently following osmotic gradient ektacytometry (which displayed the characteristic left shift) and molecular investigations that showed the presence of a known pathogenic variant, c.7367G>A (p.Arg2456His[2]), in *PIEZO1*.

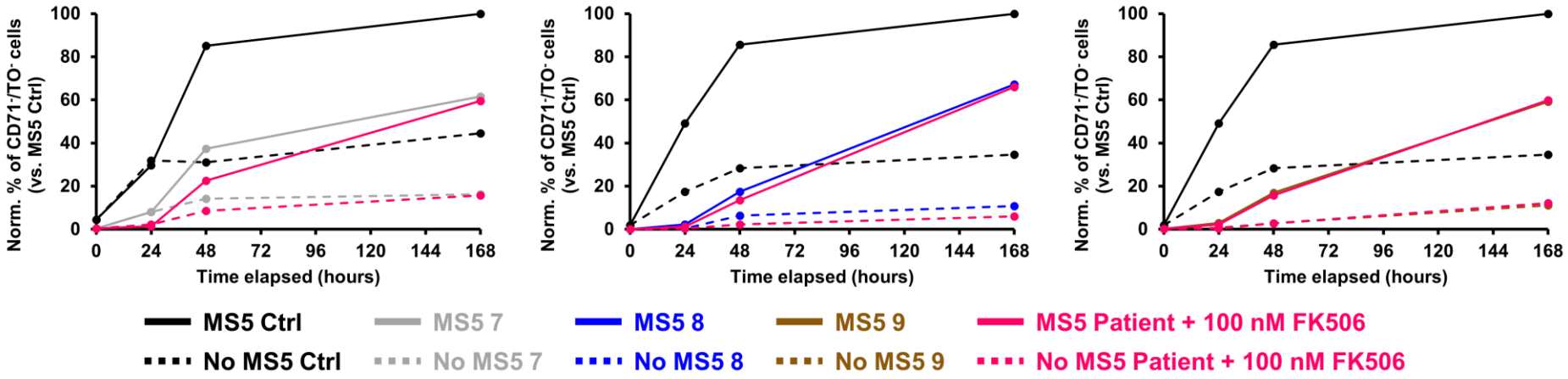
SUPPLEMENTAL FIGURES



Supplemental Figure 1 – Ektacytometry-based analysis of red blood cells from hereditary xerocytosis patients



Supplemental Figure 2 – Yoda1-induced calcium entry displays a concentration-response relationship in erythrocytes



Supplemental Figure 3 – Inhibition of calcineurin does not correct the delayed reticulocyte maturation of HX patients

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1 – Ektacytometry-based analysis of red blood cells from Hereditary Xerocytosis patients

Scatter plots of cross-sectional area plotted against the deformability index (as measured by dividing cell length by cell width), visualizing erythrocytes (CD71 negative) and reticulocytes (CD71 positive) from HX patients (annotated by patient number as per **Table 1** in the main manuscript file) separated by splenectomy status and compared to healthy reference samples (Ctrl). Cells were analyzed through use of the Automated Rheoscope and Cell Analyzer, with a minimum of 1000 cells measured per sample.

Supplemental Figure 2 – Yoda1-induced calcium entry displays a concentration-response relationship in erythrocytes

Flow cytometry histograms plotting cell count against Fluo-4 AM signal (525 nm, FITC channel) upon erythrocyte exposure to varying concentrations of Yoda1, a chemical inducer of Piezo1 activity. Fluo-4 AM serves as an indicator of the calcium concentration inside of the cell. A minimal effect on calcium entry occurs at 0.156 µM and becomes evident at 0.625 µM. Conversely, Yoda1-mediated calcium entry becomes saturated at concentrations above 5 µM.

Supplemental Figure 3 – Inhibition of calcineurin does not correct the delayed reticulocyte maturation of HX patients

Mean CD71/TO loss in reticulocytes from healthy donors and HX patients, cultured alone (dashed line) or in co-culture with MS-5 cells (solid line) and normalized against the $CD71^-/TO^-$ percentage observed in the final timepoint of the respective healthy control samples. HX patients were either left untreated or were treated with 100 nM FK506/Tacrolimus, a calcineurin inhibitor (IC_{50}: 1-3 nM). A paired two-tailed T-test comparison between untreated and FK506-treated patient samples resulted in P-values of 0.514 and 0.396 for cells cultured alone and in co-culture with MS-5 cells, respectively.

SUPPLEMENTAL REFERENCES

1. Glogowska E, Schneider ER, Maksimova Y, et al. Novel mechanisms of PIEZO1 dysfunction in hereditary xerocytosis. *Blood.* 2017;130(16):1845-1856.
2. Caulier A, Rapetti-Mauss R, Guizouarn H, Picard V, Garcon L, Badens C. Primary red cell hydration disorders: Pathogenesis and diagnosis. *Int J Lab Hematol.* 2018;40 Suppl 1:68-73.
3. Rapetti-Mauss R, Picard V, Guitton C, et al. Red blood cell Gardos channel (KCNN4): the essential determinant of erythrocyte dehydration in hereditary xerocytosis. *Haematologica.* 2017;102(10):e415-e418.
4. Fermo E, Vercellati C, Marcello AP, et al. Hereditary Xerocytosis due to Mutations in PIEZO1 Gene Associated with Heterozygous Pyruvate Kinase Deficiency and Beta-Thalassemia Trait in Two Unrelated Families. *Case Rep Hematol.* 2017;2017:2769570.