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To the editor:

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

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Dehydrated hereditary stomatocytosis 1 or hereditary xerocytosis (HX, OMIM 194380) is a rare hereditary autosomal dominant disorder characterized by hemolytic anemia and red blood cell (RBC) dehydration. The occurrence of HX is linked with gain-of-function mutations in *PIEZO1*, the gene encoding for the mechanosensitive non-specific cation channel PIEZO1(1, 2) which is activated by shear-stress and in concert with other ion channels (particularly the Gardos potassium calcium-activated channel, KCNN4) regulates cell volume homeostasis and metabolic activity in the RBC(3). Intriguingly, *PIEZO1* gain-of-function mutations have recently been reported to occur at a much higher frequency within the population than had been previously described and also implicated in malaria resistance(4), suggesting that the mechanisms underpinning HX may merit further investigation. Since reticulocytosis is one of the hallmarks of HX(5), we sought to determine whether altered reticulocyte maturation could be a causative agent of this phenotype. We characterize reticulocytes and erythrocytes from 10 HX patients in comparison to healthy controls, revealing alterations in deformability and vesicle content that implicate a maturational defect in HX. We further demonstrate that HX patients suffer from impaired reticulocyte maturation as assayed through differences in the extent and rate of loss of CD71 and RNA content over time and that this effect can be recapitulated in healthy reticulocytes upon chemically-

induced PIEZO1 overactivation, providing a functional link to the reticulocytosis phenotype present in HX.

A total of 10 samples were investigated in this study, constituting 9 patients from 6 families with one sample in duplicate. The corresponding hematological parameters can be found in **Table 1**. All patients under investigation suffered from mutations in *PIEZO1*, with the majority displaying reticulocytosis and abnormal mean cell volume values (MCV). Several patients also displayed anomalous serum ferritin content, which comprised both iron overload and deficiency. Moreover, 3 of the 9 patients had previously been subjected to splenectomy. Further detail on individual patients is provided in **Supplemental Patient Case Histories**.

Splenectomy leads to a partial loss of the body's quality assurance system for ensuring the removal of physiologically-altered circulating RBCs(6), the effects of which can be readily observed on the deformability index and cross-sectional area profiles obtained upon examination of the cells with the Automated Rheoscope and Cell Analyzer (ARCA)(7), shown in **Figure 1A**. Individual scatter plots for each sample are shown in **Supplemental Figure 1**.

Both RBCs ($CD71^-$) and reticulocytes ($CD71^+$) from splenectomized *PIEZO1*-defective patients display decreased deformability ($CD71^-$ median DI of 1.33 [Interquartile range, IQR 1.08-1.58], $CD71^+$ median DI of 1.80 [IQR 1.61-1.99]) in comparison with non-splenectomized patients ($CD71^-$ median DI of 1.70 [IQR 1.46-1.94], $CD71^+$ median DI of 1.91 [IQR 1.76-2.06]), with their RBCs displaying an enriched proportion of microcytic cells (Cross-sectional area = ~40 μm^2). Interestingly, while RBCs from non-splenectomized patients also display lowered deformability and macrocytosis in comparison with reference values (ref: $CD71^-$ median DI of 1.94 [IQR 1.78-2.11]), their reticulocytes display a relatively normal deformability index profile (ref: $CD71^+$ median DI of 1.93 [IQR 1.84-2.02]). Whilst a potential influence of $CD71^-$ reticulocytes on the total RBC population cannot be excluded, these results nonetheless illustrate that the effect of PIEZO1 overactivation on deformability only manifests in the transition from the reticulocyte to the RBC as defined by loss of CD71, implicating a potential defect in reticulocyte maturation of HX patients.

Using mitochondrial content as a surrogate measure for intracellular vesicle content, as previously described(8), we proceeded to investigate whether physiological vesicle loss is altered in reticulocytes from HX patients, observing a marked increase in vesicle in both RBCs and reticulocytes from splenectomized HX patients (**Figure 1B**). Surprisingly, a significantly increased vesicle content is also observed in the RBCs from non-splenectomized patients but not in their reticulocytes, once again indicating that defects occur in HX during reticulocyte transition to the RBC.

To investigate whether an alteration exists in reticulocyte maturation in HX, reticulocytes were isolated from patients and healthy donors and incubated in IMDM (supplemented as previously described(9)) at 37^{o}C 5% CO_2 for 7 days (168 hours), either alone or in co-culture by layering the reticulocytes onto MS-5 cells (murine stromal cell line). Transferrin receptor (CD71) expression and RNA content (as measured by thiazole orange, TO) was then examined by flow cytometry. Given that reticulocyte maturation normally occurs over a period of 24 to 48 hours after release in circulation(10), the time frame of the experiment was selected so as to ensure that the effects induced by incubation became saturating, that is, that the cells would have reached their maximum possible progression through the maturational process during the experimental conditions used. Co-culture with MS-5 cells was utilized in this study due to previously reported positive effects regarding the capacity of MS-5 cells, or of the microenvironment they generate, to facilitate reticulocyte maturation *in vitro*(11). The exact mechanism through which MS-5 co-culture induces partial reticulocyte maturation has not been reported as of yet; however, physical cell-cell interaction between MS-5 cells and reticulocytes could constitute one of the contributors to that mechanism. Importantly, since PIEZO1 channel activity (and thus mechanotransduction) is altered in HX patients, effects resulting from physical interaction could be disrupted.

Representative examples of the CD71/TO loss exhibited during maturation by healthy controls and HX patients are shown in **Figure 2A**, comprising culture both with and without the presence of MS-5 cells. Delayed reduction in the levels of both markers is evident in HX patients, and especially so at the 24 and 48-hour timepoints; however, we also observed that cells from HX patients start the maturational process with higher levels of both CD71 expression and RNA content, an observation that is consistent with the delayed erythroid differentiation recently reported in HX patients(12). Thus, the longer 168-hour timepoint is valuable from the perspective that a delay over the course of 48 hours or even longer would not disrupt the result achieved at the end of maturation. Progression through maturation also differs in both controls and patients depending on whether reticulocytes are co-cultured with MS-5 cells, with RNA being lost independently of co-culture and a more substantial reduction in CD71 content occurring in cells undergoing co-culture. Despite these differences, reticulocyte maturation is significantly delayed in HX patients irrespective of the method used for incubation (**Figure 2B, Figure 2C**). The percentage of cells negative for CD71 and RNA is significantly lower in HX patients after 7 days of incubation with both conditions, illustrating not only the existence of a delay in maturation but also an inability of patient reticulocytes to undergo complete maturation in this system.

In order to investigate whether the observed effect was a direct consequence of PIEZO1 over-activation, we examined whether this maturational phenotype could be recapitulated by

treating healthy reticulocytes with Yoda1 (a specific chemical activator of PIEZO1(13)) over an extended period of time, with the added advantage that this method enables the experiment to be initiated from a standpoint of identical starting CD71/TO profiles. We observe a significant delay in maturation rate (**Figure 2D**) upon treatment with 5 μM of Yoda1 which becomes less pronounced over time, likely due to compensatory mechanisms being engaged as a result of continuous PIEZO1 activation. The Yoda1 concentration in use was chosen due to constituting the maximal non-saturating concentration that induces calcium entry (**Supplemental Figure 2**). Notably, pharmacological treatment with FK506/Tacrolimus (a calcineurin inhibitor recently reported to abrogate Yoda1-induced effects in erythroblasts(12, 14)) did not ameliorate the delayed reticulocyte maturation phenotype of HX patients (**Supplemental Figure 3**).

Since disrupted calcium homeostasis is a prominent consequence of PIEZO1 overactivity, we hypothesize that elevated intracellular calcium levels account for the observed defects in reticulocyte maturation. However, as calcium is also known to exert widespread influence on cell signaling processes, determining the specific underlying mechanism for the detrimental effects caused by overactive PIEZO1 is a challenging proposition. Nonetheless, this work provides the first evidence that overactivation of PIEZO1 impacts reticulocyte maturation. Further investigation of the interplay between PIEZO1 activity and that of other ion channels (as well as of downstream signaling pathways) is likely to be of interest for future studies.

In conclusion, we report that hereditary xerocytosis patients with gain-of-function mutations in *PIEZO1* suffer from multiple transcriptionally-independent effects caused by PIEZO1 overactivation. These include delayed reticulocyte maturation as assayed by loss of CD71, RNA and intracellular vesicle content and significantly decreased capacity to deform upon completion of maturation, which is exacerbated in patients that have undergone splenectomy. This delay in reticulocyte maturation can be recapitulated through chemical treatment with Yoda1, demonstrating that PIEZO1 overactivation has repercussions beyond impaired hydration and volume homeostasis in the erythrocyte.

Ethics statement

The research on patient samples from University Medical Center (UMC) Utrecht was reviewed and approved by the Medical Ethical Review Board (MERB) from UMC Utrecht (METC protocol 17/450). Blood from healthy control donors was anonymously obtained using the approved medical ethical protocol of 07/125 Mini Donor Dienst, also approved by the MERB of UMCU. All source material was provided with written informed consent for research use given in accordance with the Declaration of Helsinki (NHSBT, Filton, Bristol).

The research into the mechanisms of erythropoiesis was approved by the Bristol Research Ethics committee (REC Number 12/SW/0199).

Author Contributions

PLM performed the majority of experiments, analyzed data and prepared figures; BRH and TJS optimized MS-5 co-culture and performed experiments; JGGD and GJS provided essential ARCA equipment and analysis software. MAER, PB and RvW diagnosed HX patients and provided blood samples and related data. PLM, AMT and TJS conceived and designed experiments and wrote the manuscript. TJS and AMT contributed equally to conception and supervision of the work. All authors read and edited the manuscript.

Conflict of interest statement

The authors declare no competing financial interests.

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Table 1: Hematological parameters of the Hereditary Xerocytosis patients under investigation

Patient	Age	Gender	Mutation	Splenectomy	Hemoglobin (g/dL)	MCV (fL)	Reticulocyte no. (10^9/L)	Reticulocyte %	Ferritin (µg/L)
1	33	M	p.R2088G + p.2169-2170 delK	N	14.2	105	718	18.7	94
2†	23	F	p.R2456H	Y	13.1	100	259	7.2	170
3†	47	M	p.R2456H	N	16.8	93	997	20.0	107
4	19	M	p.V598M	Y	16.1	106	472	11.0	591
5‡	47	F	p.E2496ELE	Y	10.2	77	227	5.0	67
6‡	47	F	p.E2496ELE	Y	12.5	74.4	113	2.6	-----
7	55	F	p.V598M	N	8.7	108	137	5.9	875
8#	46	M	p.R2456H	N	13.5	97	700	18.4	175
9#	43	F	p.R2456H	N	11.8	94	294	9.3	257
10†	18	M	p.R2456H	N	9.83	80	402	11.0	17
				Reference values:	*[12-16]*	*[80-94]*	*[25-120]*	*[0.8-3.0]*	*[25-250]*

The patient numbers in the table are used to identify the respective samples in the reticulocyte maturation experiment shown in **Figure 2B** and **Figure 2C**. Patient age and gender are provided. “Mutation” identifies the *PIEZO1* mutation(s) found in the patient. “Splenectomy” defines whether the patient has or has not undergone splenectomy. MCV: Mean Cell Volume (obtained through the use of a CELL-DYN Sapphire system or a Sysmex XN-9000 system). Reticulocyte no.: absolute reticulocyte number. Reticulocyte %: reticulocyte percentage as a function of all erythroid cells in circulation. Serum hemoglobin concentration and serum ferritin concentration values are also provided. Reference values are provided under each of the numerical columns. † and # indicate that the patients are relatives. ‡ indicates a repeat admission to the clinic (no serum ferritin concentration values were obtained for the second visit).

Figure legends

Figure 1: Cell defects arise upon reticulocyte maturation in Hereditary Xerocytosis patients

A) Contour plots of cross-sectional area plotted against the deformability index (as measured by dividing cell length by cell width), visualizing the probability distribution of erythrocytes (CD71 negative) and reticulocytes (CD71 positive) from HX patients compared to healthy reference samples and separated by splenectomy status. The cells were subjected to magnetic cell isolation using CD71 MicroBeads [Miltenyi Biotec] for separation of the CD71 positive and negative populations. The cells were analyzed with the Automated Rheoscope and Cell Analyzer, as previously described(8), with a minimum of 1000 cells obtained per sample. The probability density functions were generated through kernel-density estimation of data comprising 3 reference samples, 6 non-splenectomized patient samples and 4 splenectomized patient samples.

B) Comparison of mitochondrial content in cells from HX patients compared to samples from healthy donors (control) in CD71 negative and CD71 positive cells, separated by splenectomy status. Tile scans composed of 10x10 images were taken at 1024x1024 resolution of cells labelled with Mitotracker Deep Red [500nM, Thermo Fisher Scientific] using confocal imaging and analyzed manually. Sample numbers comprise 3 reference samples, 6 non-splenectomized patient samples and 4 splenectomized patient samples. Data are represented as mean ± SD. All comparisons were made with two-sample unequal variance (heteroscedastic) T-tests. n.s.s.: non-statistically significant; *: $P < 0.05$; ***: $P < 0.001$.

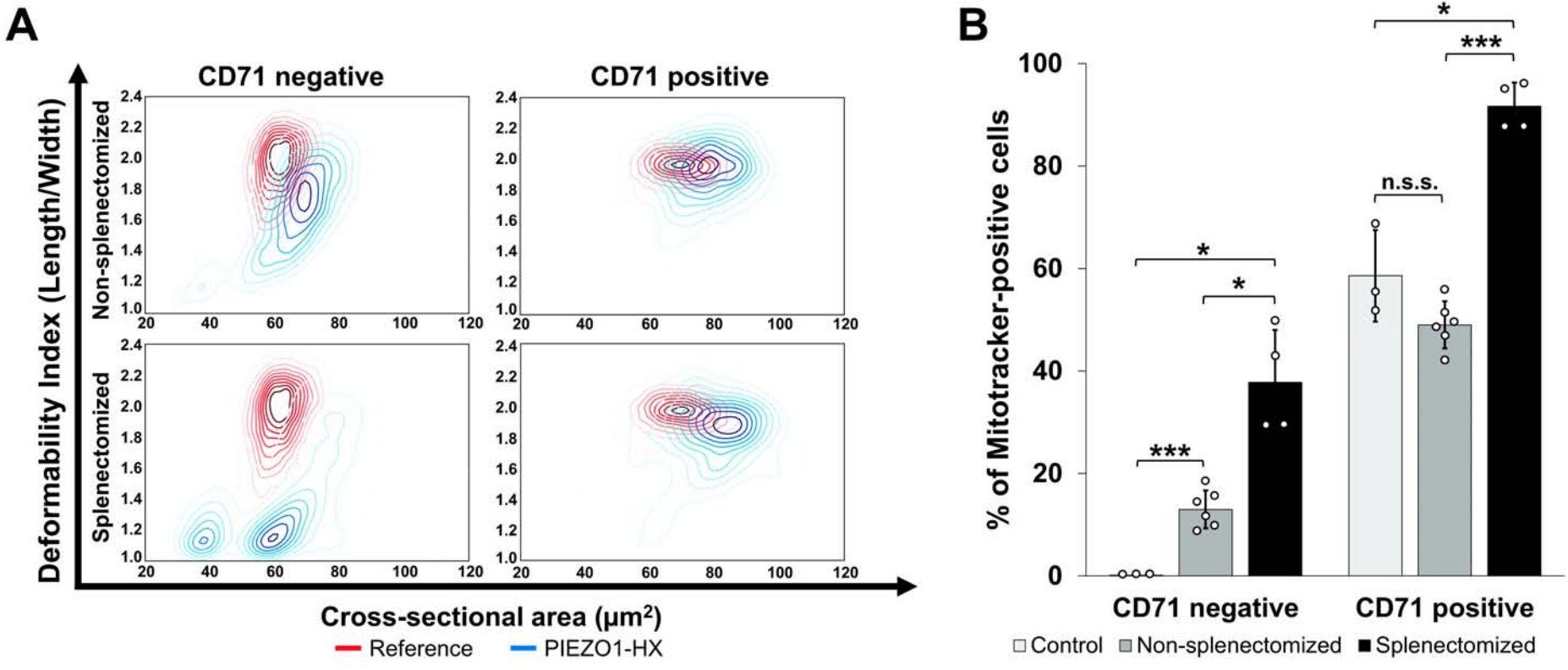
Figure 2: Delayed reticulocyte maturation is observed in HX and can be recapitulated through chemical activation of PIEZO1

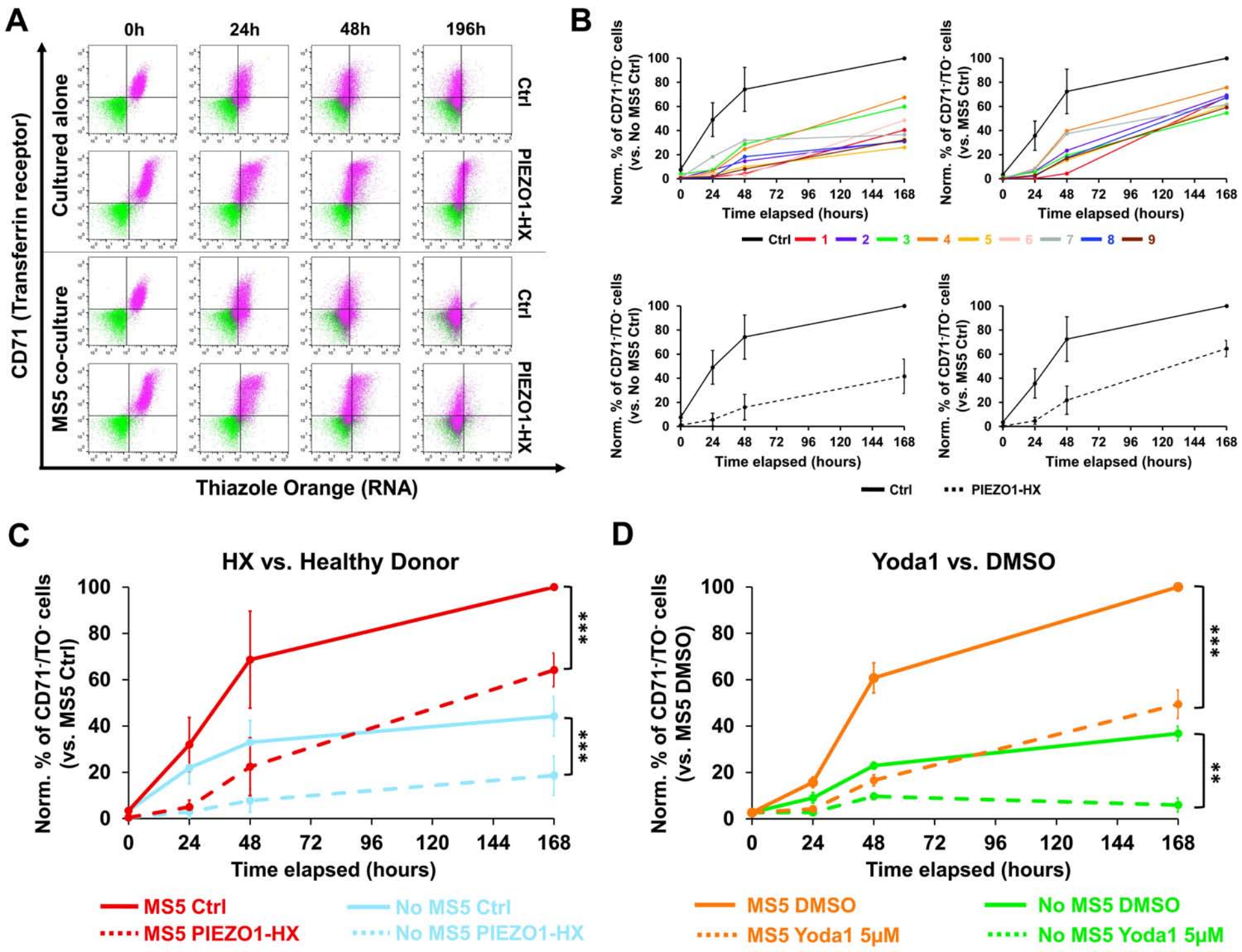
A) Representative flow cytometry diagram plotting RNA content (Thiazole Orange, TO) against membrane transferrin receptor (CD71) content (APC anti-human CD71 [CY1G4], Biolegend), displaying progression through reticulocyte maturation of cells isolated from a healthy donor (Ctrl) or an HX patient (PIEZO1-HX, patient no. 5) and kept in static cell culture conditions over the course of 168 hours (7 days), shown in magenta. Cells were either cultured alone or layered onto MS-5 cells for co-culture (details for MS-5 culture are as described in Darghouth et al(11)). CD71-negative cells from the respective samples are shown in green, defining the quadrant and respective limits of a $CD71^-/TO^-$ population.

B) Loss of CD71/TO in reticulocytes cultured alone (left) or in co-culture with MS-5 cells (right) as measured by the percentage of cells present in the $CD71^-/TO^-$ quadrant and normalized against the $CD71^-/TO^-$ percentage observed in the final timepoint of healthy control samples. N = 7 for the control samples, with error bars showing standard deviation. Each patient is represented as one separate line above, with all patients averaged below (the error bars show standard deviation). In the bottom panel, all samples were normalized to the final timepoint of MS-5 co-cultured reticulocytes from the healthy control of their respective experiment. Co-culture data was not obtainable for patient number 6. The results from patient number 10 were extreme outliers and thus excluded from this figure (available on request). Notably, this patient presented with iron deficiency, a condition which has previously been reported to decrease phenotypic severity in HX(15).

C) Average CD71/TO loss in reticulocytes from healthy donors and HX patients, cultured alone (blue) or in co-culture with MS-5 cells (red) and normalized against the $CD71^-/TO^-$ percentage observed in the final timepoint of healthy control samples. Error bars show standard deviation. N = 7 for the control samples. All comparisons were made with two-sample unequal variance (heteroscedastic) T-tests. ***: P-value < 0.001.

D) Average CD71/TO loss in reticulocytes cultured alone (green) or in co-culture with MS-5 cells (orange), with and without treatment with either 1:4000 DMSO (v/v) or 5 µM Yoda1 (chemical inducer of PIEZO1 activity; Tocris Bioscience). The data comprises three biological replicates, each with two technical replicates in MS-5 co-culture (N = 3 for the non-MS5 data, N = 6 for the MS5 data). Error bars show standard deviation. All comparisons were made with paired sample T-tests. **: P < 0.01; ***: P < 0.001.


A
CD71 negative
CD71 positive
Non-splenectomized
Splenectomized
Deformability Index (Length/Width)
Cross-sectional area (μm²)
Reference
PIEZO1-HX
B
% of Mitotracker-positive cells
n.s.s.
CD71 negative
CD71 positive
Control
Non-splenectomized
Splenectomized


A
0h
24h
48h
196h
Cultured alone
MS5 co-culture
Ctrl
PIEZO1-HX
CD71 (Transferrin receptor)
Thiazole Orange (RNA)
B
Norm. % of CD71-/TO- cells (vs. No MS5 Ctrl)
Norm. % of CD71-/TO- cells (vs. MS5 Ctrl)
Time elapsed (hours)
Ctrl 1 2 3 4 5 6 7 8 9
Ctrl
PIEZO1-HX
C
HX vs. Healthy Donor
Norm. % of CD71-/TO- cells (vs. MS5 Ctrl)
Time elapsed (hours)
***
***
MS5 Ctrl
MS5 PIEZO1-HX
No MS5 Ctrl
No MS5 PIEZO1-HX
D
Yoda1 vs. DMSO
Norm. % of CD71-/TO- cells (vs. MS5 DMSO)
Time elapsed (hours)
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MS5 DMSO
MS5 Yoda1 5μM
No MS5 DMSO
No MS5 Yoda1 5μM

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]

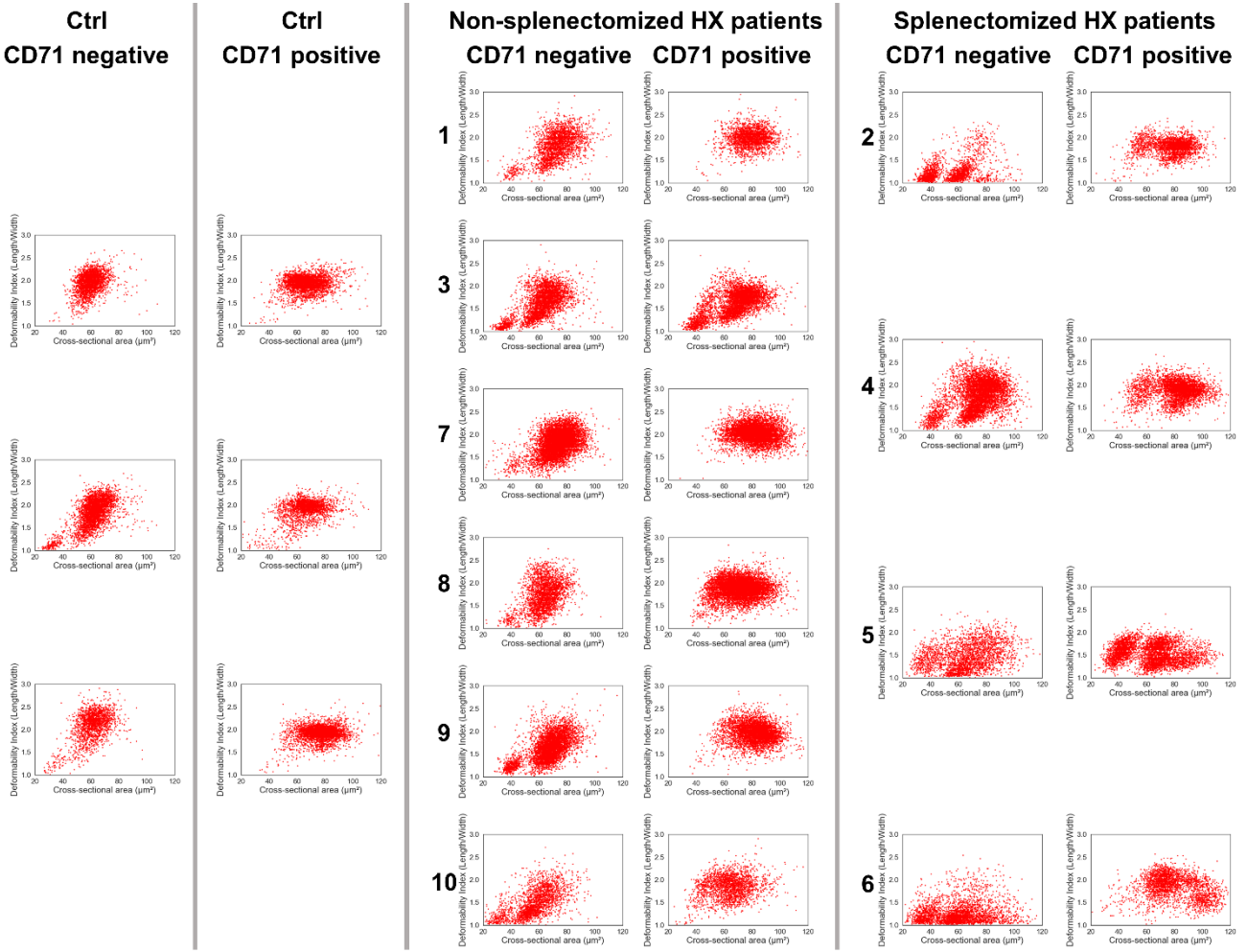
Patient 7 is a 55-year old female. The patient was asymptomatic until 24 years of age, when fatigue and abdominal pain developed accompanied by mild chronic macrocytic hemolytic anemia with reticulocytosis and splenomegaly. At the time, the patient was diagnosed with hereditary spherocytosis. There is 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

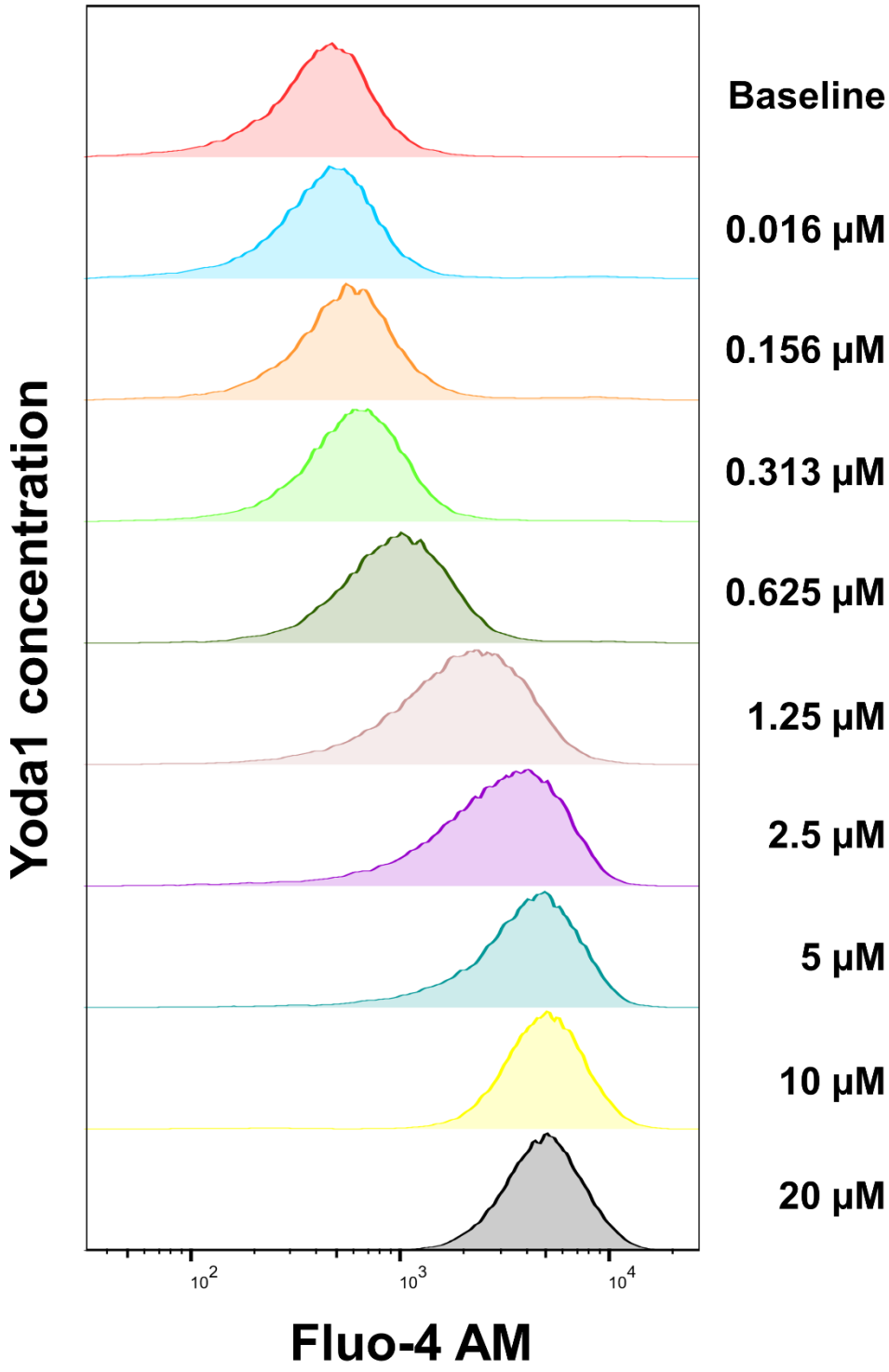
Gilbert's syndrome). Osmotic fragility tests, red blood cell membrane protein content and enzyme activity displayed normal results, thus excluding a cytoskeletal or metabolic defect. The patient was diagnosed with HX more recently, following osmotic gradient ektacytometry (which displayed the characteristic 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*.

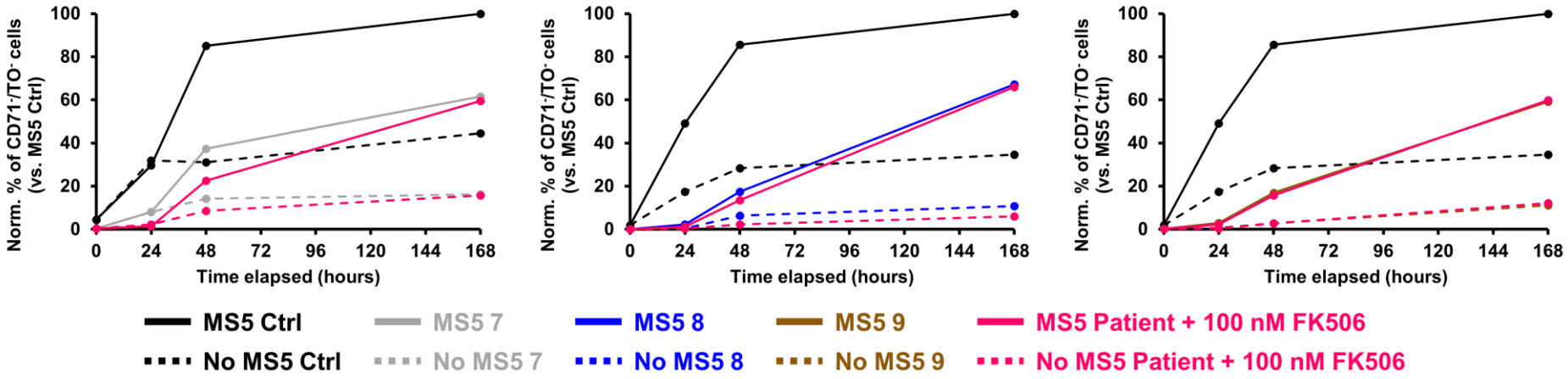
SUPPLEMENTAL FIGURES



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.
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