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Mechanistic Consequences of Piezo1 Gain-of-Function Variants for Decreased Red Cell Survival in Hereditary Xerocytosis

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To the Editor,

Hereditary xerocytosis (HX), also known as dehydrated hereditary stomatocytosis (DHS), is a rare red blood cell (RBC) disorder caused by gain-of-function (GOF) mutations in the *PIEZO1* gene [1–3], which encodes a mechanosensitive, nonselective cation channel. Activation of Piezo1 leads to, i.a., Ca^{2+} influx, opening of the Gárdos-channel, K^+ efflux, and subsequent RBC dehydration [4, 5]. Patients with HX typically exhibit compensated hemolysis, elevated reticulocyte counts, and iron overload. Mechanistic links—such as the relationship between Piezo1 activity and altered RBC lifespan, or between reticulocyte counts and erythropoiesis rate—are hypothesized but remain unconfirmed [6, 7].

We investigated a cohort of 12 genetically confirmed HX patients (see Table S1 and Figure S1 for details). Our objective was to determine how GOF variants in *PIEZO1* affect RBC physiology and erythropoietic regulation in HX. In contrast to sickle cell disease (SCD), where RBC lifespan is markedly shortened and highly variable (10–60 days) [8], much less is known about RBC survival in HX. Therefore, we first assessed the average lifespan of HX RBCs using the protein 4.1a/4.1b ratio, which functions as a molecular clock [9] and scales linearly with RBC age. Figure 1A,B depict this ratio for healthy controls, SCD patients, and HX patients, plotted against reticulocyte counts. HX patients exhibit elevated reticulocyte counts despite having a comparable RBC age to SCD patients, suggesting delayed reticulocyte maturation [10]. This "uncoupling" of reticulocyte count from RBC age accounts for its weak correlation with erythropoietin (EPO) levels (Figure 1C), indicating that here the reticulocyte count is not a reliable marker of erythropoiesis rate. Based on the established lifespan of SCD RBCs [8], HX RBCs appear to have similarly shortened lifespans (0.66 ± 0.16 vs. 0.62 ± 0.11 days; $p = 0.5$).

HX patients exhibit considerable phenotypic variability, ranging from mild hemolytic anemia to compensated hemolysis and mild polycythemia [11, 12]. This variability is reflected in the broad distribution of blood values shown in Figure 1B and Table S1. Accordingly, we adopted an approach focused on correlating specific parameters within the HX cohort. In line with this, the HX RBC age parameter shows a strong correlation with EPO levels, as illustrated in Figure 1D. Several putative mechanisms may underlie the observed increase in erythropoiesis and hypoxia sensing. A left-shifted oxygen dissociation curve in HX RBCs (Figure 1E) aligns with a previous publication [6], where reductions in 2,3-bisphosphoglyceric acid (2,3-BPG) levels, consistent with impaired glycolysis in HX RBCs were also reported. The leftward shift of the oxygen dissociation curve may stimulate erythropoiesis via hypoxia sensing. However, the P_{50} value does not

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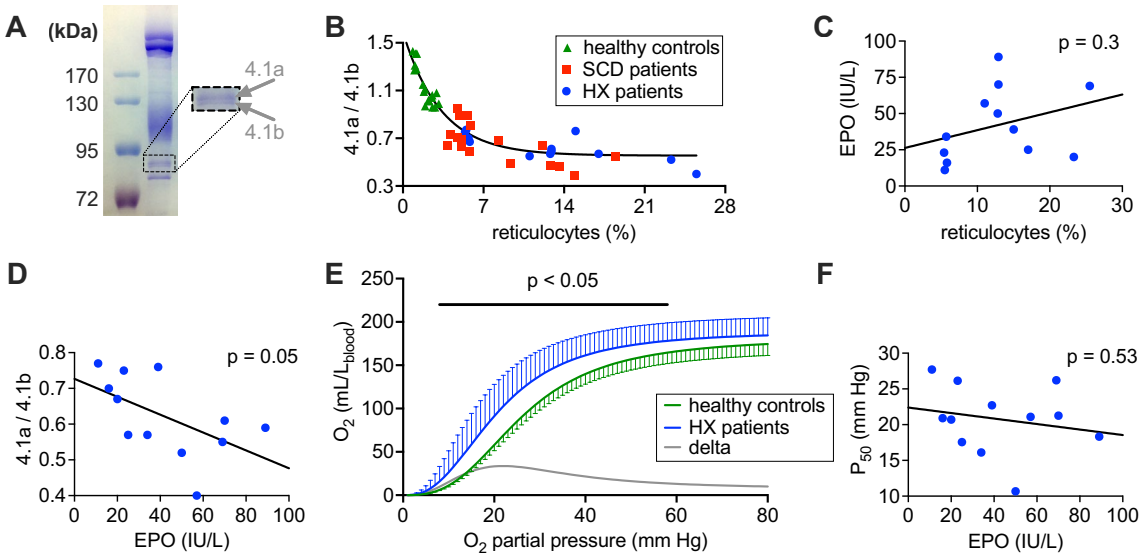

FIGURE 1 | Red blood cell age, oxygen binding, and erythropoietin levels in hereditary xerocytosis (HX) patients. Panel A shows a representative example of gel electrophoresis to determine the protein Band 4.1a to 4.1b ratio, which is known to be a molecular clock for RBC age. Both bands just differ by the deamidation of the Band 4.1 protein. Panel B depicts a plot of this Band 4.1a to 4.1b ratio against the reticulocyte count in terms of RNA positive RBCs for 12 HX patients (blue) and to allow for comparisons in days of RBC age in days (see main text) compared it with19 healthy controls (green) and 17 sickle cell disease patients (red). Panel C is a plot of erythropoietin (EPO) versus reticulocyte count (RNA positive RBCs) for the 12 HX patients. The correlation fails to be significant. Panel D shows the correlation between the average age of the RBCs (Band 4.1a to 4.1b ratio) and the erythropoietin (EPO) as a sign of increased erythrocytosis for the 12 HX patients. Panel E shows the oxygen binding curves of 14 healthy controls (green) and the 12 HX patients under investigation (blue), which are left-shifted. For clarity, error bars are drawn only in one direction—they indicate the standard deviation. In the range of the oxygen partial pressure between 8 and 58 mmHg, the curves are significantly different ($p < 0.05$). The gray line shows the differential curve. Panel F provides the plot of the P_{50} versus the EPO for the 12 HX patients. There is no significant relation ($p = 0.53$). For a detailed description of the materials and methods applied, see Supporting Information.

correlate with EPO levels (Figure 1F). Although the shift results in significantly altered oxygen dissociation, its contribution to EPO production is likely limited, as the partial pressure in the kidneys (~70 mmHg) [13] lies near the plateau of the oxygen–hemoglobin curve (Figure 1E), where no significant difference is observed.

Increased erythropoiesis may instead be directly linked to accelerated RBC clearance driven by Piezo1 activity. Given that Piezo1 activation induces dehydration through ion flux–mediated interaction with the Gárdos-channel (see above), we developed a straightforward assay (Figures 2A and S2) using flow cytometric side scatter to assess Piezo1-mediated dehydration (Figure 2B). The formation of echinocytes increases cellular granularity, thereby elevating side scatter. Direct comparison with osmotic gradient ektacytometry parameters—used to assess basal dehydration—is limited, as the polyvinylpyrrolidone solutions employed in those assays lack Ca^{2+}, which is essential for Piezo1-mediated dehydration. Notably, the side scatter–based dehydration parameter correlates strongly with EPO levels, a key marker of erythropoietic activity, supporting a link to enhanced RBC clearance (Figure 2C).

As a side note, the mechanistic interplay between Piezo1 and the Gárdos-channel helps explain the historical lack of distinction within DHS, regardless of whether the condition is driven by mutations in *PIEZO1* or the Gárdos-channel [4, 14]. Recent evidence suggests that patients with GOF mutations in the Gárdos-channel can be more precisely classified as having Gárdos-channelopathy [15].

Based on our initial findings, we hypothesize that the reduced age of HX RBCs and increased erythropoiesis in HX patients result from enhanced RBC clearance. To investigate this, we assessed phosphatidylserine (PS) exposure on the outer leaflet of the RBC membrane—a well-established "eat me" signal for macrophages [16]. Indeed, PS exposure, measured via Annexin-V binding, correlates significantly with side scatter (Figure 2D). Although the regulation of PS exposure is complex, it is likely influenced by Ca^{2+} influx into RBCs [17]. Whether Piezo1 alone provides sufficient Ca^{2+} entry or requires additional Ca^{2+} channel activity from $Ca_V2.1$ [18], potentially triggered by pseudo–action potentials [19], remains to be determined and is beyond the scope of this letter.

To support the concept of enhanced RBC clearance, we analyzed bilirubin concentration—a key marker of hemolysis—for its correlation with side scatter (Figure 2E). A significant positive correlation emerged, suggesting that more dehydrated RBCs are more likely to be cleared by the spleen. Although increased RBC clearance appears to be the primary cause of the shortened HX RBC lifespan, a trigger for enhanced erythropoiesis must still be present. This is unlikely to be anemia itself, as all but one of the HX patients studied were not anemic (Table S1). In addition, EPO does not correlate with either hematocrit or hemoglobin values (Figure S3).

During hemoglobin (Hb) degradation via heme oxygenase, carbon monoxide (CO) is produced [20]. CO binds Hb in RBCs with high affinity, reducing oxygen binding and thereby creating a

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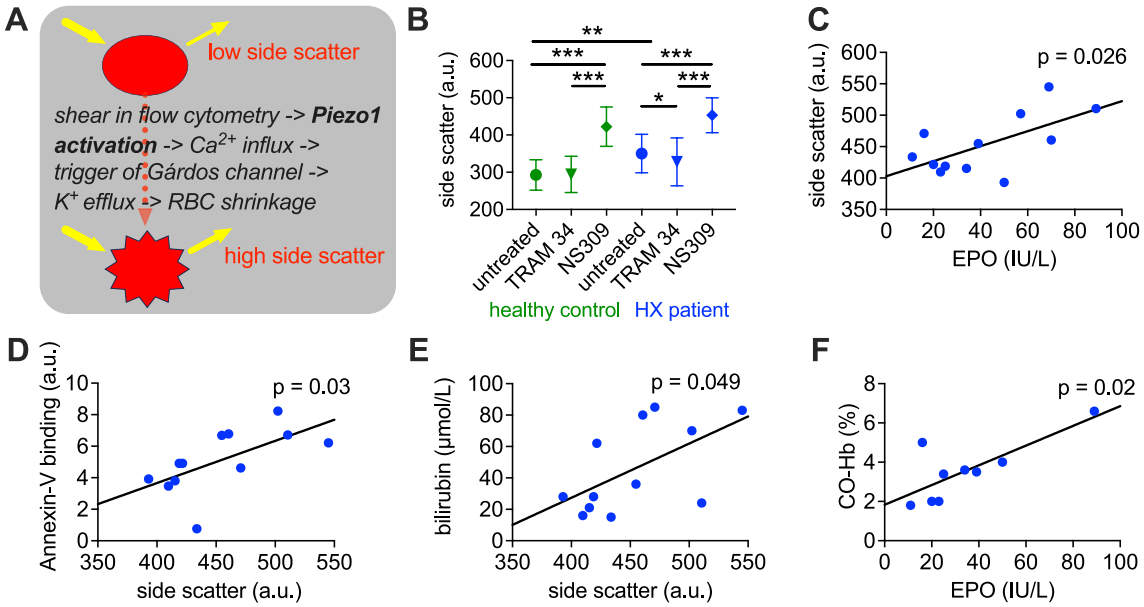

FIGURE 2 | Red blood cell dehydration and its relation to other parameters in the red blood cell life-cycle and the relation of CO-hemoglobin to erythropoietin. Panel A is the scheme behind a flow cytometry-based assay to test for the functionality of the mechanosensitive ion channel Piezo1. Panel B shows the basic measurements to establish the assay (see also Figure S2). Circles show the side scatter value of untreated cells. Triangles represent cells pretreated for 2 h with 10 µM TRAM34, a Gárdos channel inhibitor, showing that Piezo1 is not activated in healthy cells and slightly activated in HX-RBCs. Rhombuses depict the side scatter of cells incubated for 2 h with 100 µM NS309, a compound that increases the Ca^{2+} sensitivity of the Gárdos channel. As a result, the side scatter with preincubation of NS309 is the method of choice with the biggest measurable effect. All values represent the means of 12 blood samples, and the error bars represent the standard deviation. *represents $p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$. Panel C shows the correlation between the Piezo1 channel activity (side scatter) and the erythropoietin (EPO) as a sign of increased erythrocytosis for the 12 HX patients. Panel D provides the plot of the phosphatidylserine exposure in the outer membrane leaflet (Annexin-V binding) versus the EPO for the 12 HX patients. Panel E shows the correlation between bilirubin and the side scatter for the 12 HX patients. Panel F provides the plot of the CO-hemoglobin (CO-Hb) versus the EPO for the HX patients. For a detailed description of the materials and methods applied, see Supporting Information.

hypoxic stimulus. This mechanism gained attention as a doping strategy among competitive cyclists, following studies showing a significant increase in total hemoglobin mass after CO inhalation [21]. In our HX patient cohort, CO-Hb concentrations were ~2–10 times higher than in healthy individuals and correlated significantly with EPO levels (Figure 2F). These findings suggest that CO-Hb, a known stimulator of EPO via hypoxia signaling, is likely driving the erythropoietic response in HX. Unlike transient elevations seen in smokers, this signal in HX patients is persistent and sustained. Additionally, shrinkage of HX RBCs may impair oxygen delivery in the microcirculation, potentially amplifying hypoxia sensing.

In conclusion, our findings demonstrate that Piezo1 functional activity induces rapid removal of HX RBCs from circulation. This process involves Ca^{2+} signaling–mediated activation of the Gárdos-channel, presumed scramblase activity (leading to phosphatidylserine [PS] exposure), cell shrinkage, and altered deformability. A schematic overview of the involved steps is provided in Figure S4. This accelerated clearance results in hemolysis and elevated CO-Hb levels, which in turn generate a hypoxic stimulus and promote erythropoiesis—a mechanism that may also be relevant in other hemolytic anemias.

Although Piezo1-mediated depletion of 2,3-bisphosphoglyceric acid (2,3-BPG) [22] increases oxygen affinity, its impact on EPO stimulation at physiological renal oxygen tension appears limited. This contrasts with other hemolytic anemias, where elevated 2,3-BPG levels shift the oxygen dissociation curve to the right, reducing hypoxia sensing. Such shifts can be partially compensated by cardiovascular adaptations (e.g., increased heart rate, blood pressure, or cardiac hypertrophy) and pulmonary function.

Instead, chronic hemolysis—reflected by persistently elevated CO-Hb—appears to be the dominant driver of EPO upregulation in HX patients, explaining how some maintain normal or elevated hemoglobin levels. The Ca^{2+} influx via Piezo1 and K^+ efflux through Gárdos-channels necessitate upregulation of the Ca^{2+} pump (PMCA) and Na^+/K^+ pump (ATP1A), accounting for increased glucose consumption [6]. Ultimately, the molecular mechanism described here offers a compelling explanation for RBC turnover regulation in HX patients. The mechanistic link between Piezo1 activity and RBC clearance provides insight into the variability of clinical presentations, ranging from hemolytic anemia to polycythemia [11, 12].

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Ethics Statement

Patients diagnosed with hereditary xerocytosis (HX) were enrolled in the study after signing an informed consent. Patients' data were handled anonymously as outlined in the ethics agreements. These agreements were approved by the Medical Ethical Research Board (MERB) of the University Medical Center Utrecht, the Netherlands (UMCU) under reference code 15/426M "Disturbed ion homeostasis in hereditary hemolytic anemia". At the University of Zürich, the MemSID study protocol was approved by the local ethics committee of Canton Zurich (KEK-ZH 2015–0297) and the regulatory authority. The ethics vote at Saarland University was provided by the Ärztekammer of Saarland, Germany, under reference number 132/08. Exclusion criteria were erythrocyte transfusion in the past 90 days, age below 3 years, and/or body weight lower than 18 kg. 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.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

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

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** Supporting Information. **Table S1:** (attached as Excel Table): Baseline data of the Hereditary Xerocytosis patients involved in the study (Hb—hemoglobin; RBCs—red blood cells; Ht—hematocrit; MCV—mean corpuscular volume; MCH—mean corpuscular hemoglobin; MCHC—mean corpuscular hemoglobin concentration; O_{max}—osmolarity at maximal elongation index (osmoscan parameter)). The average Hb was within the healthy range (median of 13.8 g/dL for females and 14.5 g/dL for males).

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