Temporal relationship between systemic endothelial dysfunction and alterations in erythrocyte function in a murine model of chronic heart failure

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Aims
Endothelial dysfunction (ED) and red blood cell distribution width (RDW) are both prognostic factors in heart failure (HF), but the relationship between them is not clear. In this study, we used a unique mouse model of chronic HF driven by cardiomyocyte-specific overexpression of activated Gαq protein (Tgαq*44 mice) to characterize the relationship between the development of peripheral ED and the occurrence of structural nanomechanical and biochemical changes in red blood cells (RBCs).

Methods and results
Systemic ED was detected in vivo in 8-month-old Tgαq*44 mice, as evidenced by impaired acetylcholine-induced vasodilation in the aorta and increased endothelial permeability in the brachiocephalic artery. ED in the aorta was associated with impaired nitric oxide (NO) production in the aorta and diminished systemic NO bioavailability. ED in the aorta was also characterized by increased superoxide and eicosanoid production. In 4- to 6-month-old Tgαq*44 mice, RBC size and membrane composition displayed alterations that did not result in significant changes in their nanomechanical and functional properties. However, 8-month-old Tgαq*44 mice presented greatly accentuated structural and size changes and increased RBC stiffness. In 12-month-old Tgαq*44 mice, the erythropathy was featured by severely altered RBC shape and elasticity, increased RDW, impaired RBC deformability, and increased oxidative stress (gluthatione (GSH)/glutathione disulfide (GSSG) ratio). Moreover, RBCs taken from 12-month-old Tgαq*44 mice, but not from 12-month-old FVB mice, coincubated with aortic rings from FVB mice, induced impaired endothelium-dependent vasodilation and this effect was partially reversed by an arginase inhibitor [2(S)-amino-6-boronohexanoic acid].

Conclusion
In the Tgαq*44 murine model of HF, systemic ED accelerates erythropathy and, conversely, erythropathy may contribute to ED. These results suggest that erythropathy may be regarded as a marker and a mediator of systemic ED in HF. RBC arginase and possibly other RBC-mediated mechanisms may represent novel therapeutic targets for systemic ED in HF.
1. Introduction

Heart failure (HF) is an outcome of various primary and secondary incidents; in the advanced stage, it results not only in impaired cardiac function but also in the development of endothelial dysfunction (ED) in the peripheral circulation as well as alterations in red blood cell (RBC) function. However, the relationship between ED and the functional, structural, nanomechanical, and biochemical properties of RBCs in HF is not clear.

In various diseases, including HF, ED is characterized by impaired production of nitric oxide (NO) and increased production of superoxide (O$_{2}^-$) as well as other changes in the endothelial phenotype. Consistent with the oxidative stress-related mechanisms of ED, antioxidant treatment strategies, including vitamin C and NADPH oxidase 2 and xanthine oxidase inhibitors, have been shown to improve endothelial function in HF. Various mechanisms of peripheral ED have been proposed, including a decrease in shear stress linked to cardiac failure, which leads to the downregulation of endothelial nitric oxide synthase (eNOS) expression and the reduction of NO production alongside increased oxidative stress. Several contributors to ED in HF have been proposed: neurohormonal activation, with major roles of angiotensin II (Ang II) and mineralocorticoid receptor (MR)-dependent mechanisms; hyperactivation of the sympathetic system; and proinflammatory cytokines, including tumour necrosis factor alpha (TNF-$\alpha$) and interleukin (IL)-6. Notably, the most effective pharmacological treatments for HF patients, such as renin-angiotensin system (RAS) inhibitors, which include angiotensin-converting enzyme (ACE) inhibitors, Ang II type 1 receptor antagonists, and MR antagonists, improved endothelial function in the peripheral circulation of HF patients. Improvement in peripheral ED by RAS-based treatment strategies and other treatment strategies has therapeutic benefits for HF, including increased exercise tolerance in HF patients.

ED in peripheral circulation may have prognostic value independent of whether the HF is ischaemic or non-ischaemic. However, although abundant literature exists related to peripheral ED in HF of ischaemic origin, little is known about the mechanism of ED in non-ischaemic HF. Indeed, some but not all authors have confirmed the development of ED in HF of non-ischaemic origin.

Interestingly, although RBCs display physiological size heterogeneity, increased red blood cell distribution width (RDW) is an independent predictor of the short- and long-term prognosis of HF, implicating the role of altered function of RBCs in the pathophysiology of HF. Several reciprocal mechanisms between the endothelium and RBCs maintain the haemostatic balance and safeguard the cardiovascular system, whereas alterations of this balance may lead to vascular pathologies such as ED. For example, functional alterations in RBCs induced by hyperglycaemia, diabetes, a high-fat diet, malaria, and haemoglobinopathies (e.g. sickle cell diseases) contribute to the pathomechanisms of ED, and various mechanisms have been proposed for these RBC functional changes.

Despite the knowledge that ED and RDW are both prognostic factors in HF, the relationship between alterations in RBC function and the development of peripheral ED in HF is not clear. Although patients with HF present alterations in several haemorheological properties as well as
2. Methods

2.1 Animals

Female Tgαq*44 mice, a model of HF initially developed by Mende et al.,24,27 and FVB (wild-type) mice were bred in the Animal Laboratory of the Medical Research Centre of the Polish Academy of Sciences (Warsaw, Poland). Tgαq*44 mice based on the FVB strain express an αHapo protein (Tgαq*HA po) under the control of the α-MHC promoter and represent a unique model of CHF.24,27 All animal procedures were in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985) as well as with the local ethical committee on Animal Experiments in Krakow. Mice were fed a standard chow diet and kept in 12:12 light–dark conditions.

2.2 Assessment of endothelium-dependent vasodilation and endothelial permeability in vivo by magnetic resonance imaging

Endothelial function and permeability in vivo were assessed as described previously.28–30 Briefly, endothelium-dependent vasodilation in vivo was assessed by measuring the response to acetylcholine (Ach, Sigma-Aldrich, Poznań, Poland). Tgαq*44 mice were fed a standard chow diet and kept in 12:12 light–dark conditions. Depending on the applied method of analysis; the blood samples, isolated aorta by electron paramagnetic resonance spectroscopy and the brachiocephalic artery (BCA) using the variable flip angle technique31,32 before and 30 min after intravenous (i.v.) administration of a unique formulation of gadolinium contained in the liposome [gadodiamide in the liposome, concentration of formulation: (287 mg/mL, 4.5 mL/kg, i.v.)] as described before.28,29

2.3 Aorta isolation

Mice were euthanized intraperitoneally with a mixture of ketamine and xylazine in doses of 100 and 10 mg/kg body weight, respectively. Subsequently, the aorta was removed and placed in cold Krebs–Henseleit solution (KB) bubbled with a 95% O₂/5% CO₂ mixture (pH = 7.4). Aortic segments used for NO, superoxide, or eicosanoid production were immediately placed in fresh KB or frozen at -80°C.

2.3.1 Assessment of endothelium-dependent and -independent vasodilation ex vivo in wire myograph system

Aortic rings were mounted in a Muyan myograph system (620 M, Danish Myo Technology, Denmark), followed by assessment of the endothelium-dependent and independent vasodilation ex vivo, carried out as previously described.33 To study RBC-induced ED, RBCs from 12-month-old Tgαq*44 or 12-month-old FVB mice were isolated,24 diluted with serum-free culture medium to a haematocrit (HCT) of 5%, and were incubated with aortic rings isolated from 12-month-old Tgαq*44 or 12-month-old FVB mice in cell culture incubator at 37°C with 5% carbon dioxide for 1 h in the absence or presence of 100 μM of 2(S)-amino-6-borono-hexanoic acid (ABH).

2.3.2 Assessment of eicosanoid production in isolated aortic rings by liquid chromatography coupled to mass spectrometry (LC–MS/MS)

Aortic rings were added to a 24-well plate containing KB. The plate was placed into a BIO-V gas treatment chamber (Noxygen Science, Elzach, Germany), where it remained for 15 min under CO₂ flow at 37°C. Pre-incubation, the aortic rings were placed into 500 μL of fresh KB, and 100 μL samples of the incubation buffer were taken after 3 and 45 min of incubation. The concentrations of 6-keto prostaglandin F1α (6-keto PGF₁α), as well as prostaglandin E₂ and D₂ (PGE₂ and PGD₂) and 15-hydroxyicosatetraenoic acid (15-HETE) in the aorta effluents, were examined by a liquid chromatograph UFLC Nexera (Shimadzu, Kyoto, Japan) coupled to a triple quadrupole mass spectrometer QTRAP 5500 (SCIEX, Framingham, MA, USA) following the methodology previously described.35 The biosynthesis of prostacyclin (PGI₂) and thromboxane A₂ (TXA₂) were assessed based on the concentration of their stable metabolites 6-keto PGF₁α, and thromboxane B₂ (TXB₂), respectively. Results are presented as the difference between the concentration assessed after 3 and 45 min incubation.

2.3.3 Assessment of NO and O₂⁻ production in the isolated aorta by electron paramagnetic resonance spectroscopy

NO production in the isolated aorta was measured by electron paramagnetic resonance (EPR) with the cell-permeable NO spin trapping agent diethylthiocarbamate (DETC), as described previously.36

2.4 Blood and RBC analysis

Dependent on the applied method of analysis; the blood samples, isolated RBCs or RBC membranes were studied. Whole blood samples were collected from the right ventricle using a syringe containing anticoagulant (heparin). The details of RBC isolation from the whole blood and RBC membrane isolation (prepared by overnight freezing of RBCs suspended in 0.9% NaCl, HCT = 10%) are presented in the Supplementary material online.
2.4.1 Blood count, blood biochemistry, and determination of NO metabolites
A whole blood sample was used for blood count analysis using an automatic haematology analyser ABC Vet (Horiba, Kyoto, Japan). Plasma obtained after centrifugation (acceleration: 1000 x g, run time: 10 min, 4°C) was used for measuring the lipid profile with an ABX Pentra biochemical analyser (Horiba Medical Kyoto, Japan).

Measurement of nitrate (NO$_3^-$) and nitrite (NO$_2^-$) concentrations in the plasma was performed using an ENO-20 NOx analyser (Eicom Corp., Kyoto, Japan), applying a liquid chromatography method with post-column derivatization using Griess reagent, as previously described. The packed RBCs remaining after centrifugation were used for glutathione (GSH) and glutathione disulfide (GSSG) concentration measurement as well as nitrosyl haemoglobin (HbNO) detection with EPR spectroscopy.

2.4.2 Assessment of RBC shape and nanomechanics by atomic force microscopy (AFM)
Erythrocyte shape and elasticity were measured using a NanoWizard 3 (JPK Instruments, Berlin, Germany) AFM microscope. All measurements were performed using a pyramidal AFM probe attached to V-shaped silicon nitride cantilevers with a spring constant of 0.01 N/m (MLCT-C, Veeco Probes, Camarillo, CA, USA). The force mapping mode was used for both topography and elasticity measurements. The elastic moduli of RBCs were calculated using the Hertz–Sneddon model with the approximation for a paraboloidal probe. Data were analysed using JPKSPM Data Processing software. The aspect ratio was defined as the ratio between two perpendicular main axes of the RBCs (i.e. the length and width of the cell).

2.4.3 Assessment of RBC deformability by RheoScan
Erythrocyte deformability was measured using a microfluidic RheoScan AnD 300 (RheoMeditech, Seoul, South Korea) following the protocol suggested by the manufacturer. RBC deformation was quantified at a shear stress of 20 Pa in terms of the maximum elongation index.

2.4.4 Assessment of RBC flow cytometry
RBCs and reticulocytes were analysed with a BD LSR II flow cytometer (BD Biosciences, Oxford, UK) and stained against anti-mouse TER-119 PerCP/Cy5.5 (BioLegend, San Diego, CA, USA) and anti-mouse CD71 APC (BioLegend) antibodies and annexin V FITC (antibody dilution 1:100, stained for 30 min at room temperature). For each sample 100 000 000 events were acquired in log mode for forward side scatter (SSC), SSC, and fluorescent signals. Data were analysed using BD FACSDiva Software (BD Biosciences). RBC and reticulocytes were gated according to their characteristic log FCS, log SSC, and fluorescent signals.

2.4.5 Assessment of GSH and GSSG concentration in RBCs by capillary electrophoresis
GSH and GSSG concentrations were measured using a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA, USA) with 32 Karat software (ver. 8.0, Beckman Coulter) as previously described.

2.4.6 Assessment of biochemical content of RBC membranes by Raman spectroscopy and Fourier transform infrared spectroscopy–attenuated total reflectance
Isolated RBC membranes were deposited on CaF$_2$ slides, air-dried for 30 min, and examined with Raman spectroscopy (RS) followed by Fourier transform infrared spectroscopy (FTIR)–attenuated total reflectance, as previously described.

Details of methods are given in Supplementary material online.

2.5 Statistical analysis
Statistical analyses were performed using GraphPad Prism 8.4 (GraphPad Software). The results are presented as box plots (median, Q1, Q3, interquartile range, and outliers). Tgq*44 mice in different phases of HF were compared with age-matched control groups and analysed using two-way ANOVA. The normality of the distribution and homogeneity of variance were tested using the Shapiro–Wilk and F-tests, respectively. When these assumptions were violated, non-parametric tests were performed (Kruskal–Wallis and F-tests).

3. Results

3.1 Development of systemic ED in Tgq*44 mice, in vivo magnetic resonance imaging-based measurements
Magnetic resonance imaging (MRI)-based assessment of endothelium-dependent response in vivo revealed that in 8-month-old Tgq*44 mice, Ach-induced vasodilation in the AA was impaired, whereas in older mice (10- to 12-month-old Tgq*44 mice), Ach-induced vasodilation was completely lost and changed to vasoconstriction (Figure 1A, P < 0.05). ED in 8-month-old Tgq*44 mice was confirmed by increased endothelial permeability measured in vivo with MRI with the use of the Npx50 parameter of endothelial permeability. The T$_1$ signal near the BCA lumen after i.v. injection of gadolinium-containing liposomes was increased in Tgq*44 mice aged 8 months and older compared with age-matched FVB mice (Figure 1B). In contrast, flow-mediated vasodilation (FMD) in the FA was fully preserved in 8-month-old Tgq*44 mice compared with that in age-matched FVB mice. FMD in the FA was also slightly impaired in 12-month-old Tgq*44 mice, but this difference did not reach statistical significance (Figure 1C).

3.2 Development of ED in the aorta of Tgq*44 mice; ex vivo measurements
In 10- and 12-month-old Tgq*44 mice, but not in 6- and 8-month-old Tgq*44 mice, Ach-induced endothelium-dependent vasodilation was decreased compared with the age-matched FVB mice (Figure 1D–G), whereas endothelium-independent vasodilation induced by sodium nitroprusside (SNP) was fully preserved in all experimental groups of Tgq*44 mice and age-matched FVB mice (Figure 1H–K).

3.3 Systemic bioavailability of NO in Tgq*44 mice
Systemic ED in Tgq*44 mice was not associated with a reduction in the NO$_2$ concentration in plasma. However, the NO$_3$ plasma concentration decreased in 10- to 12-month-old Tgq*44 mice compared with that of FVB mice (Table 1). In contrast to the lack of decreased plasma NO$_2$...
concentration, the HbNO content in RBCs substantially decreased in 12-month-old Tg\textit{a}q*44 mice compared with that in the RBCs of age-matched FVB mice (Table 1). Tg\textit{a}q*44 mice did not display any changes in blood biochemistry compared with age-matched FVB mice until the age of 12 months, when the plasma concentration of the urea significantly increased, whereas the total cholesterol and HDL cholesterol plasma levels modestly decreased in Tg\textit{a}q*44 mice compared with the levels in FVB mice (Table 1).

### 3.4 Alterations in \textit{NO/O}_2^-/\textit{C0}_2 balance and in eicosanoid production in the aorta of Tg\textit{a}q*44 mice

ED in the aorta of 12-month-old Tg\textit{a}q*44 mice was accompanied by a decrease in stimulated NO production in \textit{ex vivo} aortas measured by EPR (Figure 2A) compared with age-matched FVB mice. Furthermore, impairment of endothelial functional response in the aorta was associated with increased O$_2^-$ production in 12-month-old Tg\textit{a}q*44 mice compared with that in age-matched FVB (Figure 2B).

In 12-month-old Tg\textit{a}q*44 mice, but not in younger Tg\textit{a}q*44 mice, the production of 6-keto PGF$_{1\alpha}$, PG$E_2$, PG$D_2$, and 15-HETE in the aorta was higher than in age-matched FVB mice (Figure 2C–F). However, TNF$\alpha$ (Figure 2G) and IL-1$\beta$ (Figure 2H) mRNA gene expression in the aorta did not differ between the 12-month-old Tg\textit{a}q*44 mice and age-matched FVB mice.

### 3.5 Basic characterization of alterations in RBCs in Tg\textit{a}q*44 mice

Alterations in RBC indices were not associated with significant differences in haemoglobin, HCT, or white blood cell and platelet (PLT) counts between Tg\textit{a}q*44 mice and age-matched FVB mice (Table 1). Tg\textit{a}q*44 mice did not display any changes in blood biochemistry compared with age-matched FVB mice until the age of 12 months, when the plasma concentration of the urea significantly increased, whereas the total cholesterol and HDL cholesterol plasma levels modestly decreased in Tg\textit{a}q*44 mice compared with the levels in FVB mice (Table 1).

Figure 1 Peripheral vascular endothelial dysfunction with the progression of HF in Tg\textit{a}q*44 mice compared with age-matched FVB mice. In vivo changes in the end-diastolic volume of the abdominal aorta 25 min after Ach administration (n = 4–6) (A). Changes in endothelial permeability described as Npx50 value, after injection of gadolinium-rich liposome contrast agent (brachiocephalic artery permeability BCA-PER) (n = 5–6) (B). In vivo changes in the volume of the femoral artery in response to flow-mediated vasodilation (FA-FMD) after 5-min vessel occlusion (n = 5–6) (C). \textit{Ex vivo}: Relaxation of the aorta rings in response to increasing doses of Ach (D–G) and SNP (H–K) (n = 6–8) in 4-, 8-, 10-, and 12-month-old Tg\textit{a}q*44 mice vs. age-matched FVB control mice. Normality was assessed using a Shapiro–Wilk test. Results are presented as box plots (median, Q1, Q3, whiskers indicate minimum/maximum), Q1 and Q3 indicate the 25th and 75th percentiles, respectively (A–C), mean ± SEM (D–K). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, Tg\textit{a}q*44 mice and age-matched FVB control mice compared using two-way ANOVA with post hoc Sidak test. SNP, sodium nitroprusside.
Table 1 Body mass, blood count, lipid profile, and NO metabolism in Tg\(a^*44\) mice compared with age-matched FVB mice

|                  | 4 months          | 8 months          | 10 months         | 12 months         |
|------------------|-------------------|-------------------|-------------------|-------------------|
| **Body mass (g)**| FVB 24.21 ± 1.47  | Tg\(a^*44\) 25.309 ± 1.391 | FVB 27.28 ± 2.46  | Tg\(a^*44\) 28.65 ± 2.28 |
| **Biochemical parameters** |                  |                   |                   |                   |
| TC (mmol/L)       | FVB 2.680 ± 0.271 | Tg\(a^*44\) 3.019 ± 0.366 | FVB 2.41 ± 0.29   | Tg\(a^*44\) 2.84 ± 0.66 |
| HDL (mmol/L)      | FVB 1.07 ± 0.11   | Tg\(a^*44\) 1.263 ± 0.192 | FVB 0.97 ± 0.14   | Tg\(a^*44\) 1.18 ± 0.24 |
| LDL (mmol/L)      | FVB 0.2 ± 0.02    | Tg\(a^*44\) 0.197 ± 0.021 | FVB 0.2 ± 0.06    | Tg\(a^*44\) 0.18 ± 0.08 |
| TG (mmol/L)       | FVB 2.03 ± 0.42   | Tg\(a^*44\) 2.581 ± 0.847 | FVB 3.02 ± 0.780  | Tg\(a^*44\) 2.73 ± 1.485 |
| Creatinine (\(\mu\)mol/L) | FVB 20.16 ± 3.93 | Tg\(a^*44\) 18.471 ± 2.766 | FVB 21.31 ± 3.76  | Tg\(a^*44\) 17.62 ± 3.65 |
| Urea (mmol/L)     | FVB 6.77 ± 2.06   | Tg\(a^*44\) 7.753 ± 0.99 | FVB 8.50 ± 0.73  | Tg\(a^*44\) 8.84 ± 1.12 |
| **Blood count**   |                   |                   |                   |                   |
| WBC (K/\muL)      | FVB 3.61 ± 0.659  | Tg\(a^*44\) 3.291 ± 1.075 | FVB 4.02 ± 1.254  | Tg\(a^*44\) 4.02 ± 1.701 |
| PLT (K/\muL)      | FVB 1209.85 ± 102.25 | Tg\(a^*44\) 1244.273 ± 95.289 | FVB 1159.08 ± 120.86 | Tg\(a^*44\) 1298.75 ± 160.3 |
| RBC (M/\muL)      | FVB 9.59 ± 0.413  | Tg\(a^*44\) 10.081 ± 0.448 | FVB 9.811 ± 0.246 | Tg\(a^*44\) 9.851 ± 0.330 |
| HGB (g/dL)        | FVB 14.21 ± 0.705 | Tg\(a^*44\) 14.453 ± 0.611 | FVB 14.91 ± 0.434 | Tg\(a^*44\) 14.3 ± 0.47 |
| HCT (%)           | FVB 52.284 ± 2.388 | Tg\(a^*44\) 54.2 ± 2.603 | FVB 54.133 ± 1.444 | Tg\(a^*44\) 53.1 ± 1.82 |
| MCHC (g/dL)       | FVB 27.185 ± 0.40 | Tg\(a^*44\) 26.67 ± 0.41 | FVB 27.53 ± 0.72  | Tg\(a^*44\) 26.89 ± 0.39 |
| **No metabolism** |                   |                   |                   |                   |
| NO\(_2\) (\(\mu\)M) | FVB 2.09 ± 0.46   | Tg\(a^*44\) 2.03 ± 0.51 | FVB 1.21 ± 0.52   | Tg\(a^*44\) 1.20 ± 0.18 |
| NO\(_3\) (\(\mu\)M) | FVB 30.71 ± 11.23 | Tg\(a^*44\) 30.63 ± 8.85 | FVB 14.70 ± 10.09 | Tg\(a^*44\) 11.22 ± 2.91 |
| HbNO (arb u)      | FVB 602.85 ± 117.14 | Tg\(a^*44\) 448.06 ± 54.994 | FVB 335.35 ± 135.2 | Tg\(a^*44\) 230.62 ± 103.19 |

*Body mass (n = 9–13), blood biochemistry (n = 7): TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglycerides; creatinine, and urea. Blood count (n = 9–13): WBC, white blood cells; PLT, platelets; RBC, red blood cells; HGB, haemoglobin; HCT, haematocrit; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration. NO metabolism (n = 5–13): Nitrite (NO\(_2\)), Nitrate (NO\(_3\)) concentration, HbNO, nitrosyl haemoglobin in 4-, 8-, 10-, and 12-month-old Tg\(a^*44\) mice vs. age-matched FVB controls mice. Normality was assessed using a Shapiro–Wilk test. Results are presented as means ± SD. Tg\(a^*44\) mice and age-matched FVB controls compared using two-way ANOVA with post hoc Sidak test or non-parametric Kruskal–Wallis test (NO\(_2\) and NO\(_3\)).

*P<0.05,
**P<0.01,
***P<0.001,
****P<0.0001.
Figure 2 Alterations in NO/superoxide balance, eicosanoid, TNFα and IL-1β gene expression in the aorta with the progression of HF in Tgαq*44 mice compared with age-matched FVB mice. NO production in the isolated aorta (n = 5–13) (A), O₂⁻ production in aortic rings (n = 5–13) (B), basal production of eicosanoid (n = 5–6) 6-keto PGF₁α (C), PGE₂ (D), PGD₂ (E), and 15-HETE (F) detected in the effluent after 45 min of incubation of isolated aortic rings. TNF (G) and IL-1β (H) gene expression (n = 5–6) in the aorta in 4-, 8-, 10-, and 12-month-old Tgαq*44 mice vs. FVB controls. Normality was assessed using a Shapiro–Wilk test. Results are presented as box plots (median, Q1, Q3, whiskers indicate minimum/maximum). Q1 and Q3 indicate the 25th and 75th percentiles, respectively. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, Tgαq*44 mice compared with age-matched FVB controls using two-way ANOVA with post hoc Sidak test. ; IL-1β, interleukin-1β; NO, nitric oxide; TNFα, tumour necrosis factor alpha.
in 12-month-old Tgαq*44 mice compared with that in age-matched FVB mice (Figure 3D).

Total glutathione, GSH, and GSSG in RBCs did not show statistically significant differences between Tgαq*44 mice and FVB mice (data not shown), but the GSH/GSSG ratio was significantly lower in 12-month-old Tgαq*44 mice compared with that in age-matched FVB mice (Figure 3E). RBC deformability measured at a high shear stress (20 Pa) displayed a marked decrease in 12-month-old
Tgαq*44 mice compared with that in age-matched FVB mice (Figure 3F).

3.6 Characterization of alterations in topography and nanomechanics of RBCs in Tgαq*44 mice by AFM

The examples of RBC images in Figure 4 reflect high variability of RBC shape during HF progression, from a normal biconcave shape to discoocytes and spherocytes with irregular symmetry. In the control sample from 4-month-old FVB mice, a characteristic biconcave or doughnut shape of RBCs was observed. In 12-month-old FVB mice (Figure 4B), the deformation of the blood cells was manifested by a slight loss of symmetry in the height of the blood cells. In Tgαq*44 mice, more pronounced changes in RBC shape were observed (Figure 4C–H). In the youngest mice, a change in the shape of the RBCs was manifested either by an increase in the central part of the blood cell or a large change in the RBC height profile symmetry. In 8-month-old Tgαq*44 mice, significant cell deformation resulting in aspect ratio modification and diminished biconcave shape were noted. These changes were even more pronounced in 12-month-old Tgαq*44 mice.

The quantitative results of the aspect ratio are presented in Figure 5A, and the measured cell elastic moduli are presented in Figure 5B. In 8-, 10-, and 12-month-old Tgαq*44 mice, both parameters were significantly increased compared with the parameters in age-matched control mice. In 4- and 6-month-old Tgαq*44 mice, the differences did not reach statistical significance. Interestingly, there was a significant negative correlation between endothelial function (measured in vivo by MRI) and RBC elasticity (measured by AFM) in Tgαq*44 mice, and this correlation was not observed in FVB mice (Figure 5C). As shown in Figure 5D–F, the increased RBC aspect ratio was correlated with increased stiffness in both Tgαq*44 and FVB mice along with aging; however, Tgαq*44 mice displayed a marked shift towards a higher aspect ratio and a higher elastic moduli value.

3.7 Effects of RBCs isolated from Tgαq*44 mice during early and end-stage HF on endothelium-dependent vasodilation, ex vivo measurements

As shown in Figure 5G, RBCs isolated from 12-month-old Tgαq*44 mice that were coincubated with aortic rings induced the impairment of endothelium-dependent vasodilation, whereas endothelial-independent vasodilation was preserved (Figure 5H). In contrast, RBCs isolated from 12-month-old FVB mice did not impair the endothelial function in the aortic rings. Interestingly, in the presence of ABH (100 μM), an inhibitor of arginase, the detrimental effect of RBCs taken from 12-month-old Tgαq*44 mice on endothelial function in the aorta was prevented. The effect of ABH was significant only for the highest concentration of Ach (10 μM), but there were no effects of ABH on SNP-induced relaxation (Figure 5I and J).

3.8 Characterization of alterations in biochemical contents of RBC membranes in Tgαq*44 mice by vibrational spectroscopy

In the RBC membranes of Tgαq*44 mice compared with age-matched FVB mice, FTIR-based analysis revealed that stretching...
vibrations of the =CH groups (band at 3013 cm⁻¹) and antisymmetric stretching vibrations of the PO₂⁻ groups (band at 1235 cm⁻¹) differed significantly, indicating a reduction in phospholipid content (Figure 6A) and a decrease in the unsaturation of membrane lipids (Figure 6D). These changes were statistically significant in 4-month-old Tgαq*44 mice compared with age-matched FVB mice, and a similar degree of difference was observed in 12-month-old Tgαq*44 mice compared with FVB mice. RS and FTIR did not reveal changes in proteins fraction (Figure 6C), total lipids to proteins ratio (Figure 6E) or the fraction of esters (Figure 6F), including cholesterol esters (Figures 6B) in RBC membranes obtained from 4- and 12-month-old Tgαq*44 compared with age-matched FVB mice.

4. Discussion

In the present work, we used a comprehensive methodology to assess the endothelial and RBC functional status and characterize the temporal relationships between the development of ED and the development of erythropathy in Tgαq*44 mice, a unique murine model of CHF with a prolonged time course of the HF progression. In contrast to most animal models, in which the transition from compensated to uncompensated CHF is relatively rapid, the Tgαq*44 murine model is characterized by a delayed progression to end-stage HF.24–26,44 The prolonged window of HF progression in Tgαq*44 mice from adaptive to end-stage HF allowed us to discover that RBC alterations occurred very early in HF pathophysiology and progressed substantially with HF progression. In particular,
HF-linked erythropathy in Tgαq*44 mice accelerated substantially and was correlated with the progression of systemic ED. Increased RBC stiffness assessed by AFM was correlated with impaired endothelial function assessed in vivo by MRI. Finally, RBCs in late-stage erythropathy induced ED when coincubated with aorta samples from FVB mice, whereas RBCs in early erythropathy stages did not. These results suggest a possible reciprocal relationship between RBC alterations and endothelial function in HF: systemic ED accelerates erythropathy and, conversely, erythropathy may contribute to ED. Such a reciprocal relationship was previously postulated to occur in other diseases but was not previously characterized in HF.

The major advantage of our experimental approach was that we assessed endothelial function by the application of an MRI-based method in vivo, a method that was validated in our previous studies. This approach appeared to be more sensitive for detecting the early phase of ED (in 8-month-old Tgαq*44 mice) compared with classical isolated vessel studies ex vivo (in 10-month-old Tgαq*44 mice) and the direct measurement of NO/O2•− balance (in 12-month-old Tgαq*44 mice). Using our comprehensive methodological approach, we demonstrated that systemic ED was present in 8-month-old Tgαq*44 mice (impaired Ach-induced vasodilation and increased endothelial permeability in vivo) and progressed further in 10-month-old Tgαq*44 mice, as evidenced by impaired Ach-induced vasodilation with preserved SNP response in the aorta ex vivo. Furthermore, in 12-month-old Tgαq*44 mice, impaired NO production and increased O2•− production, phenomena that have been reported previously in HF, were observed through biochemical measurements of the aorta.

Interestingly, in contrast to the impaired endothelial function in the aorta, the flow-mediated vasodilation in the femoral artery (FMD response in the FA) in vivo seemed to be largely preserved until end-stage HF. Previous studies have demonstrated heterogeneity in the response of the aorta and the FA in ageing and diabetes. As age increased, relaxation in response to Ach was reduced in the aorta, whereas in the FA response was fully preserved. In addition, in db/db mice, the vasodilatory response to Ach was impaired in the FA but not in the aorta. Finally, in a model of HF induced by myocardial infarction FMD in the FA was heavily impaired, while the response to Ach was only moderately affected. Comparing the results of these studies with our findings underscores the heterogeneous response of the endothelium to HF progression in the conduit vessels in ischaemic and non-ischaemic HF and reveals a difference in response depending on the vascular bed and stimulus used.

Interestingly, impaired Ach-induced NO-dependent vasodilation in the aorta was associated with a progressive reduction in plasma concentration of nitrate (8- to 12-month-old Tgαq*44 mice), whereas the concentration of nitrite remained unchanged, which suggests the activation of the nitrate–nitrite–NO reductive pathway, an alternative compensatory source that maintains NO bioavailability. However, as the HbNO content in RBCs substantially decreased in 12-month-old Tgαq*44 mice.
in late-stage HF in this model, the systemic NO bioavailability eventually fell substantially despite the activation of the nitrite reductive pathway.

Concomitantly, in 12-month-old Tgqaq*44 mice, ED was characterized by increased generation of cyclooxygenase-derived eicosanoids, such as PGD2 and PGI2 (assessed as its metabolite 6-keto PGF1α), which may play a compensatory role.50 In turn, increased vascular PGE2 could contribute to vascular inflammation and ED.37

The comprehensive nature of RBC analysis based on the numerous methods, including AFM and vibrational spectroscopy, adapted in the present work allowed us to obtain unprecedented insight into structural, functional, nanomechanical, and biochemical changes in RBCs related to the progression of ED in HF. We demonstrated that RBCs displayed mild alterations as early as in 4 months old Tgqaq*44 mice. These changes included a reduction in MCV, suggesting early RBC anisocytosis in HF; a slightly altered aspect ratio (assessed by AFM topography), indicating an early RBC shape alteration; and a decrease in phospholipid content and unsaturated lipids in RBC membranes (assessed by vibrational spectroscopy), implying early RBC membranes changes. Still, these changes did not result in statistically significant alterations in the nanomechanics or deformability of RBCs at this stage of HF. However, even at this HF stage, some RBCs displayed notable changes in elasticity. Notably, several parameters of erythropoiesis were significantly accentuated in 8-month-old Tgqaq*44 mice, including structural changes (increased AFM-based aspect ratio indicative of the presence of ellipsoidal RBCs) and nanomechanical alterations (increased RBC elasticity modulus). In 12-month-old Tgqaq*44 mice, HF-linked erythropathy was characterized by severely altered RBC shape and elasticity but also by increased RDW, impaired RBC deformability, and oxidative stress (GSH/GSSG ratio). Then, with HF progression erythropathy involved altered shape and elasticity (AFM topology and nanoindentation), altered biochemical RBC homeostasis (oxidant stress), and, finally, impaired deformability of RBCs tested using the clinically used Rheoscan system (see Figure 7). Of note, alterations in RBC status based on Rheoscan, GSH/GSSG, and RDW parameters were detected at very late stages of erythropathy, in contrast to alterations in RBC membranes and changes in their nanomechanical profile detected in the early phase of HF. Interestingly, alterations in the
biochemical profile of RBC membranes and the size of RBCs shown here displayed a distinct pattern compared with atherosclerosis, or HF of ischaemic origin. In a previous study by our group using ApoE/LDLR-/- mice, phospholipids and unsaturated lipids decreased with the progression of atherosclerosis; however, the total lipid content and MCV was higher, without significant differences in RDW, in ApoE/LDLR-/- mice compared with age-matched controls. In the current study, levels of phospholipids and lipid unsaturation in RBC membranes were significantly lower even in the early phase of HF in 4- and 6-month-old Tgq*44 mice, without changes in total lipid content. MCV values were reduced and further decreased with the progression of HF, whereas a significant increase in RDW values was observed in the late stage of HF in 12-month-old Tgq*44 mice only. These results agree with the accepted notion that the modification of the lipid composition in the RBC membranes results in changes in their shape and elasticity. Furthermore, our results revealed that HF-related erythropathy displayed different types of RBC alterations compared with atherosclerosis-related changes.

Most importantly, our results showed that HF-linked erythropathy in Tgq*44 mice was temporally linked with the progression of ED. Moreover, the progression of erythropathy seems to be correlated with progressive impairment of endothelial function (alterations in RBC elasticity were correlated with progressive impairment of Ach-induced NO-dependent vasodilation in the aorta).

Of note, in 8-month-old Tgq*44 mice, a severe pattern of HF-linked erythropathy was present. This stage of HF in Tgq*44 mice was characterized by the presence of ED, whereas cardiac function was relatively compensated compared with late-stage HF in 12-month-old Tgq*44 mice, which was characterized by severe impairment of basal cardiac function, cardiac reserve, and exercise capacity. Taken together, our findings support the notion that endothelial function plays a key role in maintaining RBC haemostasis. As such, systemic ED may accelerate erythropathy. However, erythropathy has detrimental effects on endothelial function in other diseases. This study also provided evidence supporting such a possibility. In advanced erythropathy, RBCs taken from 12-month-old Tgq*44 mice and co-cultured with isolated aorta rings induced impairment in endothelium-dependent relaxation in the ex vivo assay, and this effect was partially prevented by the inhibition of arginase. Of note, a similar experimental approach was previously used to show the contribution of diabetic erythropathy to the development of ED in diabetes. Interestingly, Pernow et al. discovered that the activation of arginase I in RBCs contributed to the development of ED in diabetes. Arginase in RBCs was also suggested to mediate ED associated with pre-eclampsia.

Interestingly, in our study, ABH (an inhibitor of arginase types I and II) prevented RBC-induced impairment of endothelial function. This effect was, however, only detectable for the highest concentration of Ach. Pernow et al. show that murine RBC expresses only arginase type I, but not arginase type II. Thus, our data suggest that impaired NO-dependent function in Tgq*44 mice may be partially due to arginase I activity in RBC, but of course, this is just one of a number of mechanisms that could contribute to systemic ED in HF. Also, erythropathy could contribute to ED by mechanisms independent on arginase.

Taken together, there seems to be a reciprocal relationship between endothelial function and RBC haemostasis in HF as suggested previously for diabetes. Systemic ED accelerates erythropathy and vice versa, erythropathy may contribute to ED. These results suggest that erythropathy may be regarded as a marker and a mediator of systemic ED in HF. Furthermore, RBC arginase and possibly other RBC-mediated mechanisms may represent novel therapeutic targets for systemic ED in HF.

Previous studies showed that RBCs express functional eNOS protein, which is closely correlated with FMD in humans, confirming a strong association between RBC functional status and endothelial function. Indeed, changes in RBC membranes could lead to increased RBC adhesion to endothelial cells for example, in diabetes, malaria, and haemoglobinopathies. Increased RBC adhesion to the endothelium may lead to impaired tissue microcirculation. Furthermore, amylin deposited on RBCs or myeloperoxidase activation on the RBC surface could also contribute to oxidative stress and impairment of NO-dependent relaxation. It remains to be established whether any of these RBC-mediated mechanisms also contribute to ED in HF. Moreover, previous studies of HF-linked erythropathy, defined as RDW, predicted a poor prognosis in patients with clinical HF. Interestingly, it was suggested that HF-linked erythropathy was independent of systemic inflammation, kidney function, and numerous other studied variables, including NT-proBNP (a good biomarker of HF progression), leaving the underlying mechanisms of the relationship between RDW and HF unclear. In agreement with these findings in Tgq*44 mice, systemic inflammation, erythropoietin, and kidney failure are also unlikely mechanisms of HF-linked erythropathy because the first two were not altered in 4-, 8-, and 12-month-old Tgq*44 mice (results not shown) and the latter was a late phenomenon, as evidenced by the fact that the plasma urea concentration increased only in 12-month-old Tgq*44 mice.

Our study has several limitations. As in previous work using this model, only female mice were used, so we cannot be sure whether the findings of this work are also relevant to male Tgq*44 mice. To confirm the detrimental effects of RBCs on endothelial function ex vivo, the isolated aorta was used similarly to in methodology described previously, but we cannot be sure to what extent RBCs contribute to ED in HF in vivo since ex vivo studies are not able to fully mimic the in vivo setting. Finally, although we provided experimental evidence that RBCs in advanced erythropathy induce impaired endothelial function, and we postulated arginase in RBC to be partially involved, further experiments with more selective arginase inhibitors and tissue-specific arginase knock-out are mandatory to delineate in detail the importance of arginase in impaired NO-dependent function in Tgq*44 mice.

In conclusion, HF-linked erythropathy in Tgq*44 mice involved progressive alterations on functional, structural, nanomechanical, and biochemical levels. The temporal relationship and correlation between the progression of HF-linked erythropathy and the progression of impairment of vascular NO-dependent function in Tgq*44 mice suggest that erythropathy may be a marker and a mediator of vascular dysfunction in HF. To the best of our knowledge, this relationship was not demonstrated previously in the context of HF, despite a wealth of evidence that RDW has prognostic significance in HF. Surprisingly, HF represents a disease in which targeting RBCs may represent a novel treatment modality to reverse systemic ED.

Authors’ contributions

T.M. and S.C. conceived and designed the study. T.M., B.P., M.T.-K., A.Bar, A.K., K.B., A.W., A.Bl, K.M.-G., M.G., A.T., M.S., R.R.-D., K.W.-L., and S.C. wrote the draft of the manuscript. K.M.M. and A.B. critically revised the manuscript. A.Kubi analysed the data. T.M. and M.T.-K. prepared the figures. T.M. and A.Bar, A.Bl, K.M.-G., M.G., A.T., M.S., R.R.-D., K.W.-L., and A.Kubi performed experiments. T.M., B.P., M.T.-K., A.Bar, A.W., and A.Bl analysed the data. T.M. and M.T.-K. prepared the figures. T.M. and S.C. wrote the draft of the manuscript. K.M.M. and A.B. critically revised the manuscript for important intellectual content. K.M.M. edited and revised the manuscript. T.M. and S.C. wrote the final version of the manuscript. All authors read and approved the final version of the manuscript.

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Data availability

The data underlying this article are available on reasonable request to the corresponding author.

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Endothelial dysfunction and erythropoiesis in heart failure

Translational perspective

Endothelial dysfunction (ED) and red blood cell (RBC) distribution width both have prognostic value for heart failure (HF), but it is not known whether these pathologies are related. We comprehensively characterized endothelial and RBC functional status in a unique murine model of chronic HF with a prolonged time course of HF progression. Our results suggest that ED accelerates erythropoiesis and, conversely, erythropoiesis may contribute to ED. Accordingly, erythropoiesis in HF reflects ED and involves various changes (in functional, structural, nanomechanical, and biochemical levels) that could have diagnostic and therapeutic significance for HF.