Erythrocytes are altered in pulmonary arterial hypertension

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To the Editor:

Erythrocyte abnormalities have been demonstrated to be associated with increased risk of cardiovascular disease development and severity, affecting morbidity and mortality [1, 2].

In particular, increased values of one of the automated complete blood count analysis components that quantifies heterogeneity in erythrocyte size, called red cell distribution width (RDW), has been shown to predict survival in pulmonary arterial hypertension (PAH), a rare and severe cardiopulmonary condition for which there is no cure at this time [3–7].

Besides the current knowledge, it is still not known whether erythrocyte abnormalities, other than RDW, are present in PAH patients and whether changes in erythrocytes are clinically relevant. We are questioning here whether erythrocytes may be altered in PAH patients and could represent a key cell type as biomarker of disease onset and/or severity. To address this issue, we studied freshly isolated erythrocytes from PAH patients and controls, as well as in the monocrotaline (MCT)-induced experimental model of pulmonary hypertension (PH) [8].

35 PAH patients and 35 anonymous healthy subjects from the French national blood centre (*Etablissement Français du Sang*) were included in our study, after signed informed consent. Adult patients with a diagnosed pre-capillary PAH (idiopathic or heritable) were eligible for inclusion if they had a stable clinical and haemodynamic status for the previous 3 months [7]. Exclusion criteria were a moderate to severe anaemia (haemoglobin <10 $g \cdot dL^{-1}$), haemoglobinopathies or any other haematological disorders. Our study complied with the Declaration of Helsinki and was approved by the local ethics committee (*Comité de Protection des Personnes Ile-de-France VII*). The data collected were anonymised, complying with the requirements of the organisation dedicated to privacy, information technology and civil rights (*Commission Nationale de l'Informatique et des Libertés*; approval number 842063). Characteristics at diagnosis and follow-up were stored in the French PH network registry (*PulmoTension*). Characteristics and variables related to PAH (physical examination, routine blood tests, New York Heart Association (NYHA) functional class, 6-min walk distance (6MWD) and right heart catheterisation were performed at the same day of blood withdrawal. Non-invasive risk assessment was performed according to the 2015 PH guidelines and low-risk criteria were defined by: 1) NYHA functional class I or II, 2) 6MWD >440 m and 3) brain natriuretic peptide (BNP) <50 $ng \cdot L^{-1}$ or N-terminal proBNP <300 $ng \cdot L^{-1}$, as previously described [9]. Results are expressed as mean±SEM.

The results of the complete automated blood cell count showed no abnormalities, except for the RDW, as shown by others. More specifically, in our study, a third of our PAH population displayed an increased RDW value, namely >14.5%, and these "high RDW" patients were in NHYA class II to IV (figure 1a). Importantly, we randomly performed peripheral smears in PAH patients and the haematological analysis of these smears excluded morphological abnormalities, independently of the RDW value (data not shown). We evaluated non-invasive risk assessment and our data clearly showed that patients with three low risk criteria display a statistically lower RDW than patients with two or one criteria (figure 1b). Interestingly, haemodynamics were more severe in the so-called high RDW PAH patients with a significantly higher mean pulmonary artery pressure (mPAP) and pulmonary vascular resistances (PVR) compared to "normal RDW" PAH patients (figure 1c and d).

Erythrocytes represent a unique anucleate cell type containing a large panel of proteins that interact with each other and that are responsible for the physiological functions needed to cross the vascular bed and

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Erythrocytes display structural and functional intrinsic defects in patients with pulmonary arterial hypertension. Their clinical relevance as potential biomarkers and their contribution in the disease pathophysiology still need to be defined. https://bit.ly/3iDYFOe

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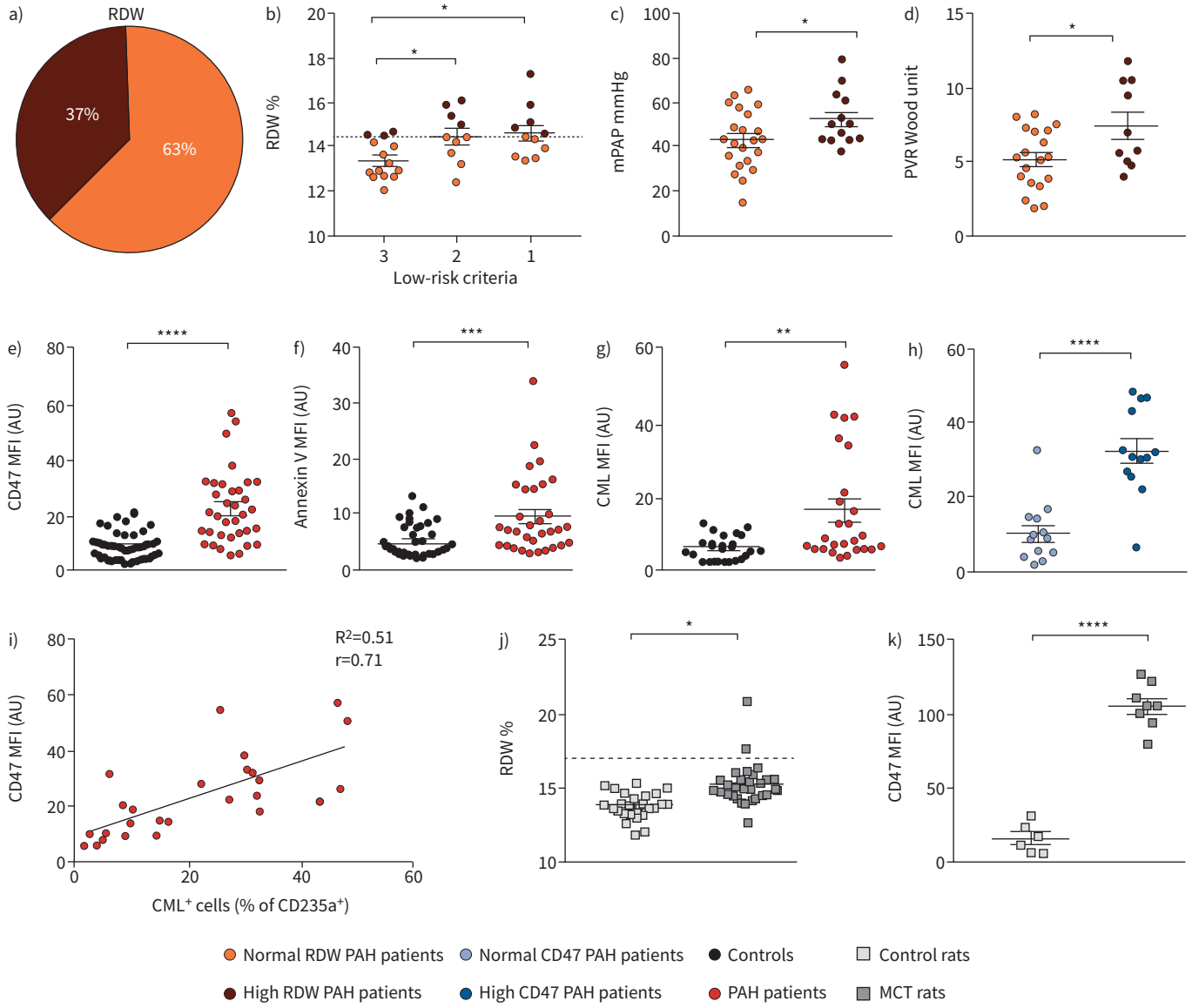

FIGURE 1 Erythrocytes alterations in pulmonary arterial hypertension. a) Red cell distribution width (RDW) value in pulmonary arterial hypertension (PAH) patients and the percentage of "normal RDW" patients (RDW <14.5%) and "high RDW" patients (RDW >14.5%). b) RDW in PAH patients based on the number of low-risk criteria (namely New York Heart Association functional class, 6-min walk distance and brain natriuretic peptide (BNP)/N-terminal proBNP). c) Mean pulmonary arterial pressure (mPAP) and d) pulmonary vascular resistance (PVR) in normal RDW patients (RDW <14.5%) and high RDW patients (RDW >14.5%). e) CD47 mean fluorescence intensity (MFI), f) annexin V MFI (reflecting phosphatidylserine exposure) and g) Nε-(carboxymethyl) lysine (CML) MFI in control subjects and in PAH patients. h) CML MFI in normal CD47 PAH patients (CD47 MFI <20.8 AU) and high CD47 PAH patients (CD47 MFI >20.8 AU); 20.8 AU is the median value for CD47 MFI. i) Correlation between CD47 MFI value and CML production in PAH patients. j) RDW value and k) CD47 MFI in control rats and in monocrotaline (MCT)-injected rats (40 mg·kg^{-1}). Flow cytometry gating conditions and MFI were set and normalised, respectively, against isotype- and fluorophore-matched non-immune IgGs. Flow cytometry data were acquired with a flow cytometer (MACSQuant) and analysed by FlowJo software program (Tree Star, Inc.). A p<0.05 level of statistical significance was used for all analyses (GraphPad Prism version 5.0, GraphPad Software Inc.). Differences between two selected groups were compared using unpaired t-test. All between-group comparisons were assessed using one way ANOVA and *post hoc* analysis of significant variables was performed using Tukey correction with all pairwise multiple comparisons. Pearson correlations were used to establish associations between dependent variables. *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001.

perform oxygen delivery. The aim of this study was to analyse intrinsic defects of erythrocytes, other than RDW: we studied one of the main membrane proteins, namely CD47, the exposure of phosphatidylserine (PS) on the outer monolayer of the erythrocytes membrane, and the production of oxidative stress by erythrocytes. Erythrocytes are known to produce oxidative stress through different compounds, in particular

via advanced glycated end-products (AGEs), a group of modified proteins and/or lipids with damaging potential, increasing reactive oxygen species formation and impairing antioxidant systems. After blood withdrawal in EDTA or ACD, erythrocytes were stained with fluorescently labelled antibodies, under non-permeabilised or permeabilised conditions to enable surface or intracellular staining, respectively. Human erythrocytes were defined as $CD235a^+$ cells, the erythrocyte specific surface marker, also called glycophorin A.

We found that CD47 mean fluorescent intensity (MFI), PS exposure and the best characterised AGE, Nε-(carboxymethyl) lysine (CML), MFI were greatly enhanced in PAH patients compared to controls (figure 1e–g). Since CD47 displayed a two-fold increase in MFI (9.7±0.9 *versus* 23.0±2.2 AU in controls and PAH, respectively; $p<0.0001$), we distinguished two populations: a "normal CD47" group, who displayed a CD47 MFI below the median value of MFI and a "high CD47" group, with CD47 MFI above the median value (20.8 AU) (figure 1h). Interestingly, in PAH patients, the high CD47 group produced three times more CML than the normal CD47 group (32.5±3.2 *versus* 10.4±2.8 AU, respectively; $p<0.01$) and CD47 expression correlated with CML production in PAH erythrocytes ($r=0.71$) (figure 1i).

Interestingly, we confirmed these results *in vivo*: we found that RDW value and CD47 MFI were greatly increased in MCT-injected rats compared to controls (approved by the animal ethics committee of the University Paris-Saclay, France) (figure 1j and k).

In this study, we characterised erythrocyte intrinsic defects in PAH patients and showed that erythrocytes are structurally and functionally altered in PAH, as reflected by anisocytosis (increased RDW), abnormal presence of trans-membrane protein (CD47), enhanced external PS exposure and by enhanced production of oxidative stress (CML) compared to healthy controls. Interestingly, patients with higher anisocytosis were more severe (increased mPAP and PVR) and those with higher CD47 membrane fluorescence intensity on erythrocytes produced more oxidative stress.

Based on our study and on data from the literature, our results point to a disruption of erythrocytes homeostasis in PAH patients. The anisocytosis that we are observing in PAH patients could be explained by an increased translocation of PS on the outer membrane, usually expressed on the membrane inner leaflet, suggesting erythrocyte senescence. In fact, this asymmetric structure of lipid bilayer anchored to the membrane skeleton has important physiological consequences.

Interestingly, CD47 (or integrin-associated protein) appears to be of particular importance as it functions as a marker of self on erythrocytes [10]. The presence of CD47 on erythrocyte surfaces prevents elimination by splenic red pulp macrophages, by binding to the inhibitory receptor signal regulatory protein (SIRP) alpha, referred as a "don't eat signal" for macrophages [10]. As erythrocytes begin the ageing process, CD47 expression is reduced or the interaction with SIRP alpha is disturbed by a conformational change in CD47 that switches the molecule from an inhibitory signal into an activating one. Thus, instead of being removed by macrophages, the erythrocytes with a reduced loss of CD47 lead to a decreased clearance from the bloodstream and may cause an exaggerated production of oxidative stress [11]. From the literature, it is well known that senescent erythrocytes produce more AGEs that bind to their receptors on endothelium, leading to nuclear factor κB activation in the systemic and pulmonary vasculature [11–13]. Erythrocyte-endothelial interaction is especially pronounced in the lung microvasculature and, besides AGEs, altered erythrocytes are also able to directly induce inflammation and pulmonary wall cell dysfunction [14], possibly contributing to PAH pathogenesis. Interestingly, the level of AGEs produced by erythrocytes, represented by glycosylated haemoglobin at time of diagnosis, has been shown to be an independent predictor of long-term prognosis in non-diabetic PAH patients [15].

We show here that erythrocytes are altered in PAH patients but further studies are needed to investigate the mechanisms underlying these abnormalities and to precise their clinical relevance as potential biomarkers, as well as their contribution in PAH pathophysiology.

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References

1 Felker GM, Allen LA, Pocock SJ, *et al.* Red cell distribution width as a novel prognostic marker in heart failure: data from the CHARM Program and the Duke Databank. *J Am Coll Cardiol* 2007; 50: 40–47.
2 Tonelli M, Sacks F, Arnold M, *et al.* Relation between red blood cell distribution width and cardiovascular event rate in people with coronary disease. *Circulation* 2008; 117: 163–168.
3 Rhodes CJ, Wharton J, Howard LS, *et al.* Red cell distribution width outperforms other potential circulating biomarkers in predicting survival in idiopathic pulmonary arterial hypertension. *Heart* 2011; 97: 1054–1060.
4 Hampole CV, Mehrotra AK, Thenappan T, *et al.* Usefulness of red cell distribution width as a prognostic marker in pulmonary hypertension. *Am J Cardiol* 2009; 104: 868–872.
5 Ulrich A, Wharton J, Thayer TE, *et al.* Mendelian randomisation analysis of red cell distribution width in pulmonary arterial hypertension. *Eur Respir J* 2020; 55: 1901486.
6 Smukowska-Gorynia A, Tomaszewska I, Malaczynska-Rajpold K, *et al.* Red blood cells distribution width as a potential prognostic biomarker in patients with pulmonary arterial hypertension and chronic thromboembolic pulmonary hypertension. *Heart Lung Circ* 2018; 27: 842–848.
7 Simonneau G, Montani D, Celermajer DS, *et al.* Haemodynamic definitions and updated clinical classification of pulmonary hypertension. *Eur Respir J* 2019; 53: 1801913.
8 Humbert M, Guignabert C, Bonnet S, *et al.* Pathology and pathobiology of pulmonary hypertension: state of the art and research perspectives. *Eur Respir J* 2019; 53: 1801887.
9 Boucly A, Weatherald J, Savale L, *et al.* Risk assessment, prognosis and guideline implementation in pulmonary arterial hypertension. *Eur Respir J* 2017; 50: 1700889.
10 Oldenborg PA, Zheleznyak A, Fang YF, *et al.* Role of CD47 as a marker of self on red blood cells. *Science* 2000; 288: 2051–2054.
11 Mangalmurti NS, Chatterjee S, Cheng G, *et al.* Advanced glycation end products on stored red blood cells increase endothelial reactive oxygen species generation through interaction with receptor for advanced glycation end products. *Transfusion* 2010; 50: 2353–2361.
12 Park L, Raman KG, Lee KJ, *et al.* Suppression of accelerated diabetic atherosclerosis by the soluble receptor for advanced glycation endproducts. *Nat Med* 1998; 4: 1025–1031.
13 Mangalmurti NS, Friedman JL, Wang LC, *et al.* The receptor for advanced glycation end products mediates lung endothelial activation by RBCs. *Am J Physiol Lung Cell Mol Physiol* 2013; 304: L250–L263.
14 Huertas A, Das SR, Emin M, *et al.* Erythrocytes induce proinflammatory endothelial activation in hypoxia. *Am J Respir Cell Mol Biol* 2013; 48: 78–86.
15 Belly MJ, Tiede H, Morty RE, *et al.* HbA1c in pulmonary arterial hypertension: a marker of prognostic relevance? *J Heart Lung Transplant* 2012; 31: 1109–1114.