Supplemental analysis:

Simulations of MCV time course during and following Epo administration

Abstract

Our work shows that EpoR signaling increases erythroblast cell size at every stage of erythroid terminal differentiation, leading to the formation of larger reticulocytes. Two human intervention studies in which Epo is administered to healthy volunteers show an increase in MCV which could not be attributed to reticulocytosis, suggesting that Epo/EpoR signaling also increases red cell (RC) size in human erythropoiesis. However, an alternative explanation is that the observed increase in MCV is the result of skewing of the circulating RC pool in favor of younger, larger RCs, since RCs loose volume with aging. Here we consider this alternative possibility quantitatively. We simulate changes in MCV that would be predicted based on published parameters for the rate of volume loss during reticulocyte maturation and RC aging, including the early release of immature reticulocytes into the circulation in response to high Epo. Our simulation suggests that these factors can only account for 30 to 50% of the overall increase in MCV observed in the human studies. Therefore, additional factors, including EpoR signaling, contribute to the observed increase in cell size in response to Epo.

I Introduction

In human intervention studies, we found that Epo administration led to increased MCV. Contrary to expectation, there was no correlation between MCV and reticulocyte count (Extended Data Figures 9,10 and Figure 6; statistical analysis supplement). Two hypotheses might account for the increase in MCV:

The first, or *null hypothesis*, is based on the known negative correlation between red cell (RC) age and volume. Although the largest loss in RC volume occurs during reticulocyte maturation, RCs continue to lose volume throughout their life (in part as a result of vesiculation)[1-4]. In the steady state, the MCV is the average of the volume of an equal number of RCs of every age. In the period following Epo administration, the immediate increase in reticulocytes is followed, as these reticulocytes mature into RCs, by an increase in the relative number of younger, and therefore larger, RCs. This skew in the age distribution of RCs is expected to increase the MCV beyond the time of increased reticulocytes and may be sufficient to account for the observed increase that we see in the human studies.

In the *alternative hypothesis,* the observed increase in MCV cannot be accounted for solely by the skewing of the RC age distribution. We propose that it is therefore also the result of EpoR-signaling - mediated increase in cell size. This hypothesis is based on our experimental findings in the mouse, which show that EpoR signaling increases cell size in an Epo- dose dependent manner at every stage of erythroid terminal differentiation (ETD), leading to the production of larger reticulocytes and RCs.

Here we simulated the null hypothesis, and then determined whether it is sufficient to account for the observations in the human studies. Specifically, we simulated the time course of expected changes in MCV based on known Epo- induced changes in circulating reticulocyte number, their time of release from the bone marrow and time in circulation. We repeated the simulation for a range of erythropoietic rates, and for multiple Epo treatment durations, as well as for different red cell life- span values. This analysis showed that the increase in MCV predicted by the null hypothesis accounts for only 30 to 50% of the observed increase. We therefore conclude that additional factors contribute to the increase in MCV. These findings are consistent with our experiments in the mouse, which show an EpoR signaling- mediated increase in cell size.

II Results

We used the null hypothesis to simulate the time course of MCV and reticulocyte number, during and following Epo treatments that increase erythropoietic rate by up to 10 fold (see 'Methods and rationale' section). The null hypothesis posits that Epo treatment leads to an increase in the number of new reticulocytes generated per day, but does not change reticulocyte or RC size. We also incorporated into the simulation the early release of immature, larger reticulocytes from the bone-marrow niche during high erythropoietic rates, which results in increased size of circulating reticulocytes and increased reticulocyte circulation time [5-7].

To compare the human intervention studies with our simulation, we estimated the increase in erythropoietic rate in each of the studies. We did this by matching the observed increase in reticulocytes to the increase predicted by the simulations.

During human intervention study #1 (**Figure 1**), participants were administered with 20 IU/kg Epoetin alpha every 48 hours for 3 weeks (~70 IU/kg Epoetin alpha / week). Circulating reticulocytes increased by an average of 1.5 fold (weekly averages were 1.68, 1.52 and 1.32 fold). These values suggested an increase in erythropoietic rate of 1.25 to 1.5 -fold, which in our simulation resulted in 1.32 to 1.68 -fold increase in reticulocytes (**Figure 1,** lower panel). The observed increase in MCV was 1.47 ±0.29 %, double the corresponding simulated increase of 0.75 ±0.25% (upper and lower bounds of the simulated values correspond to the estimated range for erythorpoietic rate (Ery rate) of 1.25 to 1.5 fold). The predicted value for the post-treatment MCV was similarly lower than the observed value (**Figure 1,** upper panel, and **Table I**).

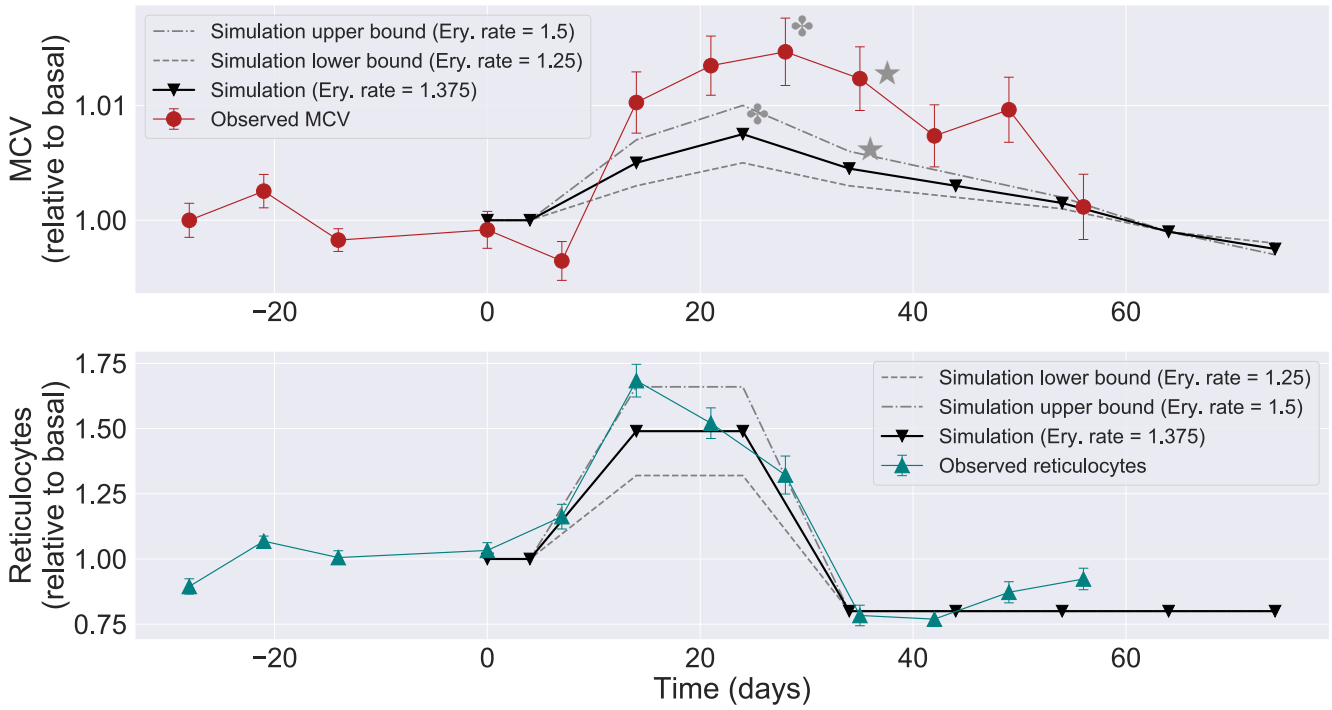

Figure 1: Observed and simulated MCV and reticulocytes, for human intervention study #1 (ED Figure 9). Simulation was for Epo treatment for 20 days, increasing erythropoietic rate by 1.25 to 1.5-fold the basal rate.

Table I	**Increase in observed MCV (mean ±sem, n=25)**	**Simulated increase in MCV Ery rate = 1.375 (range: 1.25 -1.5)**
peak ✣	1.47 ±0.29%	0.75% (0.5% - 1.0%)
post-treatment ★	1.23 ±0.28%	0.5% (0.3%-0.7%)

For the simulated increase in MCV to match the observed increase in MCV would require a 1.75 -fold increase in erythropoietic rate, generating >2-fold increase in circulating reticulocytes, well above the observed reticulocyte number (simulation: green line in **Figure 2**; observed values in Figure1, lower panel).

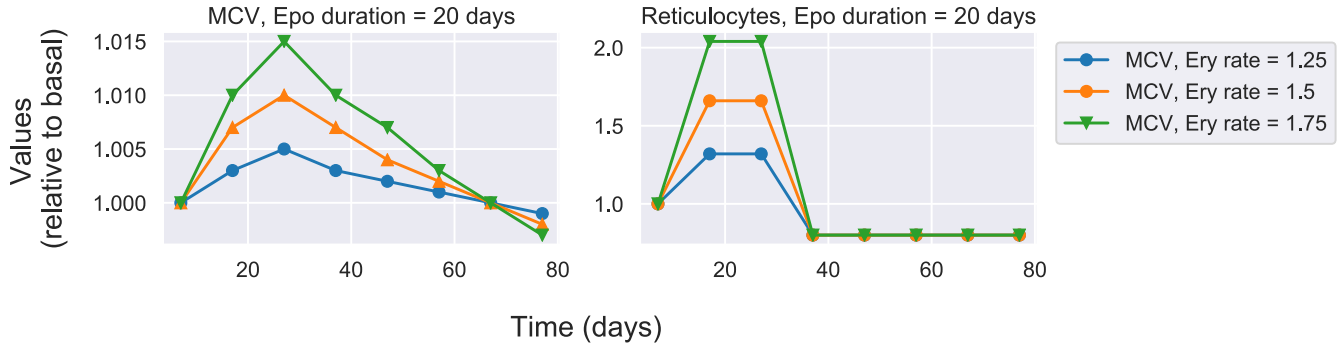

Figure 2: Simulation of MCV and reticulocyte number during and following a 20-day Epo treatment, for different levels of increase in erythropoietic rate.

In human intervention study #2 (**Figure 3**), participants were administered ~100 IU/kg Epoetin β weekly for 7 weeks. Observed reticulocyte counts increased 1.43, 1.30 and 1.24-fold, averaging a 1.32 -fold increase, which corresponds to a 1.25-fold increase in erythropoietic rate. The observed peak increase in MCV was 2.9 ±0.25 %, nearly three times the simulated increase of 1.0 ±0.3%. Similarly, the predicted post-treatment MCV was lower than the observed value (**Figure 3** and **Table II**).

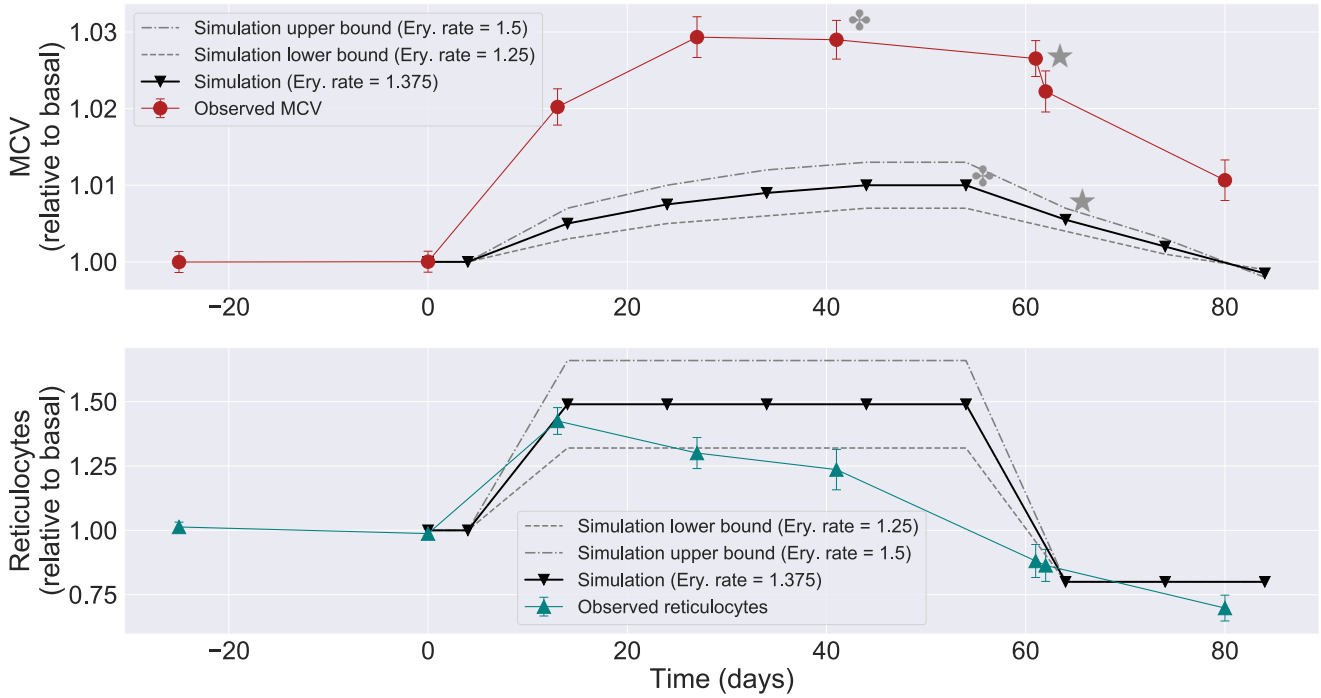

Figure 3: Observed and simulated MCV and reticulocytes, for human intervention study #2 (ED Figure 10). Simulation was for Epo treatment for 50 days, increasing erythropoietic rate by 1.25 to 1.5-fold.

Table II	**Increase in observed MCV (mean ± sem, n=24)**	**Simulated increase in MCV Ery rate = 1.375 (range: 1.25 -1.5)**
peak ✣	2.93 ±0.25%	1.0% (0.7% - 1.3%)
post-treatment ★	2.65 ±0.23%	0.55% (0.4%-0.7%)

Even a 2-fold increase in erythropoietic rate, corresponding to a far larger than observed increase in reticulocytes, would not be sufficient to account for the observed increase in MCV (red line, **Figure 4**).

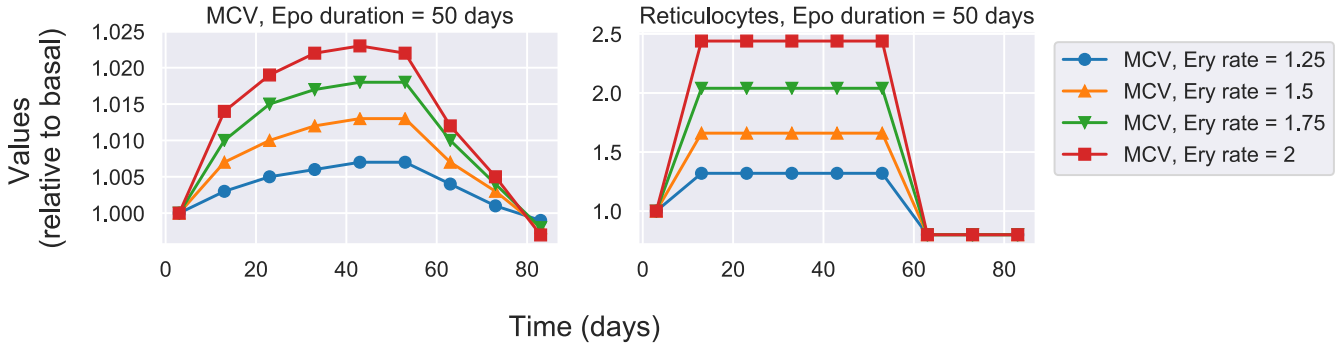

Figure 4: Simulation of MCV and reticulocyte number during and following a 50-day Epo treatment, for different levels of increase in erythropoietic rate.

The simulations considered above assumed that RC lifespan is 100 days. We repeated the simulation for a range of RC lifespans, from 95 days to 120 days (**Figure 5**). We did this in two different ways. In Figure 5, we assumed that overall RC volume decreased by the same extent over the RC lifespan, regardless of lifespan duration. We also simulated an alternative, in which the rate of RC volume loss was constant throughout the RC lifespan, so that a larger overall volume is lost if lifespan is longer. In either case, there was little effect on the MCV time course. In simulating human study #2, peak MCV was slightly higher for RC lifespan of 120, compared with RC lifespan of 95 days (peaking at a 1.3% increase instead of 1.2%) but this was still well short of the observed, 2.9% increase (**Figure 5**, lower panel).

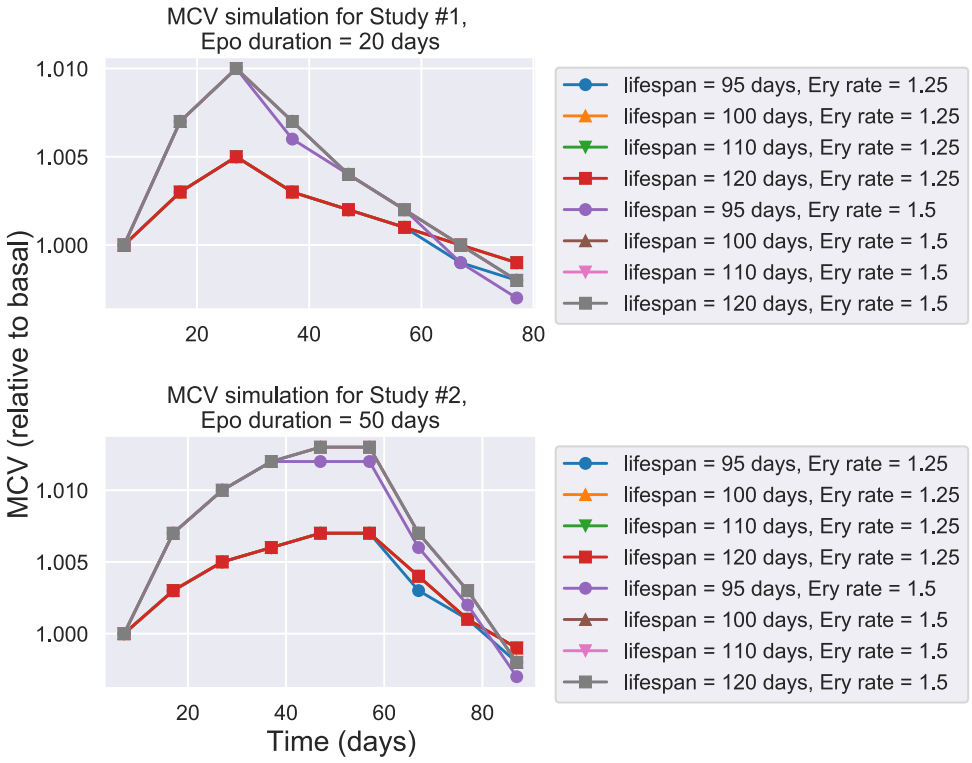

Figure 5: The effect of RC lifespan on the MCV time-course during and following Epo treatments, simulating human intervention studies #1 (top) and #2 (bottom). RC lifespan is indicated. We simulated an upper bound (Ery rate= 1.5 fold) and a lower bound (Ery rate= 1.25 fold) for each study and RC lifespan combination. Note that where MCV values are identical, overlaid symbols are hidden.

III Conclusions

Simulation of the change in MCV in response to Epo treatment using the null hypothesis accounts for only 30% to 50% of the observed increase in MCV, in two human intervention studies. We conclude that additional factors contribute to the Epo -driven increase in MCV. This finding is consistent with our experimental findings in the mouse, that in addition to increase RC number, EpoR signaling promotes an increase in erythroblast and RC size.

IV Simulation Method and Rationale

Calculation of MCV: general principle

Reticulocyte and RC volumes decrease as they age. The MCV is the average volume of all circulating reticulocytes and RCs. Therefore, to calculate the MCV, we first need to know the characteristic RC volume corresponding to each RC age over the entire life span (approximately 100 days). Second, we need to know the number of RCs of each age in the circulation. The MCV is then calculated simply by multiplying the number of cells of each age by their corresponding volume, adding this up across all RC ages, and dividing by the total number of RCs and reticulocytes.

Units used in simulation

Cell volumes, cell number and erythropoietic rate are all expressed relative to their value in the basal state. Specifically, the MCV, erythropoietic rate, and reticulocyte number in the basal state are each set to '1'. This is similar to the way we expressed observations in the human intervention studies (Figure 6 and ED Figures 9-11). We considered erythropoietic rates in the range 1 to 10, which covers the entire physiological and stress range of erythropoiesis.

Choice of parameters values

Current literature suggests that, starting with circulating reticulocytes, there is an overall ~30% decrease in volume across RC lifespan [1, 2, 8]. This loss happens in two phases: a rapid loss of 10 to 14% in circulating reticulocytes, during the course of ~1 day; and an additional, slower 16-17% loss during the rest of RC life [3] (the surface area/volume ratio remain constant [3]). The loss in volume over the life of the RC is linear, as seen from the linear decline in volume with RC Hemoglobin A1c (an indicator of RC age), and from the linear decline in flow-cytometric forward scatter with RC age [4].

We used this information to model the relationship between RC volume and age. Because there is a range of values reported in the literature for the extent of volume loss during the reticulocyte stage (10 to 14%) v. mature RCs (the remainder from the total volume loss of 30%), we set the loss in reticulocyte volume to the minimum reported experimentally (11%), and set the additional volume lost by mature RCs over their lifespan to 20%, at the very highest limit reported. This choice favors the null hypothesis, since it maximizes the chance that changes in the MCV are the result of skewing of the mature RC population. Based on published work, we set the volume of basal-state reticulocytes during their one day of maturation in the circulation to 1.25 -fold the basal MCV [9, 10].

Relationship between erythropoietic rate, reticulocyte volumes and reticulocyte circulation times

In the basal state, reticulocytes mature over 3.5 - 4 days, with only the last 24 hours of this process taking place in the circulation. Increased erythropoietic rate leads to earlier release of immature, and therefore larger, reticulocytes from the bone marrow [5-7]. In response to maximal levels of stress, as seen in aplastic anemia, or in phenylhydrazine treatment in animals, reticulocyte volumes double [10], and their maturation time in the circulation increases approximately linearly with falling hematocrit [5,11]. We used these data to determine the

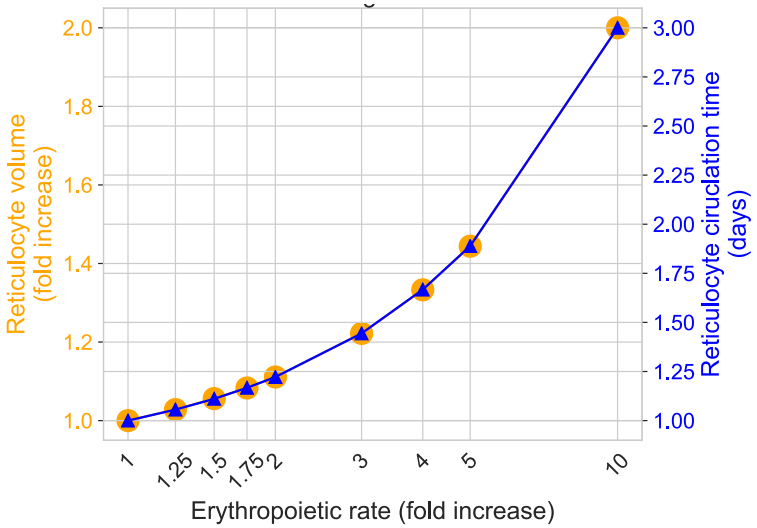

Figure 6: Increasing erythropoietic rate results in release of immature, larger reticulocytes from the bone marrow, with longer maturation times in the circulation. (NB Reticulocyte volumes here are relative to the baseline reticulocyte volumes)

relationship between erythropoietic rate, circulating reticulocyte volumes, and increased reticulocyte maturation time in the circulation (**Fig 6**).

Relationship between RC volume and RC age, in the basal state and following Epo administration

The null hypothesis posits that RC volume is not altered by Epo stimulation. The only change takes place at the reticulocyte stage. We used the relationship computed in **Figure 6** between erythropoietic rate, reticulocyte volume and reticulocyte circulation time, to set up the relationship between RC

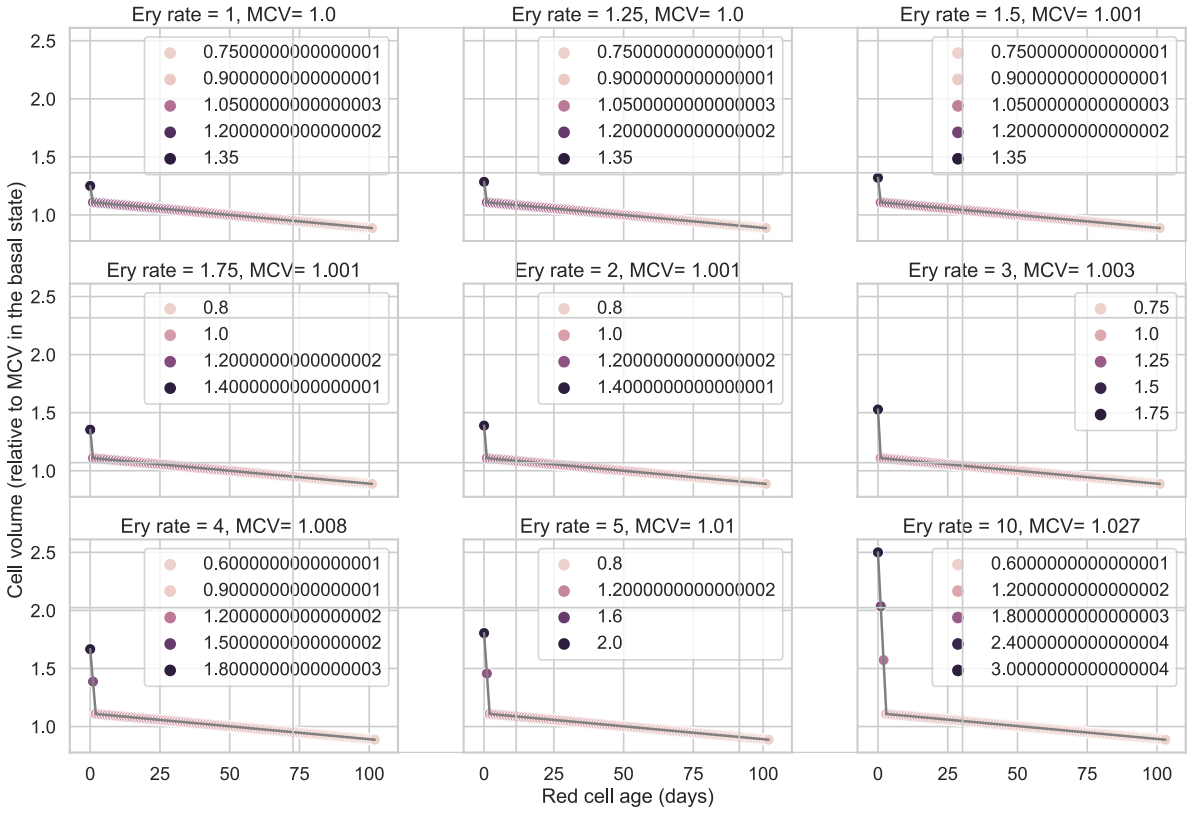

Figure 7: RC and reticulocyte volume, as a function of age, for different erythropoietic rates. Legend refers to RC volumes, relative to MCV in the basal state. In the basal state (Erythropoietic rate = 1, top left panel), rapid loss of volume at the reticulocyte stage (age = 1 day) is followed by a slower, linear loss for the remaining RC lifespan. Higher erythropoietic rates during stress lead to earlier release of larger, immature reticulocytes form the bone marrow with longer maturation times in the circulation.

volume and RC age, across reticulocyte maturation time and RC lifespan of 100 days, for the basal state and for a range of erythropoietic rates (**Fig 7**).

RC volume distributions and MCV during and following Epo administration

The MCV is the mean value of the circulating RCs at a given point in time. In the steady state, which can be defined as a constant erythropoietic rate (whether high or low) lasting for at least as long as the RC lifespan, there is an equal number of RCs of every age. The MCV is therefore the mean value of all RC volumes of every age, as depicted in **Figure 7** and the top panel of **Figure 8**.

Figure 8 illustrates the RC volume distributions during and following an Epo treatment shorter than the RC lifespan. In this example, erythropoietic rate increased by 1.5 fold for 30 days. During each of the 30 treatment days, the number of new reticulocytes increases by 1.5 fold relative to the basal state. To obtain the RC volume distribution on day 30 (last day of treatment, second panel from top), we made

use of the volume/age relationship in **Figure 8**, corresponding to an erythropoietic rate = 1.5 (the top right panel). RCs and reticulocytes whose age is ≤ 30 days are present in the circulation at 1.5 fold their number in the basal state; whereas all older cells are present in the same numbers as in the basal state. This creates a RC distribution that is skewed in favor of younger, larger cells (2nd panel, red curve), increasing the MCV to 1.012. On day 60, the number of RCs younger than 30 days is the same as would be found in the basal state, since they were generated after Epo treatment had terminated; cells older than 30 days but younger than 60 days are present at 1.5 fold the basal numbers, since they were generated during the period of Epo treatment; and finally, all cells older than 60 days are present in the same numbers as in the basal state (3rd panel, purple curve). MCV at this time point is almost back to normal, since the 'bump' in RC numbers involves cells whose volumes are near the mean. Finally, by day 90, cells whose age is >60 days are present at 1.5 -fold the number of all other cells. These older cells are smaller than the mean, bringing the MCV down below 1 (lowest panel, black curve).

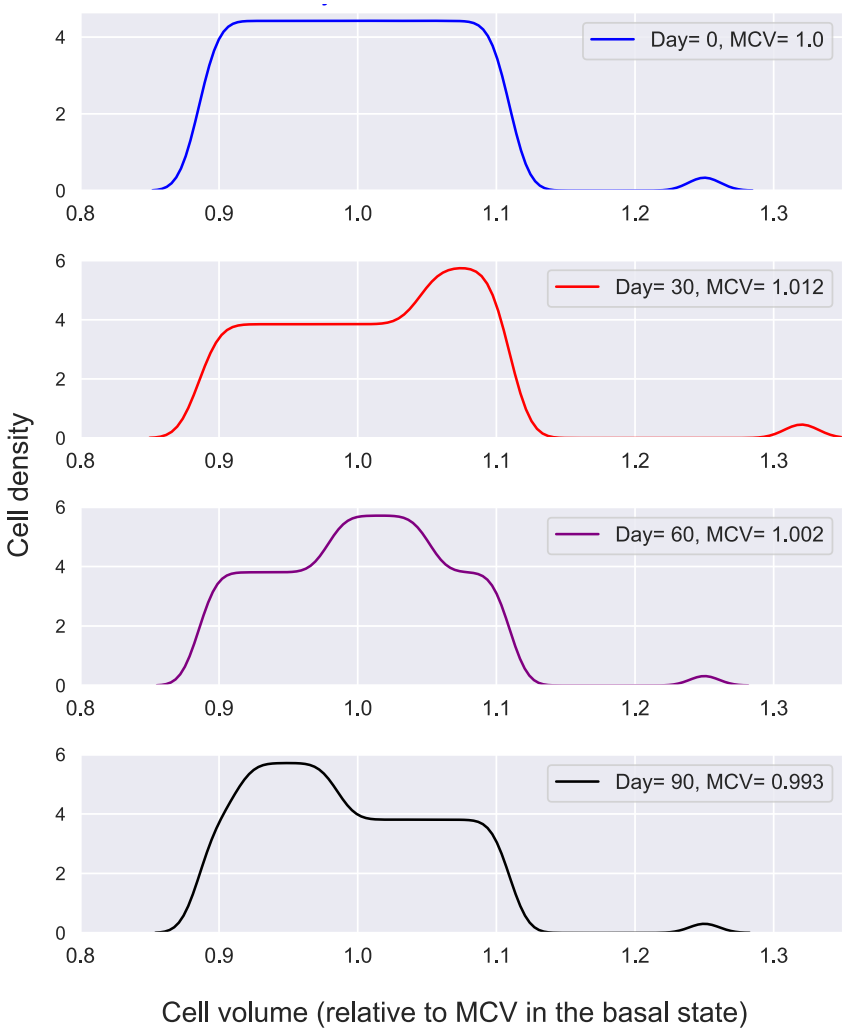

Figure 8: RC volume distributions during and following a 30 day Epo treatment in which erythropoietic rate increased by 1.5 fold. The MCV is computed as the mean of each of these distributions. Note that before the start of treatment (day 0, blue curve, top panel) the distribution is flat, since there are equal numbers of RCs of every age and volume. On day 30 (red curve, second panel from top) the distribution is skewed towards younger (≤30 days), larger cells, since there is a 1.5 fold increase in their number. By day 60 (purple curve, third panel from top), cells generated during the Epo treatment period have aged and are in the middle of the volume distribution. Finally, on day 90, these cells are skewing the distribution in favor of older cells with smaller volumes, reducing the MCV below the starting basal value. Although volume changes are continuous, for simplicity we considered them as occurring at daily intervals (see Fig 2). The large daily volume change during reticulocyte maturation therefore result in a multi-phasic distribution.

MCV and reticulocyte time course during and following Epo treatment

We used the approach illustrated in **Figure 8** to compute the MCV at 10-day intervals, during and following Epo treatments of various durations, for erythropoietic rates between 1 and 10 -fold the basal rate (**Figures 9, 10**). To simulate the expected MCV changes in the human intervention studies, we used the reticulocyte count to estimate erythropoietic rate during the study; and predicted MCV values for the relevant erythropoietic rate, treatment duration and time point.

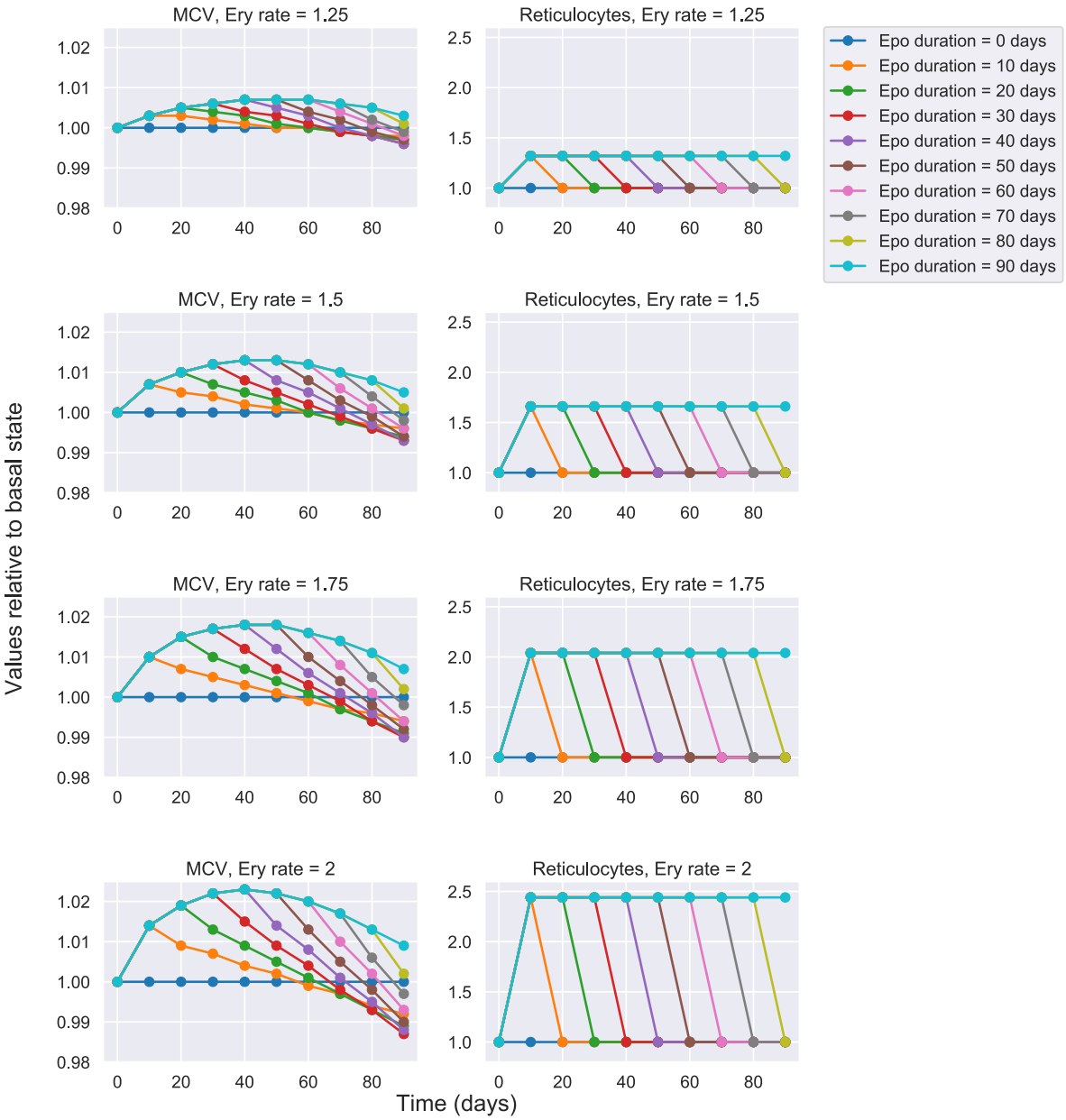

Figure 9: Time course simulation of changes to MCV and to circulating reticulocytes, during and following Epo treatment, starting on day=0 and lasting for the indicated number of days. Erythropoietic rate increased as a result of Epo treatment by 1.25 to 2 -fold.

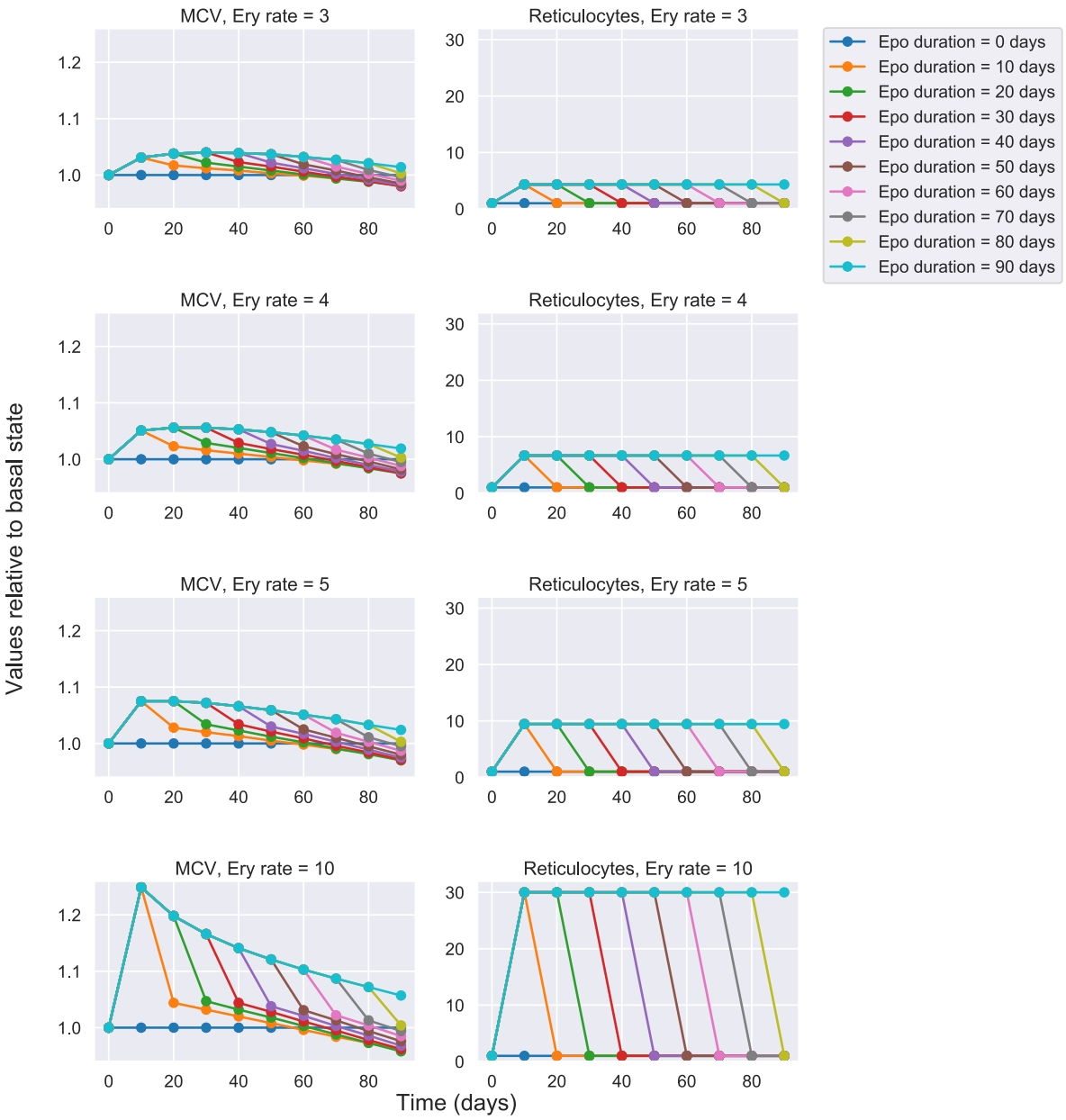

Figure 10: Time course simulation of changes to MCV and to circulating reticulocytes, during and following Epo treatment, starting on day=0 and lasting for the indicated number of days. Erythropoietic rate increased as a result of Epo treatment by 3 to 10 -fold.

V References:

1. Bosch, F.H., *et al.* Characteristics of red blood cell populations fractionated with a combination of counterflow centrifugation and Percoll separation. *Blood* **79**, 254-260 (1992).
2. Willekens, F.L., *et al.* Hemoglobin loss from erythrocytes in vivo results from spleen-facilitated vesiculation. *Blood* **101**, 747-751 (2003).
3. Gifford, S.C., Derganc, J., Shevkoplyas, S.S., Yoshida, T. & Bitensky, M.W. A detailed study of time-dependent changes in human red blood cells: from reticulocyte maturation to erythrocyte senescence. *Br J Haematol* **135**, 395-404 (2006).

4. Franco, R.S*., et al.* Changes in the properties of normal human red blood cells during in vivo aging. *Am J Hematol* **88**, 44-51 (2013).
5. Hillman, R.S. Characteristics of marrow production and reticulocyte maturation in normal man in response to anemia. *J Clin Invest* **48**, 443-453 (1969).
6. Major, A., Bauer, C., Breymann, C., Huch, A. & Huch, R. rh-erythropoietin stimulates immature reticulocyte release in man. *Br J Haematol* **87**, 605-608 (1994).
7. Rhodes, M.M*., et al.* Stress reticulocytes lose transferrin receptors by an extrinsic process involving spleen and macrophages. *Am J Hematol* **91**, 875-882 (2016).
8. Willekens, F.L., Bosch, F.H., Roerdinkholder-Stoelwinder, B., Groenen-Döpp, Y.A. & Werre, J.M. Quantification of loss of haemoglobin components from the circulating red blood cell in vivo. *Eur J Haematol* **58**, 246-250 (1997).
9. d'Onofrio, G*., et al.* Simultaneous measurement of reticulocyte and red blood cell indices in healthy subjects and patients with microcytic and macrocytic anemia. *Blood* **85**, 818-823 (1995).
10. Brugnara, C. Use of reticulocyte cellular indices in the diagnosis and treatment of hematological disorders. *Int J Clin Lab Res* **28**, 1-11 (1998).
11. Finch, C.A. Erythropoiesis, erythropoietin, and iron. *Blood* **60**, 1241-1246 (1982).