Supplemental Methods

Summary of data collected for in vitro model development

For the normal (AA) blood model, a total of 250 OECs were generated from 21 AA donors. One hundred five OECs were used to build the model while the remaining 145 curves were used to test the robustness of the model. For the sickle (SS) blood model a total of 233 OECs were generated from 27 SS donors. 132 OECs were used to build the model while the remaining 101 curves were used to test the robustness of the model.

Chemicals

For in vitro studies, voxelotor HCl or voxelotor free base synthesized at Global Blood Therapeutics (South San Francisco, CA, USA) were used.

Blood sources

For in vitro experiments, blood from healthy donors was obtained from Sanguine Biosciences, Inc (Valencia, CA, USA) or from Stanford Blood Center (Palo Alto, CA, USA). Blood from homozygous sickle cell patients was obtained from the University of North Carolina [UNC, Chapel Hill, NC, USA (IRB 88-034)] or from Sanguine Biosciences, Inc (Valencia, CA, USA). All blood samples were in sodium citrate (3.2% citrate) or acid citrate dextrose vacutainers.

In vitro whole blood oxygen equilibrium curves

To systematically assess the changes in Hb-oxygen affinity in the presence of voxelotor, OECs (483) from AA and SS blood with various % hematocrit and spiked with different voxelotor concentrations were collected using a TCS Hemox Analyzer (TCS Scientific, New Hope, PA, USA). Samples were processed as previously described[1]. Samples with <30% hematocrit were

diluted 50-fold in TES/saline buffer (30 mM TES (2-[[1,3-dihydroxy-2-(hydroxymethyl)pro-pan-2-yl]amino]ethanesulfonic acid), 140 mM saline), pH 7.4 at 25°C while those ≥30% were diluted 100-fold.

In vitro whole blood OEC analysis

To determine the optimal OEC parameters, all OEC files (either AA or SS) were analyzed using a script developed at Global Blood Therapeutics (South San Francisco, CA, USA) to determine the p50, and p20 (partial pressure of O_2 at which Hb is 50% or 20% saturated with O_2) as well as Δp50 and Δp20. In addition, changes from baseline were also assessed. Delta values for the model were obtained using Supplemental Equations 1 and 2. $p20_{control}$ and $p50_{control}$ were the values obtained from blood with no voxelotor, but with an equivalent percent of DMSO.

$$\Delta p50 = p50_{control} - p50_{sample} \qquad \text{(Supplemental Equation 1)}$$

$$\Delta p20 = p20_{control} - p20_{sample} \qquad \text{(Supplemental Equation 2)}$$

Model development:

An in vitro PK/PD correlation was developed using a method previously described for other molecules that left shift the OEC[2-4]. We developed a modified PK/PD model based upon 2 assumptions: (1) Hb bound to compound (defined as modified hemoglobin) is a distinct variant from Hb not bound to compound (defined as unmodified hemoglobin), and (2) for any unsaturated solution of compound and hemoglobin, there would exist a mixture of modified and unmodified Hb populations. We compared 4 parameters from in vitro spiking data to determine which one would be the best suited for the dosing ranges expected in the clinic. The parameters studied for the in vitro model included p50, and ∆p50 (supplemental equation 1) as well as p20

and ∆p20 (supplemental equation 2). Table S2 shows the fits for all 4 parameters examined for AA blood within the range of 0% to 40% modification. Based upon the fits and robustness of the models, p20 and ∆p20 showed a better correlation to voxelotor concentrations. Figure S1A and S1C show the correlations in AA blood between p20 or ∆p20 and spiked voxelotor levels. Using equations in model development (Table S2) we tested the 4 AA blood models with a second batch of OECs. The results indicate that ∆p20 was a better data predictor than the other parameters in AA blood. (Table S2 and Figure S2C). A similar method was used to develop an SS blood model (Table S3, Figure S3 and S4). The slopes in Table S3 for Model Robustness indicates that when using p50 or ∆p50, a 1:1 relationship is not obtained between percent Hb modification and the $[\text{voxelotor}]_{RBC}$ to $[Hb]_{RBC}$ ratio (percent Hb $\text{occupancy}_{\text{in vitro}}$). Therefore, for both AA and SS blood ∆p20 is a better data predictor than the other parameters.

AA Blood	%Hb Mod=(Δp20-0.07)/24.58	(Supplemental Equation 3)
SS Blood	%Hb Mod=(Δp20+0.16)/27.33	(Supplemental Equation 4)

Supplemental equations 3 and 4 will be used in the clinic to predict percent Hb modification, which will allow for the prediction of percent Hb occupancy.

Supplemental References

1. Oksenberg D, Dufu K, Patel MP, et al. GBT440 increases haemoglobin oxygen affinity, reduces sickling and prolongs RBC half-life in a murine model of sickle cell disease. *British Journal of Haematology.* 2016;175:141-53.

2. Beddell CR, Goodford PJ, Kneen G, White RD, Wilkinson S, Wootton R. Substituted benzaldehydes designed to increase the oxygen affinity of human haemoglobin and inhibit the sickling of sickle erythrocytes. *British Journal of Pharmacology*. 1984;82:397-407.

3. Rolan PE, Parker JE, Gray SJ, et al. The pharmacokinetics, tolerability and pharmacodynamics of tucaresol (589C80; 4[2-formyl-3-hydroxyphenoxymethyl] benzoic acid), a potential anti-sickling agent, following oral administration to healthy subjects. *British journal of Clinical Pharmacology*. 1993;35:419-25.

4. Keidan AJ, Franklin IM, White RD, Joy M, Huehns ER, Stuart J. Effect of BW12C on oxygen affinity of haemoglobin in sickle-cell disease. *Lancet*. 1986;1:831-4.

Supplemental Tables

Table S1a: Inclusion and exclusion criteria for healthy volunteers

Inclusion Criteria	Exclusion Criteria

• Adult men and women of non-childbearing potential aged 18 to 55 years inclusive and >50 kg and ≤110 kg at screening
• Women who were of non-childbearing potential were to be:
  ○ Surgically sterile (ie, bilateral tubal ligation or removal of both ovaries and/or uterus) at least 6 months prior to first dosing, or
  ○ Naturally postmenopausal for at least 24 consecutive months prior to first dosing, with a follicle-stimulating hormone level at screening of ≥40 m IU mL^{-1}
• Women were to have a negative pregnancy test at screening and check-in (day –1) for each study visit
• Subjects who were healthy as determined by a prestudy medical history, physical examination, and 12-lead electrocardiogram. Confirmed QTcF interval at screening and prior to randomization was to be ≤430 ms for men or ≤450 ms for women
• Subjects who were negative for drugs of abuse and alcohol tests at screening and admission
• Subjects who were nonsmokers and those who had not used any nicotine products for at least 3 months before screening
	• Subjects who had a clinically relevant surgical history
	• Subjects who had a clinically relevant family history
	• Subjects who had used prescription drugs within 4 weeks of first dosing (healthy subjects only)
	• Subjects who had used over-the-counter medication excluding routine vitamins but including megadose (intake of >20 times the recommended daily dose) vitamin therapy within 7 days of first dosing, unless agreed as not clinically relevant by the principal investigator and sponsor

Table S1b: Inclusion and exclusion criteria for patients with SCD

Inclusion Criteria	Exclusion Criteria
• Men or women aged 18 to 60 years and >50 kg at screening with HbSS, HbS/β^0thalassemia, HbS/β^+thalassemia, or HbSC • For subjects taking hydroxyurea, the dose had to be stable for at least 3 months prior to screening and with no anticipated need for dose adjustments during the study • Subjects who were negative for drugs of abuse, with the exception of cannabis, cotinine, and alcohol tests at screening and admission • Women of childbearing potential were to be using highly effective methods of contraception from study start to 3 months after the last dose of IMP	• Subjects who smoked >10 cigarettes per day • Subjects with HbSS or HbS/β^0thalassemia with Hb <6.0 g/dL or >10.4 g dL^{-1} at assessment just prior to dosing with study drug. Subjects with HbSC or HbS/β+thalassemia with Hb <6.0 g dL^{-1} (>ULN [appropriately corrected for gender] for subjects in cohort 15) at assessment just prior to dosing with study drug • Subjects requiring chronic transfusion therapy • Subjects receiving blood transfusion within 30 days of screening • Subjects hospitalized within 30 days of screening • Subjects with alanine aminotransferase (ALT), or alkaline phosphatase (ALP) >3x or aspartate aminotransferase (AST) >4x upper limit of normal (ULN) reference range at screening. Subjects with AST ≤4x ULN could be enrolled if, in the opinion of the investigator, this was consistent with stable chronic hemolysis • Subjects with moderate or severe renal dysfunction (calculated modification of diet in renal disease estimated glomerular filtration rate <60 mL min^{-1} 1.73 m^{2-1} appropriately corrected for race and sex) • Subjects with any history of congestive heart failure requiring hospitalization or whose 12-lead electrocardiogram at screening or prior to randomization had a confirmed QTcF interval of >450 ms for male subjects or >470 ms for female subjects

Table S1c: Inclusion and exclusion criteria for all subjects

Inclusion Criteria	Exclusion Criteria
• Subjects whose clinical laboratory test results were not clinically relevant and were accept able to the Investigator • Subjects who were negative for hepatitis B surface antigen, hepatitis C antibody and human immunodeficiency virus (HIV) I and II tests at screening • Subjects who if male were willing to use barrier methods of contraception, from study start to 3 months after the last dose of IMP • Medical history had to be verified by either a personal physician or medical practitioner as appropriate	• Subjects who did not conform to the above inclusion criteria • Female subjects who were pregnant, trying to become pregnant, or lactating • Subjects who had a clinically relevant history or presence of respiratory, gastrointestinal, renal, hepatic, hematologic, lymphatic, neurological, cardiovascular, psychiatric, musculoskeletal, genitourinary, immunologic, dermatologic, connective tissue diseases or disorders, or additional risk factors for torsades de pointe • Subjects who had a history of relevant atopy • Subjects who had a history of relevant drug hypersensitivity • Subjects who had a history of alcoholism • Subjects who had a history of drug abuse • Subjects who consume >14 (female subjects) or 21 (male subjects) units of alcohol a week • Subjects who had a significant infection or known inflammatory process on screening • Subjects who had acute gastrointestinal symptoms at the time of screening or admission • Subjects who had an acute infection such as influenza at the time of screening or admission • Subjects (females of child-bearing potential and males) who did not agree to use contraception • Subjects currently enrolled in another clinical study for another investigational drug or had taken any other investigational drug within 30 days or 5 half-lives before the screening visit, whichever was longer • Subjects who are vegans • Subjects who could not communicate reliably with the Investigator • Subjects who were unlikely to cooperate with the requirements of the study

Table S2: Models and model robustness across four potential parameters

	Model Development			Model Robustness		
	Equations	R^2	*N*	Equations	R^2	*N*
p20	y = -24.62x + 13.87	0.88	105	y = 0.99x – 5.24	0.75	145
p50	y = -15.25x + 26.13	0.46	105	y = 0.74x – 12.14	0.21	145
Δp20	y = 24.58x + 0.07	0.87	105	y = 1.09x – 0.28	0.90	145
Δp50	y = 16.09x – 0.15	0.69	105	y = 0.95x + 0.44	0.75	145

Table S3: Models and model robustness across four potential parameters in SS blood

	Model Development			Model Robustness		
	Equations	R^2	*N*	Equations	R^2	*N*
p20	y = –26.91x + 18.18	0.52	132	y = 1.11x + 4.95	0.88	101
p50	y = –11.15x + 32.52	0.07	132	y = 2.04x + 12.06	0.66	101
Δp20	y = 27.33x – 0.16	0.78	132	y = 1.04x + 1.82	0.88	101
Δp50	y = 11.50x – 0.05	0.55	132	y = 1.94x – 2.82	0.79	101

Supplemental figures

Figure S1: OECs were collected from healthy volunteers' whole blood samples spiked with voxelotor. One hundred five OECs were used to determine the correlation between p20 (A), p50 (B), ∆p20 (C), and ∆p50 (D) compared with [voxelotor]/(Hct*5 mM). Based upon the correlations, p20 (R2=0.88) and Δp20 (R2=0.87) are the optimal parameters to follow in a clinical setting where voxelotor target dosing is at 40% or below of the Hb concentration. Equation of the line, correlation and N values for all four panels can be found in Supplemental Table 2.

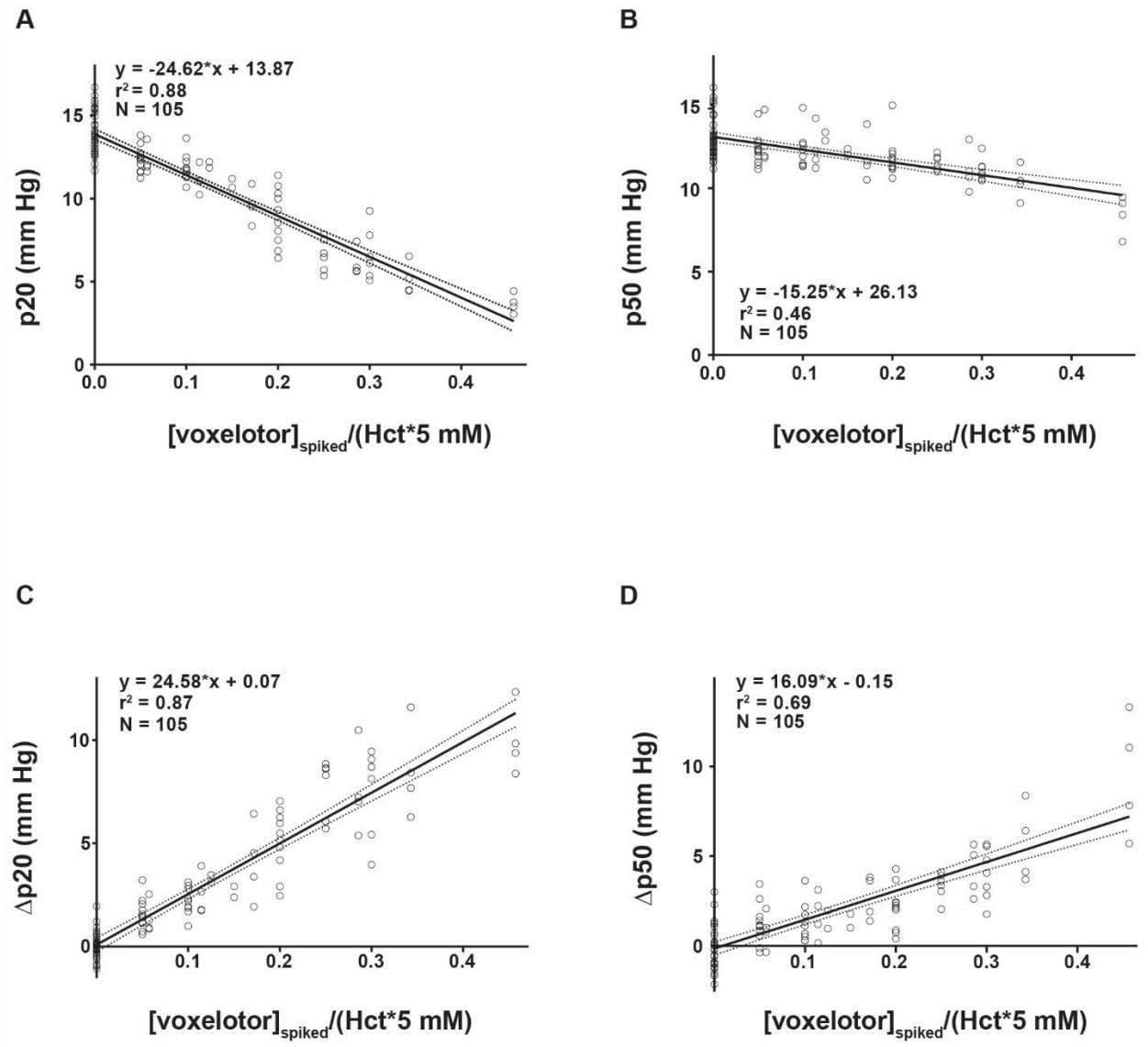

Figure S2: OECs were collected from healthy volunteers' whole blood samples spiked with voxelotor. One hundred forty-five OECs were used to test the robustness of the four parameters evaluated. The four panels show how predictive the model was of predicting the $[voxelotor]_{RBC}$ to $[Hb]_{RBC}$ ratio (also known as the %Hb occupancy). The robustness of the model based upon either p20 (A), p50 (B) ∆p20 (C) or ∆p50 (D) can be assessed. In addition, the direct relationship between percent Hb modification and %Hb occupancy can be obtained from the slope of the line. Based upon the R2 values and the slope of the lines (Found in Supplemental Table 2) it is clear that using p50 is not predictive of the %Hb occupancy.

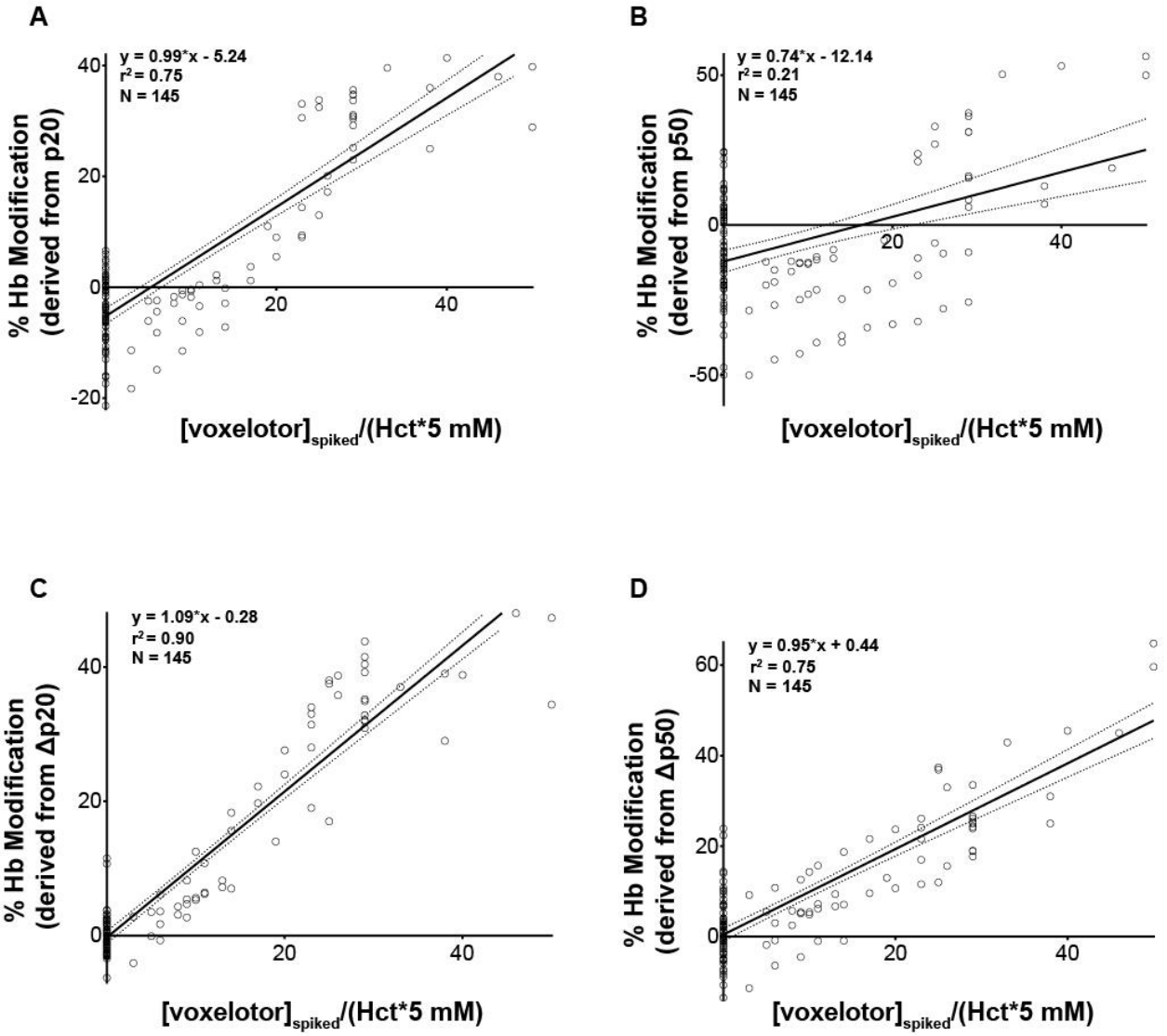

Figure S3: OECs were collected from SCD patients whole blood samples spiked with voxelotor. One hundred thirty-two OECs were used to determine the correlation between p20 (A), p50 (B), ∆p20 (C), and ∆p50 (D) compared to [voxelotor]/(Hct*5 mM). Based upon the correlations, ∆p20 (R2=0.78) is the optimal parameter to follow in a clinical setting when voxelotor target dosing is at 40% or below of the Hb concentration. Equation of the line, correlation and N values for all four panels can be found in Supplemental Table 3. The greater variability in the SCD data is likely due to varying 2,3 DPG levels in SCD patient blood, and therefore using the delta values provides an internal reference.

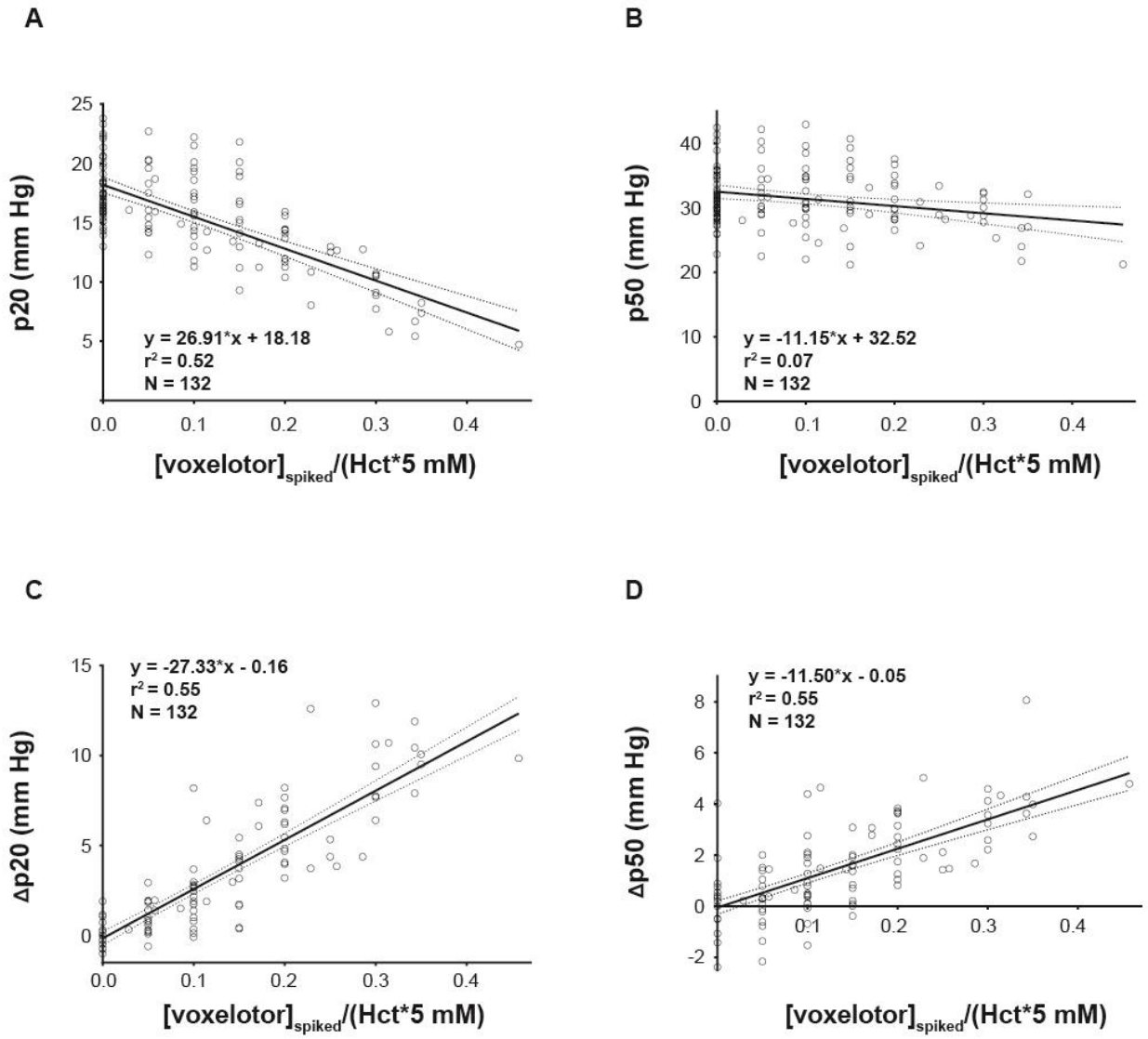

Figure S4: OECs were collected from SCD patients' whole blood samples spiked with voxelotor. One hundred and one OECs were used to test the robustness of the four parameters evaluated. The four panels show how predictive the four models were of predicting the $[voxelotor]_{RBC}$ to $[Hb]_{RBC}$ ratio (also known as the %Hb occupancy). The robustness of the model based upon p20 (A), p50 (B) ∆p20 (C) and ∆p50 (D) were assessed. In addition, the direct relationship between percent Hb modification and %Hb occupancy can be judged from the slope of the line. Based upon the slope of the lines (Found in Supplemental Table 3) it is clear that both p50 and ∆p50 are not predictive of the %Hb occupancy.