ORIGINAL RESEARCH

Progression of anemia and its relationship with renal function, blood pressure, and erythropoietin in rats with chronic kidney disease

Jacqueline K. Phillips^{1,2}, Rochelle Boyd¹, Mark B. Krockenberger³, Gaetan Burgio¹

¹Australian School of Advanced Medicine, Macquarie University, Sydney, NSW, Australia; ²School of Veterinary and Life Sciences, Murdoch University, Perth, WA, Australia; and ³Faculty of Veterinary Science, The University of Sydney, Sydney, NSW, Australia

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Correspondence

Prof. J.K. Phillips, Australian School of Advanced Medicine, Macquarie University, Sydney NSW 2109, Australia
E-mail: jacqueline.phillips@mq.edu.au

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Background: In chronic kidney disease (CKD), anemia and hypertension are significant co-morbidities that contribute to cardiovascular and renal disease progression.

Objective: The purpose of the study was to identify correlations between changes in hematologic variables against markers of renal function, blood pressure, and erythropoietin (EPO) in a naturally occurring hypertensive model of CKD, the Lewis polycystic kidney (LPK) rat.

Methods: Complete blood count, systolic blood pressure, urea and creatinine concentration, urinary protein to creatinine ratio, and plasma EPO concentration were determined in control Lewis ($n = 51$) and LPK rats ($n = 56$) aged 6–24 weeks. Renal EPO gene expression and RBC osmotic fragility were also documented. Hematopoiesis in spleen and bone marrow were assessed.

Results: Lewis polycystic kidney rats had increasing urea and creatinine concentrations, concurrent with the development of a nonregenerative normocytic/normochromic anemia and hypertension, with a significant negative correlation between both HGB and HCT with urea concentration and blood pressure ($P < .01$). HCT was also significantly negatively correlated with creatinine concentration ($P = .014$). WBC was significantly negatively correlated with urea ($P < .01$). Plasma EPO concentration was increased and renal EPO mRNA expression was significantly upregulated in LPK animals. The former was significantly positively correlated with blood pressure and platelet count ($P < .05$). RBC osmotic fragility was normal in LPK rats and there was no evidence for increased RBC elimination or extramedullary hematopoiesis.

Conclusions: Marked anemia in the LPK CKD rodent model in the presence of elevated EPO suggests inefficient erythropoiesis that is correlated with plasma urea concentration and blood pressure.

Introduction

In chronic kidney disease (CKD) patients, anemia is an independent risk factor for cardiovascular disease¹, and acts as a further driver of renal disease progression.² The mechanism for anemia of late stage CKD is classically associated with low serum erythropoietin (EPO) concentrations, likely due to impaired EPO production by the kidney as a consequence of structural damage.³ Hypertension is also a defining feature of

CKD,⁴ complicated by the relationship whereby loss of renal function leads to hypertension and vice versa.⁵ In a recent study in dogs with CKD for example, high systolic blood pressure was associated with elevated urinary albumin to creatinine ratio.⁶ The coexistence of hypertension and anemia is therefore of significant clinical relevance in the context of CKD, and indeed is considered an important risk factor for subsequent cardiovascular disease and in particular left ventricular hypertrophy.⁷ In addition, while the major

hematologic abnormalities in CKD relate to cells of erythroid lineage, changes in WBC and platelets have also been described.^{8,9} Uremia is thought to be a major contributing factor to the leucopenia associated with CKD⁹, while impaired hemostasis and platelet dysfunction in CKD have been shown to improve with EPO treatment.¹⁰

At present, while there are chemically or surgically induced models of anemia associated with CKD, and mouse transgenic and conditional models displaying anemia associated with reduced EPO concentrations¹¹, there are no well-established models of naturally progressive anemia associated with CKD in which the cardiovascular phenotype is also well characterized. We have recently established a rodent model of CKD, the Lewis polycystic kidney (LPK) rat arising from a mutation in *Nek8*¹², which is responsible for nephronophthisis 9 (NPHP9), an autosomal recessive cystic kidney disease.¹³ The NPHP genes encode for the nephrocystin protein family, the dysregulation of which constitute an overall group of autosomal recessive cystic kidney disorders that are the leading genetic cause of end-stage renal disease (ESRD) in children and young adults.¹⁴ Mouse and rat mutations in *Nek8* lead to a phenotypic presentation of cystic kidney disease that resembles human autosomal recessive polycystic kidney disease.^{15,16}

Like other forms of CKD, patients with advanced polycystic kidney disease (PKD) present with anemia and hypertension as defining features.¹⁷ However, PKD patients have been reported as having higher EPO and HGB concentration, and a higher HCT relative to their stage of CKD when compared to other ESRD patients.^{17,18} A recent study suggested that this is only true in the early stages of CKD, while in the later phases (stages 4 and 5), PKD patients' EPO concentration and HCT are decreased as in patients with other causes of CKD.¹⁹ The belief that PKD patients have relatively higher EPO and HGB concentrations is further questioned by a study in juvenile NPHP patients, which established they had lower EPO and HGB concentrations than would be predicted by the stage of CKD, and that this was apparent very early in the disease process.²⁰

Our previous studies have determined the presence of anemia in the LPK rat¹⁶; however, we have not determined the defining features of the anemia and any parallel changes in WBC or platelet counts, nor determined the relationship between the progression of anemia and both the increase in blood pressure and the decline in renal function. A key aim of this study therefore was to fully evaluate the hematologic profile of the LPK rodent model of CKD, examining RBC,

WBC, and platelet counts in animals from a young age (6 weeks) presenting with minimal changes in renal function to an advanced disease state (24 weeks). We examined the hypothesis that the anemia or changes in WBC counts could be correlated with blood pressure and variables that are commonly used as indicators of renal function (plasma urea and creatinine concentration, urinary protein/creatinine ratio), using multiple linear regression modeling. We also examined the profile of plasma EPO concentration throughout the course of the disease, and potential correlation of EPO concentration with anemia, platelet number, blood pressure, and indicators of renal function. We further examined the renal EPO mRNA expression levels, splenic and bone marrow cytology and histology, and RBC osmotic fragility to assess the erythropoietic response, RBC clearance, and RBC viability as causal mechanisms of a progressive anemia. The outcomes of this study will additionally serve to validate the LPK rodent model as a tool for investigating the mechanisms underlying anemia in CKD.

Materials and Methods

Animals and experimental design

All experiments were approved by the Animal Ethics Committees of Murdoch University and Macquarie University, and conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Rats were housed on a 12-hour light–dark cycle with access to standard rat chow and water ad libitum. Lewis animals (standard inbred LEW/CrlBR, $n = 51$) and LPK (LEW/SsNarc LPK mutant¹², $n = 56$) of mixed sex, obtained from the Animal Resources Centre, Perth Western Australia, were used for the study. There were 3 cohorts of animals used, with animal numbers and the datasets obtained from each cohort shown in Table 1. For animals from Cohort 1, rats were euthanized at age 6, 12, 18, or 24 weeks. In the week prior to euthanasia, tail-cuff blood pressure measurements were made as detailed below and voided urine samples were collected. Immediately following euthanasia with CO₂/O₂ (80/20) or sodium pentobarbitone (Thiobarb, Jurox, Rutherford, Australia; 100 mg/kg, i.p.), blood was collected by cardiac puncture into either lithium heparin for biochemical analysis and osmotic fragility determination, or K₂ EDTA for hematologic evaluation on the same day as sample collection (BD Vacutainer; Becton Dickinson and Company, Franklin Lakes, NJ, USA). Samples from a subgroup of Cohort 1 animals were used for testing of plasma EPO concentration and

Table 1. Animal cohorts, procedures, and total numbers/gender breakdown for Lewis control rats and Lewis polycystic kidney (LPK) rats.

Procedure	Age (weeks) & Strain	BP/CBC/Urine/ Biochemistry		Erythropoietin/ Reticulocytes/ Spleen		Kidney Erythropoietin mRNA		RBC Osmotic Fragility		Bone Marrow	
		Lewis	LPK	Lewis	LPK	Lewis	LPK	Lewis	LPK	Lewis	LPK
1	6	11 ^{6/5}	11 ^{5/6}	(4) ^(2/2)	(5) ^(3/2)						
	12	10 ^{5/5}	11 ^{5/6}	(4) ^(2/2)	(4) ^(2/2)						
	18	10 ^{6/4}	13 ^{9/4}	(4) ^(2/2)	(6) ^(3/3)					(5) ^(3/2)	(5) ^(2/3)
	24	6 ^{3/3}	7 ^{4/3}								
2	12					5 ^{2/3}	5 ^{2/3}				
3	12							5 ^{4/1}	5 ^{3/2}		
	18							4 ^{2/2}	4 ^{2/2}		
Strain subtotal		37 ^{20/17}	42 ^{23/19}	(12) ^(6/6)	(15) ^(8/7)	5 ^{2/3}	5 ^{2/3}	9 ^{6/3}	9 ^{5/4}	(5) ^(3/2)	(5) ^(2/3)
Subtotal		79		(27)		10		18		(10)	
Strain Total		51 ^{28/23}	56 ^{30/26}								
Total		107 ^{58/49}									

A total of 3 cohorts of animals were used for this study. Cohort 1 had 4 groups of animals of different ages (6, 12, 18, and 24 weeks) which were used to determine blood pressure (BP) and collect urine for biochemistry, and after euthanasia, blood was collected for plasma biochemistry and CBC. Samples from a subgroup of animals from Cohort 1 (in brackets) were used for determination of plasma erythropoietin (EPO) levels, reticulocyte counts, and collection of the spleen for determination of splenic index and spleen histology. Samples from a subgroup of 18-week-old animals from Cohort 1 (in brackets) were taken from the femur and ilium after euthanasia for cytologic and histologic evaluation of the bone marrow. Cohort 2 consisted of a group of 12-week-old animals whose kidneys were collected for determination of renal EPO mRNA levels. Cohort 3 consisted of animals aged 12 and 18 weeks whose blood samples were used for determination of RBC osmotic fragility. Male/female ratio is noted in superscript for each group of animals. Note in those cohorts where multiple procedures were undertaken, not all procedures/tests were performed on all animals in that cohort.

reticulocyte index at 6, 12, and 18 weeks. As the spleen is not only a site of RBC destruction and sequestration but can also be a site of extramedullary hematopoiesis in response to anemia²¹, the spleen from each of these animals was then collected and weighed to determine splenic index (spleen weight/body weight). Spleens were then fixed in 4% formalin for 24–48 h and stored in 70% ethanol until histologic processing. A complete dataset was not obtained from all animals within these subgroups. Another subgroup of 18-week-old animals from Cohort 1 was used for bone marrow collection and analysis.

For animals from Cohort 2, after euthanasia as detailed above, kidneys were collected from 12-week-old animals and stored at -80°C for RNA extraction and assessment of renal EPO mRNA expression.

For animals from Cohort 3, after euthanasia as detailed above, blood samples were collected via cardiac puncture from animals aged 12 or 18 weeks, and used for assessment of RBC osmotic fragility.

Determination of systolic blood pressure, plasma urea, & creatinine and urinary protein: creatinine ratio

Tail-cuff systolic blood pressure measurements were determined using an average of 3 measurements on

each rat after acclimatization to the procedure (NIBP controller; ADI Instruments, Bella Vista, NSW, Australia).¹⁶

Blood collected into lithium heparin after euthanasia was separated by centrifugation for biochemical analysis of plasma urea and creatinine using a Rx Daytona analyser (Randox Laboratories, Antrim, UK). Animals were manually restrained and voided urine samples were collected into sterile specimen containers (Techno Plas Pty Ltd, St Marys, SA, Australia) and processed for determination of urine protein to creatinine ratio (UPC) using a Cobas Mira analyser (Roche Diagnostics, Schweiz, AG, Switzerland) or an IDEXX VetLab analyser (IDEXX Laboratories Pty Ltd., Rydalmere, NSW, Australia) within 24 hours of sample collection. The results for urea and creatinine concentrations and UPC from a subgroup of the 6-, 12-, and 24-week-old Cohort 1 animals were part of a previous study, where they were a component of a large cohort analysis as mean $\pm$ SD data.¹⁶ The data have not been used previously for linear regression analysis against hematologic variables ($n = 17$ Lewis and 18 LPK).

Hematologic analysis

Blood collected into K₂ EDTA was used to measure RBC and RBC indices, total and differential WBC, and

reticulocyte and platelet counts using an automated cell counter (ADVIA 120 Hematology System; Siemens, Tarrytown, NY, USA). To account for reduced HCT levels noted in adult LPK animals, reticulocyte counts obtained as a percentage (%) value from the ADVIA data were corrected using the following formula to determine reticulocyte index:

$$\% \text{Reticulocytes} \times (\text{hematocrit/normal hematocrit for strain})$$

A value of 0.505 was used as the normal hematocrit for this strain. This was based on data provided by Charles River Laboratories International (Wilmington, MA, USA) as being the mean hematocrit for 8–10-week-old Lewis male and female rats using samples from animals housed in their standard production setting.²² Wright–Giemsa-stained blood smears were prepared for all samples and examined microscopically for abnormal RBC morphology such as target cells, RBC fragments, and RBC corpuscular inclusions such as Howell–Jolly bodies as indicators of splenic dysfunction.²³ Smears were also examined for WBC morphology and WBC precursors, and a manual differential WBC count was performed ($n = 100$ cells per smear) to confirm the automated differential WBC count results.

RBC osmotic fragility assay

Using lithium heparin blood samples from Lewis and LPK animals in Cohort 3 aged 12 and 18 weeks, samples were diluted 1:100 with increasingly hypotonic solutions of NaCl (0–0.9% in distilled water) and incubated for 30 min at 37°C (total $n = 18$). After centrifugation at 500*g* for 5 min, the degree of hemolysis was measured as absorbance due to hemoglobin in the supernatant at 540 nm (Pherastar FS; BMG LABTECH, Offenburg, Germany). Osmotic fragility curves were fitted with a sigmoidal curve using Prism (v 6.0a) for Mac OS X (GraphPad Software Inc. La Jolla, CA, USA), constrained to 100% hemolysis in water and 0% in 0.9% NaCl. The median corpuscular fragility, defined as the NaCl concentration (%) causing 50% of the RBC lysis, was determined as the x -value at the positive stationary point on the third derivative of the curve fit.

Cytologic and histologic evaluation of bone marrow and spleen

After euthanasia and blood collection, the left femur and ilium from the subgroup of animals from Cohort 1 aged 18 weeks were dissected, and a sample of bone

marrow was collected using a spatula. Smears were prepared on glass slides, air dried, and stained with Giemsa stain or Perl's Prussian Blue Method (counterstained with neutral red). The cells were analyzed with regard to proliferation or maturation phase for both erythroid and myeloid cell lines, and a myeloid to erythroid (M:E) ratio was determined. This was performed by identifying bone marrow particles on suitably thin areas of the smear to facilitate accurate identification of cells. Appropriate fields were examined until at least 400 cells were identified. An unpaired 2-tailed Student's *t*-test was used to compare the data. Megakaryocyte number was also assessed.

The femur and ilium were fixed in 4% formalin in 0.01 M phosphate buffer (pH 7.4) for 48–72 h at room temperature. Bone tissue was decalcified in 5% aqueous nitric acid (volume ratio 10:1) for 2–3 h and then processed for paraffin embedding. Sections of 4 μ m were cut and placed onto glass slides, stained with H&E, and subjectively evaluated for overall cellularity, confirming adequacy of the sample for assessment and the state of hematopoietic activity of the bone marrow.

Splenic tissue was collected from a subgroup of animals from Cohort 1 (Table 1). It was also paraffin embedded and 5- μ m sections were cut and placed onto glass slides, stained with H&E or Perl's Prussian Blue Method, and assessed for extramedullary hematopoiesis (EMH) and for the presence of hemosiderin as an indicator of increased RBC destruction ($n = 2$ animals per age and strain, total $n = 12$).

Determination of plasma EPO

Plasma collected from K₂ EDTA blood from a subgroup of animals from Cohort 1 ($n = 25$, Table 1) was used to measure the concentration of circulating EPO using a rat-specific ELISA Kit (USCN Life Science Inc, Wuhan, China), according to the manufacturer's instructions.

Renal EPO gene expression

RNA was extracted from the kidney of animals from Cohort 2 (Table 1) using an RNA isolation kit (Master Pure RNA Purification Kit; Epicentre Biotechnologies, Madison, WI, USA), according to the manufacturer's protocol. The AffinityScript QPCR cDNA synthesis kit (Stratagene, La Jolla, CA, USA) was used to generate cDNA using 1 μ g of extracted RNA and 15 ng/ μ L of random primers in a 50 μ L reaction volume. Real-time quantitative polymerase chain reaction (qPCR) was performed using 1 μ L of cDNA mix in a 25 μ L reaction with each forward (TACGTAGCCTCACTTCACTGCTT)²⁴ and reverse primer (TATCCGCTGTGAGTGTT

CGG) for EPO (300 nm final concentration), designed using PrimerBlast²⁵ and using SYBR green mastermix as provided by the manufacturer (Stratagene). Conditions for qPCR (40 cycles) were: 95°C for 30 s, 60°C for 1 min, then 72°C for 1 min. Each reaction was performed in 3 replicates and the average of the 3 results for each animal was used for further statistical analysis. Cycle threshold (Ct) values were determined, representing PCR cycle number at which fluorescence emission data exceeded a threshold limit, with a lower number representing a higher level of expression. Delta (Δ) Ct values were then calculated by normalizing to TOP1 (DNA topoisomerase type I), an endogenous control predetermined for stability using the geNorm reference gene selection kit as per the manufacturer's instructions (Primer Design Ltd, Southampton, UK). For statistical analysis, an unpaired 2-tailed Student's *t*-test was used to compare Δ Ct values between Lewis and LPK rats ($P < .05$). For determination of fold difference between Lewis and LPK rats (Lewis set as = 1), the $2^{(-\Delta\Delta\text{Ct})}$ ($2^{-\Delta\Delta\text{Ct}}$) method was applied, with range values determined using the formula $2^{-(\Delta\Delta\text{Ct} \pm \text{standard deviation (SD) of the } \Delta\text{Ct})}$, as detailed previously²⁶, with the data then presented as relative fold (range) values.

Statistical analysis

Results are presented as mean $\pm$ SD. Analysis was conducted using IBM Statistical Package for the Social Sciences (SPSS; v20, Chicago, IL, USA), and Prism v 6.0a (GraphPad Software) was used to create graphical representations of the data. Systolic blood pressure, plasma urea and creatinine concentration, UPC, hematologic (RBC, platelets, WBC, and reticulocyte) data, and EPO concentration were analyzed using a univariate general linear model (GLM) against the fixed factors of age and strain. The significance was set at $P \leq .05$, and the adjusted R^2 (being the relative predictive powers of the model adjusted for degrees of freedom) and level of statistical significance for between-subject effects for strain and age are provided. If any significant age $\times$ strain interactions were identified ($P < .01$), they were also reported. Distribution of the unstandardized residuals, as obtained from the GLM, was tested using a Shapiro–Wilk test of normality ($P > .01$). Extreme outliers ($> 3 \times$ interquartile range) were excluded from the data analysis. Results of the univariate analysis drove the post hoc analysis, using a Tukey's HSD test or Dunnett T3 for nonparametric datasets ($P \leq .05$).

To determine any relationship between the clinical variables blood pressure, plasma urea and crea-

tinine concentration, and UPC and the hematologic variables HGB, HCT, or WBC, and the relative strength of the relationships, multiple linear regression modeling was used. Data across all ages (6, 12, 18, and 24 weeks) and from both strains were used for the modeling. The hematologic variable was set as the dependent variable and the clinical variables as the independent variables, using a stepwise selection method of entry and listwise exclusion of missing values. The results of the final model derived from the multiple linear regression are provided with the *n* value (number of data pairs) and adjusted R^2 value for the model, and the Pearson correlation coefficient, significance, and β -standardized regression coefficient (β) for each significant independent variable, allowing assessment of the relative effect of each independent variable on the dependent variable.

To determine if the parameters of systolic blood pressure, plasma urea and creatinine concentration, UPC, HCT, HGB, or platelet count were correlated with EPO concentration, multiple linear regression modeling was again undertaken, using EPO concentration as the dependent variable and the other variables as the independent variables, with analysis from Lewis and LPK animals from 6-, 12-, and 18-week-old age groups.

Results

Systolic blood pressure, plasma urea and creatinine concentration, and UPC

Systolic blood pressure was significantly greater in the LPK rats compared to Lewis controls at all ages examined (Lewis 113.4 ± 15.1 vs LPK 185.0 ± 29.5 mmHg, total $n = 76$, adjusted $R^2 = .790$ strain effect and age effects: $P \leq .001$), and blood pressure increased in the LPK animals as they aged. As described previously¹⁶, plasma urea and creatinine concentrations were also significantly increased in the LPK rats compared to Lewis controls, and both variables steadily increased with advancing age (urea: adjusted $R^2 = .902$, creatinine adjusted $R^2 = .879$, both strain and age effect $P \leq .001$). Likewise, the UPC ratio also showed a significant strain and age dependence (adjusted $R^2 = .809$, strain and age effect $P \leq .001$).

Hematologic variables

Results presented in Table 2 show that the LPK animals demonstrated a progressive normocytic normochromic anemia by age 12 weeks, reflected by decreasing HGB concentrations from age 12 weeks

Table 2. Means ($\pm$ SD) of hematologic variables in different age groups of Lewis control rats and Lewis polycystic kidney (LPK) rats.

Rat strain Age group (weeks)	Lewis				LPK				Between subject effects		
	6 ⁸	12 ¹⁰	18 ⁹	24 ⁶	6 ⁶	12 ⁷	18 ⁷	24 ⁷	Adj R ²	Strain	Age
HGB (g/L)	136 $\pm$ 20	160 $\pm$ 9	146 $\pm$ 13	149 $\pm$ 10	118 $\pm$ 13	112 $\pm$ 22	88 $\pm$ 13	63 $\pm$ 19	.795	P < .001	P < .001
RBC ($\times 10^{12}$ /L)	7.8 $\pm$ 0.4	9.2 $\pm$ 0.5	8.5 $\pm$ 1.2	9.4 $\pm$ 0.8	6.4 $\pm$ 0.8	6.8 $\pm$ 1.4	4.9 $\pm$ 1.0	3.7 $\pm$ 1.1	.781	P < .001	P < .001
HCT (L/L)	0.5 $\pm$ 0.01	0.5 $\pm$ 0.1	0.5 $\pm$ 0.1	0.5 $\pm$ 0.03	0.4 $\pm$ 0.1	0.3 $\pm$ 0.09	0.3 $\pm$ 0.1	0.2 $\pm$ 0.06	.711	P < .001	P < .001
MCV (fL)	61.1 $\pm$ 3.6	54.9 $\pm$ 3.1	53.7 $\pm$ 3.8	54.0 $\pm$ 3.8	63.1 $\pm$ 3.8	52.6 $\pm$ 3.8	51.5 $\pm$ 2.7	52.9 $\pm$ 1.3	.541	P = .183	P < .001
MCH (pg)	18.6 $\pm$ 0.8	17.4 $\pm$ 0.5	16.8 $\pm$ 0.8	15.6 $\pm$ 1.2	18.1 $\pm$ 0.6	16.5 $\pm$ 0.2	16.7 $\pm$ 0.7	16.9 $\pm$ 0.5	.620	P = .985	P < .001
MCHC (g/L)	305 $\pm$ 8.9	318 $\pm$ 14	316 $\pm$ 35.1	288 $\pm$ 1.7	295 $\pm$ 16	319 $\pm$ 14	325 $\pm$ 18	320 $\pm$ 11	.263	P = .059	P < .001
RDW (%)	12.2 $\pm$ 1.3	10.1 $\pm$ 0.5	12.2 $\pm$ 0.5	11 $\pm$ 1	11.5 $\pm$ 0.9	10.5 $\pm$ 0.7	11.9 $\pm$ 0.7	11.9 $\pm$ 0.8	.428	P = .642	P < .001
Platelets ($\times 10^9$ /L)	692 $\pm$ 289	630 $\pm$ 66	640 $\pm$ 207	687 $\pm$ 125	735 $\pm$ 150	624 $\pm$ 228	631 $\pm$ 195	462 $\pm$ 119	.036	P = .277	P = .215
MPV (fL)	11.3 $\pm$ 2.2	9.8 $\pm$ 2.4	10.3 $\pm$ 5.3	10.7 $\pm$ 1.2	10.0 $\pm$ 3.0	8.6 $\pm$ 0.8	8.7 $\pm$ 1.2	7.8 $\pm$ 0.5	.092	P = .005	P = .243
WBC ($\times 10^9$ /L)	4.9 $\pm$ 3.1	9.1 $\pm$ 1.4	5.5 $\pm$ 3.0	8.5 $\pm$ 2.1	6.8 $\pm$ 3.1	7.8 $\pm$ 3.1	3.3 $\pm$ 1.2	2.2 $\pm$ 0.9	.431	P = .001	P < .001
Lymphocytes ($\times 10^9$ /L)	3.8 $\pm$ 2.3	7.4 $\pm$ 1.0	4.7 $\pm$ 2.5	6.5 $\pm$ 1.6	5.3 $\pm$ 2.4	5.8 $\pm$ 2.3	2.4 $\pm$ 0.9	1.6 $\pm$ 0.8	.472	P < .001	P < .001
Neutrophils ($\times 10^9$ /L)	0.7 $\pm$ 0.5	1.1 $\pm$ 0.4	0.8 $\pm$ 0.7	1.3 $\pm$ 0.4	0.9 $\pm$ 0.5	1.3 $\pm$ 0.4	0.6 $\pm$ 0.4	0.5 $\pm$ 0.1	.48	P = .066	$\S$ P = .019
Monocytes ($\times 10^9$ /L)	0.19 $\pm$ 0.1	0.28 $\pm$ 0.12	0.13 $\pm$ 0.07	0.25 $\pm$ 0.05	0.32 $\pm$ 0.17	0.21 $\pm$ 0.09	0.1 $\pm$ 0.06	0.08 $\pm$ 0.06	.51	P = .200	$\S$ P = .001
Eosinophils ($\times 10^9$ /L)	0.05 $\pm$ 0.04	0.08 $\pm$ 0.05	0.02 $\pm$ 0.02	0.05 $\pm$ 0.02	0.05 $\pm$ 0.04	0.06 $\pm$ 0.04	0.09 $\pm$ 0.05	0.02 $\pm$ 0.01	.182	P = .449	P = .110
Basophils ($\times 10^9$ /L)	0.11 $\pm$ 0.1	0.08 $\pm$ 0.05	0.01 $\pm$ 0.01	0.33 $\pm$ 0.13	0.12 $\pm$ 0.09	0.12 $\pm$ 0.15	0.01 $\pm$ 0.01	0.001 $\pm$ 0.0	.460	P = .001	P < .001

MPV indicates mean platelet volume.

Data represent mean $\pm$ SD of combined male and female data from Lewis and LPK rats of the ages identified, using animals from Cohort 1 of the study. Minimum number of animals in each group is indicated by superscript. Statistical analysis was performed using a univariate general linear model against the fixed factors of age and strain. Significance was set at $P \leq .05$ and the adjusted R^2 (Adj R^2), relative predictive powers of the model adjusted for degrees of freedom) provided. Between-subject effects of statistical significance for strain and age are provided and if present, age $\times$ strain interactions are identified ($\S$ P < .01).

onward in the LPK rats relative to Lewis control animals, concurrent with reduced RBC count and HCT, but with no significant difference in MCV or MCHC between the strains at each age point studied. Post hoc analysis indicated a 10–15% decrease in MCV in both strains between 6 and 12 weeks of age ($P < .05$). MCH and RDW also decreased moderately at age 12 weeks in both strains ($P < .05$), while a mild increase in MCHC was seen in both strains at age 6 weeks ($P < .05$). Overall, the values for MCV, MCH, and MCHC remained within published normal reference intervals.²² Microscopic examination of blood smears revealed no abnormal RBC morphology or the presence of any erythrocyte corpuscular inclusions.

Platelet count

Overall platelet counts showed no strain- or age-related effect. Mean platelet volume (MPV) showed a significant strain effect, being lower overall in the LPK animals (Lewis 10.5 ± 2.9 vs LPK 8.9 ± 1.8 fL, $n = 67$ total, $P = .005$) but there was no age effect, and values remained within published normal reference intervals for Lewis rats.²²

Reticulocyte index

Reticulocyte index demonstrated both an age- and strain-related effect (adjusted R^2 value .843, $P = .04$ strain, $P < .001$ age, $n = 25$). As illustrated in Figure 1, the reticulocyte index was greatest in the 6-week-old LPK compared to all other age and strain groups ($P < .001$). There was an age-related decline in the reticulocyte index for Lewis animals between 6 and 18 weeks ($P < .05$). At 12 and 18 weeks, there was no difference between the Lewis and LPK animals.

Figure 1. Means ($\pm$ SD) of reticulocyte index for Lewis and Lewis polycystic kidney (LPK) rats at 6, 12, and 18 weeks of age. There were a significantly greater number of reticulocytes in the 6-week-old LPK compared to all other age and strain groups. There was also an age-related decline in the Lewis animals between 6 and 18 weeks. * $P < .001$ compared to all other age/strain groups. ** $P < .05$ between indicated age/strain groups (total $n = 25$).

WBC counts

Total and differential WBC counts are provided in Table 2. Total WBC counts demonstrated a strain and age effect, with the LPK rats having slightly fewer WBC than Lewis rats at age 24 weeks ($P < .05$). Lymphocyte counts were significantly reduced in the LPK rats at 18 and 24 weeks ($P < .001$) to values close to 50% and 25% of those seen in Lewis control rats at those ages, respectively. Neutrophil and monocyte counts exhibited an age but not a strain effect; however, there was a significant age and strain interaction, with numbers trending lower in 18- and 24-week LPK rats. Eosinophil counts were not influenced by age or strain, while basophil counts were significantly greater in 24-week-old Lewis rats. Overall however, all WBC variables in both strains remained within published reference intervals for Lewis rats.²² Microscopic examination of blood smears indicated no abnormal WBC morphology, and manual differential cell counts were consistent with the data derived from the automated cell counter (data not provided).

RBC osmotic fragility assay

RBC osmotic fragility assay curves are illustrated in Figure 2B. Median corpuscular fragility values were not significantly different between any of the age/strain groups examined (Lewis: 12 weeks 0.45 ± 0.014 & 18 weeks 0.46 ± 0.012 , LPK: 12 weeks 0.46 ± 0.012 & 18 weeks 0.46 ± 0.012 , $n = 18$).

Spleen analysis

Splenic index is presented in Figure 2A. The size of the spleen relative to body weight was significantly influenced by strain and age (adjusted $R^2 = .765$, $P < .001$, $n = 27$). Post hoc analysis indicated an age-related decrease in splenic index in both strains; however, at all ages the splenic index in the LPK animals was significantly greater than in Lewis rats ($P < .05$), although this difference was not marked.

Histologic assessment revealed normal prominent white pulp with inverted follicles in both strains at all ages. In 6-week-old animals from both strains, red pulp demonstrated moderate-marked EMH, with little Perl's positive material present and either mild-moderate acid hematin or no brown pigment evident. At age 12 and 18 weeks, both strains showed minimal EMH and a moderate-marked amount of Perl's positive material present, and with no difference between strains.

Figure 2. Means ($\pm$ SD) of splenic index **(A)** and RBC osmotic fragility **(B)** for Lewis and Lewis polycystic kidney (LPK) rats. **(A):** Splenic index (spleen weight/body weight) was significantly greater in the LPK compared to Lewis rats at all ages examined (** $P < .05$ LPK vs age-matched Lewis control), and there was a significant age-related decline in both groups between 6 and 12 weeks of age (* $P < .05$ between indicated ages, total $n = 27$). **(B)** RBC osmotic fragility group data and fitted sigmoidal curves (lines) illustrating the relationship between NaCl concentration (percent saline) and degree of RBC hemolysis in Lewis and LPK rats at 12 and 18 weeks of age (total $n = 18$).

Bone marrow cytology and histology

In 18-week-old animals, the pool of both erythroid and myeloid proliferating cells were significantly reduced in the LPK animals (Lewis vs LPK: erythroid 72.37 ± 1.49 vs $43.24 \pm 16.75\%$ & myeloid 46.13 ± 6.67 vs $31.19 \pm 2.91\%$, respectively, $P < .05$, $n = 10$ animals, 533 ± 77 cells counted per smear). The M:E ratio was not significantly different between the 2 strains (Lewis vs LPK: 0.70 ± 0.2 vs 0.72 ± 0.2 ; $P = .74$). Megakaryocyte numbers were comparable between strains (data not presented). Subjective histologic analysis of bone marrow tissue sections indicated moderate to high cellularity in the LPK rats, while consistently high cellularity was noted in the Lewis controls.

Plasma and renal EPO

Plasma EPO levels were significantly influenced by strain but not age, being significantly higher in the LPK compared to Lewis controls (Lewis 476.4 ± 80.4 vs LPK 648.3 ± 76.3 pg/mL, adjusted R^2 value .523, $P < .001$ strain, $P = .486$ age, $n = 25$). Validation data for the EPO assay as used for rat serum are as follows: lower level of detection 21.7 pg/mL, analytic range 62.5–4000 pg/mL, precision (intra assay coefficient of variation [CV] $< 10\%$, inter assay CV $< 12\%$), recovery and linearity (83–91%) and (90–100%), respectively.

Renal EPO mRNA expression levels were significantly greater in the 12-week-old LPK animals compared to the Lewis animals (mean $\Delta Ct \pm SD$: LPK 8.77 ± 0.62 vs Lewis 0.87 ± 0.43 ; $P < .001$), which represented a 284-fold higher level of gene expression

(relative fold and range values: LPK 284 (211–384) vs Lewis 1 (0.54–1.83) $P < .001$, $n = 10$).

Multiple linear regression modeling

Table 3 lists the independent variables identified with multiple linear regression modeling that associated

Table 3. Results of multiple linear regression to determine parameters associated with changes in HGB, HCT, WBC, and EPO in Lewis control rats and Lewis polycystic kidney (LPK) rats.

Dependent variable	Independent variable(s)	n	Adj R^2	Pearson correlation	β	P-value
HGB*	Urea	58	.743	-.842	-0.690	<.001
	Blood pressure			-.716	-0.257	.003
HCT**	Urea	58	.786	-.806	-0.997	<.001
	Blood pressure			-.758	-0.359	<.001
	Creatinine			-.640	0.436	.014
WBC†	Urea	58	.227	-.490	-0.490	<.001
EPO‡	Blood pressure	22	.473	.619	0.566	.002
	Platelets			.457	0.378	.029

Results of multiple linear regression modeling. For the hematologic variables HGB, HCT, and WBC, data were obtained from Lewis and LPK animals aged 6, 12, 18, and 24 weeks. For erythropoietin (EPO), data were obtained from Lewis and LPK animals aged 6, 12, and 18 weeks. n indicates number of data pairs for each regression analysis after listwise exclusion of missing data; Adj R^2 , Adjusted R^2 ; β , standardized regression coefficients.

*Creatinine and urinary protein:creatinine (UPC) ratio were excluded from the final model.

**UPC was excluded from the final model.

†Blood pressure, creatinine, and UPC were excluded from the final model.

‡Urea, creatinine, UPC, HCT, and HGB were excluded from the final model.

Figure 3. Distribution of individual data points illustrating correlative relationship between plasma erythropoietin (EPO) concentration and (A) systolic blood pressure and (B) platelets in Lewis control rats (open circles) and Lewis Polycystic Kidney (LPK) rats (gray squares) aged 6, 12, and 18 weeks. $n = 22$ data pairs.

with HGB, HCT, and WBC, with results derived from Lewis and LPK animals aged 6, 12, 18, and 24 weeks. For HGB, multiple linear regression identified a strong negative association with urea concentration. HGB was also negatively associated with blood pressure, albeit to a lesser extent than urea. For HCT, linear regression modeling indicated that urea concentration, blood pressure, and creatinine concentration all contributed to the model, with urea concentration having the greatest impact. For WBC counts, urea concentration was the only variable retained in the modeling, demonstrating a significant negative correlation.

Table 3 further lists the independent variables identified with multiple linear regression modeling that associated with plasma EPO, with results derived from Lewis and LPK animals aged 6, 12, and 18 weeks. Modeling indicated a significant positive correlation of plasma EPO concentration with blood pressure and platelet count, while all other variables tested were excluded. The relationship between plasma EPO with blood pressure and platelet count for individual animals is illustrated in Figure 3.

Discussion

Anemia is associated with many negative consequences for patients with chronic renal failure, including reduced quality of life indices such as fatigue and reduced strength and critically, increased risk of cardiovascular disease.²⁷ Therefore, the study of the pathogenesis and the primary and secondary correlation of renal dysfunction with observed changes in hematology variables throughout progression of CKD may shed some light on potential mechanisms and reveal potential therapeutic approaches.

This study has shown that in the LPK rat model of CKD, a marked anemia develops that is strongly corre-

lated with plasma urea. The anemia was characterized as normocytic and normochromic, with no evidence of a regenerative response in the later phases of disease. Linear regression modeling indicated that blood pressure was also associated with anemia. Furthermore, the data presented in this study reveal an interesting discrepancy between EPO and key hematologic variables. EPO, which is produced in the renal parenchyma was increased across all stages of CKD in the LPK rat and was not correlated with the anemia nor plasma urea or creatinine concentrations.

Our study identified very strong negative correlations between urea and both HGB and HCT across the range of ages and therefore renal functions studied from both the control Lewis and LPK animal populations. Negative correlations between urea and HGB and HCT have likewise been demonstrated in people.^{1,27} Interestingly, while plasma creatinine was negatively correlated with HCT, linear regression modeling indicated that the relationship was not as strong as that for urea, and it was not correlated with HGB levels. Urinary protein:creatinine ratio was found to not correlate with either HGB or HCT. A lack of correlation between creatinine concentration and the severity of anemia has been documented in human studies;²⁸ it has been proposed to result from the insensitivity of blood creatinine in detecting a decline in glomerular filtration rate (GFR), arising from changes in both the metabolism and renal handling of creatinine, and from compensatory mechanisms that serve to maintain normal GFR despite a reduction in functional renal mass.^{27,29}

There was also a correlation between the increase in blood pressure and decline in HGB and HCT, although not as strong as that seen for plasma urea. While our work does not reveal any causal relationships, this association is consistent with studies in people that suggest HGB and blood pressure being independent predictors of cardiovascular disease, and specifically, left ventricular hypertrophy in renal disease patients.^{30,31} Left ventricular hypertrophy is also a feature of the LPK rodent model.¹⁶ Our results support recommendations that treatment of anemia and hypertension both need to be targeted as treatment outcomes in CKD patients.

One methodologic issue to be considered in the interpretation of our studies is the use of published reference intervals to compare our control and disease model data. It is in general not an acceptable practice in clinical pathology, especially in laboratory rats as the origin of the animals can affect such reference intervals.³² As we are studying a naturally progressive model of disease, no untreated control line is available

for reference data; however, this was mediated to some effect by using Lewis animals as controls for the LPK comparative analysis, being the same strain from which the LPK arose, and bred and maintained in the same facility. Furthermore, in circumstances such as the calculation of reticulocyte index, the use of a potentially inadequate HCT mean would result in a constant error and likely not affect the final statistical conclusions made.

Increased EMH was noted in the spleen of the 6-week-old LPK relative to the older animals, but this was also evident in the 6-week-old Lewis and is normal in rodents, where the diffuse hyperplasia of red pulp is both normal and more commonly present in young animals.³³ The splenomegaly we observed in the 6-week-old animals relative to older animals within both strains is consistent with this EMH. The spleens of the LPK animals were also relatively larger compared to age-matched controls at 6, 12, and 18 weeks. In the absence of histologic evidence for increased RBC destruction in the spleen, this may be associated with the genotype, with splenomegaly being described as an unusual phenotype in a number of human families with NPHP mutations.³⁴

Leukopenia is a known complication of chronic renal failure in animal models and people.⁹ In our study, we observed a reduced WBC count in the LPK rats, most evident in the later stages of the disease. Linear regression modeling indicated a significant negative correlation between WBC count and plasma urea, consistent with the hypothesis that the uremic state per se contributes to the decreased number of WBC.⁹ Interestingly, similar to our findings, a study examining PKD patients found that they had significantly lower WBC counts, and notably, reduced lymphocyte counts, when compared to patients with ESRD due to other causes.⁸

In this study, we demonstrated that the LPK animals maintained an increased concentration of plasma EPO relative to the Lewis animals at all ages studied. This was supported by our findings of markedly higher values of EPO mRNA expression in the kidneys of 12-week-old LPK animals, which also suggests that extra-renal production of EPO is not a major contributor to plasma levels.

Erythropoietin concentration and HGB are characterized by a negative feedback relationship, which despite decreasing serum EPO values, has shown to be sustained in the face of declining renal function until a critical threshold is reached.³⁵ Surprisingly however, linear regression modeling indicated that EPO concentrations were not associated with the anemia, nor the markers of renal function. EPO concentration was

however positively associated with blood pressure. Hypoxia due to lower oxygen-carrying capacity in anemic individuals is a key stimulant for increased renal EPO production. In addition, local renal hypoxia³⁶ as recently demonstrated in kidneys of LPK rats³⁷ is suspected to stimulate EPO expression and release. At the same time, hypoxia activates renal afferent nerves, which drive sympathetic nervous system activity resulting in increased blood pressure via the central nervous system.³⁸ With the recently documented evidence for increased sympathetic activity in the LPK rat model³⁹, there appears to be a common mechanism as to how hypoxia may cause/contribute to hypertension and increased EPO concentration.

While there was no overall difference in the platelet count between Lewis and LPK animals, there was a positive relationship between EPO and platelet count. This is consistent with several human studies showing that EPO induces platelet production, including in CKD patients.^{39–41} The ability of EPO to stimulate platelet production has been postulated to be due to a direct response by megakaryocytes to EPO, or a global bone marrow response to EPO driven by a putative common erythrocytic and megakaryocytic progenitor.⁴⁰ As the megakaryocyte numbers were comparable in both groups, no such effect was demonstrated in the current study.

In the absence of a low EPO concentration, the major question arising therefore is what is the underlying mechanism driving the anemia in this model of CKD? Other potential mechanisms of anemia in CKD include shortened RBC survival, anemia of chronic disease, and nutritional deficiency. Increased destruction of RBC is not a likely mechanism, as hemolytic anemias are typically accompanied by a strong regenerative erythropoietic response and the only indication of a regenerative response was in the 6-week-old LPK before the major progression of the anemia. In addition, there was no evidence for increased osmotic fragility of RBC in this study and the low levels of visible iron deposits in spleen histology also argue against a major component of anemia of chronic disease. While not directly measured, nutritional deficiencies associated with the disease phenotype such as iron, folic acid, or vitamin B12 deficiency⁴² are unlikely based on the normocytic/normochromic features of the anemia, although additional studies are required to confirm this interpretation.

An alternative possibility is a relative deficiency in EPO expression and secretion, being lower than expected for the degree of anemia in the LPK rats, a phenomena also described in human CKD patients⁴³, or an inherent resistance to EPO, such that the bone

marrow response to EPO is suppressed. Resistance to EPO is believed to be due to a reduced capacity for EPO to drive antiapoptotic pathways in proerythroblasts, with possible causes including uremic toxins, systemic inflammation, and microvascular damage in the bone marrow.^{42,44} Our demonstration of elevated EPO levels in the presence of a nonregenerative anemia, alongside reduced proliferation of the erythroid precursors in the bone marrow, and a strong negative correlation of both HGB and HCT with urea concentrations would be consistent with uremia-driven erythroid suppression in the LPK rodent model.⁴²

While the use of EPO or EPO-stimulating agents to correct anemia in CKD has been an important treatment strategy with significant impact on improving quality of life⁴⁵, its application is under serious reconsideration. Extensive evidence now exists indicating that EPO treatment can induce hypertension, increase the risk for future cardiovascular events, and altogether does not prolong survival.^{7,42} Underlying mechanisms for these adverse effects have been linked to high HGB target levels, high EPO-stimulating agent dosages, or poor hematopoietic responses.^{7,42} Early preventative treatment of anemia has been suggested as another option, aiming to prohibit the progression of CKD and cardiovascular changes, alongside an overall reduced risk of side effects.^{1,46} This hypothesis is supported by animal studies using surgically induced renal failure, where early treatment with human EPO is associated with improved renal function.⁴⁷ However, in studies where there is an acute onset of renal failure, no direct correlation with natural disease progression, and therefore no translation to the chronic disease state can be made. This is an area of need, as there are currently no clear guidelines as to what actual cut-off values of HGB, HCT, or renal function variables such as urea and creatinine concentrations should trigger initiation of treatment with EPO-stimulating agents.⁷

The LPK rodent model, now better characterized by defined variables for impaired renal function in parallel with accompanying anemia, WBC, and EPO profiles, will serve as a valuable system to further examine the underlying mechanisms, identify key intervention points, and facilitate the assessment of novel treatment strategies with particular relevance to PKD patients.¹¹ In conclusion, this study has shown that plasma urea is strongly correlated with the progression of anemia in the LPK model of CKD. The paradoxical relationship between plasma EPO concentrations and the persistent marked anemia suggest a deficient erythroid response to EPO as one possible mechanism.

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