Supporting Online Material for

A Heme Export Protein Is Required for Red Blood Cell Differentiation and Iron Homeostasis

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Published 8 February 2008, Science 319, 825 (2008)
DOI: 10.1126/science.1151133

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Materials and methods

Generation of Flvcr mutant mice. Using cDNA sequences for feline and human FLVCR, we identified the cDNA sequence of murine Flvcr from EST data in the Genbank database, and confirmed this by amplification and sequencing of exons 1, 5, and 8 from genomic DNA. We next identified BAC contigs containing the entire 18 kb murine Flvcr genomic region, confirming it was the Flvcr locus and not a paralog (S1). We then derived the intron/exon structure by aligning the cDNA and genomic sequences. Due to the presence of a gene on the opposite strand that likely shares a promoter region with Flvcr (S1), we opted to delete exon 3, which encodes the intracellular loop between transmembrane domains 6 and 7, as our knockout strategy. Structure/function analyses of two proteins structurally related to FLVCR, the lactose permease and the reduced folate carrier protein, revealed that this loop is required for protein stability, processing, and function in prokaryotic and eukaryotic cells (S2-4). Exon 3 deletion should, therefore, produce an unstable protein that cannot traffic to the membrane (S2, S3). If (unexpectedly) the protein were to successfully transport to the membrane, its structure is predicted to be severely altered, making it unlikely to retain function (S5). The construct consists of a loxP site in the intron between exon 2 and exon 3, and a PGK promoter-driven neomycin selection cassette, flanked with loxP sites in the intron between exon 3 and exon 4 (Fig. S1). Primers were designed for a PCR-based cloning strategy and we generated the targeting construct using template genomic DNA that matched the strain of the ES cells (129S4/SvJae). All exons and intron-exon boundaries were sequenced in the construct and matched sequences from a BAC clone. The ES culture, transfection, cloning, and screening were performed as before (S6). Targeted clones were transfected with a cre expression vector and recloned to identify flox and null allele containing clones by Southern blot and PCR analysis. A flox containing ES clone with a normal karyotype was used for blastocyst injection and the male chimeras were bred to female C57BL/6 mice. Heterozygous Flvcr+/flox mice were bred to either Cmv-cre+ or Mx-cre+ transgenic mice (S7, S8) to generate Flvcr+/−;Cmv-cre+ or Flvcr+/−;Mx-cre+ mice, respectively. Mice were backcrossed to C57BL/6 for 1 to 5 generations then intercrossed to obtain F1, F2, and F3 generations for use in these studies.

We then confirmed that exon 3 deletion produced a nonfunctional protein by cloning the deleted allele and appropriate controls into NRK cells which normally do not express FLVCR and performed export studies using zinc mesoporphyrin which is a fluorescent heme analogue that is transported comparably to heme (Fig. S2). Cells expressing FLVCR show export at 90 minutes. Cells expressing the exon 3 mutant fail to export ZnMP, and cells expressing both wild-type and exon 3-deleted FLVCR show heme export. This latter findings, proves that the mutated protein does not act as a dominant-negative protein. Furthermore, the lack of a dominant-negative effect suggests that FLVCR functions as a monomer, which is consistent with the known crystal structure of MFS members (S4, S9).

Breeding and analysis of mutant Flvcr mice. Animals were housed in a SPF facility at the University of Washington, Seattle. The Institutional Animal Care and Use Committee approved all studies. Embryos were obtained from timed pregnant females for genotyping, immunohistochemistry, or pathologic analysis. Embryos were staged according to standard methods; noon of the day when a copulatory plug was identified is 0.5 dpc. To induce Mx-cre
expression and delete exon 3, 7 day-old neonatal pups were treated with 50 μg of poly(I)-poly(C) (Amersham/GE Lifesciences, Piscataway, NJ) IP every other day for three doses. Animals were sacrificed 5-12 weeks post deletion for analysis. Adult-deleted animals were treated with 250 μg of poly(I)-poly(C) every other day for three doses (their bone marrow was used to generate macrophages for ZnMP and $^{55}$Fe-hemin uptake and washout studies). Flvcr deletion in adults was not as effective as in neonates, however, adult animals still became severely anemic within a similar time frame.

**Cell lines.** The cDNA encoding full length and mutant (lacking exon 3 coding elements) Flvcr were cloned from C57BL/6 bone marrow RNA using RT-PCR. These entire cDNAs were sequenced to confirm fidelity. These cDNA constructs were inserted into MSCVneo and MSCVhygro (Clontech Laboratories, Inc., Mountain View, CA) and used to generate NRK cells expressing vector alone, wild-type, mutant, or both proteins (NRK/neo, NRK/FLVCR, NRK/FLVCRΔ3 and NRK/FLVCR-FLVCRΔ3, respectively) essentially as described previously (S10).

**ZnMP and $^{55}$Fe-hemin uptake and washout studies.** These studies were performed as described previously (S10).

**In situ hybridization.** In situ hybridization of mouse embryos was performed using a 1.7 kb antisense RNA probe to murine FLVCR as described previously (S11).

**Flow cytometry.** Single-cell suspensions were prepared from freshly isolated bone marrow, spleen, or E14.5 fetal liver cells and were immunostained with anti-Ter119-PE and anti-CD71-FITC (BD Pharmingen, San Diego, CA) antibodies. Flow cytometry was performed as described previously (S12). All cells are included in the CD71-Ter119 analyses. Single-cell suspensions of cultured macrophages were immunostained with anti-GR-1-PE (BD Pharmingen), f4/80-PE (Serotec, Raleigh, NC), or Mac-1-biotin followed by strepavidin-PE (BD Pharmingen).

**Colony assays.** Assays were performed as described previously (S13). To detect BFU-E and CFU-E colonies, $8 \times 10^5$ cells/plate were plated from Flvcr-deleted mouse marrow and $1 \times 10^5$ cells/plate from control mouse marrow in duplicate in semisolid medium (Methocult™ M3434; StemCell Technologies, Vancouver BC, Canada) according to the manufacturer’s protocol.

**Blood cell analysis.** Mice were bled retro-orbitally into EDTA anticoagulated microtainer tubes (Becton Dickenson, Franklin Lakes, NJ). Blood was analyzed at Phoenix Central Laboratory, Everett, WA on a Celldyne 3500 Analyzer or at the University of Washington on a Hemavet HV950FS analyzer (Drew Scientific, Oxford, CT) programmed for mouse blood. CBC (complete blood counts) include WBC (white blood cell number), RBC (red blood cell number), HGB (hemoglobin), HCT (hematocrit), MCV (mean corpuscular volume), MCH (mean cell hemoglobin), MCHC (MCH concentration), RDW (red cell distribution width), and platelet numbers.

**Tissue staining.** Whole mouse embryos or tissues were fixed in 10% buffered formalin, sectioned, and stained with standard hematoxylin and eosin stains or Gomori’s Prussian blue stain for iron.
Murine macrophage culture. Single-cell suspensions of mouse bone marrow were collected from mice 5 days after completing poly(I)-poly(C) treatment. Cells were cultured in macrophage-differentiating media [RPMI-1640 with 30% L-cell conditioned media, 20% FBS, 10^{-4} M \beta\text{-mercaptoethanol essentially as described (S14)]. Macrophages were defined by flow cytometry (Mac-1+, f4/80+, GR-1-) and analyzed for ZnMP and ^{55}\text{Fe-hemin} export 10-24 days post in vivo deletion (after 5-19 days in culture).

Murine transplantation of retroviral vector-transduced bone marrow cells. GFP-expressing retroviral vectors, MXIG (gift from Derek A. Persons, St. Jude Children’s Research Hospital) (S15) and MFIG (generated by cloning the full length cDNA of human FLVCR into the cloning site upstream of the IRES of MXIG), were pseudotyped with ecotropic murine retrovirus (Phoenix Eco packaging line from Gary Nolan, Stanford, CA). Bone marrow was harvested from 6-12 week-old, 5-flourouracil-treated Pep3b (CD45.1) mice. Marrow cells were prestimulated with 20 ng/ml murine IL-3, 100 ng/ml human IL-6, 50 ng/ml murine stem cell factor in IMDM with 10% FBS for two days, then cultured with viral supernatant plus cytokines and 4 \mu g/ml of protamine sulfate on viral-preloaded RetroNectin-coated dishes (r-fibronectin CH296, Takara Bio Inc, Japan) for 6 hours. Viral supernatants were replaced by fresh media and the cells were allowed to recover overnight, and the treatment repeated. Transduced cells were transplanted into lethally irradiated C57BL/6 (CD45.2) mice (3 \times 10^6 cells/animal).

Transplantation studies of mice lacking FLVCR in marrow only. Bone marrow cells harvested from Flvcr^{flox/flox},Mx-cre^{+} mice or Mx-cre^{+} mice were transplanted into 6-8 week-old C57BL6 lethally irradiated (1100 CGy) female mice (10 \times 10^6 cells/animal). After stable engraftment (5-7 weeks), mice were treated with 250 ug poly(I)-poly(C) IP every other day for two doses and sacrificed 5.5-6 weeks later for analyses.

Quantitative RT-PCR and northern blot analyses. Q RT-PCR was performed using the TaqMan method as described (S10) for intact murine FLVCR (primers: ATCTGGAACCTGTGCAAGAACA and ATTGAATAAAATGCTCCAGTCATGAT; probe: CCCCTTTTCTCCTGCTGGTCAGTTATG), murine hepcidin (primers: CAGCAGAACAGAAGGCATGATG and GGCTGGCAAGGAGGAGAAG; probe: CACTCGGACCCAGGCTGCCTG), and murine \beta\text{-actin} (primers: ACGGCCAGGTCATCACTATTGA and CAAGAAGGAGGCTGGAAAAAGA; probe: CAACGAGCGGTTCGATGCC). To confirm hepcidin qRT-PCR results, we performed northern blot analysis as previously described (S10) using a probe directed against hepcidin (W12913.1) and normalized to \beta\text{-actin} expression.

Western blot analysis. Human tissues were obtained from Cooperative Human Tissues Network, Western Division at Vanderbilt University Medical Center (Nashville, TN). All procedures were approved by the University of Washington Human Subjects Review Committee. Human tissues were homogenized and proteins extracted in 50mM Tris HCL, pH 8.0, 150mM NaCl, 1% NP-40, 0.5% DOC, and 0.1% SDS. SDS-PAGE and western blot analyses were performed as described (S10). Band intensity was quantified using Image J software, version 1.37 and normalized to \beta\text{-actin} loading.
**Ferritin studies in macrophages.** Erythrophagocytosis was performed essentially as described (S16). Alternatively, macrophages were treated with 10 μM ferric ammonium citrate (FAC). After either treatment, the cells were washed and then cultured with or without hepcidin. After 24 hrs, the samples were collected in lysis buffer (150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 10 mM Tris pH 7.4), and complete protease inhibitor (Roche Diagnostics Co., Indianapolis, IN) and analyzed for ferritin levels as described (S17).

**Supporting text**

**Additional Flvcr+/− interbreedings: embryonic lethality and morphologic analyses.** We have now backcrossed Flvcr+/− mice to C57BL/6 mice for 5-7 generations (N5-7). Ongoing embryonic analyses of interbreedings between these mice differ from data from earlier generations of mice (presented in the main manuscript text and Table S1). Specifically, we are now finding dead, or live but morphologically identifiable, FLVCR null embryos at E12.5, when previously null and wild-type embryos were indistinguishable and alive (thus far we have distinguished 16 null embryos among 48 embryos genotyped at E12.5). Since backcrossing mice eliminates genetic variation, we are likely observing a strain-specific effect. This effect is also manifest in the morphologic analyses. Specifically, the facial and limb abnormalities present in E14.5 FLVCR null embryos are less apparent when fetal demise occurs prior to E14.5. Of note, C57BL/6 mice are characterized as a low iron-loading strain of mice compared to 129 and may not possess the genetic machinery to compensate during embryonic development when heme export is impaired. This could cause the embryos to die earlier in gestation before physical anomalies appear.

**Wild-type mouse embryonic expression of Flvcr by in situ hybridization.** Flvcr is expressed in both the visceral endoderm and mesoderm layers of the yolk sac at E7.5 and E8.5. Mesodermal expression is generalized, consistent with expression in both blood and endothelial components. Strikingly, Flvcr is predominantly expressed in the yolk sac at E7.5 compared to the embryo proper or to the ectoplacental cone; while at E8.5, there is an increase in expression in these other tissues. During development, chorioallantoic fusion occurs at E8.5 and the embryo’s source of nutrition shifts to the placenta. Thus, the extra-embryonic expression pattern of Flvcr may mirror the embryo’s source of nutrition during development.

The details of Flvcr expression in the placenta are as follows: Flvcr expression is absent in the chorionic plate, but present in the labyrinth, spongiosotrophoblast zone, and in the giant trophoblast cells of the placenta at E12.5 and E14.5. The outer, maternal layer of the placenta is not positive. Some but not all of the giant trophoblast cells at E8.5 express Flvcr mRNA.

**Colony assay results in Flvcr-deleted animals and controls.** CFU-E colonies are absent in marrow from Flvcr-deleted animals (deleted: 0.0 colonies/10⁵ cells plated ± 0, n= 4 vs. control: 169.7 ± 57.8, n= 3; mean ± SD, two-tailed Student’s t-test, p= 0.04). The number of BFU-E colonies in deleted mice, defined both by morphology and a criteria of a minimum of 200 cells per colony, is 12.0 ± 6.5 colonies/10⁵ cells plated (n= 4) verses 53.8 ± 29.7 in control mice (n= 4; mean ± SD, two-tailed Student’s t-test, p= 0.06). CFU-GM colonies are smaller than control colonies (1-15 cells/colony vs. >30 cells/colony). These data are consistent with a block in erythroid differentiation at the CFU-E/proerythroblast stage since the colonies counted on day 7 in a BFU-E assay contain erythroid cells that derive from BFU-E present at the time of plating. The assay thus requires that BFU-E differentiate to CFU-E and proerythroblasts and begin
hemoglobinization to be enumerated; likewise, the colonies counted on day 2 in a CFU-E assay are a representation of a CFU-E present at the time of plating, and thus require that cells proceed through the proerythroblast stage for inclusion in the CFU-E colony count. Therefore, we expect to see poorly formed BFU-E colonies or fewer BFU-E colony number (since they would not meet the criteria of ≥200 cells/colony) and no CFU-E colonies when differentiation fails at the CFU-E/proerythroblast stage. We hypothesize that the abnormal CFU-GM colony morphology in Flvcr-deleted mice is due to dysfunctional monocytes and macrophages which lack FLVCR.

**Overexpression of FLVCR results in a mild microcytic anemia.** Pep3b bone marrow (CD45.1+) was transduced with retroviral vectors, MFIG and MXIG, encoding GFP with or without human FLVCR. Transduction efficiency measured by GFP was 93.1% for MFIG and 94.1% for MXIG transduced cells. Transduced marrow was then transplanted into irradiated C57BL/6 recipients (CD45.2+). Peripheral blood and marrow mononuclear cells were collected 12 weeks after transplantation. All mice included in this analysis demonstrated excellent engraftment (percent of granulocytes expressing donor CD45.1: MFIG: 95.9 ± 3.0, n= 3, and MXIG: 86.9% ± 7.0, n= 3; mean ± SD, two-tailed Student’s t-test, p= 0.14). 56.8 ± 34.7 and 59.2% ± 28.3 of granulocytes (p= 0.93) expressed GFP in the MFIG and MXIG mice, respectively. Twelve weeks after transplantation, the MFIG mice have a mild hypochromic, microcytic anemia (MFIG: MCH 12.7 pg ± 1.7, HGB 13.2 g/dl ± 1.4, MCV 41.0 fL ± 5.2, n= 3 vs. MXIG: MCH 15.7 ± 1.7, HGB 15.4 ± 0.5, MCV 49.3 ± 1.2; mean ± SD, n= 3, one-tailed Student’s t-test, p= 0.05, 0.05, and 0.05 respectively).

**Heme toxicity and erythropoiesis failure: possible link to Diamond-Blackfan Anemia.** Diamond-Blackfan anemia (DBA) is a congenital form of PRCA in humans characterized by a macrocytic anemia, reticulocytopenia, and a block in erythroid differentiation at the proerythroblast stage ($S18$) like FeLV-C-associated PRCA and PRCA in Flvcr-deleted mice. Heterozygous mutations in RPS19, which encodes a protein that binds to the 40S (translation initiation) ribosomal subunit as one of 33 associated proteins, account for 25% of DBA cases ($S19$). Recently, mutations in two other ribosomal proteins, RPS24 and RPS17, which also bind to the 40S subunit, have been identified in a subset of DBA patients ($S20, S21$). RPS19 mutations result in defective maturation of the 40S ribosomal subunit ($S22$). These mutations should, therefore, result in a slow or late initiation of globin translation, and consequently, to transient excess in intracellular free heme, analogous to what we predict occurs in the Flvcr-deleted (or FLVCR null) mice where heme export is impaired.

While we anticipated that Flvcr-deleted mice and DBA patients would share a common erythroid phenotype, we were surprised to discover that E14.5 FLVCR null embryos exhibit the same, unique morphologic abnormalities seen in DBA, specifically, flattened faces, hypertelorism ($S23$), upper limb, and hand/digit abnormalities ($S24-26$). This convergent phenotype raises the possibility that FLVCR, heme excess, or related pathways account for both the congenital deformities and the erythroid defect in DBA patients.
FLVCR mRNA expression in multiple human tissues by quantitative RT-PCR. We confirmed that FLVCR mRNA expression does not equate to FLVCR protein levels by performing quantitative RT-PCR on the same tissue samples analyzed by western blot analysis (Fig. S4A-B). mRNA levels were comparable in all tissue samples. Specifically, mRNA copy number ranged from 1590 copies (per 50 ng RNA) in the pancreas, to 2164 in the stomach, and to 2607 in the duodenum. The discrepancy between mRNA and protein expression implies that FLVCR is regulated at the protein level.
Supporting figures

**Figure S1. Generation of Flvcr mutant mice.** A, Diagram of murine Flvcr genomic region and targeting strategy. The wild-type (wt) allele is depicted at the top with numbered exons. The position and size of the diagnostic BamHI (B) restriction sites and the location of the screening probe are indicated. The targeting construct shows the location of the neomycin resistance cassette and the 3 loxP sites (L1, L2, L3). The targeted allele (neo allele), flox allele, and null allele are also diagrammed with the size and position of the diagnostic restriction site indicated.
**B.** Southern blot analysis of *Bam*HI digested genomic DNA from the parental (AK7) ES line and two targeted clones (+/neo), probed with the probe indicated above. These clones were subsequently subjected to in vitro cre-mediated deletion and subclones were screened for the presence of a floxed allele (+/flox), and a null allele (+/-) by Southern blot with the same probe.  

**C.** PCR analysis of genomic DNA from biopsies obtained from wild-type (+/+), heterozygous floxed (+/flox), heterozygous null (+/-), or no template control samples (ntc). The primers for the L1/2 amplification, P1R (CAATAGACATTTAACACCCC) and P2F (CAAGAGTTCTATCTGGGAACC), flank the LoxP1/2 element created by the cre-mediated deletion of the neomycin cassette and generate bands of 378 bp (+) and 468 bp (flox). The primers for the L3 amplification, P3R (CGGATTTCCTCCCATACACAG) and P3F (AATTAAGGACTGGTGAGCGT), flank the LoxP3 element and generate bands of 569 bp (+) and 603 bp (flox). The primers for the L1/3 amplification, P1R and P3F, flank the LoxP1/3 element generated by cre-mediated deletion of exon 3 in the floxed allele and generate bands of 502 bp (-), 1369 bp (+), and 1497 bp (flox). The positions of molecular weight markers are indicated on the right.
Figure S2. Flvcr exon 3 is required for heme export function. Cells engineered to overexpress FLVCR (NRK/FLVCR), FLVCR with exon 3 deleted (NRK/FLVCRΔ3), FLVCR and FLVCRΔ3 (NRK/FLVCR-FLVCRΔ3), and control cells (NRK/neo) were loaded with ZnMP as before (S10), then washed and incubated for 0, 30, and 90 minutes in washout buffer. When possible, panels were chosen to contain similar cell density based upon light microscopy. The analyses of ZnMP washout after 30 and 90 minutes demonstrated a reduction in NRK/FLVCR and NRK/FLVCR-FLVCRΔ3 cell fluorescence, but not in NRK/FLVCRΔ3 cell or control cell fluorescence. Cells overexpressing both wild-type and mutant FLVCR also demonstrated a reduction in cell fluorescence at 30 and 90 minutes similar to those only expressing wild-type FLVCR.
Figure S3. Normal embryonic erythropoiesis in FLVCR null embryos. Modified Wright-Giemsa-stained cytospins of FLVCR null and control E12.5 liver cells. Scale bar, 50 μm.

Figure S4. Flvcr+/− mice have reduced Flvcr mRNA expression but normal protein levels. (A) FLVCR mRNA expression by quantitative RT-PCR. Data represent the average of three separate analyses normalized to the percent of wild-type liver mRNA levels. Flvcr+/− mice (hatched bars) n= 8, Flvcr+/− mice (solid bars) n= 8 for all tissues except bone marrow where n= 5 for both groups. (B) FLVCR protein expression in bone marrow (BM), liver, and spleen by densitometry of the 60 kB FLVCR band on western blots. Data represent the average intensity from three western blots normalized to control mice liver samples. Flvcr+/− mice n= 8, Flvcr+/− mice n= 9 for all tissues except bone marrow where n= 6 for both groups.
Figure. S5. Liver of a mouse lacking FLVCR only in the bone marrow. Representative Prussian blue (for iron) stained liver sections from a control mouse and a mouse transplanted with $Flvcr^{flox/flox},MxCre^+$ marrow and then treated to delete $Flvcr$ in hematopoietic cells. Liver sections obtained 5.5 weeks post poly(I)-poly(C) treatment (deletion). Scale bar, 100 μm.
Figure S6. Flvcr-deleted macrophages have impaired export of the heme analog ZnMP and ⁵⁵Fe-heme. (A) Macrophages from the bone marrow of adult control and Flvcr-deleted littermates (Flvcr<sup>lox/lox</sup>,Mx-cre<sup>+</sup>) were incubated with ZnMP (5 μM) for 30 minutes at 37°C, then washed and incubated for 30, and 90 minutes in buffer alone at 37°C. (B) Fe<sup>⁵⁵</sup>-heme export studies performed similarly to A. control mouse (solid) and Flvcr-deleted mouse (dashed). Radioactivity is expressed as a mean of triplicate samples (±SD).
Table S1. Genotyping of mutant Flvcr embryos and pups derived from F1, F2, or F3 intercrosses. The number of mice and percentage (%) with each genotype are indicated. The observed ratios of the genotypes are presented relative to wild-type. Chi-square value= 27.25, one degree of freedom, p< 0.001. In subsequent embryonic analyses from interbreedings between Flvcr<sup>+/−</sup> parental mice, which were backcrossed to C57BL/6 mice for 5-7 generations (resulting in a purer C57BL/6 background), the late stage embryonic death occurred at or before E12.5 (supplementary online text).
Table S2. *Flvcr*<sup>+/−</sup> mice hematologic parameters. Control mice n= 8, *Flvcr*<sup>+/−</sup> mice n= 7; mean ± SEM, two-tailed Student’s t-test.

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<th>p value</th>
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Table S3. *Flvcr*-deleted mice hematologic parameters. Control mice n= 13, *Flvcr*-deleted mice n= 11; mean ± SEM, one-tailed Student’s t-test. The absolute neutrophil and lymphocyte counts of deleted and control animals did not significantly differ.
Table S4. Hematologic parameters of mice lacking FLVCR only in the bone marrow. Control mice n= 3, mice transplanted with Flvcr1<sup>flax/flax</sup>;Mx-cre<sup>+</sup> marrow and then treated with poly(I)-poly(C) to delete Flvcr<sup>+</sup> in hematopoietic cells n= 6; mean ± SEM, one-tailed Student’s t-test. Data obtained 5.5-6 weeks post poly(I)-poly(C) treatment. White blood cell analyses excluded as the mice received sedation prior to blood draw which dramatically altered WBC parameters. CD71/Ter119 flow cytometric analyses of bone marrow and spleen from mice lacking FLVCR only in the marrow were comparable to analyses of neonatally-deleted Flvcr<sup>+</sup> mice; similarly, CFU-E colonies were absent in mice lacking FLVCR only in the marrow.

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<td>MCHC (%)</td>
<td>31.4 ± 1.7</td>
<td>32.3 ± 1.6</td>
<td>0.35</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>18.6 ± 0.5</td>
<td>29.7 ± 4.2</td>
<td>0.02</td>
</tr>
<tr>
<td>platelets (k/μl)</td>
<td>761.7 ± 92.9</td>
<td>1921.2 ± 374.8</td>
<td>0.01</td>
</tr>
</tbody>
</table>
Supporting references