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(54) Title: IMMUNOASSAY FOR HUMAN ERYTHROFERRONE

(57) Abstract: Disclosed herein are antibodies that specifically bind human erythroferrone and assay methods for detecting and/or measuring human erythroferrone, analogs of human erythroferrone, and fragments thereof. Specifically, the methods comprising using an antibody as a capture reagent and an antibody as a detection reagent for detecting or measuring a detectable label of the at least one detection reagent bound to the erythroferrone polypeptide that is bound to the capture reagent. Further disclosed are the sequences of antibodies.

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IMMUNOASSAY FOR HUMAN ERYTHROFERRONE

[0001] CROSS-REFERENCE TO RELATED APPLICATIONS

[0002] This application claims the benefit of U.S. Patent Application No. 62/515,679, filed June 6, 2017, which is herein incorporated by reference in its entirety.

[0003] REFERENCE TO A SEQUENCE LISTING SUBMITTED VIA EFS-WEB

[0004] The content of the ASCII text file of the sequence listing named "20180523_034044_175WO1_seq_ST25" which is 53.6 kb in size was created on May 23, 2018 and electronically submitted via EFS-Web herewith the application is incorporated herein by reference in its entirety.

[0005] ACKNOWLEDGEMENT OF GOVERNMENT SUPPORT

[0006] This invention was made with Government support under DK065029 and HL119893, awarded by the National Institutes of Health. The Government has certain rights in the invention.

[0007] BACKGROUND OF THE INVENTION

[0008] 1. FIELD OF THE INVENTION

[0009] The present invention relates to assays for erythroferrone.

[0010] 2. DESCRIPTION OF THE RELATED ART

[0011] Intestinal iron absorption and the release of iron from stores increase greatly within hours after blood loss or administration of erythropoietin. In murine models, the response is largely mediated by erythroferrone. Erythroferrone is a glycoprotein hormone secreted by erythropoietin-stimulated erythroblasts. Erythroferrone is made in the marrow of a subject and its production is greatly increased when the production of red blood cells is stimulated, *e.g.*, after bleeding or during recovery from anemia. Erythroferrone acts by suppressing the hepatic synthesis of the master iron-regulatory hormone, hepcidin. Pathologically increased erythroferrone contributes to hepcidin suppression and iron overload in a mouse model of non-transfused β-thalassemia.

[0012] SUMMARY OF THE INVENTION

[0013] In some embodiments, the present invention provides a method of making an antibody against human erythroferrone and/or an ERFE polypeptide, which comprises injecting a non-human animal with human erythroferrone, ELPRGPGESRAGPAARPP (SEQ ID NO: 1), GESRAG (SEQ ID NO: 2), LGSPEPGAPSRSRAR (SEQ ID NO: 34), rhERFE1

(SEQ ID NO: 3), and/or rhERFE2 (SEQ ID NO: 7), preferably rhERFE1 (SEQ ID NO: 3) or rhERFE2 (SEQ ID NO: 7), and more preferably rhERFE2 (SEQ ID NO: 7).

[0014] In some embodiments, the present invention provides an antibody produced by a method as described herein, *e.g.*, paragraph [0013]. In some embodiments, the present invention provides an antibody that comprises GIDLNDNA (SEQ ID NO: 10), IYIDTST (SEQ ID NO: 11), VREDGYRLGDV (SEQ ID NO: 12), QSLYNNNY (SEQ ID NO: 15), WAS (SEQ ID NO: 16), and AGYKSSSNDDFA (SEQ ID NO: 17). In some embodiments, the present invention provides an antibody that comprises GIDLSSYE (SEQ ID NO: 20), IGTDGTA (SEQ ID NO: 21), ARDSSGNSNYRAFDP (SEQ ID NO: 22), QSIYSY (SEQ ID NO: 25), RAS (SEQ ID NO: 26), and QQGFVISNVLNS (SEQ ID NO: 27). In some embodiments, the present invention provides an antibody comprising a VH sequence as described herein, *e.g.*, paragraph [0096]. In some embodiments, the present invention provides an antibody comprising VL sequence as described herein, *e.g.*, paragraph [0097]. In some embodiments, the antibody comprises a VH sequence as described in paragraph [0096] and a VL sequence as described in paragraph [0097]. In some embodiments, the present invention provides an antibody or an immunologically active portion thereof comprising a VH sequence as described in paragraph [0096] and/or a VL sequence as described in paragraph [0097]. In some embodiments, the antibody or the immunologically active portion specifically binds human erythroferrone or an analog thereof, ELPRGPGESRAGPAARPP (SEQ ID NO: 1), GESRAG (SEQ ID NO: 2), LGSPEPGAPSRSRAR (SEQ ID NO: 34), rhERFE1 (SEQ ID NO: 3), and/or rhERFE2 (SEQ ID NO: 7). In some embodiments, the antibody is a monoclonal antibody or a synthetic antibody. In some embodiments, the antibody is an IgG isotype.

[0015] In some embodiments, the present invention provides an immunoassay for detecting an ERFE polypeptide, such as a human erythroferrone or an analog thereof, in a sample, which comprises a1) contacting the sample with a capture reagent that specifically binds the ERFE polypeptide and then contacting with at least one detection reagent that specifically binds the ERFE polypeptide bound to the capture reagent, or a2) contacting the sample with at least one detection reagent that specifically binds the ERFE polypeptide and then contacting with a capture reagent that specifically binds the ERFE polypeptide bound to the at least one detection reagent; and b) detecting or measuring a detectable label of the at least one detection reagent bound to the ERFE polypeptide that is bound to the capture reagent. In some embodiments, the capture reagent and/or the at least one detection reagent is an antibody as described herein, *e.g.*, paragraph [0014], which if both

the capture reagent and/or the at least one detection reagent are antibodies, the antibodies may be the same or different. In some embodiments, the capture reagent or the at least one detection reagent is an antibody that specifically binds a three-dimensional epitope of the ERFE polypeptide. In some embodiments, the capture reagent or the at least one detection reagent is an antibody that specifically binds a linear epitope of the ERFE polypeptide. In some embodiments, the capture reagent is an antibody that specifically binds a linear epitope of the ERFE polypeptide and the at least one detection reagent is an antibody specifically binds a three-dimensional epitope of the ERFE polypeptide. In some embodiments, the linear epitope comprises or consists of the amino acid sequence ELPRGPGESRAGPAARPP (SEQ ID NO: 1). In some embodiments, the linear epitope comprises or consists of the amino acid sequence GESRAG (SEQ ID NO: 2). In some embodiments, the linear epitope comprises or consists of the amino acid sequence LGSPEPGAPSRSRAR (SEQ ID NO: 34). In some embodiments, the antibody was raised against rhERFE1 (SEQ ID NO: 3) or rhERFE2 (SEQ ID NO: 7). In some embodiments, the immunoassay further comprises immobilizing the capture reagent to an assay substrate. In some embodiments, the sample is obtained from a human subject. In some embodiments, the blood sample is a whole blood sample, a serum sample, or a plasma sample.

[0016] In some embodiments, the present invention provides a method of determining whether the level of erythroferrone in a subject is low or high as compared to a control, which comprises performing the immunoassay as described herein, *e.g.*, paragraph [0015] on a sample obtained from the subject to obtain a measured level of erythroferrone, and comparing the measured level of erythroferrone to a control. In some embodiments, the method further comprises characterizing the subject as having an abnormally high level of erythroferrone where the measured level of erythroferrone is more than 30 ng/ml. In some embodiments, the method further comprises diagnosing the subject as having an iron metabolism disease, wherein the measured level of erythroferrone is abnormally low or abnormally high. In some embodiments, the subject is diagnosed as having a disease or condition related to abnormally low levels of erythroferrone where the measured level of erythroferrone is abnormally low or the subject is diagnosed as having a disease or condition related to abnormally high levels of erythroferrone where the measured level of erythroferrone is abnormally high. In some embodiments, the subject is human.

[0017] In some embodiments, the present invention provides a method of treating a subject for an iron metabolism disease, which comprises administering to the subject a

erythroferrone therapeutic when the subject has been characterized has having an abnormally high level of erythroferrone using an immunoassay as described herein, *e.g.*, paragraph [0015], such as the method described at paragraph [0016]. In some embodiments, the erythroferrone therapeutic is a compound that modulates iron uptake, preferably hepcidin, a mini-hepcidins, or a modified mini-hepcidin. In some embodiments, the subject is human.

[0018] In some embodiments, the present invention provides a kit comprising one or more antibodies as described herein, *e.g.*, paragraph [0014], packaged together with one or more components, *e.g.*, detection reagents, buffers, blocking agents, assay substrates, etc., to, for example, assay an ERFE polypeptide such as human erythroferrone or an analog thereof. In some embodiments, the present invention provides a kit comprising one or more ERFE polypeptides, *e.g.*, ELPRGPGESRAGPAARPP (SEQ ID NO: 1), GESRAG (SEQ ID NO: 2), LGSPEPGAPSRSRAR (SEQ ID NO: 34), rhERFE1 (SEQ ID NO: 3), and/or rhERFE2 (SEQ ID NO: 7), packaged together with one or more components, *e.g.*, detection reagents, buffers, blocking agents, assay substrates, to, for example, capture or assay antibodies against an ERFE polypeptide such as human erythroferrone or an analog thereof.

[0019] Both the foregoing general description and the following detailed description are exemplary and explanatory only and are intended to provide further explanation of the invention as claimed. The accompanying drawings are included to provide a further understanding of the invention and are incorporated in and constitute part of this specification, illustrate several embodiments of the invention, and together with the description explain the principles of the invention.

[0020] DESCRIPTION OF THE DRAWINGS

[0021] This invention is further understood by reference to the drawings wherein:

[0022] Figure 1: Serum erythroferrone in blood donors of 2 u of erythrocytes. Lines denote individual donors. Circles and error bars show mean ± SEM for each time point. Red denotes $p<0.05$ by One Way ANOVA compared to initial baseline (day 0) concentration. The first post-donation sample was obtained at average 2.5 days after donation (range 2-4 days), and showed an increase in all samples, by 16.4 ± 8.9 ng/ml over initial sample (mean increase ± SD, $p = 0.0002$, paired t-test, $n = 24$). Further rise of serum hERFE was seen in most donors with a maximum at 8 ± 4 days (mean ± SD, $n = 24$) increasing by 26.7 ± 11.2 ng/ml over initial value. By 120 days after the blood donation, serum hERFE concentrations returned to baseline (3.9 ± 7.5 ng/ml over initial, $p = 0.28$ comparing baseline and 112 days, paired t-test, $n = 20$).

[0023] Figure 2: Serum erythroferrone in four patients with moderate anemia treated on day 0 with erythropoietin 20,000 units SC. Blood samples were obtained every 6 hours the first day, then on mornings of days 2, 3, 5, and 7.

[0024] Figure 3: Serum hepcidin in the same samples as Figure 2. The patient designated by green symbols was iron-deficient (serum ferritin 14, transferrin saturation 17%, undetectable serum hepcidin).

[0025] Figure 4: Serum hERFE in patients with β-thalassemia, non-transfused (Non), before transfusion (Pre) or after transfusion (Post). Box plots show median, box 25% to 75%, whiskers 10% to 90%, and outliers. Serum hERFE levels were massively increased in thalassemic patients, non-transfused and pre-transfusion but were closer to normal after transfusion, *$p<0.05$, One Way ANOVA on Ranks, comparing to normal reference group.

[0026] Figure 5: Serum hepcidin in the same samples, vs erythroferrone, scatterplot is fitted by an inverse third order equation, $R^2 = 0.7$.

[0027] DETAILED DESCRIPTION OF THE INVENTION

[0028] Disclosed herein is an assay for an erythroferrone, such as human erythroferrone (hERFE) or an analog thereof. The experiments herein show that the assay detects the analogous physiological hERFE increases in humans subjected to blood loss or erythropoietin administration, as well as the pathological increases of hERFE in subjects suffering from β-thalassemia.

[0029] The assay exemplified herein is the first validated immunoassay for hERFE. Applications of the assays according to the present invention include diagnosing anemia (*e.g.*, diagnosing ineffective erythropoiesis), assessing therapeutic responses to erythropoietin agonists, detecting doping with erythropoietin or erythropoietin agonists (including synthetic analogs that result in elevated serum erythroferrone), diagnosing iron metabolism diseases involving abnormal hERFE levels, and providing differential diagnoses of polycythemia.

[0030] As used herein, "iron metabolism diseases" refers to diseases where aberrant iron metabolism directly causes the disease, diseases caused by iron blood levels that are dysregulated, and diseases that can be treated by modulating iron levels, and include iron overload diseases, iron deficiency disorders, disorders of iron biodistribution, and disorders of iron metabolism, etc. Examples of iron metabolism diseases include hemochromatosis, HFE mutation hemochromatosis, ferroportin mutation hemochromatosis, transferrin receptor 2 mutation hemochromatosis, hemojuvelin mutation hemochromatosis, hepcidin mutation hemochromatosis, juvenile hemochromatosis,

neonatal hemochromatosis, hepcidin deficiency, transfusional iron overload, thalassemia, thalassemia intermedia, alpha thalassemia, sideroblastic anemia, porphyria, porphyria cutanea tarda, African iron overload, hyperferritinemia, ceruloplasmin deficiency, atransferrinemia, congenital dyserythropoietic anemia, anemia of chronic disease, anemia of inflammation, anemia of infection, hypochromic microcytic anemia, iron-deficiency anemia, iron-refractory iron deficiency anemia, anemia of chronic kidney disease, erythropoietin resistance, iron deficiency of obesity, other anemias, benign or malignant tumors that overproduce hepcidin or induce its overproduction, conditions with hepcidin excess, Friedreich ataxia, gracile syndrome, Hallervorden-Spatz disease, Wilson's disease, pulmonary hemosiderosis, hepatocellular carcinoma, cancer, hepatitis, cirrhosis of liver, pica, chronic renal failure, insulin resistance, diabetes, atherosclerosis, neurodegenerative disorders, multiple sclerosis, Parkinson's disease, Huntington's disease, and Alzheimer's disease. In some embodiments, the iron overload disease is myelodysplastic syndrome. In some embodiments, iron metabolism diseases are not typically identified as being iron related. For example, diabetes (Type I or Type II), insulin resistance, glucose intolerance, and other disorders may be ameliorated by treating underlying iron metabolism disorders. See Simcox, *et al.* (2013) Cell Metab. Mar 5; 17(3): 329–341, which is herein incorporated by reference. As such, these diseases are encompassed under the broad definition. Those skilled in the art are readily able to determine whether a given disease is an iron metabolism disease using methods in the art, including the assays of WO 2004092405, and assays which monitor hepcidin, hemojuvelin, or iron levels and expression, such as those described in U.S. Patent No. 7,534,764. In some embodiments, the iron metabolism disease is a disease or condition related to abnormally high levels of erythroferrone. In some embodiments, the iron metabolism disease is a disease or condition related to abnormally low levels of erythroferrone.

[0031] Generally, serum concentration levels of erythroferrone reflect the levels of erythropoietin (as erythropoietin stimulates secretion of erythroferrone) and/or the number of erythroblasts (which are the cells that secrete erythroferrone). Examples of a “disease or condition related to abnormally low levels of erythroferrone“ include erythropoietin deficiencies, anemia of chronic disease (also called anemia of inflammation), anemias associated with acute or chronic infections, anemia of chronic kidney disease, pure red cell aplasia, aplastic anemia, radiation, and chemicals or poisons that cause aplastic anemia (*e.g.*, pesticides, arsenic, benzene, chemotherapeutics, and chloramphenicol. Examples of a “disease or condition related to abnormally high levels of erythroferrone“ include α-

thalassemia, β-thalassemia, congenital dyserythropoietic anemias, ineffective erythropoiesis, chronic liver diseases including alcoholic liver disease and chronic hepatitis B and C, blood loss, acute hypoxia, polycythemia (*e.g.*, due to activation of the erythropoietin receptor or its pathways (*e.g.*, polycythemia vera), hemolytic anemias, treatment or doping with erythropoietin or an agonist thereof, intravenous water infusion (not half-normal saline or normal saline), exposure to chemicals or poisons that induce hemolysis (such as anti-malaria drugs (quinine compounds), arsenic, dapsone, metals (chromium/chromates, platinum salts, nickel compounds, copper, lead, cis-platinum), nitrites, nitrofurantoin, penicillin, phenazopyridine (Pyridium), rho immune globulin (WinRho), ribavirin, hemolytic toxins (*e.g.*, snake venom), sulfonamides, sulfones, etc.), and exposure to chemicals or poisons that mimic hypoxia and/or induce erythropoietin and erythroferrone (*e.g.*, cobalt).

[0032] *Properties of the Erythroferrone Assay*

[0033] The standard curve was linear after log-log transformation. Limit of blanks (64 replicates), calculated as average + 1.645* standard deviation of blanks, was 0.8 ng/ml. The limit of detection, calculated as average + 1.645* standard deviation of the concentration calculated from 64 replicates of the lowest standard (0.625 ng/ml) was 1.5 ng/ml. The lower limit of quantitation (LLQ) was determined as 14 ng/ml by analyzing at ten-fold sample dilution the CV% of 16 replicates each of 8 human samples with low hERFE concentrations, graphing CV% vs hERFE concentration, fitting the relationship with an exponential curve, and interpolating an hERFE concentration that yielded CV% = 20. The working range was therefore 14-100 ng/ml. Spike recovery was determined by adding 2.5, 5.0, or 10 ng/ml of rhERFE2 to ten-fold dilutions of human serum samples (n = 9) containing very low concentrations of hERFE (0 to 0.8 ng/ml), measuring for each sample the resulting hERFE concentration and subtracting its pre-spike hERFE concentration. The spike recovery (mean ± SD) was 92 ± 8%, 100 ± 5% and 111 ± 4% for spikes of 2.5, 5.0, or 10 ng/ml respectively, corresponding to sample concentrations of 25, 50 and 100 ng/ml.

[0034] *hERFE Response to Erythropoietic Stimulation*

[0035] hERFE concentration in male, female, and combined blood donors prior to donation (baseline) was 12 ± 9 ng/ml (mean ± SD, n = 28), 11 ± 11 ng/ml (n = 30) and 12 ± 10 ng/ml (n = 58). The distributions were skewed so that median and percentile range (25%, 75%) was 12 (7, 19), 7 (4, 9) and 8 (4, 15) for men, women, and combined genders.

Follow-up on the male donors for up to 112 days (Figure 1) showed that serum hERFE rose in the second sample in all donors (on average 2.5 days after donation, range 2-4 days) to 28 ± 11 ng/ml ($p = 3 \times 10^{-7}$, paired t-test compared to baseline). Further rise of serum hERFE was seen in most donors with a maximum reaching 38 ± 13 ng/ml ($n = 29$, $p = 5 \times 10^{-11}$) at 9 ± 4 days. At 112 days following donation, serum hERFE concentrations returned to baseline (15 ± 10 ng/ml, $p = 0.3$, $n = 23$). Mean serum hepcidin was suppressed on days 2-15 but returned towards baseline by 112 days (manuscript in preparation).

[0036] Responses to the administration of erythropoietin 20,000 units in four geriatric patients with moderate anemia of unknown etiology was also examined (Figure 2 and Figure 3). hERFE increased in all patients, reaching a maximum at 1-5 days, coincident with a decline in serum hepcidin concentrations.

[0037] *Pathological Increase of hERFE in β-thalassemia*

[0038] hERFE concentrations in non-transfused and pre-transfusion patients were greatly increased compared to the reference sample from blood donors at baseline (Figure 4, $p<0.05$, One-way ANOVA on ranks, Dunn's Method). Hepcidin concentrations inversely correlated with erythroferrone concentrations (Figure 5), consistent with the proposed role of hERFE as a pathological hepcidin suppressor in β-thalassemia.

[0039] *Antibodies against hERFE and ERFE Polypeptides*

[0040] In some embodiments, the present invention provides antibodies against one or more ERFE polypeptides, preferably hERFE. As used herein, "ERFE polypeptides" refers to an erythroferrone (preferably hERFE), analogs of hERFE, homologs of hERFE, and fragments thereof. In some embodiments, the ERFE polypeptide is a protein that comprises or consists of comprises or consists of `ELPRGPGESRAGPAARPP` (SEQ ID NO: 1), `GESRAG` (SEQ ID NO: 2), or `LGSPEPGAPSRSRAR` (SEQ ID NO: 34). In some embodiments, the ERFE polypeptide is an erythroferrone from an *Aotus spp.*, a *Cercocebus spp.*, an *Equus spp.*, a *Gorilla spp.*, *Homo sapiens*, a *Macaca spp.*, a *Microcebus spp.*, a *Neomonachus spp.*, a *Nomascus spp.*, an *Odobenus spp.*, a *Pan spp.*, a *Papio spp.*, a *Piliocolobus spp.*, a *Pongo spp.*, a *Rhinolophus spp.*, or a *Rhinopithecus spp.* In some embodiments, the ERFE polypeptide is an erythroferrone from one of the following species: *Aotus nancymaae*, *Cercocebus atys*, *Equus caballus*, *Gorilla gorilla gorilla*, *Homo sapiens*, *Macaca fascicularis*, *Macaca mulatta*, *Macaca nemestrina*, *Microcebus murinus*, *Neomonachus schauinslandi*, *Nomascus leucogenys*, *Odobenus*

rosmarus divergens, *Pan troglodytes*, *Papio anubis*, *Piliocolobus tephrosceles*, *Pongo abelii*, *Rhinolophus sinicus*, and *Rhinopithecus roxellana*. In some embodiments, the ERFE polypeptide is a human erythroferrone. In some embodiments, the ERFE polypeptide is an analog of human erythroferrone. In some embodiments, the ERFE polypeptide is a macaque erythroferrone.

[0041] As used herein, "analogs" refer to proteins (or nucleic acid molecules) of heterologous origins that display the same or substantially similar activity. As used herein, "homologs" refer to proteins (or nucleic acid molecules) of a common origin, but do not necessarily exhibit the same or substantially similar activity. Thus, ERFE polypeptides may or may not exhibit erythroferrone activity. As used herein, "erythroferrone activity" refers to the ability of the given substance to decrease hepatic hepcidin mRNA or serum hepcidin levels as compared to a negative control. As used herein, the terms "protein", "polypeptide" and "peptide" are used interchangeably to refer to two or more amino acids linked together. Groups or strings of amino acid abbreviations are used to represent peptides. Except when specifically indicated, peptides are indicated with the N-terminus on the left and the sequence is written from the N-terminus to the C-terminus.

[0042] As used herein, a given percentage of "sequence identity" refers to the percentage of nucleotides or amino acid residues that are the same between sequences, when compared and optimally aligned for maximum correspondence over a given comparison window, as measured by visual inspection or by a sequence comparison algorithm in the art, such as the BLAST algorithm, which is described in Altschul *et al.*, J. Mol. Biol. 215:403-410 (1990). Software for performing BLAST (*e.g.*, BLASTP and BLASTN) analyses is publicly available through the National Center for Biotechnology Information (ncbi.nlm.nih.gov). The comparison window can exist over a given portion, *e.g.*, a functional domain, or an arbitrarily selection a given number of contiguous nucleotides or amino acid residues of one or both sequences. Alternatively, the comparison window can exist over the full length of the sequences being compared. For purposes herein, where a given comparison window (*e.g.*, over 80% of the given sequence) is not provided, the recited sequence identity is over 100% of the given sequence. Additionally, for the percentages of sequence identity of the proteins provided herein, the percentages are determined using BLASTP 2.8.0+, scoring matrix BLOSUM62, and the default parameters available at blast.ncbi.nlm.nih.gov/Blast.cgi. *See also* Altschul, *et al.* (1997), Nucleic Acids Res. 25:3389-3402; and Altschul, *et al.* (2005) FEBS J. 272:5101-5109.

[0043] Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, Adv. Appl. Math. 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson & Lipman, PNAS USA 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection.

[0044] ERFE polypeptides of the present invention may be made using methods known in the art including chemical synthesis, biosynthesis or *in vitro* synthesis using recombinant DNA methods, and solid phase synthesis. See *e.g.*, Kelly & Winkler (1990) Genetic Engineering Principles and Methods, vol. 12, J. K. Setlow ed., Plenum Press, NY, pp. 1-19; Merrifield (1964) J Amer Chem Soc 85:2149; Houghten (1985) PNAS USA 82:5131-5135; and Stewart & Young (1984) Solid Phase Peptide Synthesis, 2ed. Pierce, Rockford, IL, which are herein incorporated by reference. ERFE polypeptides of the present invention may be purified using protein purification techniques known in the art such as reverse phase high-performance liquid chromatography (HPLC), ion-exchange or immunoaffinity chromatography, filtration or size exclusion, or electrophoresis. See Olsnes and Pihl (1973) Biochem. 12(16):3121-3126; and Scopes (1982) Protein Purification, Springer-Verlag, NY, which are herein incorporated by reference. Alternatively, polypeptides of the present invention may be made by recombinant DNA techniques known in the art.

[0045] As used herein, "antibody" refers to naturally occurring and synthetic immunoglobulin molecules and immunologically active portions thereof (*i.e.*, molecules that contain an antigen binding site that specifically bind the molecule to which antibody is directed against). As such, the term antibody encompasses not only whole antibody molecules, but also antibody multimers and antibody fragments as well as variants (including derivatives) of antibodies, antibody multimers and antibody fragments. Examples of molecules which are described by the term "antibody" herein include: single chain Fvs (scFvs), Fab fragments, Fab' fragments, F(ab')2, disulfide linked Fvs (sdFvs), Fvs, and fragments comprising or alternatively consisting of, either a VL or a VH domain.

[0046] In some embodiments, antibodies of the present invention specifically bind one or more ERFE polypeptides. In some embodiments, the antibodies specifically bind an erythroferrone or a fragment thereof. In some embodiments, the antibodies specifically bind hERFE or a fragment thereof. In some embodiments, the antibodies are raised

against rhERFE1 or rhERFE2. In some embodiments, the antibodies are monoclonal antibodies. In some embodiments, the monoclonal antibodies are obtained from rabbit-based hybridomas. As used herein, a compound (*e.g.*, receptor or antibody) "specifically binds" a given target (*e.g.*, ligand or epitope) if it reacts or associates more frequently, more rapidly, with greater duration, and/or with greater binding affinity with the given target than it does with a given alternative, and/or indiscriminate binding that gives rise to non-specific binding and/or background binding. As used herein, "non-specific binding" and "background binding" refer to an interaction that is not dependent on the presence of a specific structure (*e.g.*, a given epitope). An example of an antibody that specifically binds an erythroferrone is an antibody that binds the erythroferrone with greater affinity, avidity, more readily, and/or with greater duration than it does to other compounds. An antibody that specifically binds an erythroferrone over a specified alternative is an antibody that binds the erythroferrone with greater affinity, avidity, more readily, and/or with greater duration than it does to the specified alternative. An antibody that specifically binds a given epitope of an erythroferrone is an antibody that binds the given epitope with greater affinity, avidity, more readily, and/or with greater duration than it does to other epitopes of the erythroferrone. As used herein, an "epitope" is the part of a molecule that is recognized by an antibody. Epitopes may be linear epitopes or three-dimensional epitopes. As used herein, the terms "linear epitope" and "sequential epitope" are used interchangeably to refer to a primary structure of an antigen, *e.g.*, a linear sequence of consecutive amino acid residues, that is recognized by an antibody. As used herein, the terms "three-dimensional epitope" and "conformational epitope" are used interchangeably to refer a three-dimensional structure that is recognized by an antibody, *e.g.*, a plurality of non-linear amino acid residues that together form an epitope when a protein is folded.

[0047] As used herein, "binding affinity" refers to the propensity of a compound to associate with (or alternatively dissociate from) a given target and may be expressed in terms of its dissociation constant, Kd. In some embodiments, an antibody according to the present invention has a Kd of 10^{-5} or less, 10^{-6} or less, preferably 10^{-7} or less, more preferably 10^{-8} or less, even more preferably 10^{-9} or less, and most preferably 10^{-10} or less to its given target. Binding affinity can be determined using methods in the art, such as equilibrium dialysis, equilibrium binding, gel filtration, immunoassays, surface plasmon resonance, and spectroscopy using experimental conditions that exemplify the conditions under which the compound and the given target may come into contact and/or interact. Dissociation constants may be used determine the binding affinity of a compound for a

given target relative to a specified alternative. Alternatively, methods in the art, *e.g.*, immunoassays, *in vivo* or *in vitro* assays for functional activity, etc., may be used to determine the binding affinity of the compound for the given target relative to the specified alternative. Thus, in some embodiments, the binding affinity of the antibody for the given target is at least 1-fold or more, preferably at least 5-fold or more, more preferably at least 10-fold or more, and most preferably at least 100-fold or more than its binding affinity for the specified alternative.

[0048] In some embodiments, the antibodies of the present invention are IgG isotype antibodies. In some embodiments, the antibodies of the present invention are monoclonal antibodies. In some embodiments, the monoclonal antibodies are obtained from rabbit-based monoclonal antibodies.

[0049] *Immunoassays*

[0050] In some embodiments, the present invention provides assays for detecting ERFE polypeptides. In some embodiments, the present invention provides assays for detecting analogs and/or homologs of human erythroferrone. In some embodiments, an analog of human erythroferrone is an erythroferrone from an *Aotus spp.*, a *Cercocebus spp.*, an *Equus spp.*, a *Gorilla spp.*, *Homo sapiens*, a *Macaca spp.*, a *Microcebus spp.*, a *Neomonachus spp.*, a *Nomascus spp.*, an *Odobenus spp.*, a *Pan spp.*, a *Papio spp.*, a *Piliocolobus spp.*, a *Pongo spp.*, a *Rhinolophus spp.*, or a *Rhinopithecus spp*. In some embodiments, the present invention provides assays for detecting full-length human erythroferrone. Assays according to the present invention include any immunoassay format in the art such as enzyme immune assays (EIAs), magnetic immunoassays (MIAs), counting immunoassays (CIAs), chemiluminescent immunoassays (CLIAs), radioimmunoassays (RIAs), electrochemiluminescence immunoassays (ECLIAs), fluorescent immunoassays (FIA), enzyme-linked immunosorbent assays (ELISAs), Western blot assays, and lateral flow tests (LFTs), and the like. The assays may be automated or manual. The various assays may employ any suitable labeling and detection system. The sensitivity and specificity of the assays according to the present invention can be further improved by optimizing the assay conditions, *e.g.*, reaction times and temperatures, and/or modifying or substituting the reagents, *e.g.*, different detection and labeling system, using methods in the art. In some embodiments, the immunoassay is an ELISA assay. In some embodiments, the immunoassay is a sandwich ELISA assay. In some embodiments, the immunoassay is a lateral flow test.

[0051] Generally, to perform an assay according to the present invention, a sample to be tested is obtained. As used herein, the term "sample" includes specimens and cultures obtained from any source, as well as biological samples and environmental samples. Biological samples may be obtained from animals (including humans) and encompass fluids, solids, tissues, and gases. Biological samples include blood products, such as plasma, serum, and the like. A biological sample can be obtained from a subject using methods in the art. A sample to be analyzed using one or more methods described herein can be either an initial unprocessed sample taken from a subject or a subsequently processed, *e.g.*, partially purified, diluted, concentrated, fluidized, pretreated with a reagent (*e.g.*, protease inhibitor, anti-coagulant, etc.), and the like. In some embodiments, the sample is a blood sample. In some embodiments, the blood sample is a whole blood sample, a serum sample, or a plasma sample. In some embodiments, the sample may be processed, *e.g.*, condensed, diluted, partially purified, and the like. In some embodiments, the sample is pretreated with a reagent, *e.g.*, a protease inhibitor. In some embodiments, two or more samples are collected at different time intervals to assess any difference in the amount of the analyte of interest, the progression of a disease or disorder, or the efficacy of a treatment. The test sample is then contacted with a capture reagent and, if the analyte is present, a conjugate between the analyte and the capture reagent is formed and is detected and/or measured with a detection reagent.

[0052] As used herein, a "capture reagent" refers to a molecule which specifically binds an analyte of interest. For example, if the analyte of interest is an antibody, the capture reagent may be an antigen or an epitope thereof to which the antibody specifically binds. As used herein, a "detection reagent" refers to a substance that has a detectable label attached thereto and specifically binds an analyte of interest or a conjugate of the analyte of interest, *e.g.*, an antibody-analyte conjugate. As used herein, a "detectable label" is a compound or composition that produces or can be induced to produce a signal that is detectable by, *e.g.*, visual, spectroscopic, photochemical, biochemical, immunochemical, or chemical means. The use of the term "labeled" as a modifier of a given substance, *e.g.*, a labeled antibody, means that the substance has a detectable label attached thereto. A detectable label can be attached directly or indirectly by way of a linker (*e.g.*, an amino acid linker or a chemical moiety). Examples of detectable labels include radioactive and non-radioactive isotopes (*e.g.*, ^{125}I, ^{18}F, ^{13}C, etc.), enzymes (*e.g.*, β-galactosidase, peroxidase, etc.) and fragments thereof, enzyme substrates, enzyme inhibitors, coenzymes, catalysts, fluorophores (*e.g.*, rhodamine, fluorescein isothiocyanate, etc.), dyes,

chemiluminescers and luminescers (*e.g.*, dioxetanes, luciferin, etc.), and sensitizers. A substance, *e.g.*, antibody, having a detectable label means that a detectable label that is not linked, conjugated, or covalently attached to the substance, in its naturally-occurring form, has been linked, conjugated, or covalently attached to the substance by the hand of man. As used herein, the phrase "by the hand of man" means that a person or an object under the direction of a person (*e.g.*, a robot or a machine operated or programmed by a person), not nature itself, has performed the specified act. Thus, the steps set forth in the claims are performed by the hand of man, *e.g.*, a person or an object under the direction of the person.

[0053] In some embodiments, the antibody is one that specifically binds a three-dimensional epitope of the ERFE polypeptide of interest as present in the test sample. In some embodiments, the antibody that specifically binds a three-dimensional epitope was raised against the ERFE polypeptide of interest. In some embodiments, the ERFE polypeptide of interest is human erythroferrone. In some embodiments, the antibody was raised against a recombinantly produced human erythroferrone that comprises a FLAG-tag, and optionally comprises a trypsin-sensitive site. In some embodiments, the antibody was raised against rhERFE1 or rhERFE2. In some embodiments, the antibody is one that specifically binds a linear epitope of the ERFE polypeptide of interest. In some embodiments, the linear epitope comprises or consists of the amino acid sequence ELPRGPGESRAGPAARPP (SEQ ID NO: 1). In some embodiments, the linear epitope comprises the amino acid sequence GESRAG (SEQ ID NO: 2). In some embodiments, the linear epitope consists of the amino acid sequence GESRAG (SEQ ID NO: 2). In some embodiments, the linear epitope comprises or consists of LGSPEPGAPSRSRAR (SEQ ID NO: 34).

[0054] In some embodiments, the test sample is contacted with a first antibody and a second antibody, both of which specifically bind the ERFE polypeptide of interest. In some embodiments, the ERFE polypeptide of interest is human erythroferrone. In some embodiments, the ERFE polypeptide of interest is an analog of human erythroferrone, such as macaque erythroferrone. In some embodiments, the antibody was raised against a recombinantly produced human erythroferrone that comprises a FLAG-tag, and optionally comprises a trypsin-sensitive site. In some embodiments, the antibody was raised against rhERFE1 or rhERFE2. In some embodiments, one of the antibodies specifically binds a three-dimensional epitope of the ERFE polypeptide of interest as present in the test sample and the other antibody specifically binds a linear epitope of the ERFE polypeptide of

interest. In some embodiments, the linear epitope comprises or consists of the amino acid sequence ELPRGPGESRAGPAARPP (SEQ ID NO: 1). In some embodiments, the linear epitope comprises the amino acid sequence GESRAG (SEQ ID NO: 2). In some embodiments, the linear epitope consists of the amino acid sequence GESRAG (SEQ ID NO: 2). In some embodiments, the linear epitope comprises or consists of LGSPEPGAPSRSRAR (SEQ ID NO: 34). In some embodiments, the first antibody and/or the second antibody are raised against the ERFE polypeptide of interest. In some embodiments, the first antibody is used as a capture reagent and the second antibody has a detectable label conjugated thereto. In some embodiments, both the first antibody and the second antibody have detectable labels conjugated thereto and a third antibody that specifically binds the first antibody and/or the second antibody is used as a capture reagent.

[0055] The capture reagent may be immobilized on an assay substrate. The capture reagent may be immobilized on the assay substrate before and/or after binding the analyte of interest. As used herein, an "assay substrate" refers to any substrate that may be used to immobilize a capture reagent thereon. Examples of assay substrates include membranes (*e.g.*, a nitrocellulose membrane, a polyvinylidene fluoride (PVDF) membrane, a cellulose acetate membrane, etc.), beads, slides, multi-well plates, and the like. One skilled in the art may readily select an appropriate assay substrate for a given assay format, number of samples to be tested, and detectable label.

[0056] The assay substrate may be incubated with a blocking buffer before and/or after the capture reagent is immobilized thereon. In some embodiments, the blocking buffer comprises serum albumin, such as bovine serum albumin (BSA) or human serum albumin. In some embodiments, the blocking buffer comprises casein and/or fragments thereof. In some embodiments, the blocking buffer comprises both serum albumin and casein (or casein fragments). In some embodiments, the blocking buffer comprises PBS, 0.2% Na casein, 0.05% Tween 20, and 0.1M NaCl.

[0057] In some embodiments, the assay substrate containing the capture reagent immobilized thereon is then contacted with the sample to be tested under conditions that allow the capture reagent to form a complex with the analyte of interest. For example, an assay substrate having immobilized thereon a first antibody that specifically binds a given erythroferrone of interest is physically contacted with a serum sample under conditions that allow the first antibody to specifically bind the erythroferrone, if present in the serum sample, and then washed to remove any unbound molecules. The assay substrate is then

incubated with a detection reagent under conditions that allow the detection reagent to specifically bind the analyte of interest or conjugates of the analyte of interest, *e.g.*, conjugates comprising the analyte of interest and the capture reagent. For example, after contact with the serum sample, the assay substrate is incubated with a second antibody that has a detectable label and specifically binds any erythroferrone conjugated to first antibody. After contact with the detection reagent, the assay substrate may be washed to remove any unbound molecules. Then any detectable label that is present can be detected and/or measured using methods in the art.

[0058] In some embodiments, the immunoassays of the present invention are in the format of a sandwich ELISA, in which a first antibody that specifically binds the ERFE polypeptide of interest is immobilized on an assay substrate. The assay substrate having the first antibody immobilized thereon is then incubated with a sample to be tested for a suitable period under conditions that allow for the formation of an antibody-analyte complex. Such a complex can then be detected using a second antibody that specifically binds the ERFE polypeptide of interest. The second antibody can be conjugated to a detectable label, which can release a signal directly or indirectly. The intensity of the signal may represent the level of the ERFE polypeptide of interest in the sample. In some embodiments, both the first antibody and the second antibody have a detectable label attached thereto and a third antibody that specifically binds the first antibody and/or the second antibody is used as a capture reagent. In some embodiments, the detection reagent is first mixed with the sample to be tested and then the mixture is contacted with an assay substrate having a capture reagent immobilized thereon. In some embodiments, the ERFE polypeptide of interest is human erythroferrone. In some embodiments, one or both antibodies were raised against a recombinantly produced human erythroferrone that comprises a FLAG-tag, and optionally comprises a trypsin-sensitive site. In some embodiments, one or both antibodies were raised against rhERFE1 or rhERFE2. In some embodiments, one of the antibodies specifically binds a three-dimensional epitope of the ERFE polypeptide of interest as present in the test sample and the other antibody specifically binds a linear epitope of the ERFE polypeptide of interest. In some embodiments, the linear epitope comprises or consists of the amino acid sequence ELPRGPGESRAGPAARPP (SEQ ID NO: 1). In some embodiments, the linear epitope comprises the amino acid sequence GESRAG (SEQ ID NO: 2). In some embodiments, the linear epitope consists of the amino acid sequence GESRAG (SEQ ID NO: 2). In some embodiments, the linear epitope comprises or consists of LGSPEPGAPSRSRAR (SEQ ID

NO: 34). In some embodiments, the first antibody and/or the second antibody are raised against the ERFE polypeptide of interest.

[0059] *Diagnostic and Prognostic Applications*

[0060] The methods and kits according to the present invention may be used in the evaluation of an iron metabolism disease, preferably a disease or condition related to abnormally low levels of erythroferrone or a disease or condition related to abnormally high levels of erythroferrone. The methods and kits of the present invention may be used to monitor the progress of such a disease, assess the efficacy of a treatment for the disease, and/or identify patients suitable for a given treatment in a subject. The methods and kits of the present invention may be used to diagnose a subject as having an iron metabolism disease and/or provide the subject with a prognosis.

[0061] In some embodiments, an immunoassay according to the present invention may be used to determine whether a subject exhibits a level of erythroferrone that is low or high as compared to a control. In some embodiments, the control is a sample from a normal, healthy subject. In some embodiments, the control is a pooled sample from a plurality of normal, healthy subjects. In some embodiments, the control is a given reference level. For example, in some embodiments, the given reference level is 30 ng/ml and a concentration level above 30 ng/ml is identified as a high level. The high level may then be used to diagnose the subject as suffering from a disease or condition related to abnormally high levels of erythroferrone.

[0062] The assays exemplified herein exhibit a limit of detection (LOD) of 12 ng/ml. Therefore, in some embodiments, the sample to be tested is concentrated and then the level of erythroferrone is measured in the concentrated sample and the level of erythroferrone in the unconcentrated sample is mathematically extrapolated from the degree of concentration.

[0063] Because normal levels of erythroferrone can be below the LOD, in some embodiments, a subject can be diagnosed as having a low level of erythroferrone, by increasing the level of erythroferrone in the subject by, for example, administering a given amount of erythropoietin and then measuring the level of erythroferrone after a given time after erythropoietin administration and then comparing the level to a control. In these embodiments, the control may be a sample taken from a normal, healthy subject after the same given time after administration of the same given amount of erythropoietin, a pooled sample from a plurality of normal, healthy subjects whose samples were taken after the same given time after administration of the same given amount of erythropoietin, or a

given reference value which is an average level determined from a plurality of normal, healthy subjects whose samples were taken after the same given time after administration of the same given amount of erythropoietin. If the increased level is lower than the control, the level can be identified as a low level. The low level may then be used to diagnose the subject as suffering from a disease or condition related to abnormally low levels of erythroferrone.

[0064] A subject identified as having a low level or a high level may be subjected to a suitable treatment. For example, a subject identified as having a high level of erythroferrone or diagnosed as suffering from disease or condition related to abnormally high levels of erythroferrone may be treated with an antagonist of erythropoietin or with a mini-hepcidin or with a modified mini-hepcidin peptide such as those described in WO 2010/065815 and WO 2013/086143. As another example, a subject identified as having a low level of erythroferrone or diagnosed as suffering from a disease or condition related to abnormally low levels of erythroferrone may be treated with erythropoietin or an agonist of erythropoietin.

[0065] In some embodiments, the methods and kits according to the present invention may be used to monitor the efficacy of treatment with a therapeutic, *e.g.*, erythropoietin, that modulates the level of erythroferrone produced in the subject and the dosage of the therapeutic may be adjusted accordingly.

[0066] *Non-Clinical Applications*

[0067] In some embodiments, the methods and kits according to the present invention may be used for research purposes. For example, the methods and kits according to the present invention may be used to identify diseases that are caused by abnormal levels of erythroferrone and/or identify diseases that result in abnormal levels of erythroferrone. In some embodiments, the methods and kits according to the present invention may be used to study mechanisms, *e.g.*, mechanisms and pathways involving erythroferrone. In some embodiments, the methods and kits according to the present invention may be used to develop and screen for therapeutics that increase or decrease levels of erythroferrone in subjects.

[0068] In some embodiments, the methods and kits according to the present invention may be used for experiments to elucidate the pathophysiological interaction between erythropoiesis and iron homeostasis, including the pathogenesis of iron-loading anemias, erythropoietic response to therapy with erythropoiesis-stimulating agents in chronic

kidney disease, anemia of cancer, anemia of inflammation, and physiological adaptations to hypoxia, altitude, or blood donation.

[0069] *Kits*

[0070] In some embodiments, the present invention provides kits for use in evaluating an ERFE polypeptide of interest, *e.g.*, human erythroferrone or an analog thereof, in samples, *e.g.*, biological samples from human patients. In some embodiments, the kits comprise a capture reagent that specifically binds the ERFE polypeptide of interest packaged together with a detection reagent for detecting and/or measuring any ERFE polypeptides conjugated with the capture reagent.

[0071] In some embodiments, kits according to the present invention comprise a first antibody that specifically binds a three-dimensional epitope of human erythroferrone packaged together with a second antibody that specifically binds a linear epitope of human erythroferrone. In some embodiments, the linear epitope comprises or consists of the amino acid sequence ELPRGPGESRAGPAARPP (SEQ ID NO: 1). In some embodiments, the linear epitope comprises the amino acid sequence GESRAG (SEQ ID NO: 2). In some embodiments, the linear epitope consists of the amino acid sequence GESRAG (SEQ ID NO: 2). In some embodiments, the linear epitope comprises or consists of LGSPEPGAPSRSRAR (SEQ ID NO: 34). In some embodiments, the first antibody and/or the second antibody are raised against human erythroferrone.

[0072] Kits according to the present invention may further comprise an assay substrate for performing an immunoassay and immobilizing the capture reagent thereto. Kits according to the present invention may also comprise one or more reagents, *e.g.*, blocking buffers, assay buffers, diluents, wash solutions, etc., for performing the immunoassay. Kits may optionally provide additional components such as interpretive information, control samples, and reference levels, and standards.

[0073] In some embodiments, the kits include a carrier, package, or container that may be compartmentalized to receive one or more containers, such as vials, tubes, and the like. In some embodiments, the kits optionally include an identifying description or label or instructions relating to its use. In some embodiments, the kits include information prescribed by a governmental agency that regulates the manufacture, use, or sale of compounds and compositions according to the present invention.

[0074] In some embodiments, the kits further comprise one or more erythroferrone therapeutics, optionally in one or more unit dosage forms, packaged together as a pack and/or in drug delivery device, *e.g.*, a pre-filled syringe, for preventing, inhibiting,

reducing, or treating an iron metabolism disease in a subject. As used herein, an "erythroferrone therapeutic" refers to a compound that increases or decreases the level of erythroferrone, *e.g.*, erythropoietin, agonists of erythropoietin, antagonists of erythropoietin, or a compound that modulates iron uptake, *e.g.*, hepcidin, mini-hepcidins, and modified mini-hepcidins, in a subject.

[0075] The following examples are intended to illustrate but not to limit the invention.

[0076] METHODS

[0077] *Recombinant hERFE Production and Purification*

[0078] The hERFE sequence was cloned into pcDNA3.1 with following modifications: vector signal sequence (IL-2) was used instead of the native sequence, followed by a spacer (italics, SEQ ID NO: 4), a FLAG-tag (bolded, SEQ ID NO: 5), and a trypsin-sensitive site (dotted underline, SEQ ID NO: 6):

MYRMQLLSCIALSLALVTNSIS*AMVRS***DYKDDDDK**SPEPGAPSRSRARREPPPGNELPRGPGESRA
GPAARPPEPTAERAHSVDPRDAWMLFVRQSDKGVNGKKRSRGKAKKLKFGLPGPPGPPGPQGPPGP
IIPPEALLKEFQLLLKGAVRQRERAEPEPCTCGPAGPVAASLAPVSATAGEDDDDVVGDVLALLAA
PLAPGPRAPRVEAAFLCRLRRDALVERRALHELGVYYLPDAEGAFRRGPGLNLTSGQYRAPVAGFY
ALAATLHVALGEPPRRGPPRPRDHLRLLICIQSRCQRNASLEAIMGLESSSELFTISVNGVLYLQM
GQWTSVFLDNASGCSLTVRSGSHFSAVLLGV (rhERFE1, SEQ ID NO: 3)

[0079] Because the FLAG tag was mostly lost during cell culture, the recombinant hERFE was further modified by removing the trypsin-sensitive site which allowed the protein to be secreted efficiently with its FLAG tag:

MYRMQLLSCIALSLALVTNSIS*AMVRS***DYKDDDDK**SPEPPPPGNELPRGPGESRAGPAARPPEPTA
ERAHSVDPRDAWMLFVRQSDKGVNGKKRSRGKAKKLKFGLPGPPGPPGPQGPPGPIIPPEALLKEF
QLLLKGAVRQRERAEPEPCTCGPAGPVAASLAPVSATAGEDDDDVVGDVLALLAAPLAPGPRAPRV
EAAFLCRLRRDALVERRALHELGVYYLPDAEGAFRRGPGLNLTSGQYRAPVAGFYALAATLHVALG
EPPRRGPPRPRDHLRLLICIQSRCQRNASLEAIMGLESSSELFTISVNGVLYLQMGQWTSVFLDNA
SGCSLTVRSGSHFSAVLLGV (rhERFE2, SEQ ID NO: 7)

[0080] Freestyle 293F cells (Life Technologies) were grown in shaking flask (250 rpm) at 37°C in an 8% CO_2 humidified incubator to cell density 10^6/ml in 100 ml of FreeStyle 293 Expression medium, then transfected per manufacturer's instructions (Invitrogen Catalog # K9000-01) using 100 µg of rhERFE1 or rhERFE2 plasmid DNA and 200 µl 293fectin (Life Technologies). The transfected cells were reincubated in the shaking flask (250 rpm) at 37°C in an 8% CO_2 humidified incubator for 3-5 days in 100 ml FreeStyle 293 Expression medium supplemented with Protease Inhibitor Cocktail (Sigma) and the medium was collected. rhERFE1 was purified from supernatant using ion-exchange columns (Macro-prep, Biorad) and eluted by stepwise increasing concentrations of NaCl/

Na_2HPO_4 buffer (0.1 to 1M, pH 7.5). rhERFE2 was purified using an anti-FLAG M2 affinity gel according to the manufacturer's protocol (Sigma), eluting with 100 μg/ml FLAG peptide (Sigma). NaCl/Na_2HPO_4 buffer and FLAG peptide were removed by filtration through Amicon Ultra 30K device (Millipore) and recombinant ERFE resuspended in saline (0.9% NaCl). The purified protein was electrophoretically heterogeneous, indicating posttranslational processing and multimerization characteristic of the TNFα-C1q family of proteins (see, *e.g.*, reference #5). Predominant bands on reducing SDS-PAGE were at 52 kD and 26 kD. Antigen concentration was estimated by absorbance (1 mg/ml) at 280 nm = 0.57.

[0081] *Rabbit Monoclonal Antibody Production*

[0082] Rabbit hybridomas were generated (custom order fulfilled by Abcam, Burlingame, CA) from rabbits immunized by rhERFE1 and boosted by rhERFE2. Hybridoma supernatants were selected for reactivity against rhERFE2. After biotinylation of Mabs (EZ-Link sulfo-NHS-LC-LC-Biotin kit, Thermo Fisher Scientific), optimal pair of unbiotinylated capture Mab and biotinylated detection Mab was chosen by checkerboard testing with rhERFE1 and rhERFE2. The cDNAs encoding the final Mab pair (#9 and #42) were cloned from the hybridomas and used to produce the Mabs recombinantly. Peptide epitope scanning showed that Mab#9 bound to the peptide ELPRGPGESRAGPAARPP (SEQ ID NO: 1) but not to 6 amino acid overlap neighbors suggesting that it was specific for an epitope centered on the underlined segment GESRAG (SEQ ID NO: 2). Mab#42 did not bind to linear peptides, indicating that it probably recognized a three-dimensional epitope.

[0083] The VH nucleotide sequence of Mab#9 is:

ATGGAGACTGGGCTGCGCTGGCTTCTCCTGGTCGCTGTGCTCAAAGGTGTCCAGTGTCAGTCGGTG
GAGGAGTCCGGGGGGTCGCCTGGTCACGCCTGGGACACCCCTGACACTCACCTGCACAGTCTCTGGA
ATCGACCTCAATGACAATGCAATGAGATGGGTCCGCCAGGCTCCCGGGAAGGGGCTGGAATGGATC
GGAGTCATTTATATTGATACAAGCACATACTACGCGAGCTGGGCGAAAGGCCGATTCACCATCTCC
AAAACCTCGTCGACCACGGTGGATCTGAAAATCACCAGTCCGACAACCGAGGACACGGCCACCTAT
TTCTGTGTCAGAGAGGATGGTTATAGGCTTGGTGACGTCTGGGGCCCAGGCACCCTGGTCACCGTC
TCCTCAGGGCAACCTAAGGCTCCATCAGTCTTCCCACTGGCCCCCTGCTGCGGGGACACACCCAGC
TCCACGGTGACCCTGGGCTGCCTGGTCAAAGGGTACCTCCCGGAGCCAGTGACCGTGACCTGGAAC
TCGGGCACCCTCACCAATGGGGTACGCACCTTCCCGTCCGTCCGGCAGTCCTCAGGCCTCTACTCG
CTGAGCAGCGTGGTGAGCGTGACCTCAAGCAGCCAGCCCGTCACCTGCAACGTGGCCCACCCAGCC
ACCAACACCAAAGTGGACAAGACCGTTGCGCCCTCGACATGCAGCAAGCCCACGTGCCCACCCCCT
GAACTCCTGGGGGGACCGTCTGTCTTCATCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCA
CGCACCCCCGAGGTCACATGCGTGGTGGTGGACGTGAGCCAGGATGACCCCGAGGTGCAGTTCACA
TGGTACATAAACAACGAGCAGGTGCGCACCGCCCGGCCGCCGCTACGGGAGCAGCAGTTCAACAGC
ACGATCCGCGTGGTCAGCACCCTCCCCATCGCGCACCAGGACTGGCTGAGGGGCAAGGAGTTCAAG
TGCAAAGTCCACAACAAGGCACTCCCGGCCCCCATCGAGAAAACCATCTCCAAAGCCAGAGGGCAG
CCCCTGGAGCCGAAGGTCTACACCATGGGCCCTCCCCGGGAGGAGCTGAGCAGCAGGTCGGTCAGC

CTGACCTGCATGATCAACGGCTTCTACCCTTCCGACATCTCGGTGGAGTGGGAGAAGAACGGGAAG
GCAGAGGACAACTACAAGACCACGCCGGCCGTGCTGGACAGCGACGGCTCCTACTTCCTCTACAGC
AAGCTCTCAGTGCCCACGAGTGAGTGGCAGCGGGGCGACGTCTTCACCTGCTCCGTGATGCACGAG
GCCTTGCACAACCACTACACGCAGAAGTCCATCTCCCGCTCTCCGGGTAAATGA (SEQ ID NO: 8)

[0084] The encoded VH polypeptide sequence of Mab#9 is:

METGLRWLLLVAVLKGVQCQSVEESGGRLVTPGTPLTLTCTVSGIDLNDNAMRWVRQAPGKGLEWI
GVIYIDTSTYYASWAKGRFTISKTSSTTVDLKITSPTTEDTATYFCVREDGYRLGDVWGPGTLVTV
SSGQPKAPSVFPLAPCCGDTPSSTVTLGCLVKGYLPEPVTVTWNSGTLTNGVRTFPSVRQSSGLYS
LSSVVSVTSSSQPVTCNVAHPATNTKVDKTVAPSTCSKPTCPPPELLGGPSVFIFPPKPKDTLMIS
RTPEVTCVVVDVSQDDPEVQFTWYINNEQVRTARPPLREQQFNSTIRVVSTLPIAHQDWLRGKEFK
CKVHNKALPAPIEKTISKARGQPLEPKVYTMGPPREELSSRSVSLTCMINGFYPSDISVEWEKNGK
AEDNYKTTPAVLDSDGSYFLYSKLSVPTSEWQRGDVFTCSVMHEALHNHYTQKSISRSPGK (SEQ ID NO: 9)

[0085] The predicted CDRs of the VH chain of Mab#9 are underlined above and are as follows: GIDLNDNA (CDR1, SEQ ID NO: 10), IYIDTST (CDR2, SEQ ID NO: 11), and VREDGYRLGDV (CDR3, SEQ ID NO: 12).

[0086] The VL nucleotide sequence of Mab#9 is:

ATGGACACGAGGGCCCCCACTCAGCTGCTGGGGCTCCTGCTGCTCTGGCTCCCAGATGCCAGATGT
GCGCTTGTGATGACCCAGACTCCATCCTCCGTGTCTGCAGGTGTGGGAGGCACAGTCACCATCAAC
TGCCAGGCCAGTCAGAGTCTTTATAATAACAACTATTTATCCTGGTTTCAGCAGAAACCAGGGCAG
CCTCCCAAGCTCCTGATCTACTGGGCATCCACTCTGGCATCTGGGGTCCCATCCCGGTTCAGTGGC
AGTGGATCTGGGACACAGTTCACTCTCACCATCAGTGGCGTGGCGTGTGACGATGCTGCCACTTAC
TACTGTGCAGGCTATAAAAGTAGTAGTAATGATGATTTTGCTTTCGGCGGAGGGACCGAGGTGGTG
GTCAAAGGTGATCCAGTTGCACCTACTGTCCTCATCTTCCCACCAGCTGCTGATCAGGTGGCAACT
GGAACAGTCACCATCGTGTGTGTGGCGAATAAATACTTTCCCGATGTCACCGTCACCTGGGAGGTG
GATGGCACCACCCAAACAACTGGCATCGAGAACCGTAAAACACCGCAGAATTCTGCAGATTGTACC
TACAACCTCAGCAGCACTCTGACACTGACCAGCACACAGTACAACAGCCACAAAGAGTACACCTGC
AAGGTGACCCAGGGCACGACCTCAGTCGTCCAGAGCTTCAATAGGGGTGACTGTTAG (SEQ ID NO: 13)

[0087] The encoded VL polypeptide sequence of Mab#9 is:

MDTRAPTQLLGLLLLWLPDARCALVMTQTPSSVSAGVGGTVTINCQASQSLYNNNYLSWFQQKPGQ
PPKLLIYWASTLASGVPSRFSGSGSGTQFTLTISGVACDDAATYYCAGYKSSSNDDFAFGGGTEVV
VKGDPVAPTVLIFPPAADQVATGTVTIVCVANKYFPDVTVTWEVDGTTQTTGIENRKTPQNSADCT
YNLSSTLTLTSTQYNSHKEYTCKVTQGTTSVVQSFNRGDC (SEQ ID NO: 14)

[0088] The predicted CDRs of the VL chain of Mab#9 are underlined above and are as follows: QSLYNNNY (CDR1, SEQ ID NO: 15), WAS (CDR2, SEQ ID NO: 16), and AGYKSSSNDDFA (CDR3, SEQ ID NO: 17).

[0089] The VH nucleotide sequence of Mab#42 is:

ATGGAGACTGGGCTGCGCTGGCTTCTCCTGGTCGCTGTGCTCAAAGGTGCCCAGTGCCAGTCGCTG
GAGGAGTCCGGGGGTCGCCTGGTAACGCCTGGAGGATCCCTGACACTCACCTGCACAGTCTCTGGA
ATCGACCTCAGTAGCTATGAAATGGGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAATGGATC
GGAGTAATTGGTACTGATGGTACCGCAGTCTACGCGACCTGGGTGAAAGGCCGATTCACCATCTCC
AAAACCTCGACCACGGTGGATCTGAAAATGACCAGTCTGACAACCGAGGACACGGCCACCTATTTC
TGTGCCCGAGATTCTTCTGGTAATAGTAATTATAGGGCTTTTGATCCCTGGGGCCCAGGCACCCTG
GTCACCGTCTCCTCAGGGCAACCTAAGGCTCCATCAGTCTTCCCACTGGCCCCCTGCTGCGGGGAC
ACACCCAGCTCCACGGTGACCCTGGGCTGCCTGGTCAAAGGGTACCTCCCGGAGCCAGTGACCGTG
ACCTGGAACTCGGGCACCCTCACCAATGGGGTACGCACCTTCCCGTCCGTCCGGCAGTCCTCAGGC

CTCTACTCGCTGAGCAGCGTGGTGAGCGTGACCTCAAGCAGCCAGCCCGTCACCTGCAACGTGGCC
CACCCAGCCACCAACACCAAAGTGGACAAGACCGTTGCGCCCTCGACATGCAGCAAGCCCACGTGC
CCACCCCCTGAACTCCTGGGGGGACCGTCTGTCTTCATCTTCCCCCCAAAACCCAAGGACACCCTC
ATGATCTCACGCACCCCCGAGGTCACATGCGTGGTGGTGGACGTGAGCCAGGATGACCCCGAGGTG
CAGTTCACATGGTACATAAACAACGAGCAGGTGCGCACCGCCCGGCCGCCGCTACGGGAGCAGCAG
TTCAACAGCACGATCCGCGTGGTCAGCACCCTCCCCATCGCGCACCAGGACTGGCTGAGGGGCAAG
GAGTTCAAGTGCAAAGTCCACAACAAGGCACTCCCGGCCCCCATCGAGAAAACCATCTCCAAAGCC
AGAGGGCAGCCCCTGGAGCCGAAGGTCTACACCATGGGCCCTCCCCGGGAGGAGCTGAGCAGCAGG
TCGGTCAGCCTGACCTGCATGATCAACGGCTTCTACCCTTCCGACATCTCGGTGGAGTGGGAGAAG
AACGGGAAGGCAGAGGACAACTACAAGACCACGCCGGCCGTGCTGGACAGCGACGGCTCCTACTTC
CTCTACAGCAAGCTCTCAGTGCCCACGAGTGAGTGGCAGCGGGGCGACGTCTTCACCTGCTCCGTG
ATGCACGAGGCCTTGCACAACCACTACACGCAGAAGTCCATCTCCCGCTCTCCGGGTAAATGA
(SEQ ID NO 18)

[0090] The encoded VH polypeptide sequence of Mab#42 is:

METGLRWLLLVAVLKGAQCQSLEESGGRLVTPGGSLTLTCTVSGIDLSSYEMGWVRQAPGKGLEWI
GVIGTDGTAVYATWVKGRFTISKTSTTVDLKMTSLTTEDTATYFCARDSSGNSNYRAFDPWGPGTL
VTVSSGQPKAPSVFPLAPCCGDTPSSTVTLGCLVKGYLPEPVTVTWNSGTLTNGVRTFPSVRQSSG
LYSLSSVVSVTSSSQPVTCNVAHPATNTKVDKTVAPSTCSKPTCPPPELLGGPSVFIFPPKPKDTL
MISRTPEVTCVVVDVSQDDPEVQFTWYINNEQVRTARPPLREQQFNSTIRVVSTLPIAHQDWLRGK
EFKCKVHNKALPAPIEKTISKARGQPLEPKVYTMGPPREELSSRSVSLTCMINGFYPSDISVEWEK
NGKAEDNYKTTPAVLDSDGSYFLYSKLSVPTSEWQRGDVFTCSVMHEALHNHYTQKSISRSPGK
(SEQ ID NO 19)

[0091] The predicted CDRs of the VH chain of Mab#42 are underlined above and are as follows: GIDLSSYE (CDR1, SEQ ID NO: 20), IGTDGTA (CDR2, SEQ ID NO: 21), and ARDSSGNSNYRAFDP (CDR3, SEQ ID NO: 22).

[0092] The VL nucleotide sequence of Mab#42 is:

ATGGACACGAGGGCCCCCACTCAGCTGCTGGGGCTCCTGCTGCTCTGGCTCCCAGGTGCCAGATGT
GCCTATGATATGACCCAGACTCCAGCCTCTGTGGAGGTAGCTGTGGGAGGCACAGTCACCATCAAG
TGCCAGGCCAGTCAGAGCATTTACAGCTACTTATCCTGGTATCAGCAGAAACCAGGGCAGCCTCCC
AAGCTCCTGATCTACAGGGCATCCACTCTGGCATCTGGGGTCCCATCGCGGTTCAAAGGCAGTGGA
TCTGGGACACAGTTCACTCTCACCATAAGCGACCTGGAGTGTGCCGATGCTGCCACTTACTACTGT
CAACAGGGTTTTGTTATTAGTAATGTTCTTAATTCTTTCGGCGGAGGGACCGAGGTGGTGGTCAAA
GGTGATCCAGTTGCACCTACTGTCCTCATCTTCCCACCAGCTGCTGATCAGGTGGCAACTGGAACA
GTCACCATCGTGTGTGTGGCGAATAAATACTTTCCCGATGTCACCGTCACCTGGGAGGTGGATGGC
ACCACCCAAACAACTGGCATCGAGAACAGTAAAACACCGCAGAATTCTGCAGATTGTACCTACAAC
CTCAGCAGCACTCTGACACTGACCAGCACACAGTACAACAGCCACAAAGAGTACACCTGCAAGGTG
ACCCAGGGCACGACCTCAGTCGTCCAGAGCTTCAATAGGGGTGACTGTTAG (SEQ ID NO: 23)

[0093] The encoded VL polypeptide sequence of Mab#42 is:

MDTRAPTQLLGLLLLWLPGARCAYDMTQTPASVEVAVGGTVTIKCQASQSIYSYLSWYQQKPGQPP
KLLIYRASTLASGVPSRFKGSGSGTQFTLTISDLECADAATYYCQQGFVISNVLNSFGGGTEVVVK
GDPVAPTVLIFPPAADQVATGTVTIVCVANKYFPDVTVTWEVDGTTQTTGIENSKTPQNSADCTYN
LSSTLTLTSTQYNSHKEYTCKVTQGTTSVVQSFNRGDC (SEQ ID NO: 24)

[0094] The predicted CDRs of the VL chain of Mab#42 are underlined above and are as follows: QSIYSY (CDR1, SEQ ID NO: 25), RAS (CDR2, SEQ ID NO: 26), and QQGFVISNVLNS (CDR3, SEQ ID NO: 27).

[0095] In some embodiments, an antibody according to the present invention comprises GIDLNDNA (SEQ ID NO: 10), IYIDTST (SEQ ID NO: 11), VREDGYRLGDV (SEQ ID NO:

12), QSLYNNNY (SEQ ID NO: 15), WAS (SEQ ID NO: 16), and AGYKSSSNDDFA (SEQ ID NO: 17). In some embodiments, an antibody according to the present invention comprises GIDLSSYE (SEQ ID NO: 20), IGTDGTA (SEQ ID NO: 21), ARDSSGNSNYRAFDP (SEQ ID NO: 22), QSIYSY (SEQ ID NO: 25), RAS (SEQ ID NO: 26), and QQGFVISNVLNS (SEQ ID NO: 27).

[0096] In some embodiments, the VH sequence of an antibody according to the present invention comprises GIDLNDNA (SEQ ID NO: 10), IYIDTST (SEQ ID NO: 11), and VREDGYRLGDV (SEQ ID NO: 12). In some embodiments, the VH sequence of an antibody according to the present invention comprises GIDLSSYE (SEQ ID NO: 20), IGTDGTA (SEQ ID NO: 21), and ARDSSGNSNYRAFDP (SEQ ID NO: 22). The VH polypeptide sequences of Mab#9 and Mab#42 have significant sequence identity. Therefore, in some embodiments, the VH sequence of an antibody according to the present invention comprises

METGLRWLLLVAVLKG(X1)QCQS(X2)EESGGRLVTPG(X3)(X4)LTLTCTVSGIDL(X5)(X6)(X7)(X8)M(X9)WVRQAPGKGLEWIGVI(X10)(X11)D(X12)(X13)(X14)(X15)YA(X16)W(X17)KGRFTISKTS(X18)TTVDLK(X19)TS(X20)TTEDTATYFC(X21)R(X22)(X23)(X24)(X25)(X26)(X27)(X28)YR(X29)(X30)D(X31) (SEQ ID NO: 28)

wherein each X1 to X31 is independently any amino acid, and X18, X24, X25, X26, and X27 are each independently present or absent. In some embodiments, X1 is V or A, X2 is V or L, X3 is T or G, X4 is P or S, X5 is N or S, X6 is D or S, X7 is N or Y, X8 is A or E, X9 is R or G, X10 is Y or G, X11 is I or T, X12 is T or G, X13 is S or T, X14 is T or A, X15 is Y or V, X16 is S or T, X17 is A or V, X18 is S or absent, X19 is I or M, X20 is P or L, X21 is V or A, X22 is E or D, X23 is D or S, X24 is S or absent, X25 is G or absent, X26 is N or absent, X27 is S or absent, X28 is G or N, X29 is L or A, X30 is G or F, and/or X31 is V or P. In some embodiments, X1 is V or A, X2 is V or L, X3 is T or G, X4 is P or S, X5 is N or S, X6 is D or S, X7 is N or Y, X8 is A or E, X9 is R or G, X10 is Y or G, X11 is I or T, X12 is T or G, X13 is S or T, X14 is T or A, X15 is Y or V, X16 is S or T, X17 is A or V, X18 is S or absent, X19 is I or M, X20 is P or L, X21 is V or A, X22 is E or D, X23 is D or S, X24 is S or absent, X25 is G or absent, X26 is N or absent, X27 is S or absent, X28 is G or N, X29 is L or A, X30 is G or F, and X31 is V or P. In some embodiments, the antibody having SEQ ID NO: 28 contains GIDLNDNA (SEQ ID NO: 10), IYIDTST (SEQ ID NO: 11), and/or VREDGYRLGDV (SEQ ID NO: 12). In some embodiments, the antibody having SEQ ID NO: 28 contains GIDLSSYE (SEQ ID NO: 20), IGTDGTA (SEQ ID NO: 21), and/or ARDSSGNSNYRAFDP (SEQ ID NO: 22). In some embodiments, the VH sequence comprises a sequence having at least 79%, at least 80%, at

least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 88%, at least 89%, at least 90%, at least 91%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to METGLRWLLLVAVLKGVQCQSVEESGGRLVTPGTPLTLTCTVSGIDLNDNAMRWVRQAPGKGLEWIGVIYID TSTYYASWAKGRFTISKTSSTTVDLKITSPTTEDTATYFCVREDGYRLGDV (SEQ ID NO: 29) or METGLRWLLLVAVLKGAQCQSLEESGGRLVTPGGSLTLTCTVSGIDLSSYEMGWVRQAPGKGLEWIGVIGTD GTAVYATWVKGRFTISKTSTTVDLKMTSLTTEDTATYFCARDSSGNSNYRAFDP (SEQ ID NO: 30). In some embodiments, the VH sequence comprises or consists of at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 88%, at least 89%, at least 90%, at least 91%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 9 or SEQ ID NO: 19.

[0097] In some embodiments, the VL sequence of an antibody according to the present invention comprises QSLYNNNY (SEQ ID NO: 15), WAS (SEQ ID NO: 16), and AGYKSSSNDDFA (SEQ ID NO: 17). In some embodiments, the VL sequence of an antibody according to the present invention comprises QSIYSY (SEQ ID NO: 25), RAS (SEQ ID NO: 26), and QQGFVISNVLNS (SEQ ID NO: 27). The VL polypeptide sequences of Mab#9 and Mab#42 have significant sequence identity. Therefore, in some embodiments, the VL sequence of an antibody according to the present invention comprises

MDTRAPTQLLGLLLLWLP(X32)ARCA(X33)(X34)MTQTP(X35)SV(X36)(X37)(X38)VG GTVTI(X39)CQASQS(X40)Y(X41)(X42)(X43)YLSW(X44)QQKPGQPPKLLIY(X45)AS TLASGVPSRF(X46)GSGSGTQFTLTIS(X47)(X48)(X49)C(X50)DAATYYC(X51)(X52) (X53)(X54)(X55)(X56)SN(X57)(X58)(X59)(X60) (SEQ ID NO: 31)

wherein each X32 to X60 is independently any amino acid, and X42 and X43 are each independently present or absent. In some embodiments, X32 is D or G, X33 is L or Y, X34 is V or D, X35 is S or A, X36 is S or E, X37 is A or V, X38 is G or A, X39 is N or K, X40 is N or I, X41 is N or S, X42 is N or absent, X43 is N or absent, X44 is F or Y, X45 is W or R, X46 is S or K, X47 is G or D, X48 is V or L, X49 is A or E, X50 is D or A, X51 is A or Q, X52 is G or Q, X53 is Y or G, X54 is K or F, X55 is S or V, X56 is S or I, X57 is D or V, X58 is D or L, X59 is F or N, and/or X60 is A or S. In some embodiments, X32 is D or G, X33 is L or Y, X34 is V or D, X35 is S or A, X36 is S or E, X37 is A or V, X38 is G or A, X39 is N or K, X40 is N or I, X41 is N or S, X42 is N or absent, X43 is N or absent, X44 is F or Y, X45 is W or R, X46 is S or K, X47 is G or D, X48 is V or L, X49 is A or E, X50 is D or A, X51 is A or Q, X52 is G or Q, X53 is Y or G, X54 is K or F, X55

is S or V, X56 is S or I, X57 is D or V, X58 is D or L, X59 is F or N, and X60 is A or S. In some embodiments, the antibody having SEQ ID NO: 31 contains QSLYNNNY (SEQ ID NO: 15) and/or AGYKSSSNDDFA (SEQ ID NO: 17). In some embodiments, the antibody having QSIYSY (SEQ ID NO: 25) and/or QQGFVISNVLNS (SEQ ID NO: 27). In some embodiments, the VL sequence comprises a sequence having at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 88%, at least 89%, at least 90%, at least 91%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to MDTRAPTQLLGLLLLWLPDARCALVMTQTPSSVSAGVGGTVTINCQASQSLYNNNYLSWFQQKPGQPPKLLIYWASTLASGVPSRFSGSGSGTQFTLTISGVACDDAATYYCAGYKSSSNDDFA (SEQ ID NO: 32). In some embodiments, the VL sequence comprises or consists of at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 88%, at least 89%, at least 90%, at least 91%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 14. In some embodiments, the VL sequence comprises a sequence having at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 88%, at least 89%, at least 90%, at least 91%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to MDTRAPTQLLGLLLLWLPGARCAYDMTQTPASVEVAVGGTVTIKCQASQSIYSYLSWYQQKPGQPPKLLIYRASTLASGVPSRFKGSGSGTQFTLTISDLECADAATYYCQQGFVISNVLNS (SEQ ID NO: 33). In some embodiments, the VL sequence comprises or consists of at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 88%, at least 89%, at least 90%, at least 91%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 23.

[0098] In some embodiments, an antibody according to the present invention comprises a VH sequence according to paragraph [0096] and a VL sequence according to paragraph [0097].

[0099] In some embodiments, the present invention provides a method of making an antibody that specifically binds human erythroferrone and/or an ERFE polypeptide, which comprises injecting a non-human animal with the ERFE polypeptide. In some

embodiments, the ERFE polypeptide comprises or consists of the amino acid sequence GESRAG (SEQ ID NO: 2). In some embodiments, the ERFE polypeptide comprises or consists of the amino acid sequence ELPRGPGESRAGPAARPP (SEQ ID NO: 1). In some embodiments, the ERFE polypeptide comprises or consists of rhERFE1 (SEQ ID NO: 3). In some embodiments, the ERFE polypeptide comprises or consists of rhERFE2 (SEQ ID NO: 7). In some embodiments, the method further comprises making a hybridoma using the lymphocytes of the non-human animal, which was injected with the ERFE polypeptide and expresses antibodies against the human erythroferrone and/or an ERFE polypeptide, to obtain a monoclonal antibody that specifically binds the ERFE polypeptide. In some embodiments, the non-human animal is a rabbit.

[0100] In some embodiments, the present invention provides a method of making a recombinant antibody that specifically binds human erythroferrone and/or an ERFE polypeptide, which comprises recombinantly expressing a VH sequence according to paragraph [0096] and a VL sequence according to paragraph [0097].

[0101] *Human ERFE Immunoassay*

[0102] 96-well high binding plates (Costar #3590) were coated with Mab#9 diluted to 1 µg/ml in sodium carbonate buffer (50 mM, pH 9.6), overnight at 4°C. Plates were washed 3-times with TBS-T (TBS + 0.05% Tween 20) then blocked for 1 hour at room temperature with 200 µl/well Blocking Buffer (BB: PBS,0.2% Na casein, 0.05% Tween 20, 0.1M NaCl). Recombinant hERFE2 standard was first diluted to 1 µg/ml and then serially diluted in BB to 10, 5, 2.5, 1.25, and 0.625 ng/ml. After a 1 hour incubation at 25°C in a 300 rpm shaker, the plate was washed 4-times with TBS-T, 10 seconds, 25°C, and 300 rpm per wash and incubated 1 hour at 25°C, 300 rpm in shaker with 100 µl/well biotinylated Mab#42 (1 µg/ml in BB). After incubation for 1 hour at 25°C, 300 rpm in shaker, the plate was washed 4-times as before, incubated for 45 minutes with Neutravidin-HRP conjugate (ThermoScientific #31030) 1/5000 (100 µl/well) washed again 3-times as before and developed with 100 µl TMB Substrate System for ELISA (ThermoScientific #34028) at room temperature in the dark for 10 minutes. The reaction was stopped by adding 50 µl of 2N sulfuric acid and the plates were read on a Spectramax 250 (Molecular Devices) at 450 nm.

[0103] *Hepcidin Assay*

[0104] Hepcidin was measured by competitive ELISA using methods in the art. See, *e.g.*, reference #6.

[0105] *Human Samples*

[0106] All human studies were approved by Institutional Review Boards at respective institutions and at UCLA.

[0107] *Blood donors*—Male blood donors at the New York Blood Center (n = 30, age 19-65 years) donated 2 units of packed erythrocytes by apheresis. Samples were collected prior to and 2, 4, 7, 9, 11, 14 and 112 days following donation. Samples from female blood donors (n = 30, age 18-61) were obtained from Discovery Life Sciences, San Louis Obispo, CA.

[0108] *EPO administration*—Four geriatric patients at UCLA with moderate anemia of unknown etiology were administered 20,000 units of EPO subcutaneously and serum collected over a 1-week time course.

[0109] *β-thalassemia*—Patients were recruited at the UCSF Benioff Children's Hospital Oakland and included non-transfused (n = 11, 10 M, 1F, average 35.7 years) or transfusion-dependent patients immediately before (n = 10, 5 M, 5 F, 23 years) or 2-14 days after transfusion (n = 13, 9 M, 4 F, 17.4 years).

[0110] *Immunoassays for Other ERFE Polypeptides*

[0111] At least Mab#9 was found to bind macaque erythroferrone. BLAST sequence alignments between human erythroferrone (Accession AHL84165.1) and macaque erythroferrone (*i.e.*, *Macaca fascicularis* (Accession XP_015288524.1), *Macaca mulatta* (Accession XP_001094581.2), and *Macaca nemestrina* (Accession XP_011726193.1)) show that macaque erythroferrone contains 100% sequence identity to a portion of the linear epitope ELPRGPGESRAGPAARPP (SEQ ID NO: 1). Specifically, all three macaque sequences contain LGSPEPGAPSRSRAR (SEQ ID NO: 34). Therefore, in some embodiments, the antibody or the immunologically active portion thereof specifically binds a macaque erythroferrone, a protein comprising LGSPEPGAPSRSRAR (SEQ ID NO: 34), or LGSPEPGAPSRSRAR (SEQ ID NO: 34). Thus, in some embodiments, ERFE polypeptide being detected is a macaque erythroferrone, a protein comprising LGSPEPGAPSRSRAR (SEQ ID NO: 34), or LGSPEPGAPSRSRAR (SEQ ID NO: 34). In some embodiments, ERFE polypeptide being detected is a macaque erythroferrone, a protein comprising LGSPEPGAPSRSRAR (SEQ ID NO: 34), or LGSPEPGAPSRSRAR (SEQ ID NO: 34) and the capture reagent or the detection reagent is an antibody according to paragraphs [0083] to [0099] or an immunologically active portion thereof.

[0112] Additionally, because macaque erythroferrone has at least 91% sequence identity to human erythroferrone, Mab#42 is also expected to specifically bind macaque erythroferrone. Therefore, in some embodiments, the ERFE polypeptide being detected is a macaque erythroferrone, a protein comprising LGSPEPGAPSRSRAR (SEQ ID NO: 34), or LGSPEPGAPSRSRAR (SEQ ID NO: 34) and the capture reagent and the detection reagent are each independently an antibody according to paragraphs [0083] to [0099] or an immunologically active portion thereof.

[0113] Further BLAST searches of ERFE polypeptides containing the antigenic linear epitope GESRAG (SEQ ID NO: 2) reveal the following erythroferrone polypeptides have sequences containing SEQ ID NO: 2: *Aotus nancymaae* (Accession XP_021520750.1), *Cercocebus atys* (Accession XP_011898406.1), *Equus caballus* (Accession XP_023498471.1), *Gorilla gorilla gorilla* (Accession XP_018877321.1), *Macaca fascicularis* (Accession XP_015288524.1), *Macaca mulatta* (Accession XP_001094581.2), *Macaca nemestrina* (Accession XP_011726193.1), *Microcebus murinus* (Accession XP_012611156.1), *Neomonachus schauinslandi* (Accession XP_021534643.1), *Nomascus leucogenys* (Accession XP_012359931.1), *Odobenus rosmarus divergens* (Accession XP_012421343.1), *Pan troglodytes* (Accession XP_016806309.1 and XP_009442932.2), *Papio anubis* (Accession XP_009181726.1), *Piliocolobus tephrosceles* (Accession XP_023084478.1), *Pongo abelii* (Accession XP_024099321.1), *Rhinolophus sinicus* (Accession XP_019596439.1), and *Rhinopithecus roxellana* (Accession XP_010356276.1).

[0114] Therefore, in some embodiments, the ERFE polypeptide being detected is a protein that comprises or consists of comprises or consists of ELPRGPGESRAGPAARPP (SEQ ID NO: 1), GESRAG (SEQ ID NO: 2), or LGSPEPGAPSRSRAR (SEQ ID NO: 34). In some embodiments, the ERFE polypeptide being detected is an erythroferrone from an *Aotus spp.*, a *Cercocebus spp.*, an *Equus spp.*, a *Gorilla spp.*, *Homo sapiens*, a *Macaca spp.*, a *Microcebus spp.*, a *Neomonachus spp.*, a *Nomascus spp.*, an *Odobenus spp.*, a *Pan spp.*, a *Papio spp.*, a *Piliocolobus spp.*, a *Pongo spp.*, a *Rhinolophus spp.*, or a *Rhinopithecus spp.* In some embodiments, the ERFE polypeptide being detected is an erythroferrone from one of the following species: *Aotus nancymaae*, *Cercocebus atys*, *Equus caballus*, *Gorilla gorilla gorilla*, *Homo sapiens*, *Macaca fascicularis*, *Macaca mulatta*, *Macaca nemestrina*, *Microcebus murinus*, *Neomonachus schauinslandi*, *Nomascus leucogenys*, *Odobenus rosmarus divergens*, *Pan troglodytes*, *Papio anubis*, *Piliocolobus tephrosceles*, *Pongo abelii*, *Rhinolophus sinicus*, and *Rhinopithecus roxellana*. In some embodiments, the

ERFE polypeptide being detected is human erythroferrone. In some embodiments, the ERFE polypeptide being detected is a macaque erythroferrone.

[0115] ADDITIONAL EMBODIMENTS

[0116] Embodiment 1. An immunoassay for detecting an ERFE polypeptide, preferably a human erythroferrone, in a sample, which comprises a1) contacting the sample with a capture reagent that specifically binds the ERFE polypeptide and then contacting with at least one detection reagent that specifically binds the ERFE polypeptide bound to the capture reagent, or a2) contacting the sample with at least one detection reagent that specifically binds the ERFE polypeptide and then contacting with a capture reagent that specifically binds the ERFE polypeptide bound to the at least one detection reagent; and b) detecting or measuring a detectable label of the at least one detection reagent.

[0117] Embodiment 2. The immunoassay of Embodiment 1, wherein the capture reagent or the at least one detection reagent is an antibody that specifically binds a three-dimensional epitope of the ERFE polypeptide.

[0118] Embodiment 3. The immunoassay of Embodiment 2, wherein the capture reagent or the at least one detection reagent is an antibody that specifically binds a linear epitope of the ERFE polypeptide.

[0119] Embodiment 4. The immunoassay of Embodiment 2, wherein the capture reagent is an antibody that specifically binds a linear epitope of the ERFE polypeptide and the at least one detection reagent is the antibody that specifically binds a three-dimensional epitope of the ERFE polypeptide.

[0120] Embodiment 5. The immunoassay of Embodiment 3 or 4, wherein the linear epitope comprises or consists of the amino acid sequence GESRAG (SEQ ID NO: 2).

[0121] Embodiment 6. The immunoassay of Embodiment 3 or 4, wherein the linear epitope comprises or consists of the amino acid sequence ELPRGPGESRAGPAARPP (SEQ ID NO: 1).

[0122] Embodiment 7. The immunoassay of Embodiment 2, wherein the antibody that specifically binds a three-dimensional epitope was raised against the human erythroferrone.

[0123] Embodiment 8. The immunoassay of Embodiment 2, wherein the antibody that specifically binds a three-dimensional epitope was raised against a recombinant human erythroferrone.

[0124] Embodiment 9. The immunoassay of Embodiment 8, wherein the recombinant human erythroferrone is rhERFE1 (SEQ ID NO: 3) or rhERFE2 (SEQ ID NO: 7).

[0125] Embodiment 10. The immunoassay according any one of the preceding Embodiments, which further comprises immobilizing the capture reagent to an assay substrate.

[0126] Embodiment 11. A method of determining whether the level of erythroferrone in a subject is low or high, which comprises performing the immunoassay according to any one of Embodiments 1 to 10 on a sample obtained from the subject to obtain a measured level of erythroferrone, and comparing the measured level of erythroferrone to a control.

[0127] REFERENCES

[0128] 1. Finch C. Regulators of iron balance in humans. *Blood.* 1994;84(6):1697-1702.

[0129] 2. Kautz L, Jung G, Valore EV, Rivella S, Nemeth E, Ganz T. Identification of erythroferrone as an erythroid regulator of iron metabolism. *NatGenet.* 2014;46(7):678-684.

[0130] 3. Ganz T. Systemic iron homeostasis. *Physiol Rev.* 2013;93(4):1721-1741.

[0131] 4. Kautz L, Jung G, Du X, *et al.* Erythroferrone contributes to hepcidin suppression and iron overload in a mouse model of beta-thalassemia. *Blood.* 2015;126(17):2031-2037.

[0132] 5. Wang Y, Lam Karen SL, Yau M-h, Xu A. Post-translational modifications of adiponectin: mechanisms and functional implications. *Biochemical Journal.* 2008;409(3):623-633.

[0133] 6. Ganz T, Olbina G, Girelli D, Nemeth E, Westerman M. Immunoassay for human serum hepcidin. *Blood.* 2008;112(10):4292-4297.

[0134] All scientific and technical terms used in this application have meanings commonly used in the art unless otherwise specified.

[0135] As used herein, the terms "subject", "patient", and "individual" are used interchangeably to refer to humans and non-human animals. The term "non-human animal" includes all vertebrates, *e.g.*, mammals and non-mammals, such as non-human primates, horses, sheep, dogs, cows, pigs, chickens, and other veterinary subjects and test animals. In some embodiments of the present invention, the subject is a mammal. In some embodiments of the present invention, the subject is a human.

[0136] As used herein, "providing a diagnosis" and "diagnosing" refer to the physical and active step of informing, *i.e.*, communicating verbally or by writing (on, *e.g.*, paper or electronic media), another party, *e.g.*, a patient, of the diagnosis. Similarly, "providing a prognosis" refers to the physical and active step of informing, *i.e.*, communicating

verbally or by writing (on, *e.g.*, paper or electronic media), another party, *e.g.*, a patient, of the prognosis.

[0137] The use of the singular can include the plural unless specifically stated otherwise. As used in the specification and the appended claims, the singular forms "a", "an", and "the" can include plural referents unless the context clearly dictates otherwise. As used herein, "and/or" means "and" or "or". For example, "A and/or B" means "A, B, or both A and B" and "A, B, C, and/or D" means "A, B, C, D, or a combination thereof" and said "combination thereof" means any subset of A, B, C, and D, for example, a single member subset (*e.g.*, A or B or C or D), a two-member subset (*e.g.*, A and B; A and C; etc.), or a three-member subset (*e.g.*, A, B, and C; or A, B, and D; etc.), or all four members (*e.g.*, A, B, C, and D).

[0138] The phrase "comprises or consists of" is used as a tool to avoid excess page and translation fees and means that in some embodiments the given thing at issue comprises something, and in some embodiments the given thing at issue consists of something. For example, the sentence "In some embodiments, the composition comprises or consists of A" is to be interpreted as if written as the following two separate sentences: "In some embodiments, the composition comprises A. In some embodiments, the composition consists of A." Similarly, a sentence reciting a string of alternates is to be interpreted as if a string of sentences were provided such that each given alternate was provided in a sentence by itself. For example, the sentence "In some embodiments, the composition comprises A, B, or C" is to be interpreted as if written as the following three separate sentences: "In some embodiments, the composition comprises A. In some embodiments, the composition comprises B. In some embodiments, the composition comprises C."

[0139] To the extent necessary to understand or complete the disclosure of the present invention, all publications, patents, and patent applications mentioned herein are expressly incorporated by reference therein to the same extent as though each were individually so incorporated.

What is claimed is:

1. An immunoassay for detecting an ERFE polypeptide, preferably a human erythroferrone, in a sample, which comprises

a1) contacting the sample with a capture reagent that specifically binds the ERFE polypeptide and then contacting with at least one detection reagent that specifically binds the ERFE polypeptide bound to the capture reagent, or

a2) contacting the sample with at least one detection reagent that specifically binds the ERFE polypeptide and then contacting with a capture reagent that specifically binds the ERFE polypeptide bound to the at least one detection reagent; and

b) detecting or measuring a detectable label of the at least one detection reagent bound to the ERFE polypeptide that is bound to the capture reagent.

2. The immunoassay of claim 1, wherein the capture reagent and/or the at least one detection reagent is an antibody comprising

a VH sequence that comprises

a) GIDLNDNA (SEQ ID NO: 10), IYIDTST (SEQ ID NO: 11), and VREDGYRLGDV (SEQ ID NO: 12),

b) GIDLSSYE (SEQ ID NO: 20), IGTDGTA (SEQ ID NO: 21), and ARDSSGNSNYRAFDP (SEQ ID NO: 22),

c) METGLRWLLLVAVLKG(X1)QCQS(X2)EESGGRLVTPG(X3)(X4)LTLTCTVSGIDL(X5)(X6)(X7)(X8)M(X9)WVRQAPGKGLEWIGVI(X10)(X11)D(X12)(X13)(X14)(X15)YA(X16)W(X17)KGRFTISKTS(X18)TTVDLK(X19)TS(X20)TTEDTATYFC(X21)R(X22)(X23)(X24)(X25)(X26)(X27)(X28)YR(X29)(X30)D(X31) (SEQ ID NO: 28), wherein each X1 to X31 is independently any amino acid, and X18, X24, X25, X26, and X27 are each independently present or absent,

d) METGLRWLLLVAVLKG(X1)QCQS(X2)EESGGRLVTPG(X3)(X4)LTLTCTVSGIDL(X5)(X6)(X7)(X8)M(X9)WVRQAPGKGLEWIGVI(X10)(X11)D(X12)(X13)(X14)(X15)YA(X16)W(X17)KGRFTISKTS(X18)TTVDLK(X19)TS(X20)TTEDTATYFC(X21)R(X22)(X23)(X24)(X25)(X26)(X27)(X28)YR(X29)(X30)D(X31) (SEQ ID NO: 28), wherein each X1 to X31 is independently any amino acid, and X18, X24, X25, X26, and X27 are each independently present or absent, and at least one or more of GIDLNDNA (SEQ ID NO: 10), IYIDTST (SEQ ID NO: 11), and VREDGYRLGDV (SEQ ID NO: 12),

e) METGLRWLLLVAVLKG(X1)QCQS(X2)EESGGRLVTPG(X3)(X4)LTLTCTVSGIDL(X5)(X6)(X7)(X8)M(X9)WVRQAPGKGLEWIGVI(X10)(X11)D(X12)(X13)(X14)(X15)YA(X16)W(X17)KGRFTISKTS(X18)TTVDLK(X19)TS(X20)TTEDTATYFC(X21)R(X22)(

X23)(X24)(X25)(X26)(X27)(X28)YR(X29)(X30)D(X31) (SEQ ID NO: 28), wherein each X1 to X31 is independently any amino acid, and X18, X24, X25, X26, and X27 are each independently present or absent, and at least one or more of GIDLSSYE (SEQ ID NO: 20), IGTDGTA (SEQ ID NO: 21), and ARDSSGNSNYRAFDP (SEQ ID NO: 22),

f) at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 88%, at least 89%, at least 90%, at least 91%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to METGLRWLLLVAVLKGVQCQSVEESGGRLVTPGTPLTLTCTVSGIDLNDNAMRWVRQAPGKGLEWIGVIYI DTSTYYASWAKGRFTISKTSSTTVDLKITSPTTEDTATYFCVREDGYRLGDV (SEQ ID NO: 29),

g) at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 88%, at least 89%, at least 90%, at least 91%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to METGLRWLLLVAVLKGAQCQSLEESGGRLVTPGGSLTLTCTVSGIDLSSYEMGWVRQAPGKGLEWIGVIGT DGTAVYATWVKGRFTISKTSTTVDLKMTSLTTEDTATYFCARDSSGNSNYRAFDP (SEQ ID NO: 30),

h) at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 88%, at least 89%, at least 90%, at least 91%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 9, or

i) at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 88%, at least 89%, at least 90%, at least 91%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 19; and/or

a VL sequence that comprises

a) QSLYNNNY (SEQ ID NO: 15), WAS (SEQ ID NO: 16), and AGYKSSSNDDFA (SEQ ID NO: 17),

b) QSIYSY (SEQ ID NO: 25), RAS (SEQ ID NO: 26), and QQGFVISNVLNS (SEQ ID NO: 27),

c) MDTRAPTQLLGLLLLWLP(X32)ARCA(X33)(X34)MTQTP(X35)SV(X36)(X37)(X38)V GGTVTI(X39)CQASQS(X40)Y(X41)(X42)(X43)YLSW(X44)QQKPGQPPKLLIY(X45) ASTLASGVPSRF(X46)GSGSGTQFTLTIS(X47)(X48)(X49)C(X50)DAATYYC(X51)(X

52)(X53)(X54)(X55)(X56)SN(X57)(X58)(X59)(X60) (SEQ ID NO: 31), wherein each X32 to X60 is independently any amino acid, and X42 and X43 are each independently present or absent,

d) MDTRAPTQLLGLLLLWLP(X32)ARCA(X33)(X34)MTQTP(X35)SV(X36)(X37)(X38)V GGTVTI(X39)CQASQS(X40)Y(X41)(X42)(X43)YLSW(X44)QQKPGQPPKLLIY(X45) ASTLASGVPSRF(X46)GSGSGTQFTLTIS(X47)(X48)(X49)C(X50)DAATYYC(X51)(X 52)(X53)(X54)(X55)(X56)SN(X57)(X58)(X59)(X60) (SEQ ID NO: 31), wherein each X32 to X60 is independently any amino acid, and X42 and X43 are each independently present or absent, and QSLYNNNY (SEQ ID NO: 15) and/or AGYKSSSNDDFA (SEQ ID NO: 17),

e) MDTRAPTQLLGLLLLWLP(X32)ARCA(X33)(X34)MTQTP(X35)SV(X36)(X37)(X38)V GGTVTI(X39)CQASQS(X40)Y(X41)(X42)(X43)YLSW(X44)QQKPGQPPKLLIY(X45) ASTLASGVPSRF(X46)GSGSGTQFTLTIS(X47)(X48)(X49)C(X50)DAATYYC(X51)(X 52)(X53)(X54)(X55)(X56)SN(X57)(X58)(X59)(X60) (SEQ ID NO: 31), wherein each X32 to X60 is independently any amino acid, and X42 and X43 are each independently present or absent, and QSLYNNNY (SEQ ID NO: 15) and/or AGYKSSSNDDFA (SEQ ID NO: 17),

f) at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 88%, at least 89%, at least 90%, at least 91%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to MDTRAPTQLLGLLLLWLPDARCALVMTQTPSSVSAGVGGTVTINCQASQSLYNNNYLSWFQQKPGQPPKLL IYWASTLASGVPSRFSGSGSGTQFTLTISGVACDDAATYYCAGYKSSSNDDFA (SEQ ID NO: 32),

g) at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 88%, at least 89%, at least 90%, at least 91%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to MDTRAPTQLLGLLLLWLPGARCAYDMTQTPASVEVAVGGTVTIKCQASQSIYSYLSWYQQKPGQPPKLLIY RASTLASGVPSRFKGSGSGTQFTLTISDLECADAATYYCQQGFVISNVLNS (SEQ ID NO: 33),

h) at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 88%, at least 89%, at least 90%, at least 91%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 14, or

i) at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 88%, at least 89%, at least 90%, at least 91%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least

96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 23.

3. The immunoassay of claim 2, wherein (i) the capture reagent or the at least one detection reagent specifically binds a three-dimensional epitope of the ERFE polypeptide, (ii) the capture reagent or the at least one detection reagent specifically binds a linear epitope of the ERFE polypeptide, or (iii) the capture reagent specifically binds a linear epitope of the ERFE polypeptide and the at least one detection reagent specifically binds a three-dimensional epitope of the ERFE polypeptide.

4. The immunoassay of claim 3, wherein the linear epitope comprises or consists of the amino acid sequence ELPRGPGESRAGPAARPP (SEQ ID NO: 1), GESRAG (SEQ ID NO: 2), or LGSPEPGAPSRSRAR (SEQ ID NO: 34).

5. The immunoassay according to any one of claims 1 to 4, wherein the antibody was raised against rhERFE1 (SEQ ID NO: 3) or rhERFE2 (SEQ ID NO: 7).

6. The immunoassay according any one of the preceding claims, which further comprises immobilizing the capture reagent to an assay substrate.

7. A method of determining whether the level of an erythroferrone in a subject is low or high as compared to a control, which comprises

performing the immunoassay according to any one of claims 1 to 6 on a sample obtained from the subject to obtain a measured level of the erythroferrone, and

comparing the measured level of the erythroferrone to a control.

8. The method of claim 7, which further comprises characterizing the subject as having an abnormally high level of the erythroferrone where the measured level of the erythroferrone is more than 30 ng/ml.

9. A method of treating a subject for an iron metabolism disease, which comprises administering to the subject a erythroferrone therapeutic when the subject has been characterized has having an abnormally high level of the erythroferrone according to the method of claim 8.

10. A method of making an antibody against human erythroferrone and/or an ERFE polypeptide, which comprises injecting a non-human animal with human erythroferrone, ELPRGPGESRAGPAARPP (SEQ ID NO: 1), GESRAG (SEQ ID NO: 2), LGSPEPGAPSRSRAR (SEQ

ID NO: 34), rhERFE1 (SEQ ID NO: 3), and/or rhERFE2 (SEQ ID NO: 7), preferably rhERFE1 (SEQ ID NO: 3) or rhERFE2 (SEQ ID NO: 7), and more preferably rhERFE2 (SEQ ID NO: 7).

11. An antibody produced by the method according to claim 10.

12. An antibody comprising

a VH sequence that comprises

a) GIDLNDNA (SEQ ID NO: 10), IYIDTST (SEQ ID NO: 11), and VREDGYRLGDV (SEQ ID NO: 12),

b) GIDLSSYE (SEQ ID NO: 20), IGTDGTA (SEQ ID NO: 21), and ARDSSGNSNYRAFDP (SEQ ID NO: 22),

c) METGLRWLLLVAVLKG(X1)QCQS(X2)EESGGRLVTPG(X3)(X4)LTLTCTVSGIDL(X5)(X6)(X7)(X8)M(X9)WVRQAPGKGLEWIGVI(X10)(X11)D(X12)(X13)(X14)(X15)YA(X16)W(X17)KGRFTISKTS(X18)TTVDLK(X19)TS(X20)TTEDTATYFC(X21)R(X22)(X23)(X24)(X25)(X26)(X27)(X28)YR(X29)(X30)D(X31) (SEQ ID NO: 28), wherein each X1 to X31 is independently any amino acid, and X18, X24, X25, X26, and X27 are each independently present or absent,

d) METGLRWLLLVAVLKG(X1)QCQS(X2)EESGGRLVTPG(X3)(X4)LTLTCTVSGIDL(X5)(X6)(X7)(X8)M(X9)WVRQAPGKGLEWIGVI(X10)(X11)D(X12)(X13)(X14)(X15)YA(X16)W(X17)KGRFTISKTS(X18)TTVDLK(X19)TS(X20)TTEDTATYFC(X21)R(X22)(X23)(X24)(X25)(X26)(X27)(X28)YR(X29)(X30)D(X31) (SEQ ID NO: 28), wherein each X1 to X31 is independently any amino acid, and X18, X24, X25, X26, and X27 are each independently present or absent, and at least one or more of GIDLNDNA (SEQ ID NO: 10), IYIDTST (SEQ ID NO: 11), and VREDGYRLGDV (SEQ ID NO: 12),

e) METGLRWLLLVAVLKG(X1)QCQS(X2)EESGGRLVTPG(X3)(X4)LTLTCTVSGIDL(X5)(X6)(X7)(X8)M(X9)WVRQAPGKGLEWIGVI(X10)(X11)D(X12)(X13)(X14)(X15)YA(X16)W(X17)KGRFTISKTS(X18)TTVDLK(X19)TS(X20)TTEDTATYFC(X21)R(X22)(X23)(X24)(X25)(X26)(X27)(X28)YR(X29)(X30)D(X31) (SEQ ID NO: 28), wherein each X1 to X31 is independently any amino acid, and X18, X24, X25, X26, and X27 are each independently present or absent, and at least one or more of GIDLSSYE (SEQ ID NO: 20), IGTDGTA (SEQ ID NO: 21), and ARDSSGNSNYRAFDP (SEQ ID NO: 22),

f) at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 88%, at least 89%, at least 90%, at least 91%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to

METGLRWLLLVAVLKGVQCQSVEESGGRLVTPGTPLTLTCTVSGIDLNDNAMRWVRQAPGKGLEWIGVIYI DTSTYYASWAKGRFTISKTSSTTVDLKITSPTTEDTATYFCVREDGYRLGDV (SEQ ID NO: 29),

g) at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 88%, at least 89%, at least 90%, at least 91%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to METGLRWLLLVAVLKGAQCQSLEESGGRLVTPGGSLTLTCTVSGIDLSSYEMGWVRQAPGKGLEWIGVIGT DGTAVYATWVKGRFTISKTSTTVDLKMTSLTTEDTATYFCARDSSGNSNYRAFDP (SEQ ID NO: 30),

h) at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 88%, at least 89%, at least 90%, at least 91%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 9, or

i) at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 88%, at least 89%, at least 90%, at least 91%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 19; and/or

a VL sequence that comprises

a) QSLYNNNY (SEQ ID NO: 15), WAS (SEQ ID NO: 16), and AGYKSSSNDDFA (SEQ ID NO: 17),

b) QSIYSY (SEQ ID NO: 25), RAS (SEQ ID NO: 26), and QQGFVISNVLNS (SEQ ID NO: 27),

c) MDTRAPTQLLGLLLLWLP(X32)ARCA(X33)(X34)MTQTP(X35)SV(X36)(X37)(X38)V GGTVTI(X39)CQASQS(X40)Y(X41)(X42)(X43)YLSW(X44)QQKPGQPPKLLIY(X45) ASTLASGVPSRF(X46)GSGSGTQFTLTIS(X47)(X48)(X49)C(X50)DAATYYC(X51)(X 52)(X53)(X54)(X55)(X56)SN(X57)(X58)(X59)(X60) (SEQ ID NO: 31), wherein each X32 to X60 is independently any amino acid, and X42 and X43 are each independently present or absent,

d) MDTRAPTQLLGLLLLWLP(X32)ARCA(X33)(X34)MTQTP(X35)SV(X36)(X37)(X38)V GGTVTI(X39)CQASQS(X40)Y(X41)(X42)(X43)YLSW(X44)QQKPGQPPKLLIY(X45) ASTLASGVPSRF(X46)GSGSGTQFTLTIS(X47)(X48)(X49)C(X50)DAATYYC(X51)(X 52)(X53)(X54)(X55)(X56)SN(X57)(X58)(X59)(X60) (SEQ ID NO: 31), wherein each X32 to X60 is independently any amino acid, and X42 and X43 are each

independently present or absent, and QSLYNNNY (SEQ ID NO: 15) and/or AGYKSSSNDDFA (SEQ ID NO: 17),

e) MDTRAPTQLLGLLLLWLP(X32)ARCA(X33)(X34)MTQTP(X35)SV(X36)(X37)(X38)VGGTVTI(X39)CQASQS(X40)Y(X41)(X42)(X43)YLSW(X44)QQKPGQPPKLLIY(X45)ASTLASGVPSRF(X46)GSGSGTQFTLTIS(X47)(X48)(X49)C(X50)DAATYYC(X51)(X52)(X53)(X54)(X55)(X56)SN(X57)(X58)(X59)(X60) (SEQ ID NO: 31), wherein each X32 to X60 is independently any amino acid, and X42 and X43 are each independently present or absent, and QSLYNNNY (SEQ ID NO: 15) and/or AGYKSSSNDDFA (SEQ ID NO: 17),

f) at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 88%, at least 89%, at least 90%, at least 91%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to MDTRAPTQLLGLLLLWLPDARCALVMTQTPSSVSAGVGGTVTINCQASQSLYNNNYLSWFQQKPGQPPKLLIYWASTLASGVPSRFSGSGSGTQFTLTISGVACDDAATYYCAGYKSSSNDDFA (SEQ ID NO: 32),

g) at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 88%, at least 89%, at least 90%, at least 91%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to MDTRAPTQLLGLLLLWLPGARCAYDMTQTPASVEVAVGGTVTIKCQASQSIYSYLSWYQQKPGQPPKLLIYRASTLASGVPSRFKGSGSGTQFTLTISDLECADAATYYCQQGFVISNVLNS (SEQ ID NO: 33),

h) at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 88%, at least 89%, at least 90%, at least 91%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 14, or

i) at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 88%, at least 89%, at least 90%, at least 91%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 23,

or an immunologically active portion thereof.

13. The antibody of claim 12, wherein the antibody specifically binds human erythroferrone, ELPRGPGESRAGPAARPP (SEQ ID NO: 1), GESRAG (SEQ ID NO: 2), LGSPEPGAPSRSRAR (SEQ ID NO: 34), rhERFE1 (SEQ ID NO: 3), and/or rhERFE2 (SEQ ID NO: 7).

14. The immunoassay according to any one of claims 1 to 6, the method according to any one of claims 7 to 10, or the antibody according to any one of claims 11 to 13, wherein the antibody is a monoclonal antibody or a synthetic antibody.

15. The immunoassay according to any one of claims 1 to 6, the method according to any one of claims 7 to 10, or the antibody according to any one of claims 11 to 13, wherein the antibody is an IgG isotype.

16. The immunoassay according to any one of claims 1 to 6, the method according to any one of claims 7 to 10, or the antibody according to any one of claims 11 to 13, wherein the ERFE polypeptide and/or the erythroferrone is an analog of human erythroferrone.

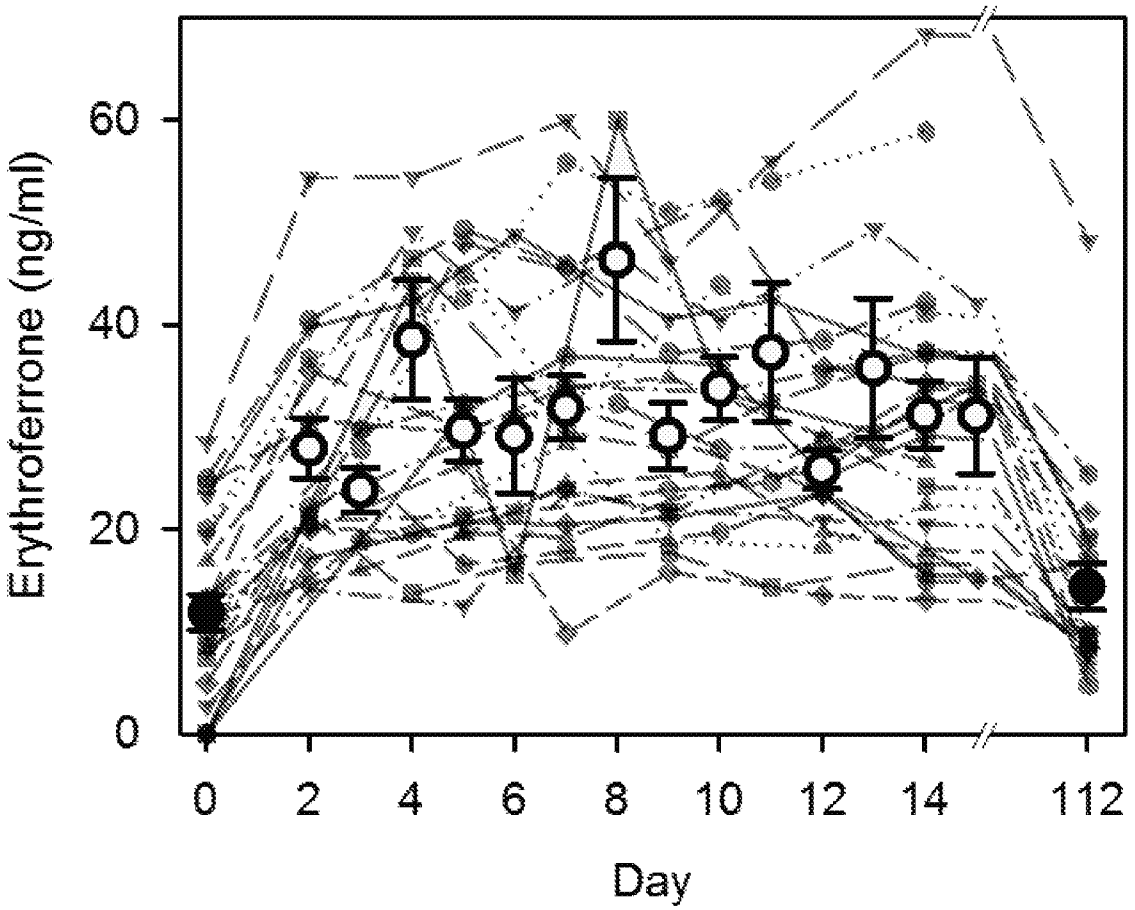

Figure 1

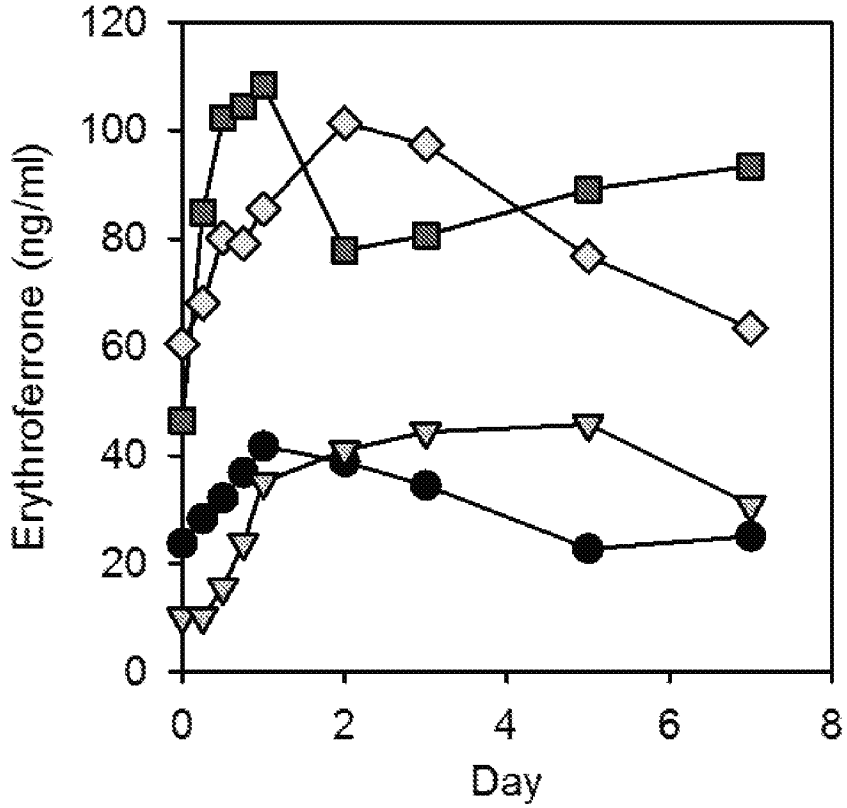

Figure 2

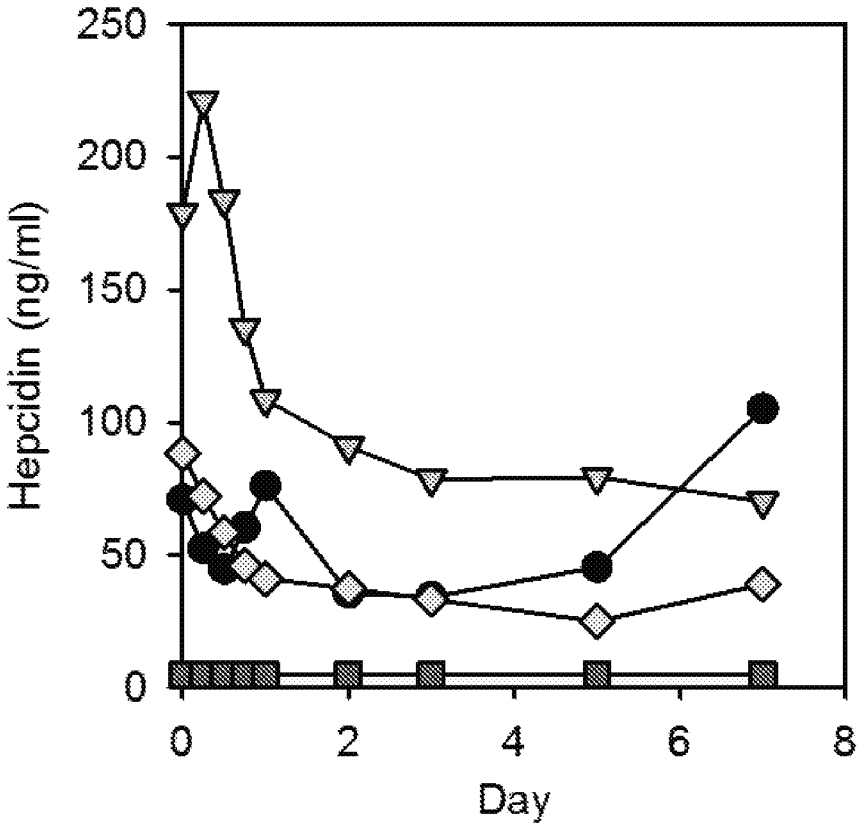

Figure 3

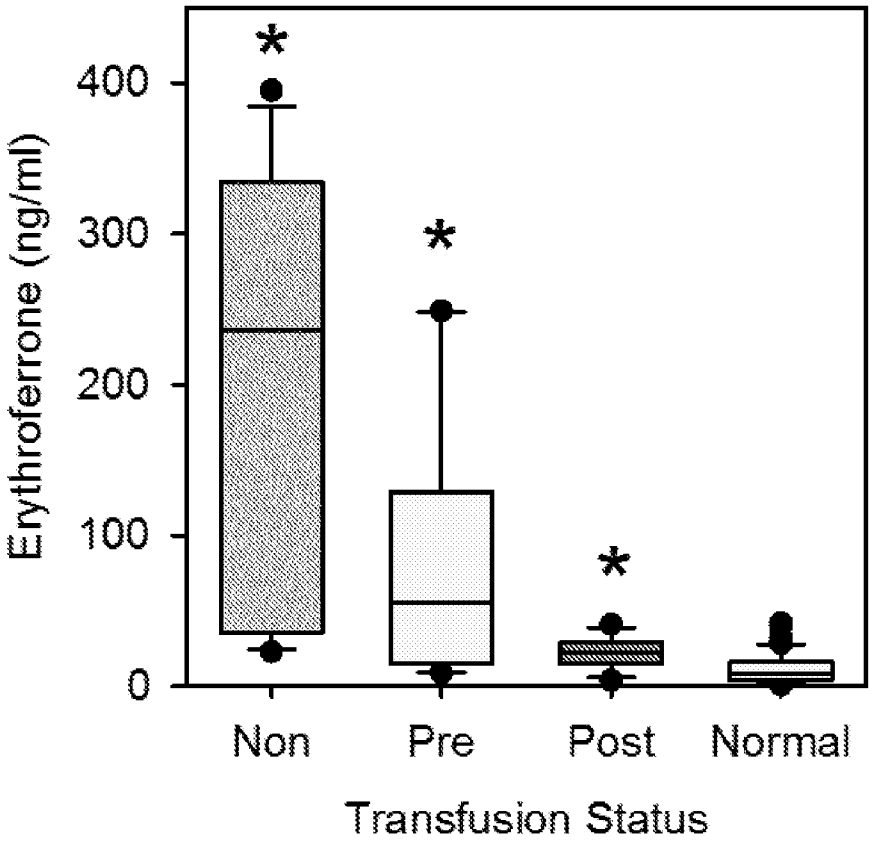

Figure 4

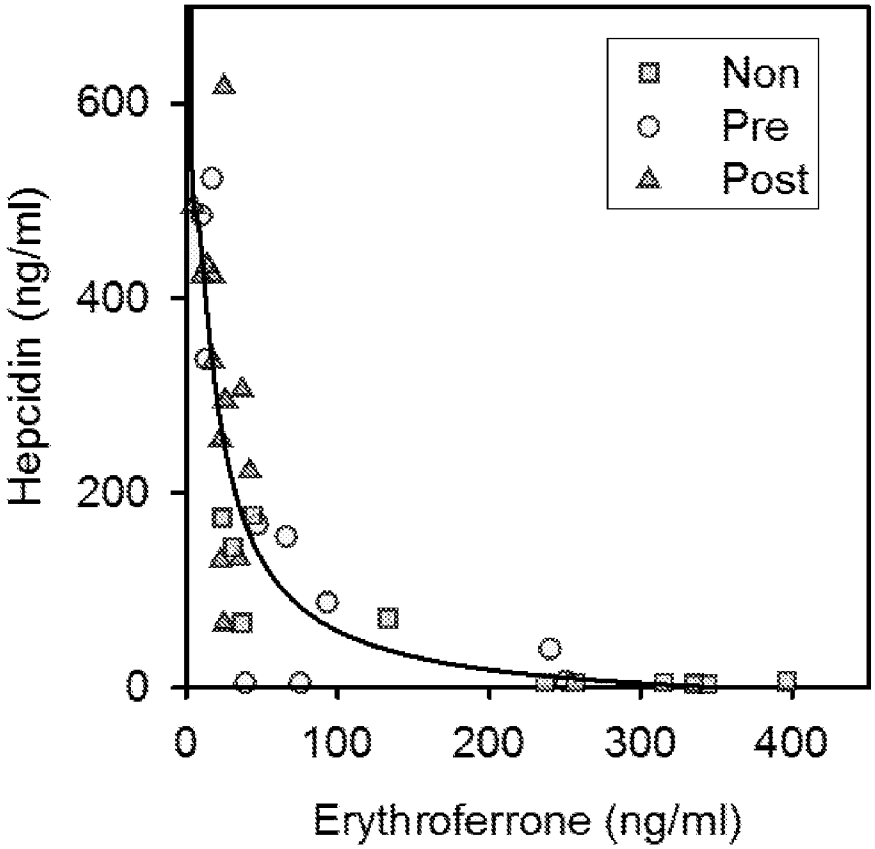
Non
Pre
Post
600
400
200
0
Hepcidin (ng/ml)
0
100
200
300
400
Erythroferrone (ng/ml)

Figure 5

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 39/00; G01N 33/00; G01N 33/566; C12P 21/08 (2018.01)
CPC - A61K 2039/505; C07K 2317/24; C07K 16/28; C07K 16/18; G01N 33/543; G01N 33/533; G01N 33/68; C07K 2317/60; C07K 2317/56; C07K 2317/21; C07K 2317/24; C07K 14/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- A	HAN et al., A Novel Dual Monoclonal Sandwich ELISA for Human Erythroferrone. Blood. 2016. 128:1272. Abstract. [online]. [Retrieved on 2018.07.27]. Retrieved from the Internet: <URL: http://www.bloodjournal.org/content/128/22/1272?sso-checked=true> PDF File: pg 1-8. Abstract: pg1, lower para; pg 2, para 1; pg 3, para 1 and Fig 1; and pg 4, Fig 3	1 --- 2-5, 12-13
A	UniProtKB_B4WSL5, Penicillin-binding Protein dimerisation domain family. Last Modified 23 September 2008. [online]. [Retrieved on 2018.08.24]. Retrieved from the Internet: <URL: https://www.uniprot.org/uniprot/B4WSL5> Accession Number; Protein; and Sequence, the region between amino acid residues 204-214	2-5, 12-13
A	US 2015/0337035 A1 (ALDERBIO HOLDINGS LLC) 26 November 2015 (26.11.2015), para [0003], [0179], [0182], and SEQ ID NO: 182	2-5, 12-13
A	US 2008/0226640 A1 (FITZGERALD et al.)18 September 2008 (18.09.2008), Abstract, para [0021], [0022], and SEQ ID NO: 2 (329 a.a.), the region between 4-329.	5/(1)
A	US 2012/0093837 A1 (GU et al.) 19 April 2012 (19.04.2012) para [0071], [0095], [0275], and SEQ ID NO: 6	12-13
A	US 2009/0028784 A1 (GARCIA-MARTINEZ et al.) 29 January 2009 (29.01.2009), para [0005], [0204], and SEQ ID NO: 219	1-5, 12-13
A	US 2016/0122409 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 05 May 2016 (05.05.2016), para [0016], [0017], [0055], [0059], [0105], [0108], and SEQ ID NO: 17 (134 a.a.), the region between amino acid residues 49-66	1-5, 12-13

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
04 September 2018	17 OCT 2018
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,A	WO 2018/027184 A1 (SILARUS THERAPEUTICS, INC..) 08 February 2018 (08.02.2018), Entire documentation, especially Abstract	1-5, 12-13
P,X --- P,A	GANZ et al., Immunoassay for human serum erythroferrone. Blood. 2017 Sep, Vol. 130(10), p. 1243-1246. Epub 2017 Jul 24. Entire documentation, especially Abstract; pg 1243, col 1, last para; and pg 1244, col 1, para 1 and Fig 1	1 --- 2-5, 12-13

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Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 6-9, 14-16
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Groups I+, Claims 1-5, 12-13, directed to an immunoassay for detecting an ERFE polypeptide, preferably a human erythroferrone, in a sample, which comprises a) contacting the sample with a capture reagent that specifically binds the ERFE polypeptide and then contacting with at least one detection reagent that specifically binds the ERFE polypeptide bound to the capture reagent; or directed to an antibody. **********Continued in the extra sheet**********

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1, 2(in part), 3-5, 12(in part), and 13, limited to SEQ ID NOs: 10-12 and 15-17, and associated SEQ ID NOs (see Groups I+ in extra sheet).

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

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Continuation of:
Box No III (unity of invention is lacking)

(Continuation of Groups I+) The capture reagent and/or the at least one detection reagent, and/or the antibody comprising a VH sequence and a VL sequence will be search to the extent that the capture reagent and/or the at least one detection reagent, and/or the antibody comprising a VH sequence encompasses a VH sequence that comprises a) GIDLNDNA (SEQ ID NO: 10), IYIDTST (SEQ ID NO: 11), and VREDGYRLGDV (SEQ ID NO: 12), which includes VH sequences of SEQ ID NOs: 9 and 28-29 comprising SEQ ID NOs: 10-12 (Specification: para [0084] - 'VH polypeptide sequence of Mab#9...SEQ ID NO: 9'; para [0085] - 'SEQ ID NO: 10...11...12'; para [0096] - 'SEQ ID NO: 10...11...12 ...SEQ ID NO: 28....SEQ ID NO: 29'); and a VL sequence that comprises a) QSLYNNNY (SEQ ID NO: 15), WAS (SEQ ID NO: 16), and AGYKSSSNDDFA (SEQ ID NO: 17), which includes VL sequences of SEQ ID NOs: 14 and 31-32 comprising SEQ ID NOs: 15-17 (Specification: para [0087] - 'encoded VL polypeptide sequence of Mab#9 is:...SEQ ID NO: 14'; para [0088] - 'CDRs of the VL chain of Mab#9 are underlined above and are...SEQ ID NO: 15...SEQ ID NO: 16...SEQ ID NO: 17'; para [0097] - 'VL sequence of an antibody...SEQ ID NO: 15...SEQ ID NO: 16...SEQ ID NO: 17... the VL sequence of an antibody according to the present invention comprises ...SEQ ID NO: 31...SEQ ID NO: 32'). It is believed that claims 1, 2(in part), 3-5, 12(in part), and 13 encompass this first named invention, and thus these claims will be searched without fee to the extent that they encompass the capture reagent and/or the at least one detection reagent, and/or the antibody comprising a VH sequence encompasses a VH sequence that comprises a) GIDLNDNA (SEQ ID NO: 10), IYIDTST (SEQ ID NO: 11), and VREDGYRLGDV (SEQ ID NO: 12); and a VL sequence that comprises a) QSLYNNNY (SEQ ID NO: 15), WAS (SEQ ID NO: 16), and AGYKSSSNDDFA (SEQ ID NO: 17). Additional capture reagent and/or the at least one detection reagent, and/or the antibody comprising a VH sequence and a VL sequence will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected capture reagent and/or the at least one detection reagent, and/or the antibody comprising a VH sequence and a VL sequence. Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be wherein the capture reagent and/or the at least one detection reagent, and/or the antibody comprising a VH sequence and a VL sequence comprising a VH sequence encompasses a VH sequence that comprises b) GIDLSSYE (SEQ ID NO: 20), IGTDGTA (SEQ ID NO: 21), and ARDSSGNSNYRAFDP (SEQ ID NO: 22); and a VL sequence that comprises b) QSIYSY (SEQ IDNO: 25), RAS (SEQ IDNO: 26), and QQGFVISNVLNS (SEQ IDNO: 27) with associated sequences (Specification: para [0090]; para [0091] - 'CDRs of the VH chain of Mab#42 are... SEQ ID NO: 20)...SEQ ID NO: 21...SEQ ID NO: 22'; para [0093]; para [0094] - 'CDRs of the VL chain of Mab#42 ... SEQ ID NO: 25... SEQ ID NO: 26... SEQ ID NO: 27)') [claims (1), 2(in part), (3-5), 12(in part), and (13)].

Group II, claims 10-11, directed to a method of making an antibody against human erythroferrone and/or an ERFE polypeptide.

The inventions listed as Groups I+ and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Feature
Groups I+ and II are related as a product (Groups I: claims 12-13) or a method of using the product (Groups I+: claims 1-5) and a method of making the product (Group II).

Groups I+ include the special technical feature of an immunoassay for detecting an ERFE polypeptide, preferably a human erythroferrone, in a sample, not required by Group II.

Group II includes the special technical feature of making an antibody, not required by Groups I+.

Among Groups I+, each SEQ ID NO associated with each specified antibody (Mab#9 or Mab#42; Specification: para [0084]-[0085], [0087]-[0088], [0090]-[0091], [0093]-[0094], [0096]-[0097]; All quotations as above) or fragment thereof represents a structurally different amino acid sequence from the respective sequence of another antibody, and vice versa.

Common Technical Features
The inventions of Groups I+ and II share the technical feature of an antibody against human erythroferrone and/or an ERFE polypeptide; and
Groups I+ further share the technical features of an immunoassay for detecting an ERFE polypeptide, preferably a human erythroferrone, in a sample, which comprises a) contacting the sample with a capture reagent that specifically binds the ERFE polypeptide and then contacting with at least one detection reagent that specifically binds the ERFE polypeptide bound to the capture reagent; and b) detecting or measuring a detectable label of the at least one detection reagent bound to the ERFE polypeptide that is bound to the capture reagent (claim 1); and
--wherein the capture reagent and/or the at least one detection reagent is an antibody comprising a VH sequence or/and a VL sequence (claims 2; and 12: an antibody comprising a VH sequence, or/and a VL sequence).

However, these shared technical features do not represent a contribution over prior art as being obvious over an article entitled 'A Novel Dual Monoclonal Sandwich ELISA for Human Erythroferrone' by Han et al. (hereinafter 'Han'; Blood. 2016. 128:1272 [online]. [Retrieved on 2018.07.27]. Retrieved from the Internet: <URL: http://www.bloodjournal.org/content/128/22/1272?sso-checked=true> PDF File: pg 1 -8) as follows:

Han discloses an antibody against human erythroferrone and/or an ERFE polypeptide (pg 2, up para - 'Two mAbs were selected, 4C1 and 2D2, as the capture and detection antibody, respectively... to quantify binding and detection of ERFE'; pg 1, para 1 - 'Erythroferrone (ERFE)'; pg 2, para 1 - 'we examined the effect of blood donation on human serum ERFE concentrations').

**********Continued in the next extra sheet**********

INTERNATIONAL SEARCH REPORT

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Continuation of:
The previous extra sheet - Box No III (unity of invention is lacking)

Han further discloses an immunoassay for detecting an ERFE polypeptide, preferably a human erythroferrone, in a sample (pg 1, lower para - 'a human ERFE assay ... first dual monoclonal sandwich ELISA for serum measurement', wherein 'a human ERFE assay.. sandwich ELISA' is 'an immunoassay for detecting an ERFE polypeptide, preferably a human erythroferrone'; pg 1, para 1 - 'Erythroferrone (ERFE)'; pg 2, para 1 - 'we examined the effect of blood donation on human serum ERFE concentrations'; Specification: para [0058] - 'immunoassays ... a sandwich ELISA'), which comprises

al) contacting the sample with a capture reagent that specifically binds the ERFE polypeptide and then contacting with at least one detection reagent that specifically binds the ERFE polypeptide bound to the capture reagent (pg 2, para 1 - 'we examined the effect of blood donation on human serum ERFE concentrations', which is done by 'contacting the sample with a capture reagent that specifically binds the ERFE polypeptide and then contacting with at least one detection reagent that specifically binds the ERFE polypeptide bound to the capture reagent', see the quotations and explanations that follow; pg 2, up para - 'We discovered at least two pairs of antibodies suitable for assay optimization. Two mAbs were selected, 4C1 and 2D2, as the capture and detection antibody, respectively', wherein '4C1' is 'a capture reagent' and '2D2' is 'at least one detection reagent that specifically binds the ERFE polypeptide bound to the capture reagent', and wherein the whole quotation encompasses 'contacting the sample with a capture reagent that specifically binds the ERFE polypeptide and then contacting with at least one detection reagent that specifically binds the ERFE polypeptide bound to the capture reagent' as it is a routine practice of sandwich ELISA; pg 1, lower para - 'a human ERFE assay ... first dual monoclonal sandwich ELISA for serum measurement'; Please see Wikipedia_ELISA: para 5 - 'ELISA involves at least one antibody with specificity for a particular antigen. The sample with an unknown amount of antigen is immobilized on a solid support ... specifically (via capture by another antibody specific to the same antigen, in a "sandwich" ELISA). After the antigen is immobilized, the detection antibody is added, forming a complex with the antigen. The detection antibody can be covalently linked to an enzyme, or can itself be detected by a secondary antibody that is linked to an enzyme through bioconjugation', the Reference is not provided, as it is a routine practice in the art); and

b) detecting or measuring the at least one detection reagent bound to the ERFE polypeptide that is bound to the capture reagent (pg 2, up para - 'Two mAbs were selected, 4C1 and 2D2, as the capture and detection antibody, respectively. We used streptavidin-HRP to quantify binding and detection of ERFE'; pg 3, para 1 - 'data we present lends strong support to the quality of the dual monoclonal sandwich ELISA we have developed. ...the assay is specific for ERFE and will allow insight into a number of hematological diseases'; pg 3, Fig 1; pg 4, Fig 3).

Although Han does not specifically teach wherein 'b) detecting or measuring the at least one detection reagent bound to the ERFE polypeptide that is bound to the capture reagent' is done by 'b) detecting or measuring a detectable label of the at least one detection reagent bound to the ERFE polypeptide that is bound to the capture reagent', one of the ordinary skill in the art at the time the invention was made would have known to do so, based on teaching of Han and the detection preference, because it is a routine choice to conjugate a detection reagent with a detectable label for detection (Please see Wikipedia_ELISA quotation above; and Under Types: the last para - 'the term "indirect ELISA" refers to an ELISA in which the antigen is bound by the primary antibody which then is detected by a labeled secondary antibody'), in order to combine methods and capture and detection reagents available in the art for facilitating detecting an ERFE polypeptide, preferably a human erythroferrone, in a sample with an expected success and without undue experimentation.

Furthermore, Han further discloses wherein the capture reagent and/or the at least one detection reagent is an antibody (pg 2, up para - 'Two mAbs were selected, 4C1 and 2D2, as the capture and detection antibody, respectively... to quantify binding and detection of ERFE'; pg 1, para 1 - 'Erythroferrone (ERFE)'; pg 2, para 1 - 'we examined the effect of blood donation on human serum ERFE concentrations').

Although Han does not specifically teach wherein the antibody comprising a VH sequence or/and a VL sequence, this limitation is determined by an inherent property of the common structure of an antibody, as evident by the Wikipedia_Antibody (Please see: Wikipedia_Antibody, The fourth Fig., and Under the fourth Figure - '3.Heavy chain (blue) with one variable (VH) ... 4.Light chain (green) with one variable (VL)').

Without a shared special technical feature, the inventions lack unity with one another.

Groups I+ and II therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Continuation of item 4: Claims 6-9, 14-16 are not drafted in accordance with the second and third sentences of Rule 6.4 (a). These claims are improper multiple dependent claims.

Note:

I) Claim 1 contains the first appeared abbreviation "ERFE" without its full name properly introduced.

II) Claim 2 contains the first appeared abbreviations "VH" and "VL" each with no full name introduced.

III) Claims 2 and 12, each comprising antibody VH and VL sequences [c)-i) for each VH and VL], that can direct to different antibodies with no relation with the claimed invention, as evident by US 2012/0093837 A1 to GU et al., which discloses an antibody heavy chain sequence that binds to a mutated epidermal growth factor receptor (EGFR) (Abstract - 'binding agents to ...the L858R point mutations in the epidermal growth factor receptor (EGFR) molecule'; para [0275] - 'amino acid sequences for the Heavy chain of the EGFR L858R (clone 43B2E11E5B2) rabbit monoclonal antibody are provided in ... SEQ ID NO:6'), which shares 94.4% of Query Match and 95.0% of the Best Local Similarity to the claimed SEQ ID NO: 9 (Specification: para [0084] - 'VH polypeptide sequence ofMab#9 is: ...SEQ ID NO: 9'); and US 2009/0028784 A1 to GARCIA-MARTINEZ et al. discloses an antibody fragment sequence that binds to interleukin-6 (para [0204] - 'fragments of the antibody having binding specificity to IL-6. ...antibody fragments of the invention comprise, ...the polypeptide sequence of SEQ ID NO: 219'; para [0005] - 'Interleukin-6 (hereinafter "IL-6"), which shares 84.1% of Query Match and 88.8% of the Best Local Similarity to the claimed SEQ ID NO: 29 (Specification: para [0096] - 'SEQ ID NO: 10...11...12 ...SEQ ID NO: 29'). Comparing antibodies by homology without specified complementarity determining regions (CDRs) sequences is MEANINGLESS, which only increases unnecessary search burden (Please See Wikipedia_Antibody for the common knowledge of an antibody structure).