CHAPTER 2

MATERIALS AND METHODS

1) Funding

Funding for the study was obtained from various sources including;

1) Post-graduate Mentor Bursary Programme, University of Pretoria, South Africa
2) Skye Foundation, South Africa
3) HF Verwoerd Research Trust, South Africa
4) Durban 2000 HIV/AIDS Research Fund, University of Pretoria, South Africa
5) NAVKOM, University of Pretoria, South Africa

2) Investigators

All determinations were performed by the candidate except for standard diagnostic evaluations which were performed by the Chemical Pathology and Haematology laboratories, National Health Laboratory Services (NHLS), University of Pretoria, South Africa. Determinations performed by the student were conducted at the Department of Physiology, Faculty of Health Sciences and the Unit for Microscopy and Microanalysis, Faculty of Natural Sciences, University of Pretoria, South Africa.

3) Patients

Forty-eight patients attending the Department of Internal Medicine, Kalafong Hospital, University of Pretoria, for treatment of chronic diseases with a high prevalence of HIV infection were included in the study. Ethical clearance for the study for this group of patients was obtained from the Faculty of Health Sciences Research Ethics Committee, University of Pretoria (ethical clearance number 118/2003). Ten patients scheduled for hip replacement at the Department of Orthopaedics, Pretoria Academic Hospital, University of Pretoria were included in the study as a group of patients with less severe immune stimulation. These patients were all diagnosed with osteoarthritis and were HIV-negative. Ethical clearance for the study for this group of patients was obtained from the Faculty of Health Sciences Research Ethics Committee, University of Pretoria (ethical clearance number 285/2003).

4) Determinations of the study

1) The expression of the H-subunit and L-subunit of ferritin in bone marrow macrophages and cells of the erythron – ultrastructural immunolocalisation of the H-subunit and L-subunit of ferritin in bone marrow macrophages and cells of the erythron.
2) Serum iron markers including serum iron, transferrin, transferrin saturation, ferritin and soluble transferrin receptor.
3) Red blood cell characteristics including red blood cell count, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, red blood cell distribution width and reticulocyte production index.
4) Prussian blue iron stains of bone marrow aspirate and core bone marrow biopsy.

5) Cytokines including Il-1β, Il-2, Il-4, Il-5, Il-6, Il-8, Il-10, Il-12, TNF-α, TGF-β1, INF-γ and GM-CSF.

6) Neopterin.

7) C-reactive protein.

8) Pro-hepcidin and caeruloplasmin.

5) Samples obtained from patients

1) A bone marrow aspirate and core bone marrow biopsy from each of the 48 patients at Internal Medicine, Kalafong Hospital were obtained at the time they had their biopsies taken for diagnostic purposes. The core bone marrow biopsy was immediately processed according to the protocol for fixation of core bone marrow tissue. A bone marrow aspirate smear was made for a Prussian blue iron stain. The bone marrow aspirates and core bone marrow biopsies were taken from the posterior superior iliac crest with a Jamshidi needle. A core bone marrow sample from each of the 10 patients at Orthopaedics, Pretoria Academic Hospital was obtained during the hip replacement procedure. The core bone marrow sample was harvested from the femur (red marrow).

2) 25 ml of coagulated blood – processed within one hour. Blood was centrifuged at 2500 g at 12°C in order to separate the serum from the blood cells. Aliquots of the serum were frozen at -75°C.

3) 5 ml of coagulated blood for standard serum iron markers including iron, transferrin, transferrin saturation and ferritin.

4) 5 ml of EDTA blood for red blood cell characteristics.

6) Materials and methods

6.1) Ultrastructural immunolocalisation of the H-subunit and L-subunit of ferritin in bone marrow macrophages and cells of the erythron

The method for the ultrastructural immunolocalisation of the H-subunit and L-subunit of ferritin was not available and therefore had to be developed and evaluated by the candidate. The theoretical background, development and evaluation of the technique for the ultrastructural immunolocalisation of the H-subunit and L-subunit of ferritin are presented in Volume 2.

6.1.1) Fixation of core bone marrow tissue

Materials

1) Fixative consisting of 4% formaldehyde (FA), 0.05% glutaraldehyde (GA) in a 0.15 M sodium phosphate buffer. The fixative was prepared fresh immediately prior to the obtainment of bone marrow tissue. A 10% paraformaldehyde (Paraformaldehyde (Trioxymethylene), SPI Supplies, cat. no. 2615, Rick Loveland & Associates, Halfway House, South Africa) solution in deionised H_2O was prepared fresh in a fume hood. The solution was heated to 60-70°C with constant stirring. Once the solution had reached the proper temperature stirring was continued for 15 minutes. At this point the solution was milky. One to two drops of 1 N NaOH was added, with stirring, until the solution cleared (1). The 0.15 M sodium phosphate buffer was prepared from two stock solutions. A 0.3 M Na_2HPO_4 stock solution (di-Sodium hydrogen phosphate Dihydrate, Fluka, BioChemika, Ultra, cat. no. 71643, Sigma-Aldrich, Aston Manor, South Africa) and a 0.3 M NaH_2PO_4 stock solution (Sodium dihydrogen phosphate Dihydrate, Fluka, Biochemika, MicroSelect, cat. no. 71505, Sigma-Aldrich, Aston Manor, South Africa). The 0.3 M NaH_2PO_4 stock solution was added to the 0.3 M Na_2HPO_4 to pH 7.25 immediately prior to the obtainment of bone marrow

tissue. This 0.3 M sodium phosphate buffer was then diluted 1:1 with the 10% freshly prepared formaldehyde stock solution and deionised H_2O. This was then followed by the addition of the GA (Pure Glutaraldehyde 25% solution, E.M. grade, SPI Supplies, cat. no. 2607, Rick Loveland & Associates, Halfway House, South Africa).

2) Ethanol 99.9% Absolute A.R., Minema, Rick Loveland & Associates, Halfway House, South Africa.

3) LR White Resin, medium grade acrylic resin, London Resin Company LTD., Rick Loveland & Associates, Halfway House, South Africa.

4) Gelatine capsules, SPI Supplies, cat. no. 2302, Rick Loveland & Associates, Halfway House, South Africa.

5) Nickel grids, 200 MESH Hexagonal grids, SPI Supplies, cat. no. 2480N, Rick Loveland & Associates, Halfway House, South Africa.

Method

1) A piece of core bone marrow was obtained during the biopsy procedure at the bedside of the patient and placed immediately in the fixative on ice.

2) The bone marrow tissue was fixed for 24 hours at 6°C whilst being rotated (TAAB rotator, Wirsam Scientific, Richmond, South Africa).

3) The bone marrow tissue was washed 3 times for 20 minutes with the sodium phosphate buffer at 6°C whilst being rotated.

4) The bone marrow tissue was dehydrated as follows 50% EtOH, 70% EtOH, 30 minutes each at 6°C whilst rotating followed by 85% EtOH, 2 times 15 minutes at 6°C whilst being rotated.

5) The bone marrow tissue was infiltrated with 1:1 85% EtOH:LR White mixture for 30 minutes at 6°C whilst being rotated. LR White dissolved in 85% EtOH but not in 80% EtOH.

6) The bone marrow tissue was infiltrated with LR White, 2 times 30 minutes at 6°C whilst being rotated.

7) The bone marrow tissue was placed in gelatine capsules in fresh LR White and was then polymerised, without any air bubbles, for 24 hours at 50°C.

8) The block of bone marrow tissue was sectioned and the sections were placed on nickel grids since copper grids can be oxidised during the immunolabelling procedures.

6.1.2) Immunolabelling of the H-subunit and L-subunit of ferritin

Materials

1) 8% $NaJO_4$, Sodium (meta) periodate, Fluka, Biochemika, Ultra, cat. no. 71859, Sigma-Aldrich, Aston Manor, South Africa.

2) Phosphate buffered saline – 20 mmol/l sodium phosphate buffer, 0.15 mol/l sodium chloride. Two stock solutions were prepared – 20 mmol/l Na_2HPO_4, 0.15 mol/l NaCl and 20 mmol/l $NaH_2PO_4 \cdot H_2O$, 0.15 mol/l NaCl. The $NaH_2PO_4 \cdot H_2O$ stock solution was added to the Na_2HPO_4 stock solution to pH 7.4. Sodium chloride, SigmaUltra, cat. no. S7653, Sigma-Aldrich, Aston Manor, South Africa. di-Natriumhydrogenphosphate, cat. no. 6586, Merck Chemicals (Pty) LTD., Germiston, South Africa, Natriumdihydrogenphosphate-1-hydrate, cat. no. 6346, Merck Chemicals (Pty) LTD., Germiston, South Africa.

3) 0.5% Glycine, Pharmacia Biotech, cat. no. 17-1323-01, AEC Amersham (PTY) LTD, Sandton, South Africa.

4) BSA, Bovine Serum Albumin, Amersham Biosciences, cat. no. RPN 412 V, Separations Scientific, Randburg, South Africa.

5) FBS, Fetal Bovine Serum, filtered and gamma irradiated, cat. no. 306, Highveld Biologicals (Pty) Ltd, Halfway House, South Africa.

6) Tween-20, Polyoxyethylenesorbitan monolaurate, Sigma for Molecular Biology, cat. no. P-9416, Sigma-Aldrich, Aston Manor, South Africa.

7) Primary monoclonal antibodies, primary monoclonal antibody specific for the H-subunit of ferritin, RH02 and the monoclonal antibody specific for the L-subunit of ferritin, LF03 were obtained from Ramco Laboratories, Inc., Stafford, Texas, United States of America.

8) Secondary antibody, Anti-mouse IgG (whole molecule), gold conjugate, 10 nm, cat. no. G-7777, Sigma-Aldrich, Aston Manor, South Africa.

9) Glutaraldehyde, Pure Glutaraldehyde 25% solution, E.M. grade, SPI Supplies, cat. no. 2607, Rick Loveland & Associates, Halfway House, South Africa.

10) Uranyl acetate, SPI Supplies, cat. no. 2624, Rick Loveland & Associates, Halfway House, South Africa.

Method

1) The sections were incubated with 8% $NaJO_4$ in H_2O for 1 hour at room temperature. All procedures were performed by placing the section on a drop of the specific solution.

2) The sections were rinsed 3 times 5 minutes with phosphate buffered saline at room temperature.

3) The sections were blocked with 0.05% glycine in H_2O for 20 minutes at room temperature.

4) The sections were blocked with 1% BSA, 5% FBS, 0.05% Tween-20 in phosphate buffered saline for 1 hour at 30°C.

5) The sections were incubated with the primary monoclonal antibodies 1:10 diluted in 1% BSA, 5% FBS, 0.05% Tween-20, phosphate buffered saline for 4 hours at 30°C.

6) The sections were rinsed 3 times 5 minutes with 1% BSA, 5% FBS, 0.05% Tween-20 in phosphate buffered saline at room temperature.

7) The sections were incubated with the secondary antibody 1:50 in 1% BSA, 5% FBS, 0.05% Tween-20, phosphate buffered saline for 1 hour at 30°C.

8) The sections were rinsed for 3 times 5 minutes in 1% BSA, 5% FBS, 0.05% Tween-20 in phosphate buffered saline at room temperature.

9) The sections were rinsed 5 minutes with phosphate buffered saline at room temperature.

10) The sections were fixed with 2% GA in phosphate buffered saline at room temperature.

11) The sections were rinsed 3 times 5 minutes with deionised H_2O at room temperature.

12) To enhance the contrast of the sections, the sections were stained for 10 minutes with 0.3% uranyl acetate at room temperature.

13) The sections were dipped 15 times in 3 separate beakers with deionised H_2O.

14) The sections were then viewed with a Philips 301 transmission electron microscope.

6.1.3) Ultrastructural characteristics of bone marrow macrophages and cells of the erythron

Figures 1 a-e contain electron micrographs to show macrophages and cells of the erythron. Furthermore, these figures illustrate ferritin aggregates found in the cytoplasm of the cells and the possible mechanisms whereby iron is taken up by red blood cell precursors.

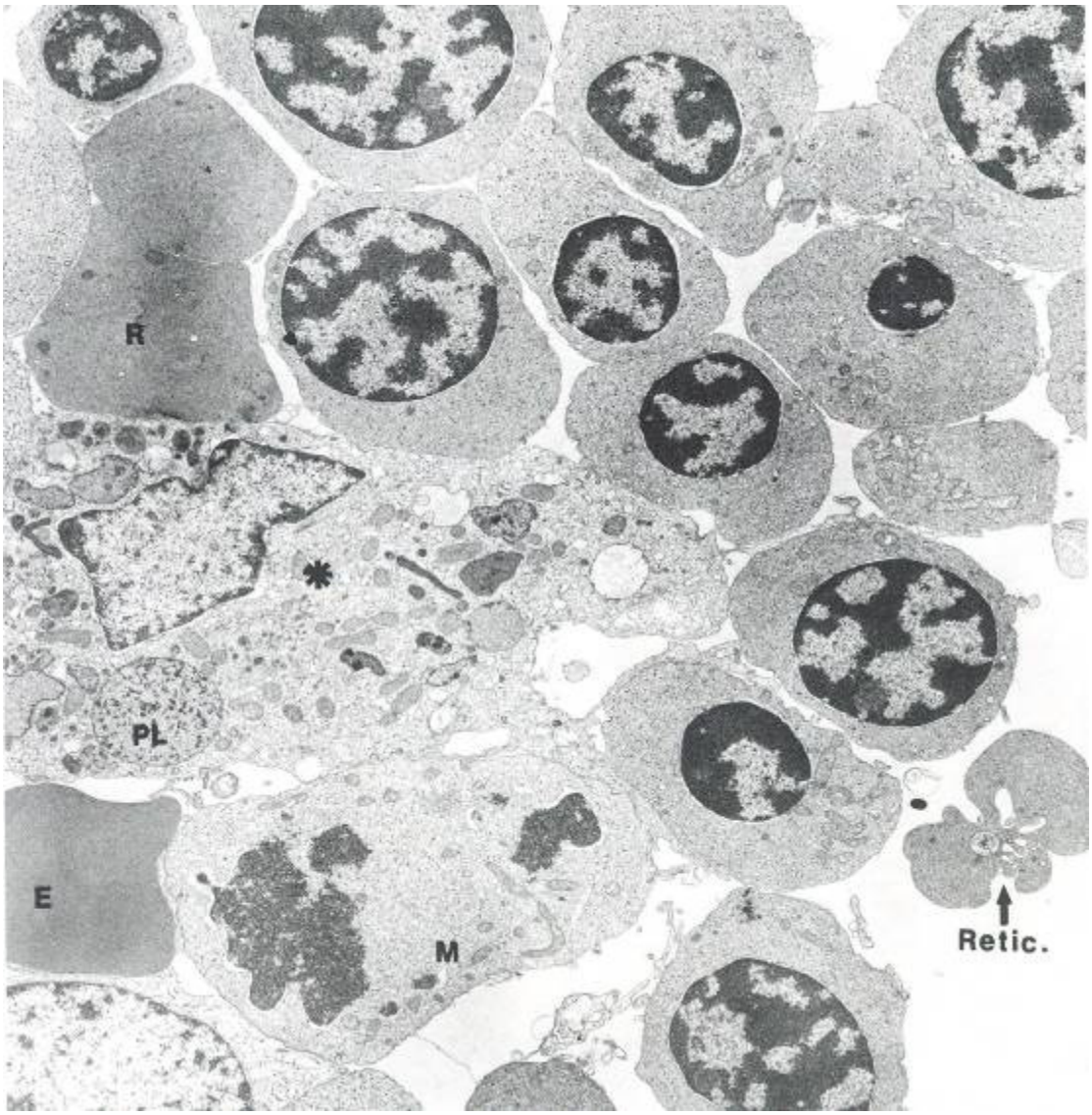


Figure 1a. Erythroblastic island. A macrophage (*) containing many inclusions probably reflecting phagolysosomes (PL) is surrounded by erythroid elements at all stages of differentiation and maturation. Note the erythroblast in mitosis (M), the young reticulocyte (Retic) shortly after nuclear extrusion, older reticulocytes (R) with a smoother contour, more electron dense cytoplasm but some residual organelles, and the mature erythrocyte (E) devoid of organelles and even more electron dense cytoplasm reflecting almost complete hemoglobinization (x 5500) (2).

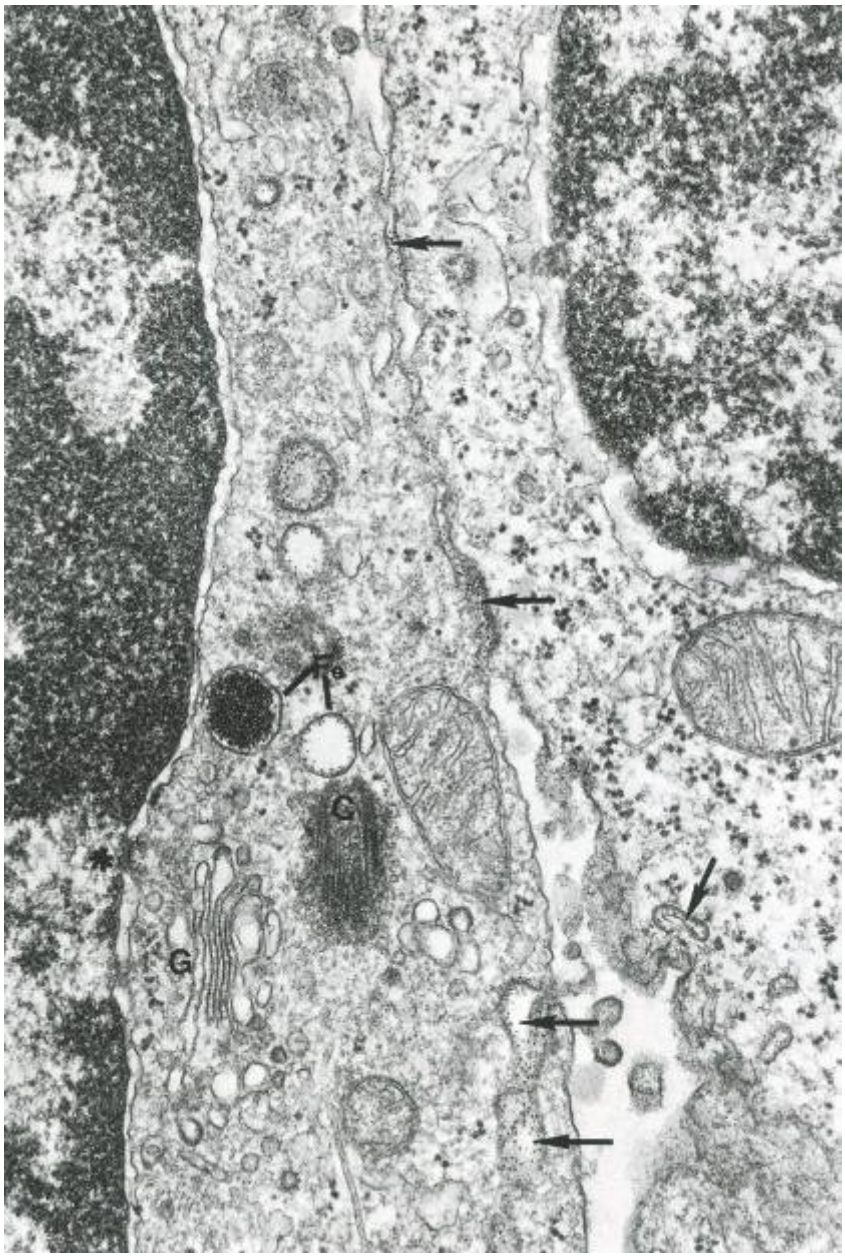


Figure 1b. Two adjacent erythroblasts from the bone marrow of a patient with increased iron storage. Note ferritin particles located on the plasma membrane of both cells and invaginations of the surface membrane (arrows). Within the cell ferritin has accumulated in membrane-bound structures (Fe), golgi zone (G), centriole (C); asterisk indicates nuclear pore (x 57000) (2).

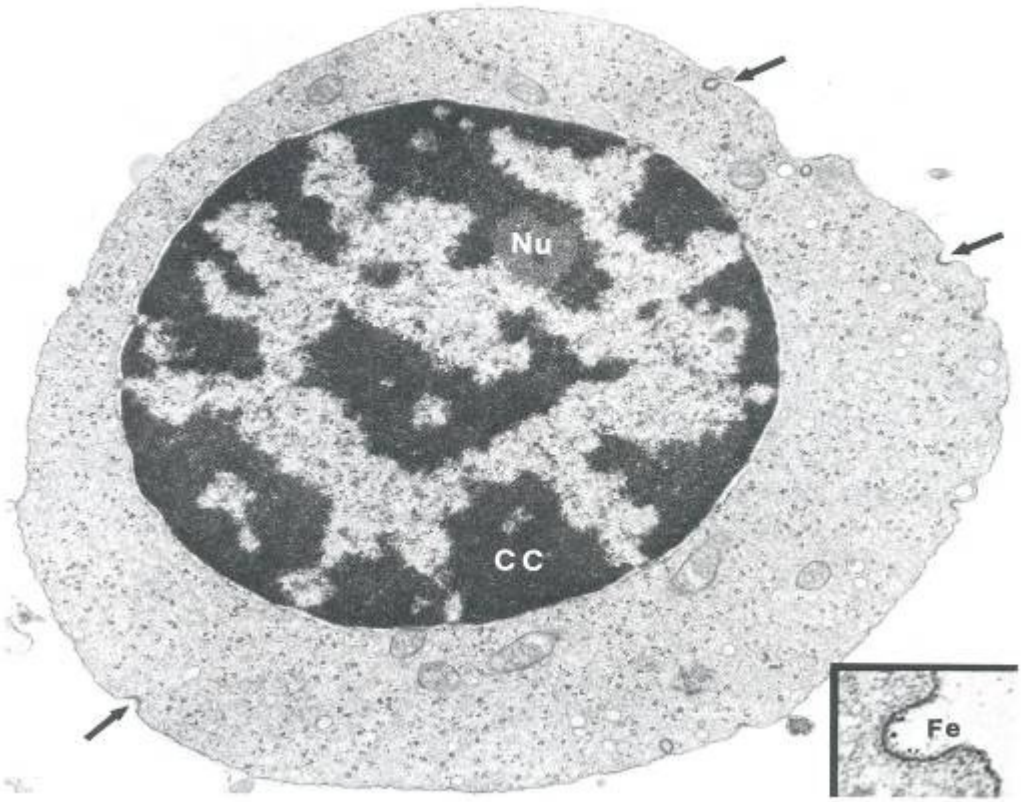


Figure 1c. Basophilic erythroblast with clumps of condensed chromatin (CC) and only a vestigial nucleolus (Nu). Although the cytoplasm is still replete with polyribosomes which would account for the degree of basophilia on light microscopy, the grey background indicates the presence of haemoglobin. Several pinocytotic vesicles of ferritin molecules (rhopheocytosis) (x 18000). Inset Fe = iron (x 40000) (2).

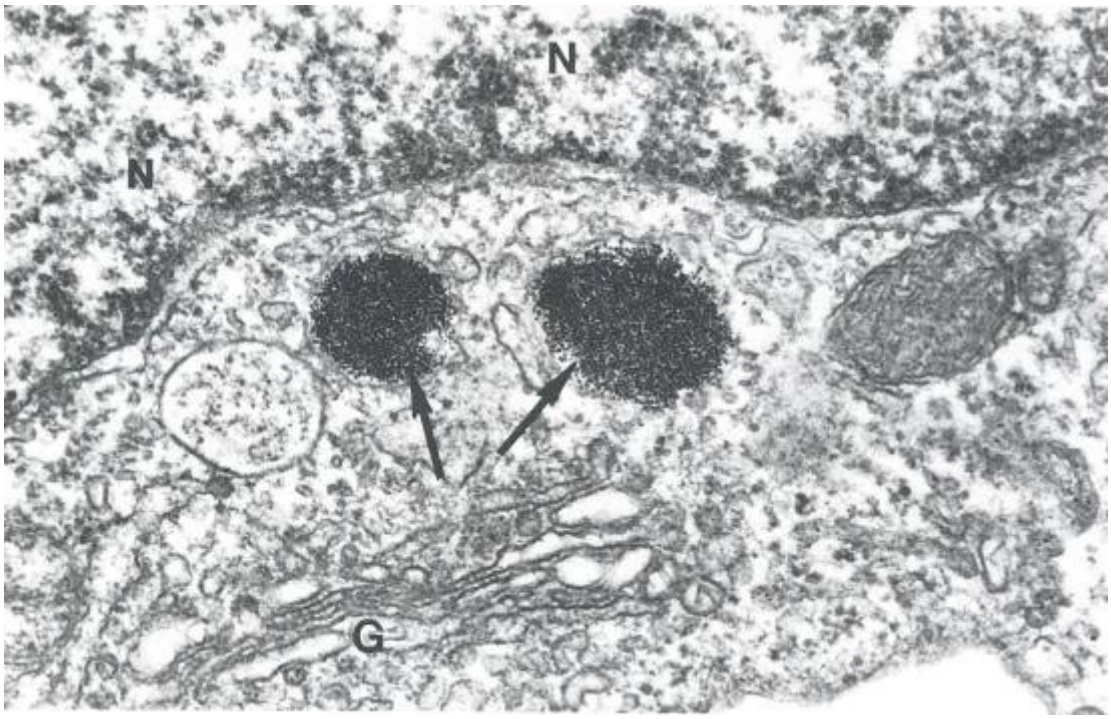


Figure 1d. Erythroblast from a patient with anaemia of chronic disease associated with "ineffective" erythropoiesis. Note the large membrane-inclusions containing ferritin (arrows), golgi apparatus (G), nucleus (N) (x 63000) (2).

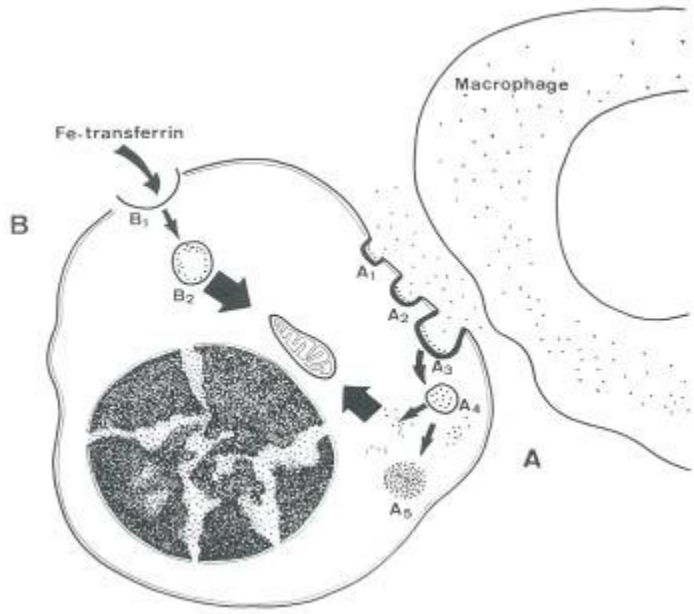


Figure 1e. Iron absorption in developing normoblasts. [The mechanisms by which iron is delivered to the mitochondria of the developing normoblasts for the incorporation into haem, are still controversial. Two principal patterns are illustrated in this schematic drawing, where the stipples represent

ferritin. According to pattern A, iron is transferred as preformed ferritin from a macrophage (the "nursing" cell) to the adjacent normoblast. The stages of this process correspond to the invagination of the cell membrane progressively enclosing ferritin molecules ($A_1 \rightarrow A_5$). According to pattern B, iron is selectively taken up by absorption to the erythroid cell surface of transferrin molecules, probably via specific cell receptors. Endocytosis of iron laden transferrin molecules or of transferrin-receptor complexes ($B_1 \rightarrow B_2$) leads to the internalization of iron for haem synthesis. Ferritin accumulates in the cytoplasm in the form of dispersed molecules or aggregates which may be either free or contained in membrane-bound vacuoles. Ferritin aggregates constitute a yellow-brown pigment known as haemosiderin and are responsible for positive staining of the erythroblasts with the Prussian blue reaction (2).]

6.1.4) Quantification of the immunolabelling of the H-subunit and L-subunit of ferritin

In order to quantify the immunolabelling of the H-subunit and L-subunit of ferritin the 10 nm gold particles were counted. The biggest area possible, consisting of only the cytosol of the cell, was demarcated. The amount of gold particles in this area was counted and expressed as count/μm^2. ImageTool was the software used to measure the area and facilitate the counting process. For each section the amount of gold particles was counted in three macrophages, not in close proximity, but on the same section. The mean was calculated and used in the statistical analysis of the data. For each patient the gold particles for three representative erythroblasts, three representative reticulocytes and three representative red blood cells were also counted. For not all patients these cells were easily distinguishable. These cells were all grouped together as cells of the erythron since no difference was shown for the expression of either the H-subunit or for the L-subunit of ferritin between these subsets of cells of the erythron. The mean for this population of cells were used as the count for the cells of the erythron and used in the statistical analysis of the data.

6.2) Serum iron markers

6.2.1) Serum iron

Serum iron measurements were determined on a BECKMAN COULTER™ SYNCHRON LX®20. Iron Reagent was used to measure the iron concentration by a timed-endpoint method. In the reaction, iron is released from transferrin by acetic acid and is reduced to the ferrous state by hydroxylamine and thioglycolate. The ferrous iron is immediately complexed with FerroZine® Iron Reagent. The system monitors the change in absorbance at 560 nm. This change in absorbance is directly proportional to the concentration of iron in the sample and is used by the SYNCHRON LX System to calculate and express the iron concentration.

Chemical reaction scheme;

Transferrin-$(Fe^{3+})_2$ → Transferrin + 2 Fe^{3+} (acetic acid pH 4.3)

Fe^{3+} + hydroxylamine + thioglycolate → Fe^{2+}

Fe^{2+} + 3FerroZine → Fe^{2+} + $(FerroZine)_3$

6.2.2) Serum transferrin

Serum transferrin measurements were determined on a BECKMAN COULTER™ SYNCHRON LX®20. Transferrin reagent was used to measure the transferrin concentration by a turbidometric method. In the reaction, transferrin combines with specific antibodies to form insoluble antigen-antibody complexes. The system monitors the change in absorbance at 340 nm. This change in absorbance is proportional to the concentration of transferrin in the sample and is used by the system to calculate and express the transferrin concentration based upon a single-point calibration.

Chemical reaction scheme;

Transferrin (antigen) + anti-transferrin antibody → antigen-antibody complex

6.2.3) Transferrin saturation

Transferrin saturation was calculated as follows;

Transferrin saturation (%) = (serum iron) / total iron binding capacity) x 100

= (serum iron) / serum transferrin x 25.1) x 100

= (serum iron) / serum transferrin) x 3.984

Where the total iron binding capacity = serum transferrin x 25.1

The total iron binding capacity (TIBC) indicates the maximum amount of iron needed to saturate serum transferrin (TRF), which is the primary iron transport protein. Theoretically, 1 mol of TRF (average molecular mass, 79 570 Da) can bind two mol of iron (55.8 Da) at two high affinity-binding sites for ferric iron. Therefore, TIBC correlates well with TRF concentration, and the theoretical ratio of TIBC (in µmol/l) to TRF (in g/l) is 25.1: TIBC (in µmol/l) = 25.1 X TRF (in g/l) (3).

6.2.4) Serum ferritin

Serum ferritin was determined by an immunometric assay, Immulite® 2000 Ferritin, for *in vitro* diagnostic use with the IMMULITE 2000 Analyzer, for the quantitative measurement of ferritin in serum.

6.2.5) Soluble transferrin receptor

The soluble transferrin receptor was determined by employing an ELISA from Ramco Laboratories, Inc., Texas, USA. The soluble transferrin receptor assay is an enzyme immunoassay based on the double antibody sandwich method. Plasma or serum samples are diluted in buffer and pipetted into microwells pre-coated with polyclonal antibody to transferrin receptor. Horseradish peroxidase (HRP) conjugated murine monoclonal antibody specific for transferrin receptor is added to the wells and the wells are incubated for two hours at room temperature. During this incubation, the transferrin receptor binds to the polyclonal antibodies adsorbed to the wells and the HRP-conjugated

secondary antibodies bind to the captured transferrin receptor. Any unbound transferrin receptor and excess HRP conjugate are removed from the wells by washing. Enzyme substrate (chromogen TMB) is added to the wells and through the action of HRP forms a blue product. Upon the addition of an acid stop solution, the blue product is converted to a yellow colour, the intensity of which is measured in a plate reader set at 450 nm. The optical density of the resulting solution is directly proportional to the concentration of transferrin receptor in the standard samples. A standard curve is generated by plotting the absorbance versus concentration of the transferrin receptor standards provided in the kit. The concentration of the transferrin receptor in the sample is then determined by comparing the sample's optical density reading with the standard curve graph.

6.3) Red blood cell characteristics

6.3.1) Red blood cell count

Red blood cell counts were determined by a Coulter Counter®. Red blood cells are suspended in a conductive liquid (diluent) and acts as an insulator. Each cell passes through an aperture and momentarily increases the resistance of the electrical path between the submerged electrodes on either side of the aperture. This causes a measurable electronic pulse. These pulses are counted and correlate to the amount of cells.

The red blood cell count is the number of erythrocytes measured directly, multiplied by the calibration constant and expressed as;

$RBC = n \times 10^6$ cells/µl

6.3.2) Haemoglobin concentration

Weight (mass) of haemoglobin determined from the degree of absorbance found through photocurrent transmittance is;

Hb (g/dl) = constant x $\log_{10}$ reference %T / sample %T

6.3.3) Haematocrit (Hct)

This is the relative volume of packed erythrocytes to whole blood, calculated as;

Hct (%) = RBC x MCV / 10

6.3.4) Mean corpuscular volume (MCV)

This is the average volume of individual erythrocytes derived from a RBS histogram. The system;

- Multiplies the number of RBCs in each channel by the size of the RBCs in that channel.
- Adds the products of each channel between 36 fl and 360 fl.
- Divides that sum by the total number of RBCs between 36 fl and 360 fl.
- Multiplies by a calibration constant and expresses MCV in femtoliters.

6.3.5) Mean corpuscular haemoglobin (MCH)

This is the weight of haemoglobin in the average erythrocyte count, calculated as;

MCH (pg) = Hb / RBC x 10

6.3.6) Mean corpuscular haemoglobin concentration (MCHC)

This is the average weight of haemoglobin in a measured dilution, calculated as;

MCHC (g/dl) = Hb / Hct x 100

6.3.7) Red blood cell distribution width (RDW)

RDW represents the size distribution spread of the erythrocyte population derived from the RBC histogram. It is the coefficient of variance (CV), expressed in percentage, of the RBC size distribution.

6.3.8) Reticulocyte production index (RPI)

Whole blood samples are incubated with a supravital dye, New Methylene Blue, in a special solution (reagent A). The dye precipitates the basophilic RNA network found in reticulocytes. This is followed by the addition of reagent B, a hypotonic clearing reagent, which clears haemoglobin and unbound stain from the cells. Stained reticulocytes differ from mature erythrocytes and other cell populations by light scatter, direct current measurements and opacity characteristics. Reticulocyte counts can then be measured in the Coulter Counter.

RPI = (Hct / 0.45 x reticulocyte %) / shift correction factor for haematocrit

- shift correction factors for hct; 45 = 1, 35 = 1.5, 25 = 2, 15 = 2.5
- reticulocyte % = the number of reticulocytes per 100 RBCs
- > 2.5 = blood loss with normal bone marrow response, < 2.5 = suppressed bone marrow

6.4) Prussian blue iron stain of bone marrow aspirate and core bone marrow biopsy

6.4.1) HCl-ferrocyanide iron stain of bone marrow aspirate smears

1) The smears were fixed in methanol for 10 minutes.

2) The smears were air-dried.

3) The smears were stained for 10 minutes in 2% potassium ferrocyanide/0.2 N HCl in a Coplin jar at 58°C.

4) The smears were rinsed in distilled water.

5) The smears were air-dried.

6) The smears were counter-stained with nuclear fast red for 10 minutes.

7) The smears were rinsed in distilled H_2O.

6.4.2) HCl-ferrocyanide iron stain of core bone marrow LR White plastic sections

Materials

1) Menzel-Glaser Superfrost® Plus Microscope Slides, Labotec, Halfway House, South Africa.

2) 10% ferrocyanide, Potassium hexacyanoferrate (II) Trihydrate, Fluka, Biochemika Ultra, cat. no. 60279, Sigma-Aldrich, Aston Manor, South Africa; 10% HCl, Hydrochloric acid 30%, Riedel-de-Haën, cat. no. 30053, Sigma-Aldrich, Aston Manor, South Africa.

3) 1% eosin, Eosin yellowish, Gurr®, Microscopy Material, cat. no. 45380, BDH Chemicals Ltd., England, in 70% ethanol (acidified with acetic acid).

Method

1) The 2 µm thick sections were placed on microscope slides.

2) The slides were rinsed in deionised H_2O.

3) The sections were stained for 1 hour in 10% ferrocyanide/10% HCl prepared just before use in Coplin jars at 25°C.

4) The slides were rinsed in deionised H_2O.

5) The sections were counter-stained with 1% eosin in 70% ethanol (acidified) for 10 minutes.

6) The slides were rinsed in deionised H_2O.

7) The slides were air-dried on a hot plate.

8) The sections were mounted with immersion oil and a cover slide.

6.5) Cytokines Il-1β, Il-2, Il-4, Il-5, Il-6, Il-8, Il-10, Il-12, TNF-α, TGF-β1, INF-γ and GM-CSF

6.5.1) Il-8, Il-1β, Il-6, Il-10, TNF-α and Il-12p70

Il-8, Il-1β, Il-6, Il-10, TNF-α and Il-12p70 were determined by employing the Human Inflammation Kit, BD™ Cytometric Bead Array (CBA) (The Scientific group, Midrand, South Africa). The BD™ Cytometric Bead Assay employs a series of particles with discrete fluorescence intensities to simultaneously detect multiple soluble analytes. The BD™ CBA is combined with flow cytometry to create a powerful multiplexed assay. The BD CBA system uses the sensitivity of amplified fluorescence to measure soluble analytes in a particle-based immunoassay. Each bead in a CBA provides a capture surface for a specific protein and is analogous to an individually coated well in an ELISA plate. The BD CBA capture bead mixture is in suspension to allow for the detection of multiple analytes in a small volume sample. Six bead populations with distinct fluorescence intensities have been coated with capture antibodies specific for Il-8, Il-1β, Il-6, Il-10, TNF-α and Il-12p70 proteins. The six bead populations are mixed together to form the BD™ CBA which is resolved in the FL3 channel of a flow cytometer such as the BD FACScan™ or BD FACSCalibur™ flow cytometer. The capture beads, PE-conjugated detection antibodies, and recombinant standards or test samples are mixed together to form sandwich complexes. Following acquisition of sample data using the flow cytometer, the sample results are generated in graphical and tabular form using the

BD™ CBA Analysis Software. The BD™ Cytometric Bead Assay Human Inflammation Kit can be used to quantitatively measure:

Interleukin-8 (Il-8)

Interleukin-1β (Il-1β)

Interleukin-6 (Il-6)

Interleukin-10 (Il-10)

Tumor Necrosis Factor-α (TNF-α)

Interleukin-12p70 (Il-12p70)

6.5.2) Il-2, Il-4, Il-5, Il-10, TNF-α and IFN-γ

Il-2, Il-4, Il-5, Il-10, TNF-α and IFN-γ were determined by employing the BD™ Cytometric Human Th1/Th2 Cytokine Kit (The Scientific group, Midrand, South Africa). The methodology is similar to that of the Inflammation Kit, BD™ Cytometric Bead Array (CBA). The BD™ Cytometric Bead Assay Human Th1/Th2 Cytokine Kit were used to quantitatively measure:

Interleukin-2 (Il-2)

Interleukin-4 (Il-4)

Interleukin-5 (Il-5)

Interleukin-10 (Il-10)

Tumor Necrosis Factor-α (TNF-α)

Interferon-γ (IFN-γ)

With the Th1/Th2 CBA cytokine kit the IFN-γ standards were lost and the measurement of IFN-γ was done by employing the human IFN-γ ELISA Kit, DRG Diagnostics, Germany.

IFN-γ determinations

The BD OptEIA™ test (The Scientific group, Midrand, South Africa) is a solid phase sandwich ELISA. It utilizes a monoclonal antibody specific for the IFN-γ coated on a 96-well plate. Standards and samples are added to the wells, and any IFN-γ present binds to the immobilised antibody. The wells are washed and streptavidin-horseradish peroxidase conjugate mixed with biotinylated anti-human IFN-γ antibody is added, producing an antibody-antigen-antibody "sandwich". The wells are again washed and TMB substrate solution is added, which produces a blue colour in direct proportion to the amount of IFN-γ present in the initial sample. The stop solution changes the colour from blue to yellow, and the microwell absorbances are read at 450 nm.

Il-10 and TNF-α

Il-10 and TNF-α were measured in both the Human Inflammation kit and the Human Th1/Th2 kit. The mean of these values were calculated and used for statistical analysis.

6.5.3) Transforming growth factor β1 (TGF-β1)

TGF-β1 measurements were performed by employing a TGF-β1 ELISA (DRG Diagnostics, Germany, Orb Diagnostics, Modderfontein, South Africa). The TGF-β1 ELISA kit is a solid-phase enzyme-linked immunosorbent assay which is based on the sandwich principle. Prior to testing, the standards and patient samples are diluted in assay buffer, acidified with HCl and then neutralised with NaOH. Afterwards, the neutralised standards and samples are added to the antibody-coated micotiter wells. After the first incubation the unbound sample material is removed by washing with diluted wash solution. Then a monoclonal mouse anti TGF-β1 antibody, a biotynilated anti mouse IgG andtibody and the Streptavidin-HRP enzyme complex are incubated in succession. An immuno-enzyme sandwich complex is formed. The unbound conjugate

is removed by washing. Subsequently substrate solution is added. After a definite time colour development is stopped by addition of stop solution and the absorbance at 450 nm is measured with a microtiterplate reader. The intensity of the colour development is proportional to the TGF-β1 concentration in the sample.

6.5.4) Granulocyte macrophage colony stimulating factor (GM-CSF)

GM-CSF measurements were performed by employing a GM-CSF ELISA (DRG Diagnostics, Germany, Orb Diagnostics, Modderfontein, South Africa). The DRG GM-CSF ELISA is a solid phase enzyme amplified sensitivity immunoassay (EASIA) performed on microtiter plate. The assay is based on an oligoclonal system in which a blend of monoclonal antibodies (MAbs) directed against different epitopes of GM-CSF is used. The use of a number of distinct MAbs avoids hyperspecificity and allows high sensitive assays with extended standard range and short incubation time. Standards or samples containing GM-CSF react with capture monoclonal antibodies (MAbs 1) coated on the micotiter well and with a monoclonal antibody (MAb 2) labelled with horseradish peroxidase (HRP). After an incubation period allowing the formation of a sandwich:coated MAbs 1 – GM-CSF – Mab 2 – HRP, the microtiter plate is washed to remove unbound enzyme labelled antibodies. Bound enzyme-labelled antibodies are measured through a chromogenic reaction. Chromogenic solution (TMB + H_2O_2) is added and incubated. The reaction is stopped with the addition of Stop solution (H_2SO_4) and the micotiter plate is then read at the appropriate wavelength. The amount of substrate turnover is determined colorimetrically by measuring the absorbance which is proportional to the GM-CSF concentration. A standard curve is plotted and the GM-CSF concentration in a sample is determined by interpolation from the standard curve.

6.6) Neopterin

Neopterin measurements were performed by employing a neopterin ELISA (DRG Diagnostics, Germany, Orb Diagnostics, Modderfontein, South Africa). The neopterin ELISA is a solid phase enzyme-linked immunosorbent assay (ELISA) based on the basic principle of a competitive ELISA. An unknown amount of antigen in the sample and a fixed amount of enzyme labelled antigen compete for the antibody-binding sites (rabbit-anti-neopterin). Both antigen-antibody complexes bind to the wells of the microtiter strips coated with a goat-anti-rabbit antibody. Unbound antibody is removed by washing. The intensity of the colour developed after the substrate incubation is inversely proportional to the amount of antigen in the sample. Results of samples can be determined directly using the standard curve.

6.7) C-reactive protein (CRP)

CRP measurements were performed by employing a CRP ELISA (DRG Diagnostics, Germany, Orb Diagnostics, Modderfontein, South Africa). The CRP ELISA is an enzyme immunoassay for the quantitative determination of CRP in human plasma and serum. Microtiterstrips coated with anti-CRP antibody are incubated with diluted standard sera and patient samples. During this incubation step CRP is bound specifically to the wells. After removal of the unbound serum proteins by a washing procedure, the antigen-antibody complex in each well is detected with specific peroxidase-conjugated antibodies. After removal of the unbound conjugate, the strips are incubated with a chromogen solution containing tetramethylbenzidin and hydrogen peroxide: a blue colour develops in proportion to the amount of immunocomplex bound to the wells of the strips. The enzymatic reaction is stopped by the addition of 2 N H_2SO_4 and the absorbance values at 450 nm are determined. A standard curve is obtained by plotting the standard absorbance values versus the corresponding standard values. The

concentration of CRP in patient samples is determined by interpolation from the standard curve.

6.8) Pro-hepcidin and caeruloplasmin

6.8.1) Pro-hepcidin

Pro-hepcidin measurements were performed by employing a hepcidin prohormone ELISA (DRG Diagnostics, Germany, Orb Diagnostics, Modderfontein, South Africa). The Hepcidin Prohormone ELISA Kit is a solid phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding. The microtiter wells are coated with a polyclonal antibody directed towards an antigenic site on the Hepcidin Prohormone molecule (28-47 aa). Endogenous Hepcidin Prohormone of a patient sample competes with a Hepcidin Prohormone-biotin conjugate for binding to the coated antibody. After incubation the unbound conjugate is washed off. The amount of bound biotin conjugate is reverse proportional to the concentration of Hepcidin Prohormone in the sample. After addition of the substrate solution, the intensity of colour developed is reverse proportional to the concentration of Hepcidin Prohormone in the patient sample.

6.8.2) Caeruloplasmin

The caeruloplasmin assay is a measurement based on rate nephelometry. This measures the rate of increase in light scattered from particles suspended in solution as a result of complexes formed during an antigen-antibody reaction.

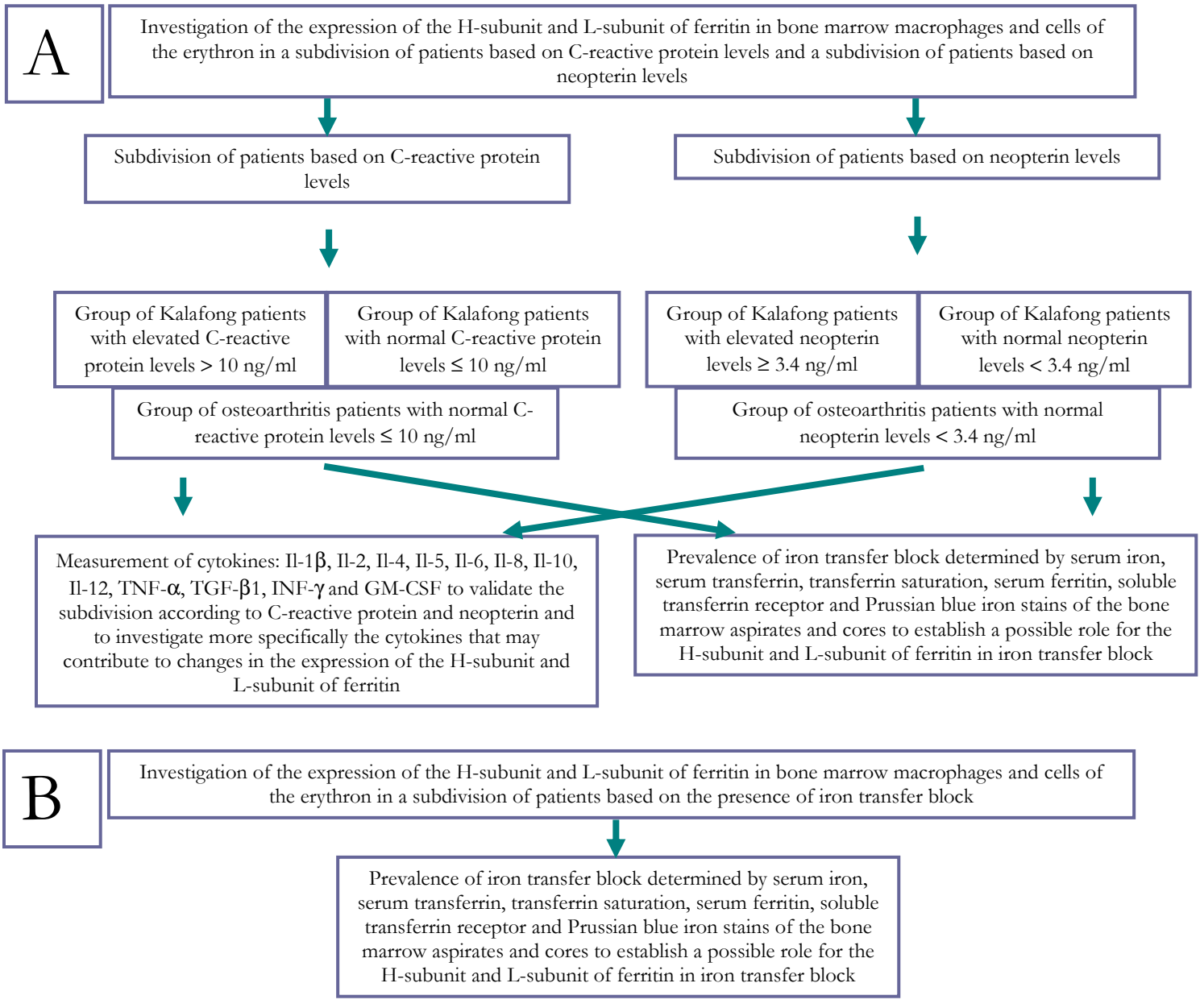
Chemical reaction scheme;

Caeruloplasmin(sample) + antibody → [caeruloplasmin(sample)-antibody (aggregates)]

7) Statistical analysis

Statistical analysis employed in this study included the Welch t-test and since groups were relatively small and variances could be large, use was also made of the ranksum (Mann-whitney) test. The p-values for both tests were reported and when interpreted, preference was given to the p-value of the ranksum test when the Welch t-test was not significant. Pearson's product-moment correlation coefficient (r) was employed to assess dependence between study parameters. Testing was done at the 0.05 level of significance.

8) Study design



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