Normal Erythrocytes Inflammation

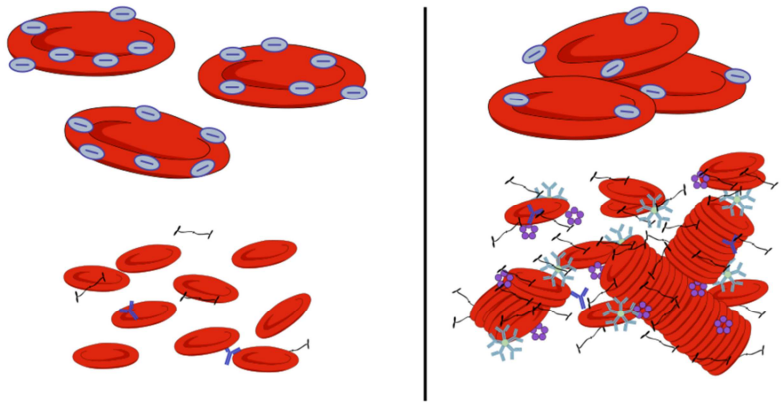


Fig 1: Normal: Negatively charged erythrocytes; low sedimentation rate.
Inflammation: Less negatively charged erythrocytes; sedimentation occurs, stimulated by all the different factors that increase rouleaux formation (Fibrinogen, CRP, Immunoglobulin).

The sedimentation process can be divided into three stages:

A. Lag stage-rouleaux formation (0-20 min) Erythrocytes start to aggregate and form rouleaux. The presence of acute phase proteins encourages rouleaux formation. During this phase, no sedimentation occurs.

B. Decantation stage-sedimentation (15-30 min) Erythrocyte aggregates fall to the bottom under influence of gravity at a constant rate. Large aggregates fall faster than small aggregates or single cells. Falling aggregates induce an upward plasma current that slows down sedimentation.

C. Packing stage (25-60 min) The rate of sedimentation slows down to zero and cells start to pack in the bottom of the tube.

Rouleaux formation

Fig. 2: At low or no flow condition, RBC's adhere side to side and form stacks called rouleaux, followed by end to end connections creating 3D aggregates (the rouleaux formation)

The Westergren method is the gold standard

In the 1920's, Swedish practitioners Robert Fårhæus and Alf Westergren developed a systematic method for ESR measurement. Although several alternative methods were developed in that era, the Fårhæus-Westergren method, or Westergren method as it became known in the English-speaking world, quickly gained dominance. In 1973, the Westergren method was adopted as the reference method for ESR measurement by the International Council for Standardization in Hæmatology (ICSH). The Westergren gold standard was reconfirmed in 2011 by the ICSH and by the Clinical and Laboratory Standards Institute (CLSI). It remains the gold standard that all other ESR measurement methods and techniques are evaluated against.

The Wintrobe method: An alternative method for ESR measurement

The sixth edition of Gradwohl's Clinical Laboratory Methods and Diagnosis, published in 1963, mentions five different methods to measure ESR. These were the Westergren method, the Linzenmeier method, the Graphic or Cutler method, the Wintrobe-Landsberg method and the Landau method, which was a modification of the Linzenmeier method.

Of these methods, only the Westergren method and Wintrobe method are still in use today. The Wintrobe method uses tubes of only 100 mm long with a smaller diameter than standard Westergren tubes. EDTA blood without extra diluent is added to the tube and allowed to sediment for 60 minutes. After 60 minutes the distance that the blood cells have fallen is registered in mm.

Because the Wintrobe tubes are shorter than the Westergren tubes, the method is less sensitive than the Westergren method.

Procedure

The Westergren method as referenced by the ICSH consists of the following steps:

Blood collection

Non-hemolyzed blood is anti-coagulated with EDTA at collection.

It is recommended that the EDTA sample is tested within 4 hours after collection, but it has been reported that storage for up to 24 hours at 4°C still results in a stable ESR value. When ready to test, the blood sample is thoroughly mixed and diluted 4:1 using a sodium citrate solution.

Tube handling

The Westergren method uses standardized colorless, circular glass or plastic tubes, with an inner diameter of at least 2.55 mm and sufficient length to include a 200 mm sedimentation scale. The inner diameter should be constant (± 5%) over the whole length; a so called Westergren tube.

The diluted sample is aspirated and transferred to the Westergren tube. The Westergren tube is then placed in a stable, vertical position at a constant temperature (± 1°C) between 18°C and 25°C in an area free from vibrations, drafts and direct sunlight.

Reading the result

After 60 ± 1 minute, the distance from the bottom of the plasma meniscus to the top of the descended erythrocytes is read and recorded in mm. The buffy coat that is made up of leukocytes should not be included in the erythrocyte column.

The Westergren method

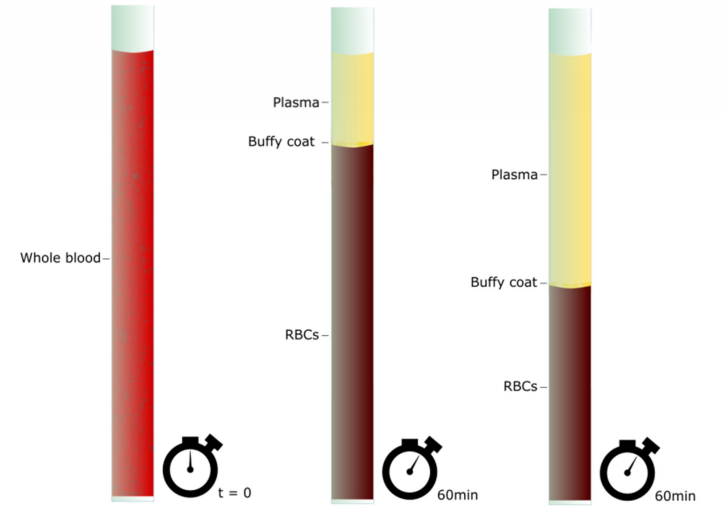


Fig 3: a. The diluted sample is aspirated and transferred to the Westergren tube.
b. A normal ESR after 60 minutes; <20 mm plasma.
c. An elevated ESR after 60 minutes.

Technical issues affecting ESR

Although the procedure is a simple one in theory, there are several technical factors that can affect the ESR result.

Quality of the Westergren tubes

Tubes with inadequate bore holes can be sensitive to erratic plugs of tightly packed cells that cause undue variation in the ESR results, especially in blood samples of high ESR and with a high hematocrit volume.

Because of contamination risks, disposable, tubes are sometimes used. These need to be replaced after use and generate a lot of waste. Some plastic tubes can have a strong attractive force on erythrocytes or release plasticizers that can affect sedimentation rate. Mold-release agents used in the manufacturing process may sometimes alter sedimentation characteristics. Reusable tubes need to be cleaned thoroughly after use.

Sample storage

The storage conditions of the sample are permitted, conform the CLSI standard (CLSI, 2011), to be up to 4 hours at room temperature or up to 24 hours at 4 degrees Celsius in cold storage. These storage conditions are in line with the sample storage requirement for the hematology analyzers.

Incorrect blood preparation

Correct blood preparation is important for reliable results. Whole blood should be anti-coagulated with EDTA without significant dilution of the sample. Alternatively, blood can be collected and diluted 4:1 in special sodium citrate tubes suitable for ESR measurement. Heparin can lead to falsely increased ESR readings (Penchas, Stern, & Bar-Or, 1978).

Changes in plasma viscosity and hematocrit can cause variable plugging of the long Westergren tube by rapidly falling erythrocyte aggregates. Correct dilution (4:1) of the blood sample in sodium citrate prevents this and makes the measurement independent of viscosity and hematocrit differences.

Deviation in vertical placement

Westergren tubes need to be placed perfectly vertically. Angles of more than 2 degrees off the vertical can accelerate sedimentation and result in a false increase in ESR. A deviation of 3 degrees can accelerate ESR up to 30%.

Temperature variation

The sedimentation process is substantially influenced by temperature variations. One example would be when sunlight would shine on some tubes but not others. The ICSH recommends a constant temperature (± 1°C) between 18°C and 25°C.

Vibrations

Vibrations can artificially increase sedimentation rate and should therefore be avoided.

Problematic samples

In some cases, erythrocyte abnormalities can result in hazy, cloudy samples that are difficult to read. Also hemolytic or lipemic samples can cause difficulties in the accurate reading of ESR. (CLSI, 2011) (Hardeman, Levitus, Pelliccia, & Bouman, 2010)

In some cases **sampling** errors, e.g. a low sample volume, can lead to foam or bubbles in the sample. In these cases, the reduced quality of the sample can lead to incorrect results and false clinical interpretation, if the analyzing system does not perform internal quality controls and flags low quality samples and unreliable readings.

Other possible sedimentation results

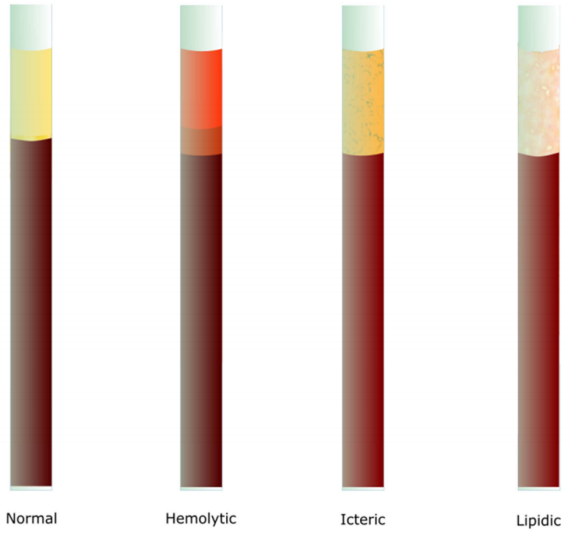


Fig.4: *- Normal sample*
- Hemolytic sample: Due to hemolysis; hemoglobin leaks from the damaged erythrocytes and turns the plasma red.
- Icteric sample: Mostly due to liver problems, elevated bilirubin turns the plasma darker yellow.
- Lipidic sample: Because of too much fat, the plasma turns opaque white and thickens.

Using tilt to speed up ESR

A tube that is not held completely vertical can lead to increased sedimentation rates and is one of the technical factors that can affect ESR readings. But could this knowledge be used to increase ESR and develop a rapid ESR method?

DM Dissanayake of the University of Peradenya in Sri Lanka has tested whether it was possible to use an inclined tube to get a faster reading of the ESR.

Dissanayake tilted tubes at an angle of 45 degrees and registered sedimentation distances every 30 seconds from 4 to 13 minutes by reading the lowest level of the meniscus. These results were compared with a traditional Westergren reading of the same sample in another tube that was kept vertically. The experiment contained a wide range of ESR readings, from 0 mm to well over 150 mm. The correlation between the traditional Westergren reading and the tilted tube was maximal between 10 and 11.5 minutes (correlation coefficient=0.985-0.986) for both low and high ESR readings.

The accuracy of the results was considered acceptable. It demonstrates however that a tilted tube has a strong influence on the optimal testing time.

Reducing analysis time

In the original Westergren method, the ESR is read after 60 minutes, which puts practical limitations on the workflow in clinical laboratories. A laboratory investigation comparing the Westergren ESR method readings of a wide range of blood samples at 30 minutes and 60 minutes showed that 30 minute ESR readings correlate highly with the corresponding 60 minute ESR readings over a wide range of blood samples (correlation coefficient = 0.984). Thus, an ESR reading after 30 minutes can reliably be extrapolated to the corresponding ESR reading at 60 minutes.(Rogers, 1994)

Correlation coefficient:

- The value of a correlation coefficient ranges between -1 and 1.
- The strongest linear relationship is indicated by a correlation coefficient of -1 or 1.
- The weakest linear relationship is indicated by a correlation coefficient equal to 0.

NB: The correlation coefficient 0.984 is considered a very strong linear relationship. The accuracy of the results was considered acceptable. It demonstrates however that a tilted tube has a strong influence on the optimal testing time.

Comparison between 30 and 60 minutes Westergren method

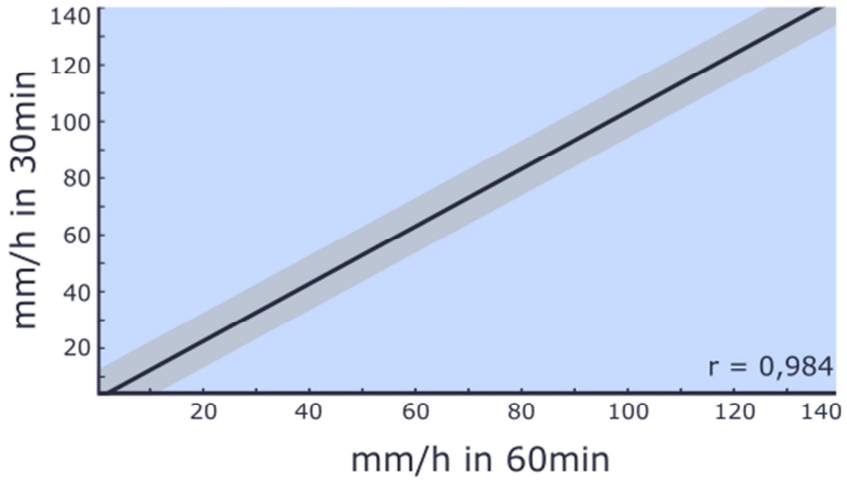


Fig. 5: The 30 minute ESR readings correlate highly with the corresponding 60 minute ESR readings over a wide range of blood samples (correlation coefficient = 0.984).

Aggregation versus sedimentation

Alifax Test-1 and Alcor iSED are ESR analyzers that produces ESR reading results within 20 seconds after sampling. Erythrocyte sedimentation is influenced by aggregation properties as well as plasma viscosity and hematocrit volume. It takes approximately 10 minutes before sedimentation starts at a constant rate. This means that the Test-1 and the iSED analyzers don't actually measure sedimentation, but rather calculates a mathematically derived ESR based on aggregate

measurements in the first, rouleaux forming stage only. Thus, these test results need to be manipulated to an ESR value according to the Westergren method in order to be clinically useful.

The Diesse VES-Matic Cube line of instruments is like Westergren a sedimentation based test. To run VES-Matic test it uses the original EDTA tube that was used for drawing the blood from the patient. No sample is taken from the tube and nothing is added. Not consuming any sample seems very attractive but has some serious drawbacks.

1. In order not to lose accuracy a relatively full sample tube is required. This puts some constraints on the testing order and logistics in the lab. Also the tube is occupied for at least 20 minutes before any other hematology test can be done.

2. A more serious drawback is that the sample is not diluted, nor is the result adjusted for the viscosity of the sample. The hematocrit level will have a significant influence on the measured sedimentation.

The hematocrit level of a sample will, among others, even vary with the individuals hydration level. Not adjusting for a variation in hematocrit (as is prescribed in Westergren) will make it impossible to truly compare the readings from the VES-Matic instruments with the ESR measures that are in accordance with the international standard as referenced by the ICSH.

Comparison of Westergren with other methods (X)

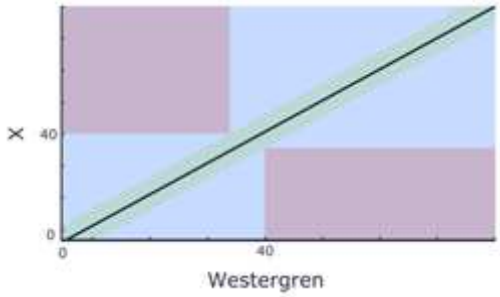
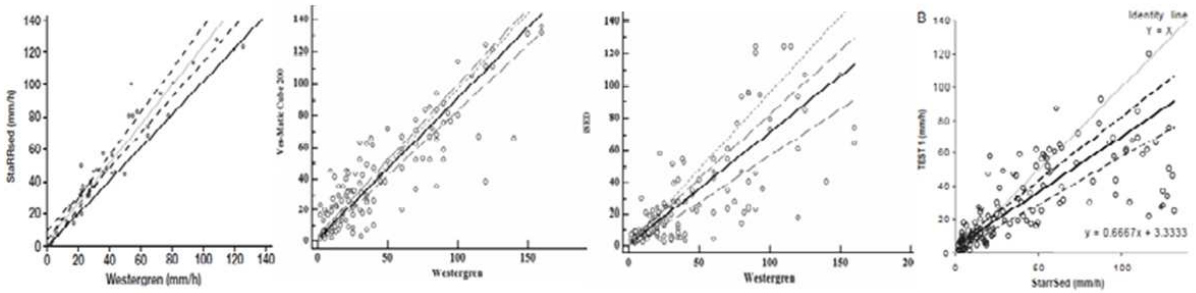


Fig. 6: Results reported into the upper left quadrant are considered normal according to the Westergren gold standard, but high according to method X. These so called "false positives" will lead to additional costs for supplementary testing or unnecessary treatment. Results reported into the lower right quadrant are considered high according to the Westergren gold standard, but low according to method X. These so called "false negatives" may lead to missed diagnosis.

In addition, sedimentation characteristics of the second and third stage can be relevant for some diseases, e.g. multiple myeloma. Test-1 was not as sensitive to the presence of paraproteins as the Westergren method (Raijmakers, Kuijper, Bakkeren, & Vader, 2008) and could produce significantly different results, especially in the higher ESR readings (Hardeman, Levitus, Pelliccia, & Bouman, 2010). In one comparison it was found that in 11.5% of the samples, the differences in results could lead to either a missed diagnosis (false negative) or additional testing costs (false positive)

The figures below are Passing Bablok regression plots taken from three independent publications. By evaluating and connecting the dots of three publications it is possible to compare the Test1 and iSED with the Starrsed and the gold standard Westergren. It again articulates the quality of the original

Westergren method and the Westergren related methods in determining ESR. The Test1, the iSED and the VES-Matic demonstrated clear flaws compared to the original Westergren and the Starrsed, leading to an important number of false negatives.



Regression Plots: comparing methods

Fig. 7: Higher than 40 on the Westergren scale and lower than 40 on the compared instrument scale are potentially missed diagnoses.

Recent ESR instrument evaluation articles

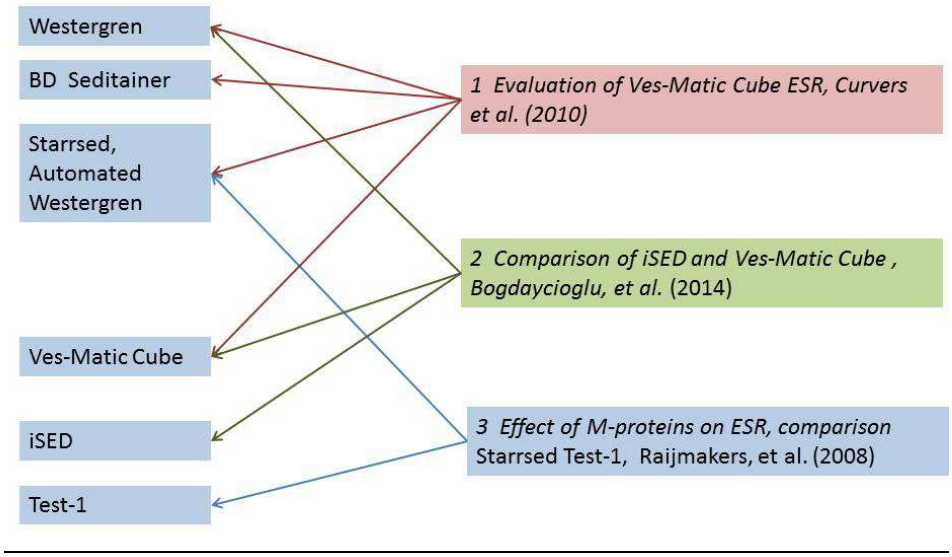


Fig. 8: Different publications evaluating different methods.

Quality equipment ensures reliable results and reduced cost of operation

The Starrsed ESR analyzers from RR Mechatronics are automated ESR analyzers that use the reference Westergren method as recommended by the ICSH and CLSI (2011). Starrsed analyzers perform fully automated ESR measurements in 30 or 60 minutes.

Pre-mixing, sampling and dilution of standard whole-blood EDTA samples in sodium citrate is fully automated, which ensures accuracy and frees up time for the operator, who only needs to load the samples into the analyzer. The analyzers contain a built-in barcode reader that automatically

identifies and registers the correct blood samples. Starrsed analyzers use a specifically designed needle for sampling that minimizes damage to the rubber stopper and ensures that blood vials can be sampled reliably multiple times.

Correct placement of a Starrsed analyzer guarantees a vertical position, a vibration-free environment and shielding from sunlight and drafts. The Starrsed analyzers use infrared light to read the ESR results and the optical reader is, in combination with built-in algorithms, even capable of detecting the relevant plasma-blood cell interface in hazy samples. The results are temperature corrected to 18.3°C and enable reliable clinical interpretation of the result.

Starrsed analyzers use standardized, reusable glass tubes that are specially made and tested. The tubes are cleaned using detergent and protease enzymes, rinsed and dried after each cycle, ensuring that the tubes are clean before use. This reduces waste and minimizes biohazard risks and the cost of operation.

The washing process of the Westergren tubes

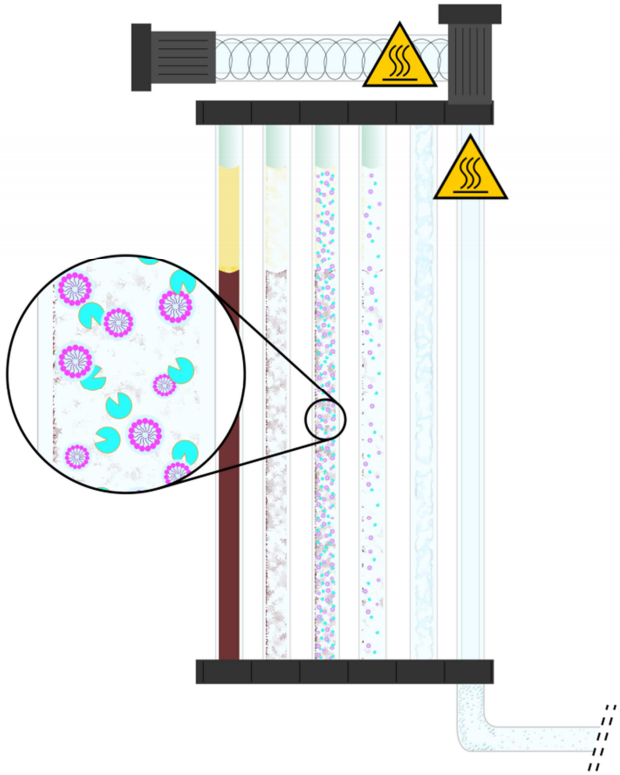


Fig. 9: The tubes are cleaned using detergent and protease enzymes. The inside of the tube is dried and disinfected by air that has passed through a heating element.

ESR in clinical analysis

Normal reference values for the Westergren ESR method are ≤15 mm for men and ≤20 mmr for women. The ESR increases slightly with age with the highest values found in 65-74 years of age. Reference values should be established locally and it is recommended to establish a reference value for each decade of adult life. The probability for disease becomes significant when ESR >50 mm.

Reference values Westergren ESR method

	Newborn	Neonatal to puberty	20y	55y	90y
Men - 5% exceed	0 -2	3 - 13*	12	14	19
Women - 5% exceed			18	21	23

Table 1. ESR (mm/h) reference ranges for various ages (years)
*Some laboraties use an upper limit of 20 mm

Mean and upper limits of Normal (CLSI. 2011)

ESR			Upper Limit of Normal	
Age (years)	mean Male	mean Female	Male	Female
18-30	3,1	5,1	< 7,1	< 10,7
31-40	3,4	5,6	< 7,8	< 11,0
41-50	4,6	6,2	< 10,6	< 13,2
51-60	5,6	9,4	< 12,2	< 18,6
61-70	5,6	9,4	< 12,7	< 20,2
over 70	5,6	10,1	< 30	< 35

Physiological and clinical factors that increase ESR

ESR values are higher for women than for men and increase progressively with age. Pregnancy also increases ESR.

During acute phase reactions, macromolecular plasma proteins, particularly fibrinogen, are produced that decrease the negative charges between erythrocytes and thereby encourage rouleaux formation.

Paraproteins are positively charged molecules that are abundantly present in multiple myeloma and Waldenström's macroglobulinemia patients. Like fibrinogen, paraproteins decrease the negative charges between erythrocytes and increase rouleaux formation. As described earlier the aggregation based ESR tests (Test-1 and iSED) typically miss detecting these disorders, see paragraph above on "Aggregation versus Sedimentation". (Raijmakers, Kuijper, Bakkeren, & Vader, 2008)

High protein concentrations increase plasma viscosity, which slows down the fall rate and thus ESR. However, the effects of fibrinogen and paraproteins on the negative charges between erythrocytes and rouleaux formation far outweigh the effect of increased plasma viscosity, resulting in a strong net increase of ESR.

In anemia, erythrocyte numbers are reduced, which increases rouleaux formation. In addition, the reduced hematocrit affects the velocity of the upward plasma current so that erythrocyte aggregates fall faster.