SEED Haematology

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Automated haematology sample interferences, flagging and results interpretation - Part 1

The purpose of this newsletter is to provide an overview of pre-analytical variables and other possible interferences during full blood count and differential count analysis, resultant flagging of results, interpretation and possible solutions. This is part 1 of a review of this topic and will focus on RBC and haemoglobin measurement interferences.

Key words:
HB, RBC, interference.

Introduction and background.

Haematological investigations concern themselves with analysis of living cells. This alone already indicates that the blood samples for haematological testing must be in as "lifelike" a state as possible to accurately represent the patient's physiological or health status at the time of collection. Apart from the difficulty in trying to preserve the cells until analysis, there is the challenge of accurately analysing, measuring and counting the living cells and blood constituents like haemoglobin whilst suspended in whole blood. Methods introduced to improve sample stability such anticoagulants may also distort the results.

While haematological laboratory testing technologies have advanced greatly to deal with these challenges over the years analytical limitations still exist. For accuracy and reliability of haematological results a measurement system has to be specific. This means only the analyte or parameter of interest should form part of the results. Any other detectable substance that affects the accuracy of the results constitutes so-called interference. An attempt must always be made to not only minimise or avoid possible interferences, but to be aware of or detect them when they exist in order to correctly interpret the results.

What is interference?

When substances other than the measurement parameter are included or affect the final result, this is obviously undesirable as the results are erroneous and may not represent the true biological status of the patient. This is referred to as interference. The purpose of this discussion is to explain the possible causes of interference, share possible laboratory corrective solutions and to explain the possible implications for clinical diagnosis.

What are the possible causes of analytical interferences?

Erroneous results may be due to a number of influences which may include but are not limited to the variables described below. These can be broadly divided into pre-analytical and analytical factors.

Pre-analytical influences.

From the above discussion, it is already clear that it is possible to compromise sample quality, and therefore the results, even before processing. Acceptable sample collection technique, tube filling, choice of anticoagulant and sample storage before processing should all be strictly observed to ensure quality of results.

1. *Phlebotomy technique*

   Care must be taken to avoid prolonged application of the tourniquet as this may result in haemoconcentration and false high cell counts.

2. *Choice of anticoagulant*

   The EDTA anticoagulant is the most appropriate anticoagulant for full blood count (FBC) analysis. Both formulations of EDTA, K_2 and K_3 are acceptable but K_2EDTA is currently preferred as it is more soluble and thus causes less cell swelling in older samples. However it is known that EDTA may cause platelet clumping and leuco-agglutination. Therefore should platelet clumping be detected during automated analyser processing, the sample must be checked microscopically to confirm if there is frank thrombocytopenia. Samples with platelet clumping can be recollected in trisodium citrate or heparin and retested. Doing so commonly remedies the platelet clumping although this may not always be the case. Please note that only the platelet count should be used from the citrated blood sample analysis.

3. *Tube filling*

   It is important that tubes are adequately filled in order to ensure the correct ratio between anticoagulant and blood. Under-filling may cause cell shrinkage and degeneration with falsely low haematocrit (HCT) and elevated mean cell haemoglobin concentration (MCHC). Furthermore, the tubes must be adequately mixed to ensure that the sample does not clot which would lead to erroneous results. As a general rule of thumb, cellular changes only become noticeable if the fill volume is less than 2ml.

4. *Sample handling prior to analysis*

   Storage conditions have significant impact on the nature of results obtained from automated analysers. As indicated above, cellular changes may have qualitative and quantitative effects. Automated analyser principles rely on examination of structural properties of cells for cell count, volume measurement and morphological differentiation.

   As samples age, the cells start to swell. When cells swell excessively they may fragment into smaller particles. This may happen to both RBCs and platelets resulting in low mean cell volume (MCV) and mean platelet volume (MPV) respectively. Furthermore platelet fragmentation may result in a false high platelet count. The speed at which morphological changes occur with delay in analysis is compounded if the sample tube is additionally under-filled.

   Poor phlebotomy technique may cause haemolysis of red blood cells (RBC) within the collection tube and give rise to erroneous results.

   a) Qualitative changes.

   Due to possible combined effect of sample aging and anticoagulants, peripheral smears should always be made from samples that are as fresh as possible, preferably less than 1 hour after collection. This is because EDTA induced morphological changes start to become apparent within 3 hours and become increasingly severe beyond 18 hours when stored at room temperature. Sample storage at 2-8°C does slow down the changes but does not eliminate them. As indicated earlier sample tube under-filling will speed up these changes.

   - **White blood cell morphology:** the expected changes include loss of nuclear detail while the cytoplasm becomes more vacuolated with blurred edges. Neutrophil structural changes are most profound, with nuclei becoming separated and shrunken. Monocytes show prominent vacuolation which should not be misinterpreted as active phagocytosis. Lymphocyte changes include nuclear budding into 2 to 3 lobes which may be confused with apoptosis.
   - **Red blood cell morphology:** red cells are generally better preserved with significant changes only setting in at about 6 hours from time of collection. The changes are progressive and usually include cellular crenation where the cell outline appears spiked (should not be confused with renal disease) and sphering (must be distinguished from genuine spherocytes from burns and haemolysis).

   b) Quantitative changes.

   Here it also follows that the changes that occur become more significant with time and are compounded by high ambient temperatures. So if care is taken to keep

sample at temperatures within 2-8°C most parameters will be stable up to 24 hours.

Parameter specific changes are indicated below:

- **MCV and HCT:** As cells start to and progressively swell with time, the MCV and HCT will increase. HCT will be falsely low if the sample is haemolysed during collection.
- **White blood cells and platelets:** White blood cell (WBC) and platelet (PLT) count rapidly decline as the EDTA and ageing related changes take effect. For the white blood cell count the morphological changes previously described will also affect automated differential count. Please note that the extent or severity of the resulting inaccuracy will also depend on the analyser technology used. Analysers using standard optical technologies, for example, are more affected by volume related white cell changes than those using fluorescence flowcytometry involving DNA staining dyes. This is because DNA remains more stable and intact over a longer time while other structural properties, like volume and nuclear changes appear earlier. The Sysmex fluorescence flow cytometry principle used on the X-Class and XN series analysers allows for reliable automated differential count up to 48 hours post collection (provided the samples are kept at 2-8°C).
- **Reticulocyte counts** will start to drop after 6 hours
- **Nucleated red blood cells (NRBCs)** will disintegrate at room temperature after 1 to 2 days.
- **Haemoglobin (HB)** is the most stable parameter and will still be stable for up to 2 days, but please note that risk of RBC lysis will becomes higher with time and higher temperatures. Haemolysis will result in possible drop in HCT.

Analytical influences

Apart from morphological changes that will normally manifest on smear examination, some changes will be detected by or affect automated analysis. Here it should be stated that it is the combination of the analyser's inherent limitations together with other interfering blood constituents which may provide added challenges in terms of the accuracy and reliability of results. The analyser operator must therefore be sufficiently aware of their analyser's operating capabilities in order to deal with possible aberrant results emanating from these interferences.

While manufacturers may use different measurement principles for particular parameters, problem samples pose similar challenges to most. Where possible interferences are detected the analyser must be able to alert the operator and provide as much information as possible about the nature of the interference and the affected parameters. Indeed some analysers like the modern Sysmex XT, XE and now the more technically advanced XN series go even further to even suggest possible solutions and identify possible pathologies.

This goes a long way to improve the speed of diagnosis and to minimise the risk of missing serious pathologies

What are the specific interferences and related flags to look out for?

Most 3 and 5 part differential count analysers use the impedance method whereby cells are counted using volume as the main discriminator.

White blood cells (WBC) are included when red blood cell (RBC) and platelet (PLT) count measurements are performed. This normally does not pose a problem as the WBC, in relative terms, is too low to make an impact on the overall RBC or PLT count, although there are exceptions (see below).

On Sysmex analysers the flags are divided into "abnormal" and "suspect" flags. Abnormal flags are based on laboratory's own patient reference ranges and can thus be changed to be specific for that particular laboratory. These include count related flags like *anisocytosis, leukocytosis, thrombocytopenia, anaemia, neutrophilia, monocytosis* and others. On the other hand the suspect flags such as *PLT clumps?, RBC Agglutination?, HGB interference?, HGB defect?* and *Iron deficiency?* are based on arithmetic algorithms that cannot be changed by the user.

Red blood cell related flags and interferences

In case of 3 part diff and 5 part diff analysers RBC and PLT counting relies on size measurement and as such any particles detected beyond the discriminators, marked LD and UD in figure 1, will cause the instrument to alarm, indicating the possiblity of unreliable results.

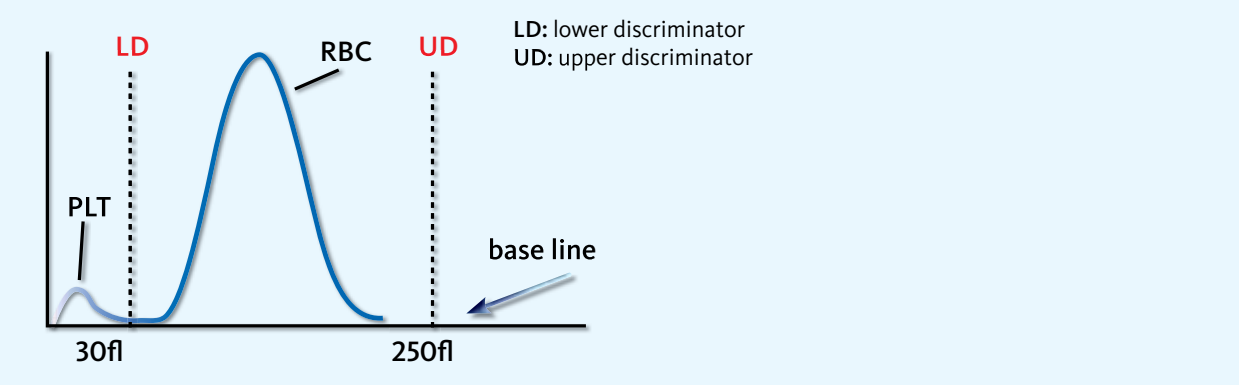

Fig. 1 *PLT and RBC histograms showing the size discriminators used to identify each population.*

Interference may affect either the lower (LD) or the upper (UD) discriminators and the appropriate alarm message will appear beside the relevant result (see figure 2).
In the Sysmex 3 part diff analysers the following flag messages may appear during red cell measurement:

- RL – abnormal height at lower discriminator
- RU – abnormal height at upper discriminator
- MP – multiple peaks
- DW – distribution width

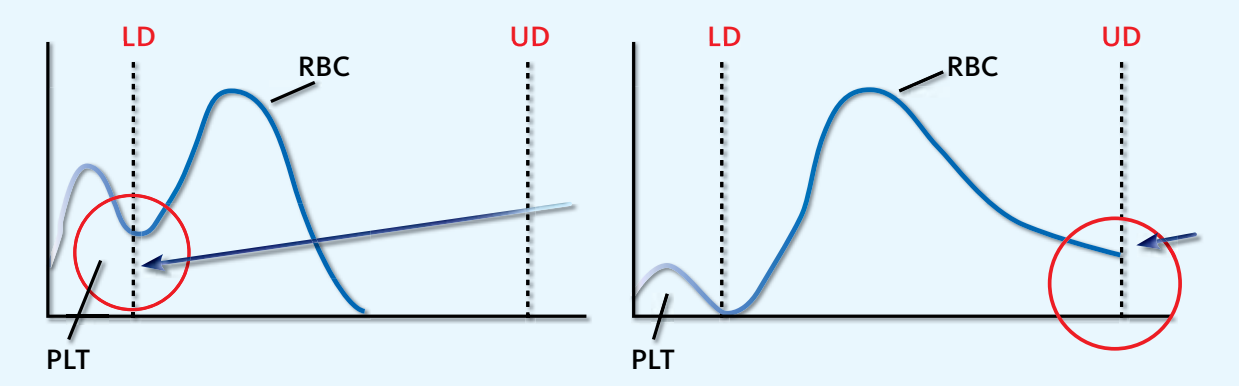

Fig. 2 *RBC histograms showing an abnormality at the lower and upper RBC discriminators respectively.*

Possible causes of interferences at the lower discriminator of the RBC histogram with recommended actions are shown in Table 1. In such cases, an RL flag will appear.

Table 1 *Possible causes of interferences at the RBC histogram lower discriminator which may give rise to false low or high RBC and/or platelet counts*

Cause	Recommended action
Giant platelets	Confirm on peripheral smear review
Microerythrocytes	Smear review to assess severity. Extreme microcytosis may point to a possible underlying inherited condition. Iron deficiency must be excluded.
RBC fragments or dysplastic RBC	Slide review recommended. Fragments may provide early warning for a possible haemolytic episode.
Platelet clumps	Smear review to assess degree of clumping and to provide an estimate of correct platelet count. Suggest recollection in trisodium citrate or heparin and repeat analysis. Manual chamber platelet count may be required.

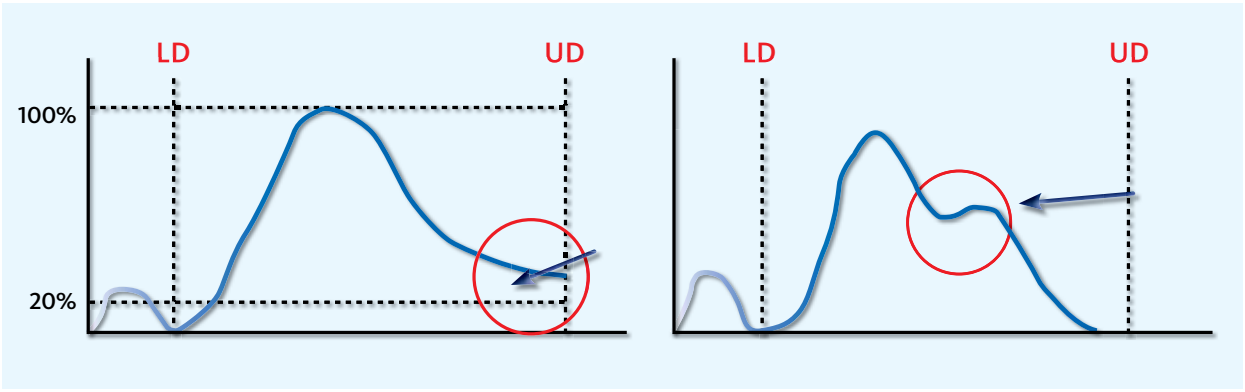

Fig. 3 *RBC histograms depicting a double peak and violation of the upper discriminator respectively.*

In addition to the RL and RU flags just indicated the flags DW and MP may occur.

The DW flags (see right hand histogram in figure 3) will be generated when the RL or RU do not go below the 20% mark above the base. In this case the RDW-SD and RDW-CV will not be displayed. The clinical background would be helpful in determining the cause of action to take, if any. The MP flag will occur where there are double peaks on the RBC histogram.

Possible causes of DW and MP flags are shown in tables 2 and 3 respectively.

Table 2 *Possible causes of interferences with the red cell distribution width measurements*

Cause	Recommended action
Cold agglutinins (RBC clumping)	Warm sample to 37°C and rerun.
Rouleaux formation (seldom)	Confirm on smear
Extreme anisocytosis (very high RDW)	Slide review

Table 3 *Possible causes of multiple peaks within the RBC histogram*

Cause	Recommended action
Iron deficiency currently under iron replacement therapy. The same pattern can occur if an iron deficient patient receives a blood transfusion.	The first population is generated by the microcytic iron deficient cells and the second by the more recently produced cells with adequate iron content (normocytic). In the case of blood transfusion the second peak would represent the transfused cells.
Vitamin B12/folate deficiency currently under treatment. The same pattern can occur if a Vitamin B12/folate deficient patient receives a blood transfusion.	The first peak is generated by the more recently produced healthy cells (normocytic). The second peak represents the macrocytic cells produced during the nutritional deficiency. In the case of blood transfusion the first peak would represent the transfused cells.
Extreme leukocytosis (WBC>600 X 10^3/µL) (e.g. Chronic lymphocytic leukaemia)	In this case the second peak is often smaller, but is important to always view the results in total to see if there is any correlation between possible high white cell count and the flag. It is advisable to review the slide unless it is a known patient.

If more than one peak is detected in the RBC histogram, the RBC, RDW-CV, RDW-SD, MCV will be flagged. If a dimorphic red cell population is the cause of the additional peak, the RBC value will be accurate. Extreme leukocytosis may result in incorrect RBC count. In that case results must be treated with caution.

On the Sysmex X Class range of analysers, the same interferences may occur but the instruments often give the RBC or PLT Abnormal distribution flags or both. The advantage here is that in the service screen there is provision of more detail about the RBCs. For both the RBC and PLT additional specific flags may be generated depending on the source of interference (see figure 4).

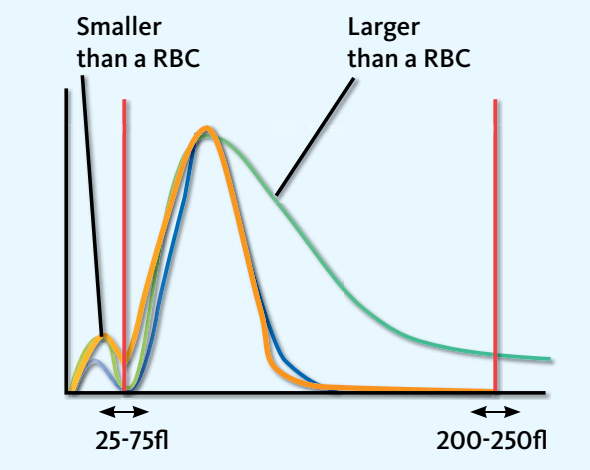

Fig. 4 *Histogram illustrations showing the effect of interferences and resulting abnormal histogram distributions for red cells. (blue curve = RBC normal distribution, orange curve = RBC curve showing interference at the low discriminator, green curve = RBC curve showing presence of interference at the upper discriminator)*

Some specific examples of interferences and suggested corrective measures

a) RBC histogram double peak due to extreme leukocytosis.

The presence of a second peak in the RBC histogram is commonly observed in association with extreme leukocytosis. The WBC count is generally 600 x 10^9/L or higher in order to be evident as a second population in the RBC histogram. Such high WBC counts generally only occur in the chronic leukaemias, with interference in the RBC histogram generally being limited to chronic lymphocytic leukaemia, where the cells are generally uniformly small in size in contrast to a much wider size of white blood cells in chronic myelogenous leukaemia (CML). The origin of the second peak can be confirmed to be due to leukocytosis by noting the high WBC count and by reviewing the differential count where an extremely high lymphocyte count will be observed. Please note that in addition to RBC interferences, extremely elevated WBC may also result in more interferences that may affect other parameters. Examples include HGB turbidity and WBC Abnormal scattergram flags, which may point to possible HB measurement or differential count discrepancies. These will be discussed in detail in respective sections or in subsequent SEED articles.

So it is important to examine all the results, including the differential count and related flags. In order to obtain the correct RBC count in the case of interference by extreme leukocytosis, the sample can be diluted with an appropriate diluent (normal saline or the analyser specific diluent e.g. CellPack® for Sysmex analysers). This diluted sample must then be reanalysed and the RBC value obtained multiplied by the dilution factor. On Sysmex 5 part differential analysers, the correct RBC count can be obtained from the service screen of the original analysis (please refer to the relevant instrument user manual).

b) Incorrect RBC values due to red blood cell agglutination.
RBC agglutination is caused by immune antibodies that bind to the membranes of adjacent RBCs resulting in large aggregates. Both warm and cold antibodies may be responsible, although cold antibodies are more commonly involved. Red blood cell agglutination may result in increased MCV and MCHC. In addition, RBC agglutination may also lead to falsely increased WBC in 3 part differential analysers. This is because the red cell agglutinates are more resistant to the lysing reagent and any unlysed aggregates may be counted as WBC instead of RBC. This is because in impedance RBC counting, agglutinated RBCs stick together and form large cell clumps which pass through the counting aperture as large single units. This phenomenon may give rise to erroneous results as shown in table 4.

Table 4 *Interference caused by presence of RBC agglutination*

Abnormal RBC histogram curve – very wide RDW
Falsely low RBC count and HCT
Falsely high MCV and MCHC (>37g/dl)
Falsely low HB (If lysing insufficient)
Falsely high WBC count

c) Interference with haemoglobin measurement
The spectrophotometric principle is used for haemoglobin measurement. A lysing agent is used to destroy RBC membranes thereby releasing Hb from the cells. The Hb concentration is determined from the intensity of the colour of the diluent in which the Hb is freely suspended. Consequently any substance (other than Hb) that may interfere with or contribute to light absorption may give rise to a false Hb value. Analysers have various built in mechanisms to alert the user to the possible interference. In the case of Sysmex analysers a HGB turbidity? Flag will be displayed.

Possible interferences are shown in table 5.

Table 5 *Causes of possible erroneous Hb measurement*

Haemolysis
RBC agglutination
Lipids
Abnormally high WBC count

An MCHC of > 36.5 g/dl will automatically trigger the HGB turbidity? flag. The reason for this is that MCHC is generally a stable parameter with few clinical causes for high values, hence when deviations occur analytical errors generally need to be excluded. MCHC is a calculated parameter (MCHC=Hb/HCT) hence it can be elevated if HCT is falsely low or Hb is falsely high.

The presence of haemolyis may cause a false high MCHC for two reasons:

- *In vivo haemolysis*
  Here the patient's RBCs are breaking up and releasing free Hb into the blood. Consequently the Hb measurement will include the plasma Hb plus what was released from the intact RBCs during analysis. The HCT (a measure of the intact red cell mass) will be low relative to the measured Hb. This combination may give rise to an elevated MCHC as the free Hb has been included erroneously.

- *Ex vivo haemolysis*

  This is much more common and generally occurs due to poor phlebotomy technique or old samples. Here red blood cells breakdown inside the collection tube. In this case the measured Hb is correct (as the RBCs were intact at the time of sample collection) but the HCT will be low as RBCs that have disintegrated in the tube will not be counted. Again the proportion of Hb to HCT will be wrong potentially giving rise to false high MCHC. Haemolysed samples, if due to aging or traumatic venipuncture should be recollected.

  RBC agglutination may also give rise to an elevated MCHC due to a false low HCT (as previously described). If the RBCs are tightly clustered, the lysing agent may not penetrate adequately and hence may give rise to a falsely low Hb due to incomplete lysis of the RBCs within the sample. Post incubation at 37°C, this should be corrected.

  In both lipaemia and extreme leukocytosis (>100 X 10^3/µL) the Hb result may be falsely increased.

- *Lipaemia*

  In the case of lipaemia, a plasma replacement procedure is usually used to obtain a corrected haemoglobin measurement. This involves centrifugation of the sample and replacing the supernatant plasma with a suitable saline solution (in the case of Sysmex analysers the standard diluent CellPack® should be used). Care must be taken not to remove the white cells and platelets. It is advisable that only the HB should be reported from this repeat analysis as the spinning process often results in discrepancies of other parameters. Please note that all the calculated indices involving the HB result may no longer correlate. The doctor responsible for the patient should always be consulted when such cases are encountered.

- *Leukocytosis*

  In the case of extremely high WBC counts diluting the sample may help improve the results. The Hb correction is often small, but may be important in cases of severe anaemia.

  All corrective steps undertaken, where appropriate, must be indicated in the report if they have potential impact in clinical interpretation and therefore the clinical intervention.

Take home message

- Whist automated full blood count analysis has greatly advanced, limitations still exist and these should be taken into account during sample processing and reviewing of results.
- Proper care must be taken when collecting, transporting and storing samples to avoid introducing variables that may result in incorrect results.
- During results review, all results must be thoroughly checked, including numerical values, flags, histograms and scattergrams.
- Where interferences have been identified, relevant corrective steps, according to the laboratory's protocols must be followed.

References:

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