Changes in red blood cell hemoglobinization during pregnancy

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Introduction
Decreased hemoglobin concentration (Hb) is a common feature in the third trimester of pregnancy, partly due to physiologic hemodilution. As the degree of hemodilution display considerable inter/individual variation, Hb concentrations show similar variation. Therefore it is complicated to establish reliable cut-off limits for anemia. Several diagnostic guidelines are used in obstetric practice. Koninklijke Nederlandse Organisatie voor Verloskundigen (KNOV) and World Health Organization (WHO) practise Hb values of 6.3 and 6.8 mmol/l respectively for anemia discrimination and providing an indication for subsequent iron supplementation (1-4).

The aim of the study was to gain insight into the additional value of advanced red blood cell parameters, particularly immature reticulocyte count (IRF) and reticulocyte hemoglobin content (Ret-He) to establish deviations in hemoglobinization (5) and appropriate Hb discrimination levels.

Ret-He reflects a ‘short term’ indication concerning the status of reticulocytes hemoglobinization. In contrast zinc protoporphyrin (ZPP) reflects a ‘long term’ impression corresponding with the lifespan of red blood cells (RBCs) (6).

An appropriate biomarker for detection of iron deficient erythropoiesis (IDE) is zinc protoporphyrin. The parameter reflects the extent to which zinc rather than iron, has been chelated with protoporphyrin. This process functions as a homeostatic mechanism by inhibiting excretion of iron following hemolysis macrophages. Thus, it is a highly sensitive functional indicator of IDE (7).

Regarding clinical interpretation Ret-He is similar to ZPP. It is a cellular measure of IDE that on its own does not distinguish between functional and true iron deficiency. In contrast to ZPP in RBCs, Ret-He is measured in reticulocytes. Therefore it is considered to be a more sensitive marker for short term changes in Hb-production (6).

Pregnancy is associated with a physiological increase in inflammatory biomarkers, especially during the 1st and 3rd trimester. Serum ferritin will be elevated during inflammation because of its role as an acute-phase reactant, and therefore may overestimate body iron stores (7).

It should be stressed that functional and true IDE are not mutually exclusive processes and may co-exist, especially during late pregnancy when low-level inflammation with depleted iron stores is likely to occur (7).

Concerning ZPP/heme ratio results in our study we apply an opinion based cut-off level of >75µmol/mol heme as an indication for iron deficient erythropoiesis.

Subjects, Materials and Methods
Blood samples (K<sub>2</sub>EDTA, Becton Dickinson, Plymouth UK) were selected from 114 pregnant women in the third trimester within a Hb range suspicious for anemia in pregnancy (Hb ≤7.0 mmol/l, MCV 80-100 fL). Apparently healthy women (n=35) were selected as reference subjects’ group. Hb, IRF and Ret-He were determined within 4 hours after sample collection on a Sysmex XE2100 hematology analyzer with additional dedicated software (Sysmex Corporation, Kobe, Japan).

The methodology of IRF and Ret-He measurements is based on application of automated fluorescent flow cytometry utilizing a polymethine dye for binding cytoplasmic RNA in reticulocytes. The mean forward light scatter intensity in the reticulocyte channel is estimated as a measure for volume and Hb content of red blood cells and reticulocytes. Several algorithms are applied in order to transform original data into IRF and RET-He dedicated results. Measurements of RBC Zinc protoporphyrin heme ratio (ZPP) were performed on a haematofluorometer (AVIV Biochemical Inc., Lakewood, USA) using front surface illumination fluorometry.

Statistical evaluation
The statistical software package SPSS/PC, version 14.0 for Windows, was applied for statistical analysis of results (SPSS, Chicago, IL). Independent Samples T-test was performed in order to detect statistically significant deviations between the groups of subjects.

Results
Discrimination concerning anemia in pregnancy based on application of the KNOV guideline (Hb <6.3 mmol/l) resulted in 21% of subjects with decreased Hb concentrations and in case of application of the WHO guideline (Hb <6.8 mmol/l) even in 69%. Hb values in 48% of the subjects were in the debatable range of 6.3-6.8 mmol/l; in 33% of these subjects poor RBC-hemoglobinization occurred (Ret-He <1850 amol).

Establishment of ZPP/heme ratio revealed increased
results (>75 µmol/mol heme) in 45% of the subjects in the inconclusive Hb concentration range (Hb 6.3-6.8 mmol/l) (figure 1). IRF counts demonstrated increased results (0.011 ± 0.007 x 10¹²/L) if compared to the reference group (0.003 ± 0.002 x 10¹²/L, p = <0.001). Hemoglobinization parameters Ret-He, RBC-He and Ret-He/RBC-He ratio showed significantly decreased results if compared to the reference group (mean ± standard deviation: 1921 ± 240 amol (p = <0.001), 1882 ± 129 amol (p = <0.001) and 1.01 ± 0.05 (p = <0.001) respectively).

With respect to clinical interpretation of results in the indicated grey area (Hb 6.3-6.8 mmol/l) 4 groups are considered (figure 2):

1. ZPP <75 µmol/mol heme, Ret-He > 1850 amol (n = 24, blue).
   Interpretation: normal hemoglobinization (low Hb due to haemodilution).

2. ZPP <75 µmol/mol heme, Ret-He < 1850 amol (n = 1, blue).
   Interpretation: ineffective hemoglobinization? Follow-up after 2-4 weeks is advised.

3. ZPP > 75 µmol/mol heme, Ret-He < 1850 amol (n = 17, red).
   Interpretation: ineffective hemoglobinization.

4. ZPP > 75 µmol/mol heme, Ret-He > 1850 amol (n = 13, red).
   Interpretation: ineffective hemoglobinization due to iron suppletion or increased erythropoiesis.

   It is recommended to check Ret-He/RBC-He ratio in advance. Ret-He/RBC-He ratio > 1.05 is indicative for increased erythropoiesis.

**Discussion and conclusion**

Anaemia is the most common haematological problem in pregnancy (8). What is referred to as the physiologic anaemia of pregnancy is a dilution process secondary to an increase in plasma volume. During pregnancy the demand for micronutrients, especially iron and folate, is increased and maternal body stores and dietary intake may be insufficient for adequate erythropoiesis. Appropriate assessment is complicated by disproportionate expansion of plasma volume compared with RBC mass (2, 3, 7).

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Figure 1. (A) Scatterplot representing Hb and ZPP concentrations. (B) Scatterplot representing Hb and RET-He content. Research group = red; Reference group = green; x-axis: (left, black) KNOV diagnostic guideline, (right, grey) WHO diagnostic guideline; y-axis: (grey) upper (A) or lower (B) level of reference value; (black) discrimination level for absolute iron deficiency.

Figure 2. Scatterplot representing Hb and Ret-He content. ZPP > 75 µmol/mol heme = red; ZPP ≤ µmol/mol heme = blue; x-axis: (left, black) KNOV diagnostic guideline, (right, grey) WHO diagnostic guideline; y-axis: (black) discrimination level for impaired hemoglobinization.
Anaemia screening during pregnancy only based on Hb measurements is inappropriate and inconclusive in many subjects. A decreased Ret-He result is considered to be indicative for insufficient RBC haemoglobinization. Increased IRF counts are indicative for increased erythropoiesis during the third trimester of pregnancy (9). Increased IRF results combined with increased ZPP and decreased Ret-He results are indicative for functional iron deficiency. With establishment of ZPP iron deficient erythropoiesis can be detected with reasonable accuracy. However, ZPP cannot discriminate between true and functional iron deficiency. The latter phenomenon occurs when body iron stores are adequate but iron is not available to the bone marrow, such as may happen in case of infection and inflammation. Many pregnant women demonstrate inconclusive Hb values between KNOV and WHO discriminant values (figures: grey area). In the grey area, Hb is an unreliable indicator for haemoglobinization. In pregnant women, MCV is a poor marker for detection of iron deficiency for at least two reasons. Firstly, the physiological increase in MCV during gestation will counterbalance the microcytosis resulting from iron deficiency in an early stage. Secondly, RBCs have a mean survival of approximately 120 days. Consequently, it takes a large number of RBCs with a small volume to reveal a decreased MCV value. Therefore, MCV reduction can be observed only in late pregnancy when the RBC population has been partially replaced by young RBCs (8). The opinion is favoured that iron deficiency anaemia during pregnancy is an unphysiologic event. That is the reason why discriminatory values for cut-off levels should be derived from an iron treated, iron depleted population (8).

Ret-He is considered to yield a useful tool for diagnostic screening and follow-up of iron availability during the second half of pregnancy. It is recommended to evaluate ZPP and Ret-He in addition.

References