Establish Reference Intervals of Complete Blood Count for Twin Pregnancy

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Abstract

Background: Twin pregnancy is of high risk, which increased in recent years. Establishment of reference intervals of complete blood count (CBC) for twin-pregnant women during pregnancy might help for the properly prognosis of adverse outcomes in twin pregnancy.

Methods: We screened out 253 cases of twin pregnancy reference cohort from 1153 twin pregnancy after the complications and adverse pregnancy outcomes were excluded. Complete blood count data were collected during the mid- and late-term of pregnancy and analyzed by SPSS to establish the reference intervals of peripheral blood of twin pregnancy.

Results: The RBC, HGB, HCT, and PLT were lower in twin pregnant women than those in healthy nulligravida women during gestation, while the levels of WBC, NEU, and NEU% increased, especially in the mid-term. The reference intervals of late-term pregnancy validated by using 20 samples of twin pregnancy, then utilized for figuring out the distinctive characteristics of CBC with preterm birth (PTB) pregnancy. Absolutes of WBC and NEU increased in PTB pregnancy using our established reference intervals, implying they might be prognosis indicators of this adverse outcome.

Conclusion: Establishing the reference interval of blood cell-related indicators of normal twin pregnancy is helpful for the monitoring and prognosis of PTB.

1. Introduction

In China, as the one-child policy had been gradually relaxed during recent decades(1), there has been an increase in multiple pregnancies, which is mainly related to progress in the treatment of infertility and assisted reproductive technologies (ART)(2, 3). Multiple pregnancy is associated with adverse maternal outcomes, including increased rates of pre-eclampsia, pregnancy-induced hypertension, maternal anemia, and venous thrombosis(4, 5). Twin pregnancy is determined as one of the top ten risk factors causing stillbirth, the number of which in China in 2015 remained high and ranked in the top fifth of the world(6).

The blood routine test has essential diagnostic value in disease judging at the clinic, such as anemia and infection. However, without correct reference intervals, there is both an increased risk of missing significant changes due to pathological conditions and to erroneously interpret regular changes as a pathological event(7, 8). Although the physiological and biochemical changes in pregnancy, influence many of laboratory tests and the changes in normal laboratory values induced by pregnancy are well known(9, 10), few studies have been conducted to determine blood reference intervals for normal singleton pregnant women at present(11-13). The changes in blood routine parameters of peripheral blood in the single pregnancy are reported. While the red cell mass increases during pregnancy, the plasma volume increases more, resulting in relative anemia. This leads to dilution anemia with lower hemoglobin (HGB) level, hematocrit (HCT), and red blood cell (RBC) count(9, 10, 14). A stable higher upper reference limit of white blood cell (WBC) count during pregnancy had been reported either. WBC count is known to increase in pregnancy, thus limiting the use of this parameter as a marker for infection...
During pregnancy (10, 14). Using the normal unpregnant woman as the reference interval, which may lead to the diagnosis of a normal pregnancy with anemia or infection as having abnormal values and escalating medical costs, even unnecessary and potentially dangerous therapeutic actions.

Though it is known to have a high risk of adverse outcomes and increasing incidences, twin pregnancy has not been involved in the above studies. Therefore, it is essential to develop reference intervals for the normal twin pregnancies. As early in 1995, Blickstein had described the difference of hemoglobin levels between twin and singleton pregnancies (15). However, no comprehensive blood reference interval established yet for twin pregnancy without complications or adverse outcomes at present. Establishing accurate blood references for twin pregnancy can be extremely important for not only correct clinical decisions such as ablation placentae, appendicitis, premature rupture of membranes and preeclampsia, but also timely access to risk-appropriate obstetric and neonatal care which help reduce mortality and morbidity.

The correct interpretation of laboratory tests requires accurate reference intervals from an appropriate population. In this paper, we sought to develop laboratory blood reference intervals from 120 normal twin pregnancy Chinese women in mid-term and late-term in a single institution with virtually identical stuff, equipment, and methodology. We used those results to create reference intervals and tested our reference intervals by using 20 twin pregnancy women blood. After confirmed the correctness of the reference interval, we found that RBC, HGB, HCT, and PLT decreased in twin pregnancy, while WBC and NEU increased, implying that they might be prognosis indicators of some adverse outcomes.

2. Materials And Methods

2.1. Patients

In this retrospective section of this study, 1153 women with twin pregnancy ranging from 20 to 40 years old, admitted to the obstetric units between January 2015 and December 2018, were consecutively recruited and studied.

Maternal and neonatal outcomes considered in this study were as described by Sun. (16), with some modifications for these twin pregnant cases. A group whose delivery time was below 36 weeks of pregnancy or weight of newborns was less than 2000g are also considered as an adverse pregnancy outcome group, while the remaining are approximately considered with a good outcome. The intensity associated with the adverse pregnancy outcomes was analyzed by calculating the factors OR (Odd Ratio).

2.2. Statistical analysis

The complete blood count (CBC) data of the selected reference cohort were retrieved from Sysmex XN9000 analysis system, were then analyzed using Statistical Package for Social Sciences (SPSS)
version 23.0 software (IBM Corp., Armonk, NY, USA). Based on the CLSI guidelines recommendation of 120 reference subjects for establishing reference intervals, 158 twin pregnant women were enrolled. The CBC parameters of these pregnancies at each time point were entered into an Excel database and sorted in ascending order. Reference intervals were established using nonparametric analysis (17-20). The identification of possible outliers was used by the box plot. According to CLSI/NCCLS C28-A2, outliers were excluded from each group if the D/R ratio was over 1/3, where D is the absolute difference between an extreme value and the next largest value, and R is the range of all observations.

The normal distribution assumptions for CBC data were ascertained with the Kolmogorov–Smirnov test. For normal distribution data, \( x \pm 1.96s \) can be used to represent the data distribution range (21). For skewed distribution data, the percentile method is used, and P2.5 and P97.5 are used as the lower and upper limit of the reference interval, respectively. Thus, the reference interval of particular indexes (RBC, HGB, HCT, PLT, WBC, NEU, NEU%, LY, LY%) in mid and late-term pregnancy of twin pregnancy was obtained. The descriptive statistics are presented as mean and standard deviation (SD). These values were compared to well-established reference intervals (WS/T 405-2012) developed for the Chinese population. Differences with \( p<0.05 \) were considered significant.

### 2.3. Validation of established reference intervals

Twenty clinical samples with the normal outcome of gestation at random were processed for validation of the established reference intervals. The index of these 20 samples is compared with the establishing reference intervals that need to be verified. If no more than two measured value is out of the reference range, the reference intervals can be used directly. If three or more than three values are out of range, it is necessary to refilter the other 20 subjects of the population, reconduct the above comparison to see if validation passed. If there are still three or more than three measured values out of range, the reference interval should be reestablished (22).

### 2.4. Analysis CBC data of PTB and PPH group based on the established reference intervals

According to the delivery time and maternal blood loss, PTB group (live deliveries during 30~35 weeks of gestation) and PPH group (blood loss >600mL) were generated. The late-term CBC of these two groups were re-analyzed based on the established reference intervals to reveal some potential indicators of the adverse outcomes' predicting.

### 3. Results

#### 3.1. Clinic characteristics of twin pregnancy and definitions of exclusion criteria
The incidence of complications was analyzed in 1153 cases of twin pregnancy (Table 1). Twin pregnancy combined with diabetes, premature rupture of membranes, placental abruption, preeclampsia, anemia, hepatitis B carrying, and thyroid dysfunction were of higher incidence comparing to a singleton pregnancy, indicating that twin pregnancy was one of the high-risk factors in pregnancy. Diabetes mellitus was the most prevalence complication, accounting for 21.3%, while a group of premature rupture of membrane and anemia followed at 16.0%. The third rank was scarred uterus (10.8%).

All these women with twin pregnancy were delivered by planned cesarean. The outcomes were then grouped into the adverse ones and the better ones (the remaining). According to single-factor analysis, it was found that TTTs, hypertension, preeclampsia, selective fetal growth restriction, premature rupture of membranes and placental abruption, scar uterus, chorioamnionitis, and pelvic inflammation are the high-risk factors for the adverse outcome of twin pregnancy (Table 1). Chorioamnionitis shows the most significant positive correlation to adverse outcomes, whose OR value was 5.40. Premature rupture of membrane and pelvic inflammatory disease were also observed high positive correlation to adverse outcomes with OR values of 3.31 and 3.81, respectively. However, the OR value of anemia was 0.79, indicating that the diagnosis of anemia based on the existing reference intervals appeared to be a protective factor.

After screening the clinical figure of these pregnant women, 253 cases without following exclusion criteria were then selected as the reference cohort (Fig. 1).

**Exclusion criteria:**

(1) with Twin-to-twin transfusion syndrome (TTTs), preeclampsia, diabetes, hypertension, G6PD deficiency, hyperthyroidism, hepatitis B carriers, hyper-viscosity, anemia, intrahepatic cholestasis,

(2) fetal malformation, infection, proteinuria, uterine tumor/cancer, selective fetal growth restriction, chorioamnionitis, umbilical cord around the neck, premature rupture of fetal membrane, scar uterus, and amniotic fluid,

(3) time of delivery is under 36 weeks of gestation,

(4) the weight of either neonate is below 2000 grams,

(5) neonate with major congenital anomalies.

However, when looking up the complete blood count records of these 253 cases in LIS informatic system, only 158 records of them had that of 30-35w (late-term) and 98 records of 20-25w (mid-term) of gestation.
3.2. Clinical laboratory analyses of complete blood count of the reference cohort

The CBC data of reference cohort within mid-term and late-term pregnancy were retrieved from the Sysmex XN9000 analysis platform. It is found that only 98 and 158 cases could be tracked, respectively. The mean pregnancy duration of this group was 36.8 weeks, and the mean dates of the CBC data from specimens were 23 0/7w and 32 4/7w on average. The above parameters are all normal distribution through the analysis of the Kolmogorov–Smirnov test, and the mean values ± SD were shown in Table 2.

As the table indicated, both the mid-term and late-term pregnant women of twin pregnancy have the lower level of RBC, HGB, HCT and PLT than those in healthy nulligravida and the difference was statistically significant (P<0.05), and RBC, HGB and HCT increased as the gestation developing from mid-term to late-term. The count of white blood cells, neutrophils, and NEU% in the mid-term and late-term pregnancy increased than those in the healthy nulligravida group, and statistical significance (P<0.05) showed in the mid-term. The absolute count of WBC and NEU were 11.36±4.92 and 9.76±5.00 in mid-term, and drop to 8.54±4.17 and 7.17±4.17 when entered into late-term gestation. During the pregnancy, we found that the absolute count of LY was stable, but the LY% of reference decreased compared to that of the healthy nulligravida both in mid-term and late-term, and the difference of LY% in the mid-term was statistically significant (P<0.05). In conclusion, according to Chinese healthy unpregnant females, the reference intervals of RBC and PLT in twin pregnancy decreased; on the contrary, the WBC and neutrophils increased during pregnancy.

The requirements of Clinical and Laboratory Standards Institution (CLSI) suggest that any laboratory has its own reference intervals if no more than two results were outside the proposed reference interval when 20 samples are adopted to evaluate a new reference interval. Thus, a small group of 20 samples chosen at random, as described in methods, was tested for the reference interval of the late-term pregnancy (Fig. 2). The HGB index and HCT index of different two samples exceeded the upper and lower limits of the reference range of the twin pregnancy individually, while the RBC index of one sample was lower than the lower limit of that. Besides, all the other values fell within the established reference range. The verification passed, and the reference interval can be applied directly.

However, when using the Chinese normal female population reference intervals, 12 (60.0%) of the group would have out of range values of RBC count, 13 (65.0%) women would have out-of-range hemoglobin and hematocrit value. All of these out-of-range values were below the lower limit of the reference range. From the perspective of WBC, 10 (50.0%) of the group would have out of range WBC count, 12 (60.0%) and 11 (55.0%) women would have out-of-range count and percentage of neutrophils, 12 (60.0%) women would have out-of-range lymphocyte using the Chinese normal female population reference intervals (Fig.2).
3.3. Indexes of CBC might be potential indicators for adverse outcome of twin pregnancy

After establishing the reference intervals, we then further assessed its application potential in clinical diagnosis. Eighty-nine cases of twin pregnancy had a PTB during the period of 30-35 weeks of gestation, and 16 cases who suffered PPH were analyzed, while 3 of them were overlapped (Fig. 3). We excluded the three overlapped cases in order to see a single characteristic indicator relating to the particular outcome. The mean dates of first CBC records as they admitted to our center were 32 5/7w and 35 0/7 w of gestation, respectively of PTB and PPH groups, and the mean dates of delivery were 33 4/7w and 37 4/7w. Based on the statistical analysis of CBC among group REF, PTB, and PPH (Table 3), we found that the level of HGB and HCT significantly drop in the PTB group (p<0.05), while absolute WBC and NEU increased in PTB group. In the PPH group, it showed a significant decrease in HGB (p<0.05), but no statistical differences in other indexes (Fig. 4).

Using the established reference intervals for twin pregnant women in this study, 10.47% (9/86) of PTB population had beyond-upper limit WBC values during late pregnancy, while 12.79% (11/86) had beyond-upper limit NEU values. Conversely, 17.44% (15/86) of PTB population had the out-of-range value of HGB and/or HCT, 14 of which were below of lower limit and should be considered to have any grade of anemia (Fig. 5A). Otherwise, of the PPH population, 30.77% (4/13) were below the lower limit of established reference intervals of HGB value (Fig. 5B).

4. Discussion

Laboratory tests are often requested during pregnancy to exclude pathological complications that may affect maternal or fetal health(23, 24). Diagnostic accuracy is based on the evaluation of results in relation to accurate reference values(25). Although changes in normal laboratory values induced by pregnancy are well known, few studies have been conducted to establish reference intervals for pregnant women, especially twin pregnancy(7, 10-13). In this study, we report x ±1.96s as reference intervals for complete blood count (CBC) parameters in twin pregnancy. We found that the level of RBC, HGB, HCT, PLT and LY% in the mid-term and late-term pregnant women of twin pregnancy were lower than those in healthy nulligravida, while the count of white blood cell, neutrophils and NEU% in the mid-term and late-term pregnancy were higher(7, 10, 13). We used those results to create reference intervals and tested our reference intervals by using 20 twin pregnancy women blood. After confirmed the correctness of the reference interval, we found that RBC, HGB, HCT, and PLT decreased in twin pregnancy, while WBC and NEU increased, implying that they might be prognosis indicators of this adverse outcome. This was a longitudinal study that followed twin pregnant women from the mid-term to late-term pregnancy. The establishment of suitable reference intervals for twin pregnant women has the potential to improve diagnostic quality, which could lead to increased survival, reducing unnecessary treatment and cost savings.
In the beginning, we found that 16.0% of twin pregnant women were diagnosed with anemia, the OR value of which to adverse outcomes was 0.79(Table.1). These figures strongly suggested that using the HBG level of normal Chinese nulligravid might cause incorrect interpretation in the clinic. Developing an accurate set of CBC reference intervals for a twin pregnancy is of urgent need. As a result, we found that RBC, HGB, HCT, and PLT decreased during normal twin pregnancy during mid-term and late-term, consistent with findings from other studies for a singleton pregnancy(10, 11). The MCV was unaffected by twin pregnancy and remained the same as the levels in healthy nulligravida, indicating that RBC, HGB, and HCT were affected by hemodilution rather than nutritional deficiencies(9). Consistent with other studies for a singleton pregnancy, we refer the reason was due to the expansion of plasma volume during pregnancy with a concomitant lower expansion in red-cell volume(14). In the mid-term, RBC, HGB, HCT showed the lowest value while the late-term increased slightly, the reason may be with the pregnancy developed from mid-term to late-term, the body activates the metabolic system to compensate for the physiological change, and the blood dilution stabilized(18, 26).

Oppositely, according to our data, although HCT and RBC count decrease with increasing gestational age, the absolute WBC count reference interval elevated during pregnancy, which was due to the significant increase in neutrophil count during normal twin pregnancy, possibly related to stress response, redistribution of WBCs between the marginal and circulating pools or pain, nausea, vomiting, and anxiety in the absence of infection(19, 27). WBC count and NEU count are expected to increase during pregnancy; therefore, the reference intervals for these parameters should be especially considered with caution in the clinic. The use of this parameter should be limited as a marker for infection during pregnancy in case of proper reference intervals lack(20, 28). We found that the increase in WBC count resulted primarily from an increase in neutrophil counts and peaked at the mid-term, and decreased at late-term, which was a little different from the other studies. The absolute count of WBC and NEU drop from 11.36±4.92 / 8.54±4.17 to 9.76±5.00 / 7.17±4.17 when entered into late-term of gestation and NEU% was stable. We found the absolute count of LY is stable during twin pregnancy and LY% was lower than that of the healthy nulligravida, while the lowest was detected in the mid-term with statistically significant (P<0.05), consisted with the data that neutrophil absolute counts and peaked at the mid-term(14, 20, 28).

Investigation of potential risk markers of adverse outcomes had been performed at molecular, biochemical index, and metabolism level(29-31). Multiple pregnancies are at increased risk of adverse maternal and perinatal outcomes, mainly due to obstetric complications, preterm birth, and birth weight discordance(32). Cirvicle length, fetal fibronectin, and uterine activity monitoring, which used to be potential risk factors of interest, were not recommended now since no new evidence identified suggesting their accuracy(33). Based on ultrasound screening, crown-rump length and placental location were measured to show their association to adverse pregnancy outcome(34-36). In the current study, we aimed to identify risk factors in more routine laboratory tests and figured out NEU absolute in CBC might be a predictor of PTB. According to the established reference intervals for a twin pregnancy, it's found that NEU increased to above the upper limit (>11.31×10^9/L) in 12.79% of the PTB population. The association between NEU count and PTB needs to be further confirmed by the evidence of a bigger sample size.
While the strength of this study is that a large number of screening populations (1153 cases) in one institute, the limitations of the present study should not be overlooked. First, the sample size for reference interval establishment is small due to the high incidence of adverse outcomes in twin pregnancy, though it met the requirement of CLSI guidelines already. Second, CBC data of candidates were obtained mainly from examinations in the second and third trimester due to the retrospective nature of this study; thus, we could have missed the earlier changes in maternal hematology responding to twin pregnancy. However, compared to using standard whole blood reference intervals from non-pregnant women, which might result in an increase in out-of-range values among twin pregnant women, these limitations of the current study may hardly hamper the suitability of accurate reference intervals for this population.

To summarized, in the present study, we have established reference intervals for several hematological variables in healthy twin pregnant women that can be used for clinical management in our center. These values should be considered for the development of region-specific reference intervals for twin pregnancy women in China. Further validation and practice in the clinic of these established reference intervals are warranted. We hope that the presentation of these reference values may assist the clinician in distinguishing between physiological changes and pathological states during twin pregnancy.

**Abbreviations**

CBC: Complete Blood Count; RBC: Red Blood Cell; WBC: White Blood Cell; HGB: Hemoglobin; HCT: Hematocrit; NEU: Neutrophils; PLT: Platelets; PTB: Preterm Birth; PPH: Postpartum Hemorrhage; ART: Assisted Reproductive Technologies; TTTS: Twin-Twin Transfusion Syndrome; PROM: Premature Rupture Of Membrane; PID: Pelvic Inflammatory Disease.

**Declarations**

**Ethics approval and consent to participate**

Ethical approval was obtained from the ethical committees of The Third Affiliated Hospital of Guangzhou Medical University, Guangdong, People’s Republic of China. The informed consent from all subjects was obtained at the department of prenatal diagnosis of The Third Affiliated Hospital of Guangzhou Medical University. Patient records/information were anonymized and de-identified before analysis. All methods in this study were performed in accordance with the Declaration of Helsinki and relevant regulations.

**Consent for publication**

Not applicable.

**Availability of data and materials**
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Authors' contributions
ZYF and HWY conducted most of the experiments, analyzed the results, and wrote most of the paper. ZYF and LXH conducted sample collection and statistics. LL and MM conducted validation. CM was responsive to the clinic consultant. XY conceived the idea for the project and wrote the paper with HWY.

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Tables

Due to technical limitations, tables are only available as a download in the Supplemental Files section.
FIGURE 1

Flow diagram of reference subjects in the retrospective cohort study.

Figure 1

Flow diagram of reference subjects in the retrospective cohort study.
Validation of the established reference intervals. The X-axis indicates different individuals in the validation population. The black dotted line indicates the upper limit and the lower limit of the reference range for each index of twin pregnancy. The solid point represents subjects who regard values were within the range of reference intervals. Hollow point represents subjects who are regarding values that were out of the range of reference intervals. The slender blue dotted line indicates the upper limit and the lower limit, respectively, of the reference range for each index of normal nulligravid.
Figure 3

Outcomes analysis of twin pregnancy. (a) Venn Diagram of the population with adverse outcomes of PTB and PPH. (b) Characteristics of the population with different gestation outcome. REF, pregnancy with a good outcome; PTB, pregnancy with preterm-birth; PPH, pregnancy with postpartum hemorrhage.
Figure 4

Box plot of gestational outcome-specific CBC ranges for RBC, HGB, HCT, PLT, WBC, NEU, NEU%, LY, and LY%. Every box plot contains the middle 50% of the data. The upper edge of the box indicates the 75th percentile, and the lower edge indicates the 25th percentile. The horizontal bar in the middle of each box plot represents the median value. The whiskers extending from the box plot represent the range of values obtained, excluding outliers. Circles and asterisks outside the ends of the whiskers indicate outliers (1.5× the interquartile range) and extreme values (3.0×the interquartile range), respectively. Bold black dotted
line indicates the upper limit and the lower limit, respectively, of the reference range for each index of twin pregnancy. The slender blue dotted line indicates the upper limit and the lower limit, respectively, of the reference range for each index of normal nulligravid.

**Figure 5**

(a)

![Figure 5a](image)

(b)

![Figure 5b](image)

**Figure 5**

Individual distribution of responding CBC indexes with a significant difference between diverse gestation outcomes. The X-axis indicates different individuals of PTB(a) or PPH(b) population. The black dotted line indicates the upper limit and the lower limit of the reference range for each index of twin pregnancy.
The black point represents subjects who are regarding values were within the range of reference intervals. The red point represents subjects who are regarding values that were out of the range of reference intervals.

**Supplementary Files**

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- TABLE1.tif
- TABLE2.tif
- TABLE3.tif