RESEARCH ARTICLE

Correlation of Maternal Serum Hepcidin, Soluble Transferrin Receptor (sTfR) and Cholecalciferol with Third Trimester Anemia: Findings from A Nested Case-control Study on A Pregnancy Cohort

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Abstract

BACKGROUND: Cholecalciferol, hepcidin, and soluble transferrin receptor (sTfR) interaction play an essential role in iron hemostasis. Anemia in pregnancy contributes to morbidity and mortality both for the mother and baby. In this study, we assessed the correlation between hepcidin, sTfR and cholecalciferol in third trimester maternal anemia. We aimed to find the cut-off for hepcidin and sTfR.

METHODS: A case-control study involving 56 pregnant women in each anemia and healthy group was nested on a previous larger cohort study in Indonesia. Serum hepcidin, sTfR and cholecalciferol level were measured by enzyme-linked immunosorbent assay (ELISA) method.

RESULTS: Serum hepcidin and sTfR level were significantly higher in case group, while serum cholecalciferol level has no difference between the two groups. New cut-off points were found for hepcidin (<15.93 ng/mL) and sTfR level (>2234.45 ng/mL). Low level of hepcidin (OR=5.32) and high level of sTfR (OR=8.28) increase the risk of anemia. High level of sTfR (adjusted OR=4.725; CI 95%=1.730-12.904; *p*=0.02) was the most important factor contributes to anemia, followed by the low level of hepcidin (adjusted OR=3.677; CI 95%=1.363-9917; *p*=0.01).

CONCLUSION: The high level of sTfR is the most important factor related to anemia in the third trimester, followed by the low level of hepcidin. Low cholecalciferol level tends to favor the incident of anemia. The new cut-off point of third trimester sTfR and third trimester hepcidin were established in this study and may be useful for risk assessment and treatment monitoring for anemia in pregnancy.

KEYWORDS: anemia, cholecalciferol, hepcidin, pregnancy, soluble transferrin receptor

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Introduction

Anemia in pregnancy has become a global health concern in many countries, as increases disabilities, morbidity, mortality, and of course, the cost of healthcare.(1,2) Anemia in pregnancy is associated with low birth-weight, premature birth, intrauterine growth restriction and neonatal anemia. (3,4) The global prevalence of anemia in pregnancy in year 2010 was 32.9%, and contributed to 8.8% of total morbidity. (2) While in Indonesia, as reported in Riset Kesehatan Dasar (Basic Health Research) in 2013, the prevalence reached

37.1%, although iron supplementation consumption has covered 89.1% among pregnant women.(5)

Vitamin D sufficiency is correlated with anemia, specifically anemia of inflammation, as recorded in one cohort study involving both adult men and women in USA. (6,7) Previous study showed that women with vitamin D insufficiency in the first trimester had the highest proportion of anemia.(4) The comorbidities such as preeclampsia, gestational diabetes, and the signs of intrauterine growth restriction were found in anemia that is related to low vitamin D.(2,3,5) Our previous studies also showed a positive correlation between first trimester level of vitamin D and ferritin.(4,8)

Some parameters including hemoglobin, hematocrit, iron, erythrocyte, and platelet count were lower in pregnant women, especially in their third trimester, compared to non-pregnant. In contrast, the soluble transferrin receptor (sTfR), total iron binding capacity, and interleukin-6 (IL-6) levels were higher. The IL-6 was significantly correlated with both ferritin and high-sensitivity C-Reactive Protein (hs-CRP), supporting the pregnancy as an inflammatory state.(9)

The high prevalence of vitamin D deficiency in Indonesian pregnant women challenges the clinician on finding the recommended dose of vitamin D supplementation. A single oral dose of vitamin D (100,000 IU vitamin D2) supplementation significantly increased the serum level of vitamin D. However it suppressed hepcidin level by 34%. (10) Similar finding in a randomized-controlled trial shows the hepcidin level was reduced without a change in plasma pro-inflammatory cytokine or ferritin concentrations.(7) The two studies indicate vitamin D as a potent regulator of the hepcidin-ferroprotein axis in humans. On the other hand, they are also alarm for a better strategy of anemia in pregnancy management related to vitamin D deficiency.

Hepcidin's biological actions are initiated by its binding with ferroprotein. Once hepcidin is bound, the protein will be internalized within one hour, and soon after that degraded in the duodenum epithelium. Afterwards, macrophages and hepatocytes stimulate the iron efflux channel to maintain the plasma iron level, and therefore facilitate the hemoglobin production. Hepcidin feedback mechanism was negatively regulated by iron concentrations in the plasma and liver, while positively regulated by erythropoietic demand for iron.(11)

Treatment of cultured hepatocytes or monocytes with prohormone 25-hydroxyvitamin D or active 1,25-dihydroxyvitamin showed a 50% reduction of hepcidin mRNA expression due to direct transcriptional suppression of hepcidin gene (HAMP) expression mediated by 1,25-dihydroxyvitamin D binding to the vitamin D receptor. This suggests the association between HAMP suppression with the increasing expression of ferroprotein, and decreasing expression of ferritin.(10)

The information regarding to the regulatory changes of hepcidin in pregnancy were very limited until currently. Prohepcidin is known to be significantly higher in pregnant women compared to healthy non-pregnant ones, suggests as an adaptive mechanism toward low iron plasma, associated with iron deficiency anemia in pregnancy. When the plasma iron level is low, the sTfR would normally raise. One may hypothesize that when vitamin D level is low, hepcidin would increase and then facilitates the iron efflux to promote erythropoiesis.(9)

World Health Organization (WHO) recommends a dose of 600 IU vitamin D supplementation for pregnant women.(12) However, this recommendation has never been put on a clinical trial in Indonesia. Thus, we performed this study as a nested case-control from our previous study with some additional markers to find out the correlation between the first trimester vitamin D level and third trimester maternal hepcidin and sTfR levels. To the best of our knowledge, this is the first nested cohort study conducted in Indonesia to find the cut-off point of anemia risk assessment.

Methods

Study Design

This was a follow-up case-control study nested from our previously published cohort study which was conducted from July 2016 until March 2018.(4,8) Subjects with any congenital anomaly in their pregnancy were excluded from the study. The subjects were divided into two groups, case and control group, with 56 subjects in each group. Case group consisted of of pregnant women with anemia in the third trimester, while the control group consisted of randomly selected pregnant women without anemia. This study has been approved by the Committee for Health Research Ethics Universitas Padjadjaran (No.330/ UN6. KEP/EC/2018).

Biomarkers Analysis

All laboratory examinations were performed in Dr Hasan Sadikin Hospital, Bandung, Indonesia. Frozen serum (-80^{o}C) was thawed in room temperature before used for biomarkers' assessment according to each kit inserts. Serum cholecalciferol level was measured in the first trimester,

while hemoglobin, serum sTfR and hepcidin levels were measured in the third trimester. Maternal age, education, parity and pre-pregnancy nutritional status were recorded. The nutritional status was classified by WHO.

To determine whether the subject had anemia or not, subjects' hemoglobin was measured using automated hematology analyzer with impedance method measurement (Sysmex XP-100, Sysmex Corporation, Kobe, Japan). According to WHO and Center for Disease Control (CDC) classification, anemia in third trimester was defined as hemoglobin <11 g/dL.(13)

Cholecalciferol was measured by enzyme-linked immunoassay with final fluorescent detection (ELFA) method (VidasR, bioMérieux, Midrand, South Africa) according to the kit. This method is a sequential competitive immunoassay. The sample was mixed with pre-treatment reagent to separate 25(OH)D from its binding protein, then collected and transferred into the well that contains an alkaline phosphatase (ALP)-labelled anti-vitamin D antibody (conjugate). The vitamin D antigen present in the sample and the vitamin D antigen coating the interior of the SPR were competed for binding sites on the anti-vitamin D antibody-ALP conjugate. During the final detection step, the substrate (4-methylumbelliferyl phosphate) was cycled in and out of the SPR. The conjugate enzyme catalyzes the hydrolysis of this substrate into a fluorescent product (4-methylumbelliferone), the fluorescence of which is measured at 450 nm. The intensity of the fluorescence was inversely proportional to the concentration of vitamin D antigen present in the sample. Results were automatically calculated by the instrument in relation to the calibration curve. The VIDAS® 25-OH Vitamin D Total assay was standardized to internal controls that are traceable to a LC-MS/MS method calibrated with NIST SRM972a. The detection dose is between 8.1-126 ng/mL.

Hepcidin (Cat. No. E-EL-H0077) and sTfR (Cat. No. E-EL-H1646) examinations were performed by enzyme linked immunoassay (ELISA) method (Elabscience, Houston, Texas, USA) according to the kit. The assay's principle was based on antigen-antibody binding. The method of the assay involves immobilization at the surface of the microplate well, and combined with the specific antibody for hepcidin or sTfR. Then a biotinylated detection antibody specific for Human Hepc or sTfR, and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. Enzyme-substrate reaction was terminated by the addition of stop solution and the color change. The optical density (OD) was measured spectrophotometrically at a wavelength of 450±2 nm. Minimum detectable dose of hepcidin was 195.2 pg/mL, while the minimum detectable dose of sTfR was 31.25 ng/mL.

Statistical Analysis

Statistical data analysis was performed using R v.3.3.1. statistical software (DataCamp, Inc., New York, USA). Pearson Chi-Square, Mann-Whitney and Independent T-test were performed to compare the subjects' characteristics from both groups. Multivariate regression analysis was performed to determine which factors can independently predict the thirdtrimester maternal anemia development. Cut-off points and Odd Ratio (OR) (confidence interval (CI) = 95%) determination for cholecalciferol, hepcidin and sTfR were performed by MedCalc (MedCalc Software Ltd, Ostend, Belgium).

Results

Maternal demographic characteristics were presented in Table 1. The body mass index (BMI) mean value was significantly lower in case group compare to control, but the nutritional status of both groups was not different. All subjects were routinely taking iron 90 tablet supplementation, but were not considered as confounder in this study.

Subjects' biochemical characteristics could be found in Table 2. There was no significant difference in serum cholecalciferol level between both groups. The mean values for serum hepcidin, and sTfR level were significantly higher in case group compared to control group.

Areas under the ROC curves and cut-off points for cholecalciferol, hepcidin and sTfR were presented in Figure 1. Cholecalciferol showed the smallest area under curve (AUC) (59.8%, p=0.074) with no significance for anemia in the third trimester. The cut-off point of sTfR level for anemia in the third trimester was 2234.45 ng/mL, (AUC=66.1%, p<0.001), with sensitivity 64.29% and specificity 82.4%. While the cut-off point of hepcidin level in the third trimester for anemia was unexpectedly low, which was <15.93ng/mL (AUC=0.736, p<0.001), with 66.1% sensitivity and 73.2% specificity.

Further analysis was performed using those cut-off points to find the OR of hepcidin and sTfR to anemia with 95% CI, as shown in Table 3. Pregnant women with low level of hepcidin were 5.32 times more likely to have anemia in their third trimester, and those with high level of sTfR were 8.28 times more likely to have anemia in their third trimester.

Table 1. Demographic characteristics of the study population.

Characteristics	Anemia (n=56)	Non-Anemia (n=56)	*p*- value
Age (years)			
Mean (SD)	27.6 (6.3)	28.5 (5.4)	0.400[a]
Median (range)	27.5 (16-43)	29.0 (17-39)	
Age group, n (%)			
<20 years old	5 (8.9)	2 (3.6)	0.492[b]
20-35 years old	46 (82.1)	48 (85.7)	
>35 years old	5 (8.9)	6 (10.7)	
Education level, n (%)			
Primary school	19 (33.9)	20 (35.7)	0.317[b]
Junior high school	17 (30.4)	23 (41.1)	
Senior high school	17 (30.4)	9 (16.1)	
Bachelor degree	3 (5.4)	4 (7.1)	
Parity, n (%)			
Primiparity	48 (85.7)	39 (69.6)	0.100[b]
Multiparity	8 (14.3)	16 (28.6)	
High-parity	0 (0)	1 (1.8)	
Pregestational body mass index (kg/m^2)			
Mean (SD)	22.4 (6.82)	23.2 (4.45)	0.031[c]
Median (range)	20.3 (15.6-50.9)	22.4 (15.9-3.8)	
Nutrition status, n (%)			
Undernutrition	12 (21.4)	7 (12.5)	0.141[b]
Normal	35 (62.5)	32 (57.1)	
Overnutrition	9 (16.1)	17 (30.4)	

[a]Tested with Independent T-test ; [b]Tested with Pearson Chi Square, [c]Tested with Mann-Whitney.

The multivariate analysis results as shown in Table 4 indicated that the high level of sTfR (adjusted OR 4.725 CI 95% 1.730-12.904; *p*=0.02) was the most important factor related to anemia in the third trimester, followed by the low level of hepcidin (adjusted OR 3.677 CI95% 1.363-9917; p=0.01). Cholecalciferol had no significant risk to anemia, while low pregestational BMI has no impact on anemia in pregnancy.

Discussion

Bivariate analysis performed in current study as presented in Table 2, showed that lower maternal cholecalciferol level in the first trimester is associated to the presence of anemia in the third trimester. Maternal iron status is important for fetal heath, since human placenta has functions to protect a fetus through regulating the expression of iron transporters. (14) Thus, managing hypovitaminosis D, iron deficiency and anemia in pregnancy is essential.

Knowing the primary causes of anemia would allow selection of interventions, hence characterizing the performance of available markers for iron deficiency is very important.(13) Serum ferritin level has been reported as a sensitive and specific marker for iron deficiency anemia, as it remained unaffected by inflammation and less affected by biological variability.(12,15) Serum ferritin level <12 ng/mL and <15 ng/mL were used in one systematic review for

Table 2. Biochemical characteristics of the study population.

Markers	Anemia (n=56)	Non anemia (n=56)	*p*- value[a]
Cholecalciferol in trimester I Median (range) (ng/mL)	15.7 (8-31.2)	18.1 (8-43.6)	0.074
Hepcidin in trimester III Median (range) (ng/mL)	14.1 (1.20-84.8)	7.5 (32.6-96.52)	<0.001
sTfRb in trimester III Median (range) (ng/mL)	2557.6 (403-78.900)	1447.6(126.2-10.900)	<0.001

[a]Tested with Mann-Whitney test.

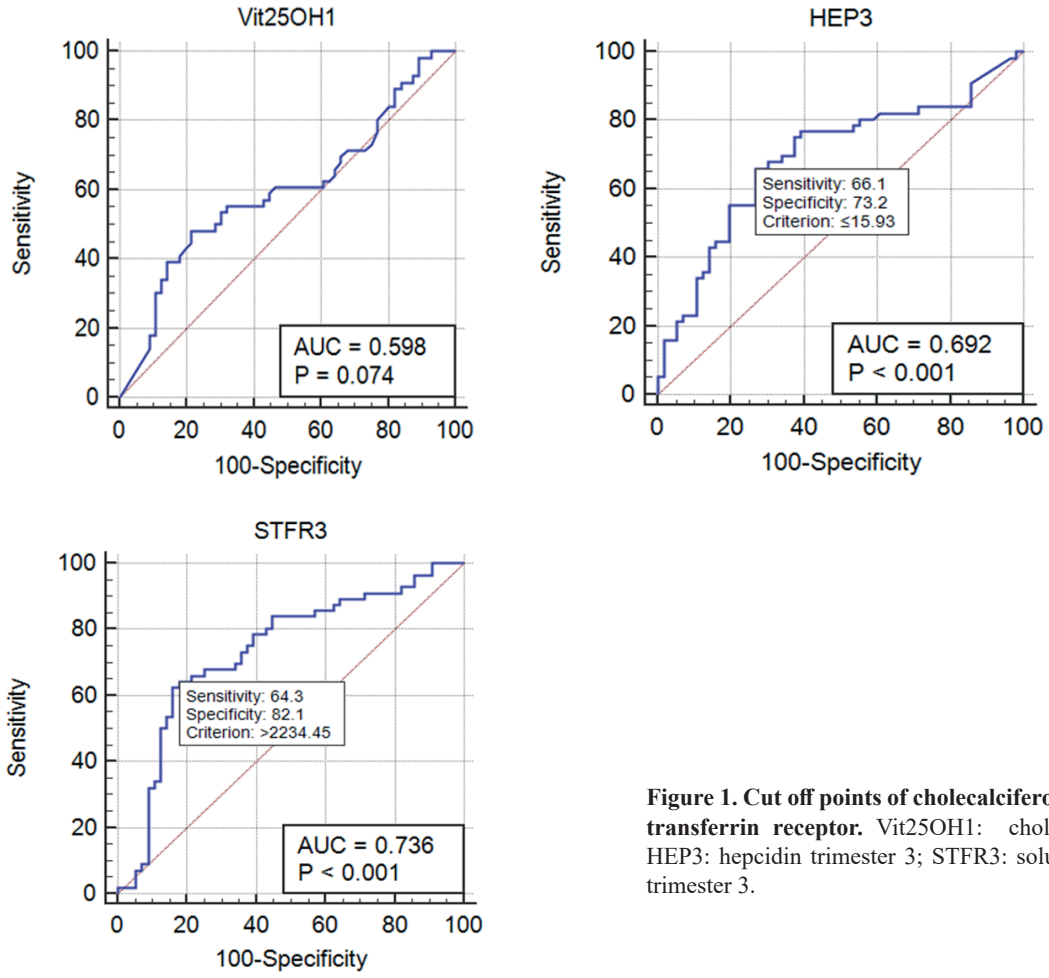

Figure 1. Cut off points of cholecalciferol, hepcidin and soluble transferrin receptor. Vit25OH1: cholecalciferol trimester 1; HEP3: hepcidin trimester 3; STFR3: soluble transferrin receptor trimester 3.

iron deficiency anemia diagnosis in pregnant women.(15) Our previous cohort study showed that the first trimester ferritin level mean value is 67.81±53.81 ng/mL, and we found the cut-off value for trimester serum ferritin level as <27.23 ng/mL (LR (+) 3.07 (95% CI 1.8-5.3), specificity 86.29% 95% CI 79.0%-91.8%) for anemia in pregnancy. Twenty-four point nine percent of the subjects have ferritin levels <30 ng/mL, but only 7.5% of them have anemia.(8)

In our previous cohort study, all subjects routinely took iron 90 tablets during their pregnancy, however, the iron deficiency still occurred among the subjects.(4,8,16) We also unexpectedly found the high sTfR level (2557.6 ng/mL in anemia group *vs.* 1447.6 ng/mL in non-anemia group). Thus the iron tablet supplementation needs to be evaluated if we want to achieve a 50% reduction in the prevalence of anemia among women of reproductive age by 2025.(17).

The level of transferrin receptors increase in iron deficiency anemia, while the sTfR/ferritin index decrease. (18) sTfR and ferritin level were the potential markers for early detection and therapy monitoring in iron deficiency anemia.(19) However, the first trimester hepcidin and sTfR level have a weak correlation with the third trimester anemia

Table 3. The association between low hepcidin and high sTfR to anemia in pregnancy.

Marker Cut-off	Anemia	Non-Anemia	Total	OR (95%CI)
Hepcidin in trimester 3 (ng/mL)				
≤15.93	37	15	52	5.323 (2.368-11.963)
>15.93	19	41	60	
TfR in trimester 3 (ng/mL)				
≥2234.45	36	10	42	8.280 (3.450-19.872)
<2234.45	20	46	66	

Table 4. Multivariate analysis of risk factors for anemia in pregnancy.

Variables	Coefficient B	S.E (B)	Adjusted OR (95% CI)	*p*-value
Pregestational Body Mass Index	-0.035	0.044	0.965 (0.886-1.052)	0.418
Cholecalciferol trimester I	0.966	0.525	2.627 (0.939-7.354)	0.066
Hepcidin trimester III	1.302	0.506	3.677 (1.363-9.917)	0.01
sTfR trimester III	1.553	0.513	4.725 (1.730-2.904)	0.002

in this study, while the anemia keep progressing along the pregnancy age.(4,8) Therefore, the first trimester ferritin level will be more suitable for assessing the risk of maternal anemia in the third trimester, while the early third trimester sTfR level could be utilized as a marker for monitoring the management of anemia.

We assumed that the inflammatory state in pregnancy plays a role in increasing the cases of anemia to almost four-fold in the subjects.(4,8,16) The process of chronic inflammation is associated with functional iron deficiency, so the normal iron level and elevated serum ferritin level do not always meet the iron needs in the bone marrow. Another study suggested this as the effect of increased hepcidin transcription.(20) Nevertheless, the third trimester hepcidin level in our study's subjects was significantly lower (14.15 ng/mL) in the anemia group compare to 17.55 ng/mL in the non-anemia group. Subjects with low level of hepcidin also found to have 5.32 times higher risk of having anemia in their third trimester. Hepcidin plays a role in maintaining plasma iron level.

In accordance with previous study, we found a significantly higher level of sTfR in maternal anemia compare to non-anemia.(21) The study correlated the sTfR level to the severity of anemia. While in another study, the risk level of iron deficiency anemia in early pregnancy can be varied, depending on which parameter is used. They found the proportion of 19.6%, 15.3% and 15.7% of iron deficiency anemia respectively, while using the cut off value of ferritin (SF<12 µg/L), sTfR (TfR≥21.0 nmol/L) and total binding iron (calculated from SF and sTfR).(22)

The use of sTfR measurement for maternal anemia monitoring is clinically important, to determine if the patient needs more observation or treatment. The measurement of sTfR, ferritin, and the sTfR/log ferritin ratio (TrF-F index) as a treatment monitoring tool for adults with iron deficiency. Their study showed a significant change after iron supplementation in female subjects, but not in male. (18). Unfortunately, there were no pregnant subjects in the study.

sTfR and hepcidin were both found to be correlated with anemia in pregnancy in this study. The new cut-off point of third trimester sTfR>2234.45 ng/mL and third trimester hepcidin<15.93 ng/mL were established, with relatively low sensitivity but acceptable specificity (>70%). Pregnant women with low level of hepcidin are 5.32 times more likely to have anemia in their third trimester, and those with high level of sTfR are 8.28 times more likely to have anemia in their third trimester. While cholecalciferol has no significant risk to anemia.

Conclusion

This study found that the high level of sTfR is the most important factor related to anemia in the third trimester, followed by the low level of hepcidin. Low cholecalciferol level tends to favor the incident of anemia. The new cut off point of third trimester sTfR and third trimester hepcidin were established in this study and may be useful for risk assessment and treatment monitoring for anemia in pregnancy.

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