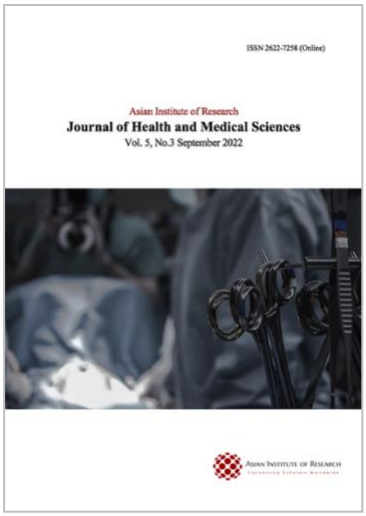

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A Study on Distribution and Association of CD320, a Receptor for Active Vitamin B12, in Pregnant Mothers and their Newborns Cord Blood

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Abstract
Objective: Our objective was to study the levels and relation between CD320 receptors in anaemic mothers in their last trimester of pregnancy and their newborns after birth. Methods: Association between CD320, VitB12, active B12 levels in mothers with anaemia were analysed before they underwent labor and in blood collected from their newborns after labor. Regression analysis was performed in order to assess the relation between the mothers and their neonatal vitamin CD320 levels in relation to their Vit B12 status. Results: Among the 200 pregnant mothers recruited, 59% were anaemic. CD320 levels were significantly higher in anaemic mothers (414.09 ±10.75 pg/ml) when compared with cord blood (372.25 ± 7.81 pg/ml). CD320 levels were higher in both mother and cord blood with Vit B12 deficiency, however, were lower in mothers with active B12 deficiency. CD320 levels in cord blood were positively associated with haemoglobin (r value 0.8, *p* value 0.42) of mothers and negatively with serum folate (r value -0.15; *p* value 0.23), Vit B12(r value -0.21; *p* value0.28) and active Vit B12 levels (r value -0.25; *p* value0.03) in mothers. Conclusion: CD320 levels in cord blood positively correlated with haemoglobin of mothers and negatively with folic acid and B12 status. A negative trend (p=0.0024) was observed between maternal and neonatal CD320 levels.

Keywords: CD320, Anaemia, Pregnant Mothers, Cord Blood, Vitamin B12, Active B12

1. Introduction

Deficiency of Vitamin B12 (Vit B12) is common in India and is found to be more widespread than it is assumed, especially among pregnant women (Pathak et al. 2007; Laxmaiah 2015). Apart from beneficial effects of Vit B12 in mothers, it also helps in the development of the foetus (Pepper 2011 & Greibe 2011). Newborns with Vit

B12 deficiency are known to develop neural tube defects and intrauterine growth retardation (Molloy et al 2009).Vit B12 after absorption in the intestinal cells of the mother (Nielsen et al 2012), gets partially bound to the protein transcobalamin (TC), is called holo- transcobolamin or holo-TC and the remaining with apoTC (Nielsen et al 2012). HoloTC is also called active B12 and is the form through which cellular uptake of B12 takes place with the help of a receptor (Nielsen et al 2012) called CD320, which was recently purified from human placenta. This holoTC receptor mediates holoTC uptake in most cells in the body (Quadros et al 2009). The CD320 receptor was found to bind mostly to holoTC, only to a lesser degree to apoTC and is a low-density lipoprotein receptor. Abuyaman et al 2013, had reported for the first time that sCD320 was found in both serum and urine, its urinary concentration exceeded those of the serum and its levels increased with progression of pregnancy.

Apart from the discovery of the new Vit B12 receptor, there are no further studies on it and whose novelty lies in the fact that it is expressed both in serum as well as urine. Hence, there is an unmet need to explore the diagnostic potential of this new receptor. We thus hypothesized that Vit B12 deficiency in the mothers leads to Vit B12 deficiency in the cord blood and thus leading to increased expression of the CD320 receptors in the placenta to compensate for the deficiency in the cord blood and replenish Vit B12 levels in the foetus. As total Vit B12 levels are not reliable indicator of B12 status and also Vit B12 deficiency is very common in our country, especially in pregnant women we intended to study this newly discovered receptor of active Vit B12 both in the mothers and newborns blood, its expression in the placentas and their association with total Vit B12 levels.

2. Methods

Ours was a cross-sectional study that was carried out in accordance with Ethical committee clearances from both our Institute and the collaborating Hospital, which is a tertiary level hospital where patients from both lower as well middle strata of the society come for their treatment. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects/patients were approved by the Institutional Ethical committee. A written informed consent was obtained from all subjects. We recruited 200 mothers who were either in labor or admitted for delivery and who agreed to participate in the study. Upon admission and after undergoing routine complete blood picture (CBP) estimation, the mothers were requested to participate in the study. They were made to understand the details of the study including its benefit for the society and science, before asking for their consent. The study was initiated after we gave information about the study to the subjects. Mothers in the reproductive age group of 18–45 years, primiparous, were recruited, while those suffering from high blood pressure or high blood sugar and positive for hepatitis or AIDS were excluded from the study.

Information on the mother's sociodemographic profile, clinical and obstetric history, dietary intake was obtained through a questionnaire, by trained staff recruited for the study. Maternal anthropometry was also performed in which, the height, weight and BMI of mothers were noted. The body mass index (BMI) of the mothers was calculated in kg/m^2.

2.1. Study groups

There were two study groups based on mother's hemoglobin (Hb) levels. All the mothers whose Hb values were less than 11g/dl were assigned to anaemic group and those whose Hb levels were ≥ 11g/dl were assigned to normal group (WHO).

2.2. Sample collection and processing

2.6.1. Blood samples

After enrollment into the study, about 5ml of non-fasting sample of blood was drawn from the ante-cubital vein of mothers in labor or who was about to deliver, while 5ml of blood from the umbilical cord of the newborns was collected after delivery, in lavender cap ethylene diaminetetraacetate (EDTA) and red cap vacutainer tubes, which were transported in ice. In the lab, blood in EDTA tubes, was processed within 6 hours of collection, in automated hematology analyzer (ADVIA 120, Seimens) and analyzed for Hb and red cell indices. The serum after separation from blood, was stored in a −80°C refrigerator till it was analyzed for ferritin, vit B12, holo-transcobolamin (holo-TC) or active B12 and soluble CD320 receptors.

2.6.1.1 Serum ferritin was estimated by using SA ELISA kit from Calbiotech, Inc. based on the streptavidin-biotin principle.

2.6.1.2 **S**erum Vit B12 and folic acid analysis was performed by using the RIA kit from MP Biomedicals (USA).

2.6.1.3 Serum holo-TC (Active B12) was estimated by using ELISA method (Axis-Shield Diagnostics, Dundee, Scotland, UK).

2.6.1.4 CD320/8D6A ELISA Kit was used to determine serum CD320 receptor levels by sandwich ELISA method.

2.6.2 Placentas

After delivery, placentas were collected in 10% buffered formalin solution. In the lab, they were weighed after removing the umbilical cord (UC) and the amniotic and chorionic membranes and their morphology along with weight and size were noted. After overnight fixation in the buffered formalin they were grossed and 4 bits were taken from the four quadrants, about 2-5cms from the center, away from the margins and including both maternal and fetal surfaces. Two sections from the UC and one from the membranes were also taken. The tissues were next processed overnight in an automatic tissue processor, their paraffin blocks prepared, sectioned to 5 microns and stained with hematoxylin and eosin stain (H&E). The stained placental tissue sections were viewed under a brightfield microscope and the histopathological findings were noted.

2.6.2.1 Immunohistochemical expression of the placental tissues for CD320 protein expression was studied on the paraffin embedded tissues by using the CD320 primary antibody from CUSABIO technology Ltd (Product code: CSB-PA865096DSR1HU). The secondary antibodies used were 2-step plus Poly-HRP Anti-Mouse/Rabbit IgG Detection system with DAB solution by Elabscience (Catalog No. E-IR-R217). The placental tissue sections after dewaxing and hydration, incubated with E-IR-R217 C (3% H_2O_2) to eliminate endogenous peroxidase activity and washed in phosphate buffered saline (PBS) followed by incubation in E-IR-R217 A normal goat serum. Next the primary CD320 antibody at 1:900 dilution, raised in rabbit was added to the slides and incubated at 4^0C overnight. This was followed by incubation at room temperature in E-IR-R217 B (Polyperoxidase antirabbit IgG), followed by DAB solution.

2.6.2.2 Immunohistochemical stained sections analysis

The immunohistochemical analysis was performed by counting the percent (P) of trophoblastic cells of placenta, exhibiting positive stain for the marker, which was graded from not-detected with grade 0%, to total or homogenous staining graded as 100%. The intensity of immunohistochemcial staining (I) was also graded as follows: grade 1 for weak intensity of stain, 2 was given for moderate intensity and 3- for strong intensity of staining. The final results were then obtained by multiplying the percentage of stained cells (P) with staining intensity (I), which was called quick score (Q) where Q was = P × I with as maximum score of 300) (Abuyaman et al 2013).

2.7 Cut-off values for defining deficiency of parameters

Serum ferritin deficiency was stated as <15ng/ml (WHO), Vit B12 deficiency was stated, after using the standards of Centre for Disease Control and Prevention (CDC) definition, as < 150 pmol/L or 203 pg/ml (Yetley 2011). Folate deficiency as <6.8 nmol/L or 4.4ng/ml (10), holo-TC deficiency as < 35 pmol/l (Lindgren 1999). CD320 being a relatively newly discovered receptor, we could not come across any cut off values for defining deficiency state both for the mothers and cord blood. Maternal BMI was ranged from 18.5 to 30.0 kg/m^2, based on classification by World Health Organization (WHO) and CDC(WHO 1995).

2.8 Statistical Analyses of data

Processing of the data and its analyses was carried out with SPSS software (19.0 version). Constant or continuous data were expressed in means ±standard deviation or SD and absolute or definite or categorical data as numbers (%). We examined the relation between mothers and newborn cord blood Vit B12 levels including CD320 levels. All the mean values of constant or continuous data variables were compared in the mother's blood and newborn cord blood by using Student's *t* test. Non-parametric *t*-test was used to compare the data between serum ferritin, VitB12, active B12, CD320 levels and CD320 expression level score by immunohistochemistry in placentas. Multiple comparisons within the groups were carried out with the help of ANOVA. Correlations between various data variables in mother's blood and newborn cord blood were examined with Spearman's test. Multivariable backward regression analyses was performed to study the different variables that could predict change in the levels of CD320 in maternal and newborn cord blood. All the variables were compared with low CD320 levels. The characteristics found significant in the univariate analysis with $p < 0.2$ were analysed for multivariate analysis. Variables which had $p<0.05$ were analysed further by final adjusted regression model if they had a p value <0.05. Statistical significance was given to a *p* value of less than 0.05.

3. Results

Table 1 shows the general features of the pregnant mothers included in our study. The average age of the subjects was 23 years while BMI was 24.53± 2.39. While 45.5% of them were illiterate, majority were non-working mothers (90%).61.5% of the mothers were gravida >1 with 32.6% being multiparous. The mean placental weight was 416.35 ± 89.45 grams and mean newborn birth weight was 2.82 ±0.43 kgs.

Table 1: Characteristic features of pregnant mothers and their newborns

Variables	**Values**
Maternal	
Age (years) Mean ± SD (Range)	23.84 ± 3.10 (18-35)
Height,(feet) Mean ± SD (Range)	5.31±0.20 (4.11-5.80)
Weight (kg) Mean ± SD (Range)	63.23 ±7.47 (38-86)
BMI (kg/m^2) Mean ± SD (Range)	24.53± 2.39(17.2-30.6)
Education % (n/total)	
Illiterate	45.5 (91/200)
Schooling	45 (90/200)
College	9.5 (19/200)
Occupation % (n/total)	
Working	10 (20/200)
Non-working	90 (180/200)
Monthly income % (n/total)	
<Rs 5000	6.5 (13/200)
Rs 5000-10,000	70 (140/200)
Rs10,000-50,000	23.5 (47/200)

Gravida >1 % (n/total)	61.5 (123/200)
Parity >1 % (n/total)	32.6 (45/138)*
Previous place of delivery % (n/total)	
Home	7.2 (15/158)*
Hospital	68.4 (143/158)*
Number of antenatal visits ≥5% (n/total)	73.5(147/200)
Placenta Weight(gm) Mean ± SD (Range)	416.35 ± 89.45(223-703)
Newborn	
Birth Weight(kg) Mean ± SD (Range)	2.82 ±0.43(1.8-5.1)
Birth C-R length(cms)Mean ± SD (Range)	30.62 ±1.71(27-38)
Skinfold thickness(cms)Mean ± SD (Range)	1.24 ±0.24 (0.8-2.5)
Head circumference(cms)Mean ± SD (Range)	30.8 ±1.64 (26-36)
Mid circumference(cms)Mean ± SD (Range)	12.75 ±1.32 (8-17)

*History not available for the remaining cases

Kg-Kilograms, BMI-Body mass index, kg/m^2 -Kilograms per meter square, SD- Standard deviation, Rs- Rupees, gm- grams, C-R- Crown-rump , cms-centimeters

Figure 2 shows distribution of the study subjects according to Hb levels. While 41% were non-anaemic, 59% were anaemic, among whom 16% had mild, 35% moderate and 8% severe anaemia.

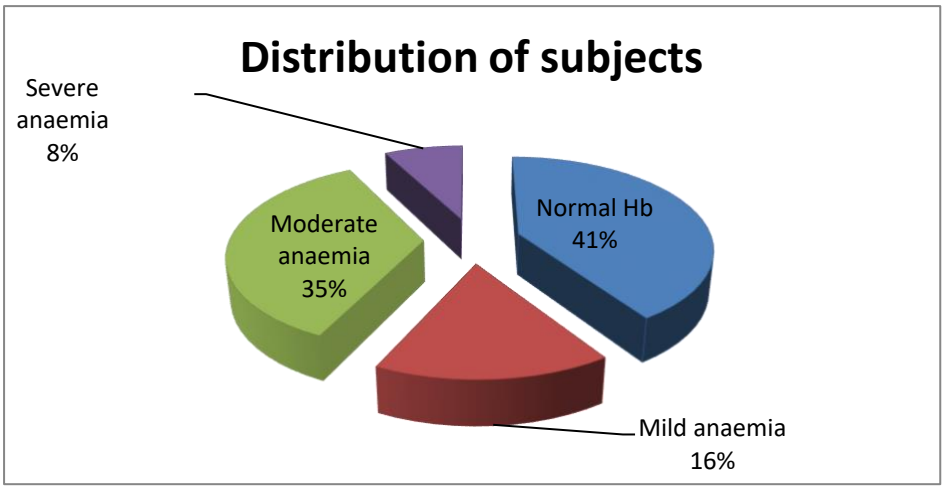

Figure 2: Pie chart showing distribution of subjects with different grades of anaemia.

Table 2 shows the different hematological and biochemical parameters in maternal and cord blood. Hb showed significant ($p<0.001$) and higher levels in the cord blood than mother's blood. Similarly, micronutrients like folate, total Vit B12, Active B12 except CD320 receptor levels (levels significantly lower in cord blood), were found to be higher ($p<0.001$) in cord blood when compared to maternal blood.

Table 2: Comparison of Hb, Vit B12, its active form and its receptors and folate levels in mother's and cord blood

Parameter	**Mothers blood Mean ± SE (Range)**	**Cord blood Mean ± SE (Range)**	***p*-value**
Hb (g/dl)	10.30 ± 0.15 (3.7-15.4)	15.75 ± 0.15 (7.40-23.30)	<0.001
CD320 receptors (pg/ml)	414.09 ±10.75 (155.7-736.2)	372.25 ± 7.81 (231.80-554.70)	0.0024 (<0.01)
Folate (ng/ml)	5.66 ± 0.97 (0.55-74.3)	10.84 ± 0.53 (1.33-23.93)	<0.001
Vitamin B12 (pg/ml)	147.23 ± 26.54 (28.6-1472.2)	286.06 ± 30.18 (21.9-1684.9)	<0.001
Active B12 (Holo-TC)	48.48 ± 3.35	106.67 ± 6.44	<0.001

(pmol/l)	(11.1-228.30)	(19.10-315.10)	

Hb- Hemoglobin, SE- Standard Error, Holo-TC- Holo-transcobolamin, fl- fentolitre, ng/ml-nanogram/milliliter, pg- picogram, pmol-picomoles.

Figure 3 shows the number of mothers and newborns cord blood with different micronutrient levels. While 59.3% mothers were anaemic with Hb levels<11g/dl, only 7.8% of newborns cord blood was deficient in Hb. 54% mothers showed low ferritin levels while only 1.6% of newborns were ferritin deficient. In case of folate, 66% mothers were folate deficient while 7.2% of newborns cord blood was folate deficient.89% of mothers showed Vit B12 deficiency while 53% of newborns cord blood was Vit B12 deficient. With Holo-TC samples, 36.7% mothers showed deficient levels while cord blood samples showed only 9.3% deficiency.

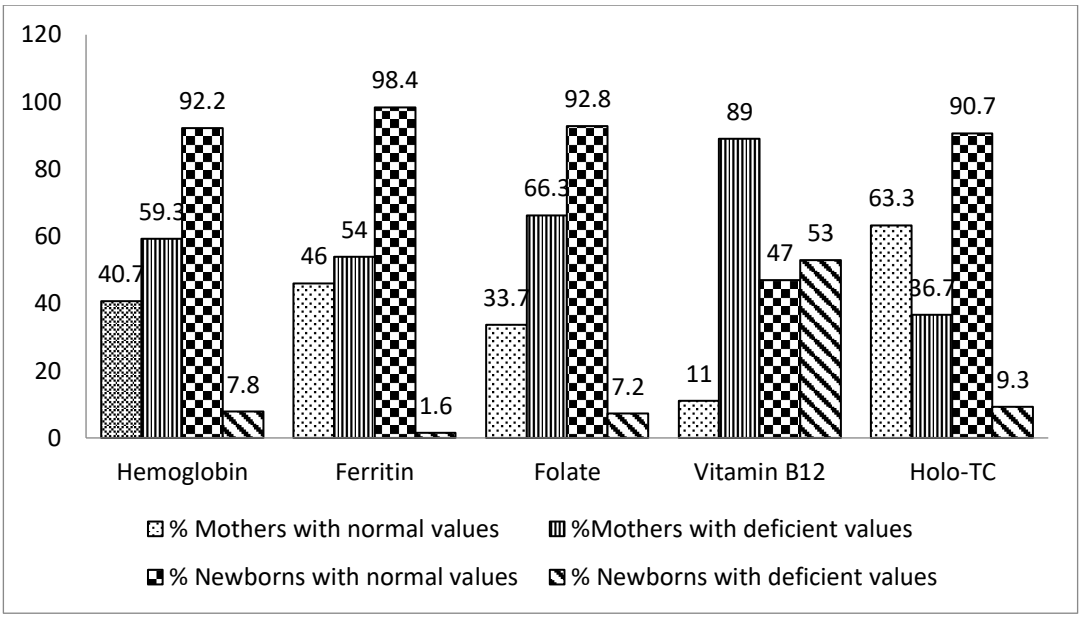

Figure 3: Bar diagram shows the number, in percentage, of mothers and newborns with different micronutrient levels.

Table 3 shows that CD320 levels were higher, albit non-significantly (p=0.19), in mothers who had anaemia when compared those who were normal , but in cord blood the CD320 levels were observed to be low in newborns of mothers suffering from anaemia (p=0.18). Similarly CD320 levels were higher in both mothers and cord blood with Vit B12 deficiency in comparison to those with normal B12 levels. However, CD320 levels were lower in mothers with Active B12 deficiency while in cord blood the levels were higher.

Table 3: CD320 levels in mothers and cord blood in relation to other micronutrient status in mothers blood

Parameter	**Status**	**CD320 in Mothers (pg/ml)**			**CD320 in Cord blood (pg/ml)**		
		Mean± SE	***p*- value**	**Median**	**Mean± SE**	***p*- value**	**Median**
Hb Mothers (g/dl)	**Normal**	391.09± 17.68	0.188	357.70	389.25 ±15.14	0.177	394.70
	Anaemic	422.84± 13.14		412.50	365.67± 9.05		365.10

Ferritin Mothers (ng/ml)	**Normal**	398.97 ±15.19	0.294	381.15	371.83± 12.10	0.908	372.30
	Deficient	421.21± 14.63		405.75	373.68± 10.38		366.65
Folate Mothers (ng/ml)	**Normal**	404.84± 20.58	0.63	369.95	365.13 ±15.68	0.561	364.95
	Deficient	416.45 ±13.64		412.50	376.19± 11.12		373.65
Vit B12 Mothers (pg/ml)	**Normal**	397.94 ±46.64	0.635	332	336.29 ±30.44	0.115	328.30
	Deficient	415.31 ±11.62		407.50	379.42 ±9.19		374.40
Active B12/Holo-TC Mothers (pmol/l)	**Normal**	422.1 ±14.69	0.321	412.05	359.30± 12.62	0.163	363.10
	Deficient	399.98 ±15.43		393.50	385.16± 12.17		387.10

Hb- Hemoglobin, RBC- Red blood cells, PCV- Packed cell volume /hematocrit, MCV – Mean corpuscular volume, RDW- Red cell distribution width, SE- Standard Error of mean, Holo-TC- Holo-transcobolamin, fl- fentolitre, ng/ml- nanogram/milliliter, pg- picogram, pmol- picomoles

Table 4 shows spearman correlation between different blood parameters in mothers and cord blood. CD320 levels in the mothers correlated positively with age, height and weight of mothers but negatively with BMI.CD320 levels of cord blood showed positive correlation with age and height of mothers but negative correlation with weight and BMI of mothers.CD320 levels of mothers showed a negative correlation with Hb, B12, active B12 and folate of the mothers.CD320 levels of the cord blood although showed a positive correlation with Hb of mothers, it showed a significant negative correlation with folate and Vit B12 and a significant negative correlation with active B12 of mothers.Similarly,CD320 levels of cord blood positively correlated with Hb and negatively with folate, Vit B12 and active Vit B12 in cord blood.

Table 4: Spearman correlation between maternal and cord blood parameters

		Hb (M)	Folate (M)	Vit B12 (M)	Active B12 (M)	CD32 0 (M)	Hb (B)	Folate (B)	VitB12 (B)	Active B12 (B)	CD320 (B)
Age (M)	Correlation	.032	.120	-.071	-.002	.009	-.050	-.049	-.121	.092	.048
	Significance	.644	.280	.529	.985	.930	.492	.661	.277	.369	.662
	N	209	83	82	98	98	192	83	83	97	86
Ht (M)	Correlation	.051	-.078	-.153	-.129	.071	-.006	-.051	-.307**	-.122	.026
	Significance	.467	.484	.169	.206	.486	.937	.649	.005	.234	.815
	N	209	83	82	98	98	192	83	83	97	86
Wt (M)	Correlation	.067	.061	.029	.045	.000	-.055	-.050	-.029	.052	-.138
	Significance	.337	.582	.797	.663	.997	.448	.652	.797	.612	.204
	N	207	83	82	98	98	191	83	83	97	86
BMI (M)	Correlation	.044	.110	.126	.109	-.075	-.114	-.033	.126	.077	-.195
	Significance	.523	.322	.258	.286	.463	.116	.768	.257	.452	.072
	N	209	83	82	98	98	192	83	83	97	86
Hb (M)	Correlation	1.00	.536**	.286**	.346**	-.060	.058	.511**	.250*	.174	.088
	Significance	.	.000	.009	.000	.559	.427	.000	.023	.089	.423
	N	209	83	82	98	98	192	83	83	97	86
Folate (M)	Correlation		1.000	.271*	.418**	-.060	.074	.667**	.214	.322**	-.150
	Significance		.	.014	.000	.592	.514	.000	.062	.005	.231

	N		83	82	82	81	81	77	77	76	66
B12 (M)	Correlation			1.000	.686**	-.036	.075	.101	.516**	.496**	-.141
	Significance			.	.000	.754	.508	.384	.000	.000	.262
	N			82	81	80	80	76	76	75	65
Active B12 (M)	Correlation				1.000	-.016	.093	.229*	.593**	.463**	-.256*
	Significance				.	.877	.377	.045	.000	.000	.036
	N				98	95	93	77	77	78	67
CD320 (M)	Correlation					1.000	.064	.126	-.051	-.050	.189
	Significance					.	.545	.278	.661	.665	.125
	N					98	93	76	76	78	67
Hb(B)	Correlation						1.000	-.065	.219*	.137	.130
	Significance						.	.564	.050	.191	.246
	N						192	81	81	93	82
Folate (B)	Correlation							1.000	.180	.172	-.033
	Significance							.	.104	.121	.787
	N							83	83	82	71
Vit B12 (B)	Correlation								1.000	.508**	-.078
	Significance								.	.000	.517
	N								83	82	71
Active B12 (B)	Correlation									1.000	-.027
	Significance								.000	.	.806
	N								82	97	83
CD320 (B)	Correlation										1.000
	Significance										.
	N										86

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).
Hb- Hemoglobin, M- Mother, B- baby, Wt- weight, Ht- height, BMI- Body mass index.

Linear regression (Table 5) analysis was performed to study the predictors of CD320 levels in mothers and cord blood. Maternal CD320 levels were found to be associated with several maternal and neonatal features like education status, occupation and Active B12 status of mothers and MCV, MCH, MCHC, HDW and folate of cord blood. In the multivariate analyses, after adjusting for confounding factors like age, height, weight, BMI, education, occupation, income etc, it was found that poor education, better occupation and higher active B12 values were significantly associated with higher CD320 values in mothers and lower HDW values in cord blood were significantly associated with higher CD320 levels in mothers.

Table 5: Multivariate regression analysis

Parameters	**CD320 of mothers**			
	Univariate (p <0.2)		**Multivariate (p<0.05)**	
	β (95% CI)	***p*-value**	**β (95% CI)**	***p*-value**
Age of mothers	-2.93 (-9.49-3.63)	0.378	-	-
Height of mothers	26.8 (-71.94-125.54)	0.591	-	-
Weight of mothers	-0.302 (-2.87-2.27)	0.816	-	-

BMI mothers	-4.04 (-12.31-4.24)	0.335	-	-
Education status mothers	-33.17 (-65.11--1.22)	**0.042**	-46.52 (-80.2-12.82)	**0.008**
Occupation mothers	88.53 (25.24-151.83)	**0.007**	83.1 (23.17-143.0)	**0.007**
Monthly family income	-1.70 (-40.65-37.25)	0.931	-	-
Parity	13.52 (-28.86-55.89)	0.526	-	-
Hb of mothers	0.420 (-8.97-9.81)	0.929	-	-
RBC mothers	-2.21 (-27.52-23.11)	0.863	-	-
MCV mothers	0.91 (-1.30-3.11)	0.417	-	-
MCH mothers	0.195 (-4.41-4.80)	0.933	-	-
MCHC mothers	-1.50 (-6.07-3.07)	0.516	-	-
HDW mothers	7.83 (-8.46-24.13)	0.341	-	-
Ferritin mothers	-0.18 (-0.96-0.60)	0.654	-	-
Folate mothers	-1.32 (-3.88-1.23)	0.306	-	-
B12 mothers	-0.03 (-0.13-0.06)	0.461	-	-
Active B12 mothers	0.442 (-0.20-1.09)	**0.177**	0.76 (0.06-1.45)	**0.033**
Cord blood Hb	2.76 (-7.66-13.17)	0.601	-	-
Cord blood RBC	-5.81 (-47.91-36.29)	0.784	-	-
MCV cord blood	2.55 (0.31-4.78)	**0.026**	-	-
MCH cord blood	5.84 (-2.67-14.35)	**0.176**	-	-
MCHC cord blood	-6.91 (-14.36-0.54)	**0.069**	-	-
HDW cord blood	-7.29 (-16.55 -1.97)	**0.121**	-8.13 (-16.23—0.031)	**0.049**
Ferritin cord blood	-0.14 (-0.41-0.13)	0.302	-	-
Folate cord blood	3.86 (-0.92-8.65)	**0.112**	-	-
B12 cord blood	-0.002 (-0.09-0.08)	0.971	-	-
Active B12 cord blood	-0.07 (-0.45-0.31)	0.717	-	-
CD320 cord blood	0.19 (-0.17-0.56)	0.300	-	-

Values in bold are statistically significant

BMI- body mass index, Hb- Hemoglobin, RBC- Red blood cells, PCV- Packed cell volume /hematocrit, MCV – Mean corpuscular volume, MCH- mean corpuscular hemoglobin, MCHC- mean corpuscular hemoglobin concentration, HDW- hemoglobin distribution width.

Table 6 and Figures 4 & 5 shows the results of CD320 immunohistochemical expression scores in placentas in the background of different micronutrients levels in mothers. Placental CD320 expression was found to be

higher in mothers **(Fig 4)** with deficient Hb, folate, Vitamin B12 and active B12 levels when compared to those with normal levels**(Fig 3)**, albeit non-significantly.

Table 6: CD320 Immunohistochemistry score in relation to different micronutrients status

Parameter	**Status**	**Minimum score**	**Maximum score**	**Mean(SD)**	**95% CI**	***p*-value**
Hb status	Normal	2	12	6.42 (3.8)	-0.48 to 4.33	0.45
	Anaemia	4	12	8.34 (3.17)		
Folate	Normal	3	12	6.75 (2.60)	-0.17 to 4.44	0.06
	Low folate	3	12	8.89 (3.41)		
Vit B12	Normal	4	12	7.75 (3.45)	-2.40 to 3.08	0.8
	Low Vit B12	3	12	8.09 (3.32)		
Active B12	Normal	3	12	7.59 (3.15)	-1.14 to 3.63	0.6
	Low Active B12	4	12	8.83 (3.56)		

Hb- haemoglobin, SD- standard deviation, CI- confidence interval

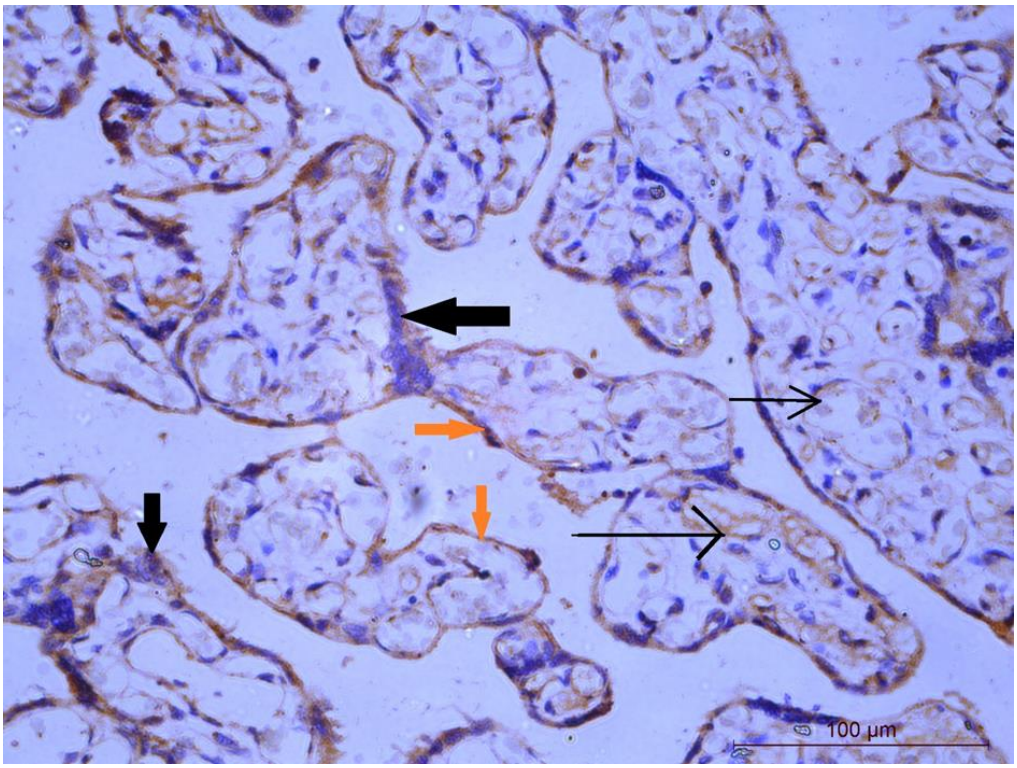

Figure 4: Shows the placental chorionic villi lined by the trophoblasts and which show mild intensity of the immunohistochemical staining for CD320. The bold black arrow points to the syncytiotrophoblasts which are stained mildly, while the bold orange arrows point to the cytotrophobblasts showing similar mild staining. The thin black arrows show mild staining of the fetal capillaries for CD320 immunostain. Original magnification X20.

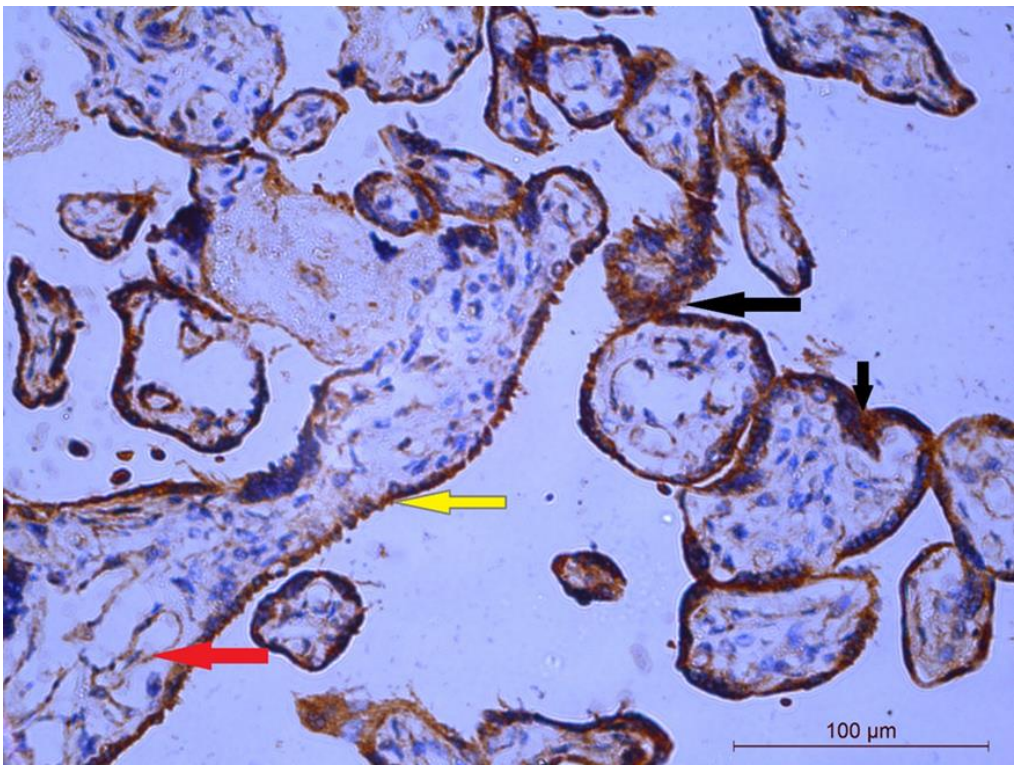

Figure 5: Shows the placental chorionic villi lined by the trophoblasts showing intense immunohistochemical staining for CD320. The bold black arrow points to the syncytiotrophoblasts which are stained intensely, while the bold yellow arrow points to the cytotrophobblasts showing similar strong staining. The bold red arrow shows mild staining of the fetal capillaries for CD320 immunostain. Original magnification X20

4. Discussion

In the present study, pregnant mothers in last trimester of pregnancy were recruited along with their corresponding newborns. They were evaluated for important micronutrients like Vit B12, folate and ferritin with specific reference to the newly discovered Vit B12 receptor CD320.

Anaemia among pregnant women is highly prevalent in our country with the latest National Family Health Survey (NFHS-5) data showing 53.2% pregnant mothers being anaemic (Hb<11g/dl) (NFHS-5 2019-21). Our study too showed an almost similar prevalence of 59% of anaemia in the study participants with 35% of them having moderate anaemia, thus indicating that anaemia was highly prevalent in this vulnerable population despite the distribution of free iron and folic acid tablets by the Indian Government. Figure2 shows the distribution of various micronutrient deficiencies. Although the role played by iron deficiency is considered to be most important in development of nutritional anemia and is also the commonest micronutrient deficiency in developing countries (Krafft 2003), in our study, ferritin deficient mothers were lesser (54%) when compared to folate deficient (66.4%) and total VitB12 deficient (89%). Thus, a high prevalence of Vit B12 deficiency was a notable finding along with folate deficiency in our study. Literature search of different studies from the world and also from India showed that Vit B12 deficiency was highly prevalent in the range of 40-70% [Pathak 2007, Van den Broek 2000, Milman 2006, Katre 2010, Veena 2010). Dr Yajnik et al,2008 have also found folic acid deficiency to be highly prevalent in their study conducted in pregnant women (Yajnik et al,2008) . This could be due to low Vit B12 intake in our country due to vegetarian diet and also that pregnant women are administered only iron and folic acid tablets in pregnancy. But in our study, compared to total VitB12 deficiency, lesser number of women (36.7%) were deficient in active B12.However, the figure shows that in contrast to mother's micronutrient status, except for total VitB12, cord blood showed sufficient levels of Hb, ferritin, folate and active B12 in majority of subjects which is in corroboration with other studies [Rima Obeid 2006, Rima Obeid 2005, Muthayya 2006, Adaikalakoteswari 2015, Murphy 2007, Koc A 2006) and thus indicating that like iron,

Vit B12 and folate are transferred across placenta against a concentration gradient to maintain sufficient levels in the fetus.

CD320 has been recently discovered as the receptor for active B12. As VitB12 deficiency is very common in our country, and despite extensive literature search we couldn't come across any studies till date on the CD320 receptors in the world, we intended to study these receptors in both mothers and cord blood. Total Vit B12 is not considered to be a suitable predictor of Vit B12 levels in pregnant mothers as it gradually declines during pregnancy (Milman 2006, Koebnick 2002). CD320 circulates in the blood mostly bound to active B12 or holo-TC and which is available for tissue uptake and is also suggested to be more sensitive to early changes in the levels of total Vit B12 (Nexo 2002). Our study found higher CD320 levels in mothers with anaemia in comparison to normal mothers but was reverse in cord blood where the CD320 levels were lower in newborns of anaemic mothers. This increased levels of CD320 could be to meet increased requirement due to anaemia in mothers while cord blood had sufficient Hb levels. In case of total Vit B12, folate and ferritin, in both mothers and cord blood, CD320 levels were higher in deficiency state, which could be the compensatory mechanism to meet the increased requirement. However, in contrast, CD320 levels were lower in mothers with active B12 deficiency while in cord blood, the levels were higher from deficient mothers which could be due to the fact that active B12 deficiency was observed to be more in mothers in comparison to cord blood which showed normal active B12 levels in majority. However, there were no other studies elsewhere to compare our study results.

Spearman correlations were performed to study the relation between CD320 levels and various maternal and cord blood parameters. CD320 levels in mothers and cord blood correlated positively with age, height and weight, but negatively with BMI of mothers.CD320 levels of the cord blood were negatively correlated with weight of mothers thus indicating the role of maternal anthropometrical parameters on cord blood CD320 levels although not significantly. With respect to blood parameters, CD320 levels of mothers negatively correlated with Hb , total B12, active B12 and folate of the mothers while those of cord blood positively correlated with Hb of mothers and negatively with folate and total B12 and significantly negatively correlated with active B12 of mothers. However, one study on non-pregnant women showed that the sCD320 levels were positively related to both total B12 and active B12 (Arendt 2012 & Arendt 2012). This is in contrast to our study which was conducted in pregnant women. Similarly, CD320 levels of cord blood positively correlated with Hb and negatively with folate, Vit B12 and active B12 of cord blood. These findings indicate that CD320 levels are mostly negatively related with micronutrient levels, which could be the response to deficiency state probably by increasing the expression of genes encoding CD320 receptors in order to meet the deficient levels. Studies on the genes coding CD320 receptors could be planned to throw light on this matter.

Better occupation of the mothers and higher active B12 values were significantly associated with increased CD320 values in mothers and lower HDW values in cord blood were significantly associated with higher CD320 levels in mothers in multivariate regression analyses while adjusting for maternal age, BMI, gravida, parity, BMI, occupation, family income, Vit B12 and folate levels. Our study being the only study on CD320 in both pregnant women and cord blood, there were no other studies to compare and analyze these findings. However, our study definitely shows the effect of both maternal and cord blood parameters on CD320 levels of both mothers and their newborns.

Expression of CD320 in placentas by immunohistochemistry shows increased expression of CD320, albeit non-significantly, in mothers with deficiency of iron, Vit B12, folate and active B12, thus pointing to definite role played by CD320 in their uptake and transfer from mothers to the developing foetus. CD320 receptors have been stated to be associated with cellular active B12 uptake and their association in our study with all the other micronutrients however point to their non-specific role in relation to active B12 and in turn point to the role of another receptor. Even Arendt et al could not find any association of CD320 levels with clinical findings and they also they did not find any evidence with regard to sCD320 acting as a novel biomarker for VitB12 deficiency [29]. Instead a study by Moestrup et al shows megalin to be the receptor having a high-affinity for the TC-B12 complex. They found that cells take up the Vit B12 after the TCB12 complex binds to megalin, which is followed by uptake of TC by the cells and degradation of TC. Thus, this finding proves the novel role of megalin receptor in Vit B12 homeostasis (Moestrup 1996). Thus, it needs to be followed up by further studies.

There are however a few limitations in our study. Unfortunately, we could not measure urine CD320 levels and also the metabolite of Vit B12, methylmalonic acid (MMA). As CD320 levels are supposed to be more in urine in comparison to serum, its study in urine and also in relation to MMA would have added strength to our study.

To conclude, we found that Vit B12 deficiency was highly prevalent in the pregnant women in our study. CD320 receptors levels were for first time studied in the cord blood and although not significant, a definite correlation was observed between CD320 levels in the mothers and newborn cord blood which needs further evaluation.

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