RESEARCH ARTICLE

GESTATIONAL IRON DEFICIENCY ANEMIA IS NOT SIMPLY A CONSEQUENCE OF IRON DEFICIENCY: AN OBSERVATIONAL STUDY FOR COFOUNDERS.

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Manuscript Info	*Abstract*
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	Objectives: To evaluate serum levels of vitamin D (VD), hepcidin, interleukin (IL)-6 and iron indices in pregnant women **Patients & Methods:** 88 pregnant women fulfilling the inclusion criteria underwent full clinical examination and gave blood samples for determination of hemoglobin concentration (Hb. Conc.), and ELISA estimation of serum 25-hydroxy VD (25OH-VD), ferritin concentration (SFC), hepcidin and IL-6 at the 6th wk gestational age (Booking time) and underwent re-determination of Hb. Conc. at start of the 3rd trimester and Hb. Deficit was calculated. Study outcome included determination of the frequency of iron deficiency (ID), iron deficiency anemia (IDA) and VD deficiency (VDD) and evaluation of the relations between these parameters. **Results:** At booking time, 17 women were anemic (Hb. Conc. <11 gm/dl), while 14 women had SFC<15 ng/ml, for a frequency of anemia, ID and IDA of 19.3%, 15.9% and 11.4%, respectively. At the 3rd trimester, 35 women had Hb. Conc. <11 gm/dl, thus the frequency of IDA was doubled during pregnancy with significantly (p=0.0029) higher frequency of anemic women and significantly (p=0.0007) lower mean Hb. Conc. compared to booking time with a median decrease of Hb. deficit by 4.96% (95% ICR: -7 to -3.23). At booking time, 17 women had insufficient VD levels and 64 women had VDD. At booking Hb. Conc. and deficit was significantly correlated with serum FC, 25OH-VD, hepcidin and IL-6. Serum 25OH-VD level showed significant correlation with SFC, hepcidin and IL-6 levels, and serum hepcidin levels was significantly correlated with serum FC and IL-6. Regression analysis defined at booking high serum hepcidin and IL-6 as significant negative, while high serum 25OH-VD level as significant positive predictor for oncoming 3rd trimester Hb. conc. For prediction of the extent of Hb. deficit, at booking high serum 25OH-VD was negative (β=-0.307, p=0.004), while serum IL-6 level was positive (β=0.235, p=0.026) significant predictors for high deficit. **Conclusion:** Gestational IDA is a progressive condition weakly responding to iron supplemental therapy. IDA is a multi-factorial condition strongly associated with VDD, disturbed immune milieu and deregulated iron homeostasis. VDD plays a crucial role in pathogenesis of ADI.

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Introduction:-

Anemia is a common problem in obstetrics and perinatal care. Hemoglobin concentration below 10.5 g/dl could be considered as true anemia [1]. Iron deficiency (ID) constitutes the main cause of anemia, however, among pregnant women, the frequencies of ID and iron deficiency anemia (IDA) and between the reported prevalence of ADI are highly discrepant, where **Sukrat et al.** [2] documented that according to the WHO criteria (Hb. conc. <11 g/dl) the highest prevalence of anemia and IDA during pregnancy was 14.1% and 6%, respectively. Thereafter, **Siridamrongvattana et al.** [3] reported ID and IDA prevalence of 20.1% and 6%, respectively, of their series of pregnant women. On contrary, **Breymann** [1] documented that worldwide prevalence of ADI ranged between 20%-80% and consists of a primarily female population.

Despite of these figures for ID and IDA during pregnancy, the U.S. Preventive Services Task Force Evidence Syntheses documented that the clinical significance of routine supplemental iron during pregnancy for both pregnant women and infants remains unclear, but could improve maternal hematological indexes [4]. Moreover, there is probable evidence that high intake of heme iron is associated with increased risk of type-2 diabetes and gestational diabetes [5] and **Kinnunen et al.** [6] reported glucose intolerance-related outcome of pregnant women of 13% and 11% with selective or routine iron supplements and the frequency of large-for-gestational-age was 8.3% and 8.2%, respectively.

Hepcidin is a primary phase antibacterial peptide hormone produced in the liver [7]. Hepcidin is the master regulator of systemic iron bioavailability in humans [8]. Inappropriate hepcidin production contributes to the pathogenesis of common iron disorders as its deficiency causes iron overload, while elevated hepcidin levels especially during inflammatory conditions causes iron restriction [9]. Hepcidin regulates iron intestinal absorption, tissue distribution, macrophage iron release and extracellular concentration through its effects on ferroportin (Fpn)-mediated export of cellular iron [10].

Vitamin D (VD) plays a key role in development of gynecological/obstetric diseases [11] and the maternal-fetal unit is under the influence of VD, as the breakdown in VD homeostasis may underlie the development of pre-eclampsia, and gestational diabetes with its subsequent effects [12] and has been associated with a plethora of adverse health effects on the offspring [13].

Supplemental VD therapy during pregnancy is a matter of debit as the current evidence base could not allow definite conclusions regarding the optimal maternal circulating concentration of 25-hydroxyvitamin D (25OH-VD) during pregnancy, and how this might best be achieved [14]. Also, **Pérez-López et al.** [15] found VD supplementation during pregnancy was associated with increased its circulating levels, birth weight, and length, but was not associated with other maternal and neonatal outcomes.

Hypothesis:-
Gestational anemia is a multi-factorial disorder that could not be considered as a mal-nutrition disorder to be corrected by iron supplements. Pregnancy induces disturbed maternal immune milieu that may have an impact on iron homeostasis. Moreover, VD deficiency (VDD) may have an impact on both iron homeostasis and maternal immune milieu.

Objectives:-
The current study targets to evaluate serum levels of VD, hepcidin, IL-6 and iron indices in pregnant women at 1st trimester

Design:-
Clinical prospective observational study

Setting:-
Faculty of Medicine, Benha & Tanta Universities

Patients & Methods:-
The study protocol was approved by the Local Ethical Committee and all enrolled women signed a written fully-informed consent to participate in the study. All women attending the antenatal care unit at Department of Obstetrics and Gynecology, Benha University Hospital for assurance of being pregnant during the period since March 1st till June 30th each year for two years (2016 & 2017) were eligible to evaluation and were asked to attend the clinic fasting on the start of the 6th week gestational age (GA) and then on the start of 3rd trimesters for clinical evaluation and giving fasting blood samples for investigations. At the 6th wk GA, all women had complete history taking and full clinical examination including body height and weight determination and body mass index (BMI) was calculated as weight (kg)/height (m^{2}) [16].

Exclusion criteria included presence of multiple pregnancy, fetal congenital anomalies, current DM or essential hypertension, obesity-inducing endocrinopathy, evident manifestations of hypo-parathyroidism, thyrotocixosis, renal or hepatic diseases and women lost during the course of pregnancy were excluded from the study.

Diagnosis of iron deficiency (ID) was dependent on estimation of serum ferritin level and diagnosis of iron deficiency anemia (IDA) relied on estimation of hemoglobin concentration (Hb conc.). A serum ferritin concentration (SFC) ≤15 ng/ml with Hb conc. ≥11, 10.5 and 11 g/dl during 1st, 2nd and 3rd trimesters, respectively are diagnostic of ID, while Hb conc. <11, 10.5 and 11 g/dl during 1st, 2nd and 3rd trimesters, respectively coupled with SFC< 15 ng/ml indicates IDA but if coupled with SFC ≥15 ng/ml points to an inflammatory state inducing iron stores depletion [17]. All women with Hb conc. <11 gm/dl received iron supplemental therapy in the form of once daily oral ferrous fumarate 350 mg caps (HAEMOTON cap; containing ferrous fumarate 350 mg, vitamin B$_{12}$ 7.5 µg, folic Acid 2 mg, ascorbic acid 50 mg and other vitamins; Glaxo Smith Kline Co., Egypt). Hb conc. was re-estimated at the start of the 3rd trimester and Hb deficit was calculated in relation to Hb conc. estimated at the 1st trimester and number of patients showed down-progression of Hb conc. was determined.

Vitamin D sufficiency status was defined according to 25-OHD concentration as follows: ≥75 nmol/L sufficient level, 50-75 nmol/L insufficient level and <50 nmol/L deficient level. Vitamin D deficiency was categorized as mild, moderate and severe if 25-OHD concentration was 25-50 nmol/L, 12.5-25 nmol/L and <12.5 nmol/L, respectively [18].

Investigations:-
Venous blood samples (5 ml) were collected from the antecubital vein under complete aseptic conditions at booking time and were divided into three parts:
1. The first part was put in EDTA tube (about 1.8 mg trik EDTA/ 1 ml blood) for at once Hb conc. estimation by cyanomethemoglobin method [19].
2. The second part of the sample obtained at booking time was put in a tube containing sodium fluoride (2 mg sodium fluoride/ ml blood) to prevent glycolysis for estimation of blood glucose levels using glucose oxidase method [20].
3. The third part of the sample obtained at booking time was kept in a plane container and allowed to clot then serum was separated by centrifugation at 3000 rpm for 10 min. Serum was removed and placed in pyrogen-free Eppendorf tubes and stored at -70°C until ELISA assayed for
   a. Fasting serum 25OH-VD levels, using an ELISA kit from Cayman Chemical, Ann Arbor, MI, USA [21].
   b. Serum ferritin concentration (SFC), using an ELISA kit from Eurogenetics UK [22].
   c. Serum hepcidin level using ELISA kit from Quantikine r&d systems Inc. Minneapolis MN USA [23].
   d. Serum IL-6 using ELISA kit from Pelikine™ Inc., Concord, USA [24].

Study outcome:-
1. The frequency of ID and IDA among newly pregnant women and the extent of deficit through out duration of pregnancy.
2. The frequency and severity of hypovitaminosis D among newly pregnant women.
3. Evaluation the relation between serum levels of IL-6, pro-inflammatory cytokine, 25OH-VD and hepcidin, a modulator of iron storing process, and frequency of IDA and pregnancy-induced deficits in ID.
4. The impact of iron supplemental therapy on the studied parameters.

Statistical analysis:-
Obtained data were presented as mean±SD, numbers and percentages. Results were analyzed using paired t-test and Chi-square test (X^2 test). Possible relationships were investigated using Pearson's linear regression. Data were analyzed using Regression analysis (Stepwise Method) to define the persistently significant predictors for oncoming Hb conc. and Hb deficit in pregnant women. Statistical analysis was conducted using the IBM SPSS (Version 23, 2015) for Windows statistical package. P value <0.05 was considered statistically significant.

Results:-
The study included 117 pregnant women eligible for evaluation; 29 were excluded and 88 were enrolled in the study. Enrolment data of studied women are shown in table 1.

Table 1:-Patients' enrolment data

Data			Findings
Age (years)			28.1±2.7
Body mass index data	Weight (kg)		78.3±7.9
	Height (cm)		168.8±3.3
	BMI (kg/m^2)	Average (<24.9)	27 (30.7%)
		Overweight (25-29.9)	54 (61.4%)
		Obese (30-34.9)	7 (7.9%)
		Mean (±SD)	27.5±2.8
Obstetric history	Gravidity	Primi	23 (26.1%)
		Multi	65 (73.9%)
	Parity	Para-1	27 (30.7%)
		Para-2	38 (43.2%)
Blood pressure (mmHg)	Systolic		114.3±9
	Diastolic		72±4.6
Fasting blood glucose (mg/dl)			98.8±5.1

According to Hb conc. estimated at booking time, 17 women were anemic with Hb. Conc. <11 gm/dl, while 71 women had Hb. Conc. >11 gm/l. As regards ID, 14 women had SFC <15 ng/ml and 74 women had SF ≥15 ng/ml. Thus, 10 women had IDA with SF<15 ng/ml and Hb. Conc. <11 gm/dl, 4 women had ID with SF<15 ng/ml and Hb. Conc. >11 gm/dl, 7 women had anemia with Hb. Conc. <11 gm/dl, but SF ≥15 ng/ml and 67 had accepted levels with SF≥15 ng/ml and Hb. Conc. >11 gm/dl.

At the start of the 3rd trimester, 35 women had Hb. Conc. <11 gm/dl, while 53 had Hb. Conc. >11 gm/dl, (Fig. 1) thus the frequency of women developed or progressed from ID to IDA was doubled throughout the duration of pregnancy with significantly (p=0.0029) higher frequency of anemic women compared to at booking time. Mean estimated Hb. Conc. at the start of 3rd trimester was significantly (p=0.0007) lower Hb. Conc. compared to that estimated at booking time. Concerning change of Hb. Conc., 8 women had higher estimates, while 80 women had decreased estimates at the 3rd trimester compared to their booking estimates with a median decrease by 4.96% (95% ICR: -7 to -3.23).

Figure 1: Consort Flow sheet
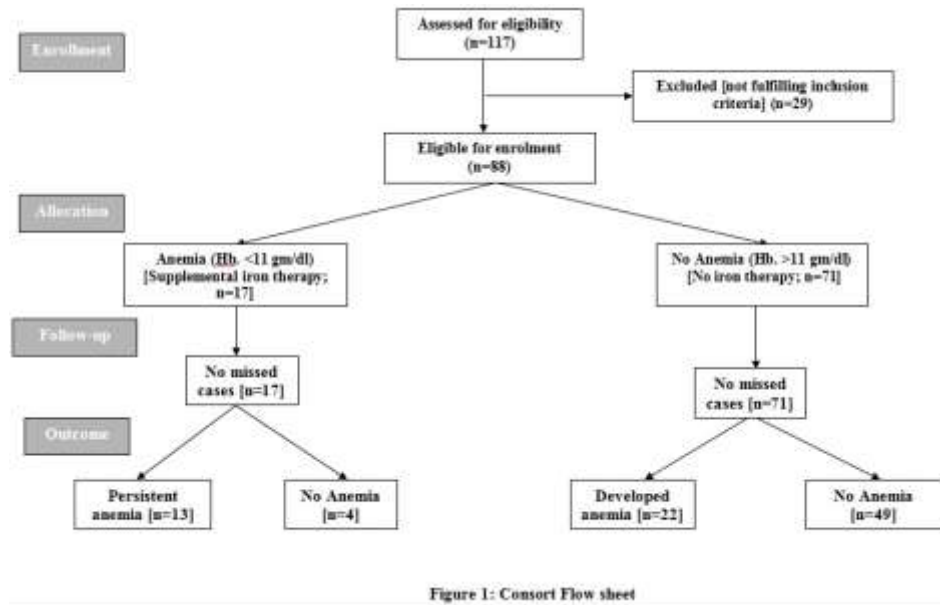
At booking time, estimated serum 25OH-VD defined 7 women with sufficient levels (>75 nmol/ml), 17 women had insufficient levels (50-74.9 nmol/ml), 61 women had mild deficiency (25-49.9 nmol/ml) and three women had moderate deficiency (<25 nmol/ml) with a mean serum 25OH-VD level of 43.94±16.46; range: 19.5-79.4 nmol/ml. Mean estimated serum hepcidin was 20.87±2.98; range: 16.72-28.96 ng/ml and mean level of IL-6 was 32.56±13.33; range: 10-65 ng/ml.

At booking Hb. Conc. showed positive correlation with serum FC and 25OH-VD levels, while showed negative significant correlation with serum hepcidin and IL-6. On contrary, Hb. deficit between Hb. conc. at start of 3rd trimester to at booking time showed positive significant correlation with serum hepcidin and IL-6, while showed negative significant correlation with serum 25OH-VD level and negative non-significant correlation with SFC levels. Interestingly, serum 25OH-VD level showed significant correlation with SFC, hepcidin and IL-6 levels that was positive with SFC, but was negative with hepcidin and IL-6 levels. Serum hepcidin levels showed significant correlation with both SFC and IL-6 levels that was negative and positive, respectively (Table 3).

Table 3:-Pearson's correlations between studied variables

Variables	SFC		25-OH VD		Hepcidin		IL-6	
	r	p	r	p	r	p	r	p
At booking Hb. Conc.	0.580	0.0006	0.338	0.001	-0.507	0.0008	-0.362	0.001
Hb. Deficit	-0.110	0.307	-0.388	0.0008	0.248	0.020	0.341	0.001
SFC			0.293	0.006	-0.504	0.0007	-0.192	0.073
25OH-VD					-0.307	0.004	-0.346	0.001
Hepcidin							0.326	0.002

Hb. Conc.: Hemoglobin concentration; SFC: Serum ferritin concentration; 25OH-VD: 25-hydroxy vitamin D; IL-6: Interleukin 6

Evaluation of booking time estimated parameters as predictors for 3rd trimester Hb. Conc. using Regression analysis defined high serum hepcidin as the persistently significant predictor, followed by high serum 25OH-VD level as a positive significant predictor, and high serum IL-6 levels as negative predictors for oncoming 3rd trimester Hb. conc. For prediction of the extent of Hb. deficit Regression analysis defined at booking high serum 25OH-VD as the negative (β=-0.307, p=0.004), while serum IL-6 level as the positive (β=0.235, p=0.026) significant predictors for high deficit (Table 4).

Table 4:-Regression analysis for at booking estimated parameters as predictors for the 3rd trimester Hb. Conc.

Analytical model	At booking parameters	Standardized coefficient	p
Model 1	Hepcidin	-0.219	0.031
	25-OH VD	0.217	0.020
	IL-6	-0.247	0.008
	SFC	0.256	0.010
Model 2	Hepcidin	-3.641	0.0007
	25-OH VD	2.754	0.007
	IL-6	-2.595	0.011
Model 3	Hepcidin	-4.272	0.0005
	25-OH VD	3.485	0.001
Model 4	Hepcidin	-5.282	0.0003

25OH-VD: 25-hydroxy vitamin D; IL-6: Interleukin 6

Kaplan-Meier regression analysis for defining the cutoff point of parameters estimated at booking time for prediction of hazard for development or progression from ID to IDA defined SFS at <16 ng/ml (Fig. 2), serum 25OH-VD at <50 nmol/ml (Fig. 3), serum hepcidin and IL-6 at >20 ng/ml (Fig. 4) and 27 ng/ml (Fig. 5) as cutoff points for prediction of high possibility for decreased Hb. conc. at the 3rd trimester, development or progression from ID to IDA.
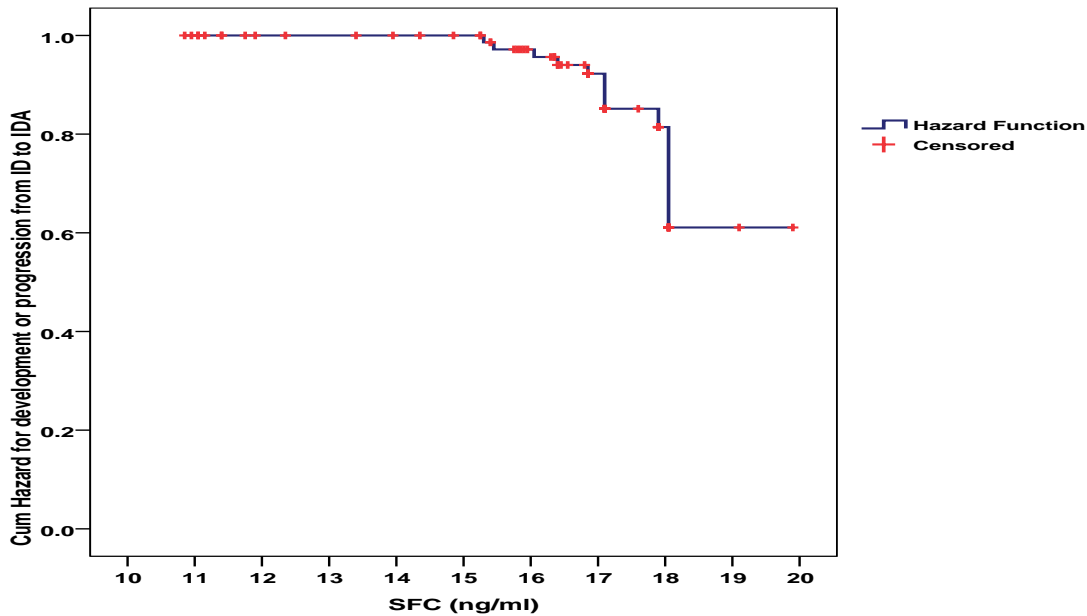
Fig. 2:-Kaplan-Meier regression analysis for defining the cutoff point of SFC for prediction of for development or progression from ID to IDA.
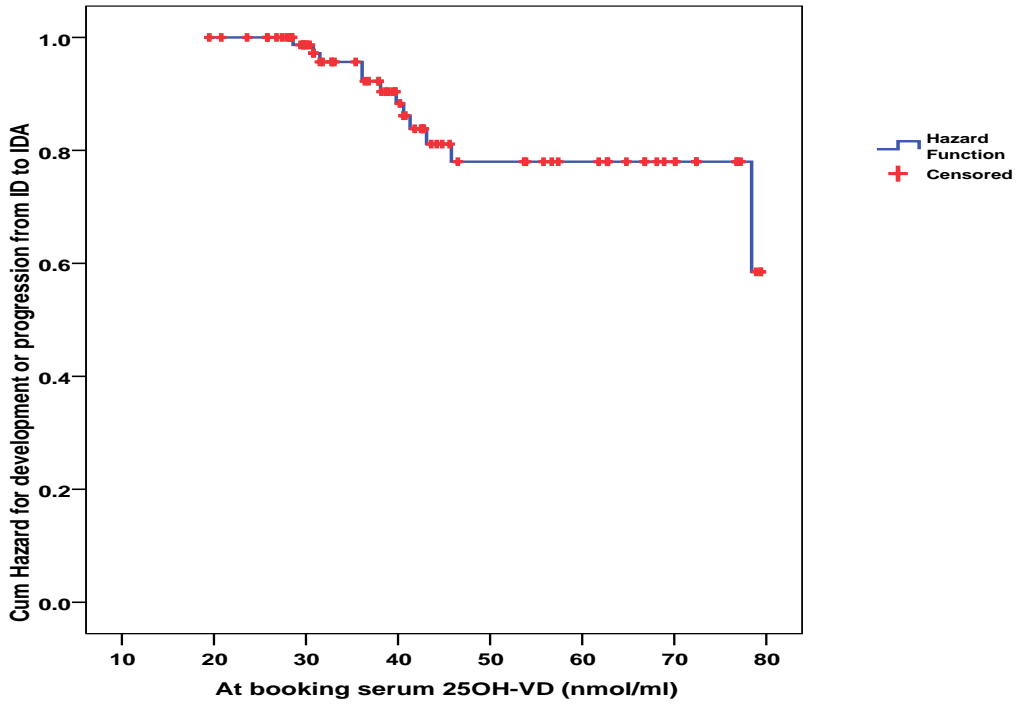
Fig. 3:-Kaplan-Meier regression analysis for defining the cutoff point of serum 25OH-VD for prediction of for development or progression from ID to IDA.
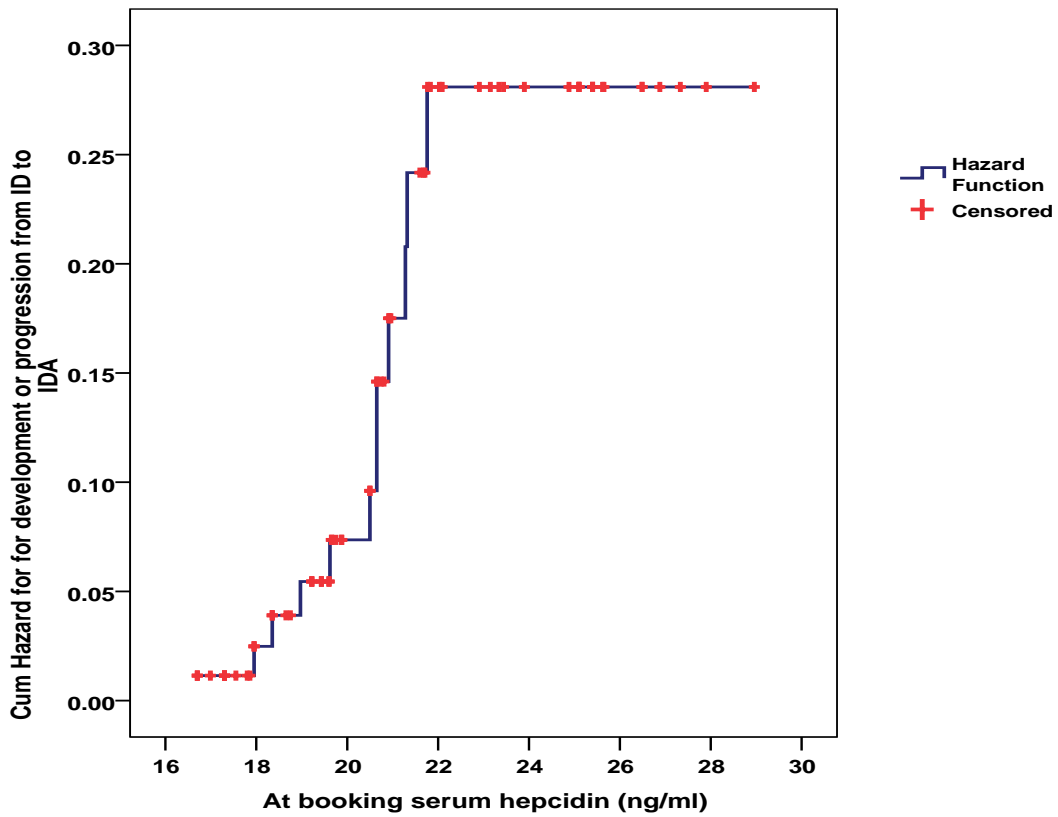

Fig. 4:-Kaplan-Meier regression analysis for defining the cutoff point of serum hepcidin for prediction of development or progression from ID to IDA.
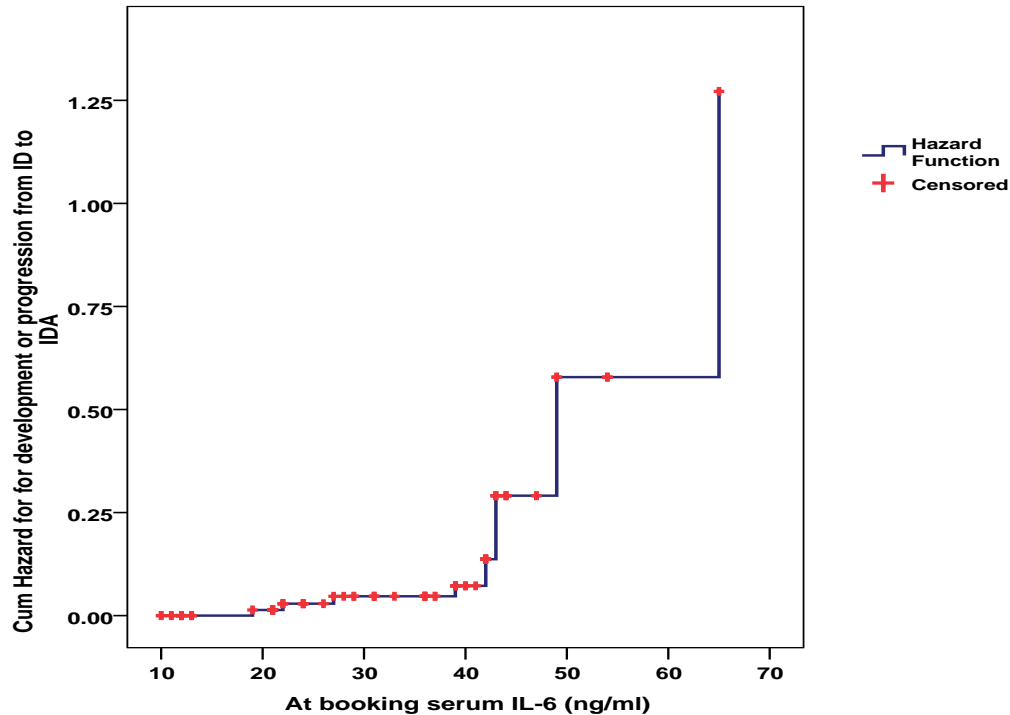
Fig. 5:-Kaplan-Meier regression analysis for defining the cutoff point of serum IL-6 for prediction of for development or progression from ID to IDA

Discussion:-

At booking time, during 1st trimester, 17 women were anemic (Hb. Conc. <11 gm/dl), while 14 women had SFC<15 ng/ml, for a frequency of anemia, ID and IDA of 19.3%, 15.9% and 11.4%. These figures coincided with that previously reported by **Sukrat et al.** [2] and **Siridamrongvattana et al.** [3] and with the recently reported by **Nguyen et al.** [25] who found 20% of pregnant women were anemic, 14% had low iron stores and 3% had IDA and by **Okafor et al.** [26] who detected ID and IDA in 20% and 17.9% of pregnant women.

Despite of supplemental iron therapy, 35 women (39.8%) had Hb. Conc. <11 gm/dl at the 3rd trimester, thus the frequency of women developed IDA or progressed from ID to IDA was doubled during pregnancy and mean estimated Hb. Conc. was decreased by a median value of 4.96% (95% ICR: -7 to -3.23). Only 8 women (9.1%) showed good response to supplemental therapy by increased Hb. conc. in comparison to their previous estimated.

These findings point to the interplay of multiple factors, other than ID, for development of gestational IDA, so fulfilling the study hypothesis. In support of this assumption, the reported high prevalence of VDD, elevated serum hepcidin and IL-6 levels in the studied women at time of booking. Moreover, at booking Hb. Conc. was positively correlated with serum levels of 25OH-VD, but negatively correlated with serum hepcidin and IL-6 levels. Moreover, Hb. deficit showed positive association with serum hepcidin and IL-6, while showed negative association with serum 25OH-VD levels.

In line with these findings, **Thomas et al.** [27] detected a positive correlation between maternal 25OH-VD and hemoglobin at both mid-gestation and at delivery. Recently, **Yuan et al.** [28] found 25OH-VD concentrations was significantly lower in women affected with anemia than controls and the risk of anemia was significantly increased with decreasing serum 25OH-VD concentrations in a dose-dependent manner up to 80% increase in anemia risk in women with 25OH-VD concentrations <50 nmol/L.

Multiple studies tried to investigate the relation between VDD and IDA, **Zittermann et al.** [29] supposed that VDD contributes to decreasing bone marrow local production of calcitriol with increasing calcium membrane permeability, so erythropoiesis declines. These changes as evidenced by *In vitro* studies occur at mRNA and protein

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levels [30]. **Sharma et al.** [31] supposed that hyperparathyroidism secondary to VDD increases erythroid progenitor cells proliferation. Moreover, **Thomas et al.** [27] found maternal 25OH-VD was inversely associated with erythropoietin at both mid-gestation and delivery and attributed the effect of VDD on Hb. conc. to both a direct effect and an indirect effect mediated by erythropoietin.

Interestingly, the current study detected a negative association between levels of 25OH-VD and hepcidin. On basis of hepcidin regulatory action on iron homeostasis through its effects on ferroportin (Fpn)-mediated export of cellular iron through binding to Fpn and inducing its internalization [10], thus the reported positive association between 25OH-VD levels and both Hb conc. and SFC levels could be attributed to a modulatory effect for VD on hepcidin/Fpn pathway inducing more iron release, thus increasing SFC, to be available for more synthesis of hemoglobin, so increasing Hb conc. These findings and assumption supported that previously reported by **Bacchetta et al.** [10] who documented that VD is a potent regulator of the hepcidin-Fpn axis in humans and **Azizi-Soleiman et al.** [32] who found VDD is associated with higher hepcidin level in the body. **Smith & Tangpricha** [33] attributed the regulatory effect of VD on hepcidin/Fpn axis to a direct suppression of hepcidin mRNA transcription.

The reported data concerning IL-6 points to the possibility for the presence of evident or hidden infection or simply representing disturbed immune response to pregnancy. In accordance with this assumption, **Wirth et al.** [34] studied cross-sectional, nationally representative data from 10 surveys and detected anemia prevalence of ~40% among women in the reproductive age in countries with high infection burden and 12% and 7% in moderate and low infection burdens countries and found the proportion of ID anemic women was lower in high-infection (35%) than in moderate- (65%) and low-infection (71%) countries, so concluded that the contribution of ID to anemia varies according to a country's infection burden.

Moreover, estimated IL-6 levels were positively correlated with serum hepcidin, but negatively correlated with serum 25OH-VD, thus closing the triangle of danger inducing and maintaining IDA despite of supplemental iron. In line with these data, **Paesano et al.** [35, 36] found bovine lactoferrin established iron homeostasis by modulating serum IL-6 and pro-hepcidin synthesis, whereas ferrous sulfate increased IL-6 and failed to increase hematological parameters and prohepcidin. Also, **Lee et al.** [37] detected high ferritin and hepcidin concentrations and considered these high levels as a consequence of inflammation because IL-6 concentrations at delivery were 1.6-fold higher than mid-gestation levels with a positive association between IL-6 and both hepcidin and ferritin.

The pathogenic mechanism for inflammatory anemia was explored in vitro and in vivo by **Cutone et al.** [38] who found increased IL-6 and IL-1β is associated with up-regulation of cytosolic ferritin and down-regulation of Fpn leading to intracellular iron overload leading in vivo to higher host susceptibility to infections and ID in the blood and anemia of inflammation. Furthermore, **Rosa et al.** [39] reported that bovine lactoferrin through its anti-inflammatory activity against IL-6 cures anemia of inflammation through up-regulating Fpn and transferrin receptor 1, while down-regulating ferritin and consequently inhibits intracellular iron overload with increased availability of iron for erythropoiesis

Conclusion:-
Gestational iron deficiency anemia is a progressive condition weakly responding to iron supplemental therapy. IDA is a multi-factorial condition strongly associated with VDD, disturbed immune milieu and deregulated iron homeostasis. VDD plays a crucial role in pathogenesis of ADI, so wider scale studies were mandatory to evaluate the effect of supplemental VD therapy on ADI and if this effect is dose-dependent and whether additional supplemental iron therapy is needed or not.

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