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Original Research Article

Role of Luteal Phase Serum Biomarkers in Predicting Pregnancy Outcome in ICSI Cycles: A Prospective Cohort Study

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Conflict of interest: Nil

Abstract:

Introduction: Pregnancies achieved by IVF-ET cycles are at increased risk of adverse outcomes when compared with natural pregnancies or those achieved by ovum donation cycles. The aim of this study is to evaluate the prognostic value of serum biomarkers progesterone and estradiol and to determine their optimal cut-off values at seven days post ET in predicting pregnancy outcome in women undergoing ICSI cycles.

Methods and analysis: This is a prospective cohort study that was conducted at KJIVF. All patients with definite indication for ICSI are included. Standard protocol and procedures were observed as per patient protocol. Inclusion criteria: All patients undergoing ICSI. Exclusion criteria: All patients undergoing FET, Embryo Donor or Egg Donor Programs. All patients received progesterone and estrogen support after ET. Serum progesterone levels and estradiol levels were measured at Day 7 post ET.

Hormone assays: Serum progesterone and estradiol is measured by ELISA in units of ng/ml and pg/ml respectively.

Results: AUC of E2 is .722, AUC of P4 is .784 well within 95% confidence interval which emphasizes on significance of predicting the pregnancy outcome.

Conclusion: A significant correlation was found between day 7 level of progesterone and estradiol and pregnancy outcome post ET.

Introduction

Couples undergoing ART are under lot of stress and anxiety about the treatment outcome. It is well known that pregnancies achieved by IVF-ET cycles are at increased risk of adverse outcomes when compared with natural pregnancies or those achieved by ovum donation cycles.

Thus, in ART centers where counseling of patients is so much emphasized on, prediction of early pregnancy outcome is of

interest both to clinician and couples. Until date, Serum beta hcg has been widely used as a hormone predictor. Therefore, there is need for any other marker and prove its predictive accuracy.

Corpus luteum plays an important role in early pregnancy and its activity can possibly be a marker. Progesterone being the hormone of uterine receptivity and a strong marker in natural conception cycle but in IVF cycles, its role is little studied. Serum estradiol after ET can too be investigated as a predictor of pregnancy outcome. For a normal endometrium, an E2 priming is necessary which up-regulates the progesterone receptors.

Both serum progesterone and estradiol helps in degranulation of mast cells, releasing cytokines and growth factors increasing the endometrial receptivity. In stimulated cycles there is iatrogenic luteal phase defect. Any early estrogen rise in conception cycles in luteal phase denotes stimulated embryos. To study the prognostic value of serum biomarkers progesterone and estradiol 7 days post ET in predicting pregnancy outcome in women undergoing ICSI cycles. To determine optimal cut-off values for serum progesterone and oestradiol levels for predicting pregnancy outcome after ICSI cycles at day 7 post embryo transfer.

Methods

Study Design

Type of study: Prospective single centre cohort study

Place of study: KJIVF and Laparoscopy Centre, Delhi.

Study period: 6 months (7th August 2017 - 31st January 2018).

Literature search:

Systematic reviews, meta-analyses databases, articles were searched including Medline, Pubmed, Cochrane Library as references. Both the studies, those who reported and those who did not report a cut off value or proposed results only by giving elevated or non-elevated levels of progesterone and estradiol for predicting outcome of pregnancy were included.

Inclusion criteria-

All patients undergoing ICSI.

Exclusion criteria-

All patients undergoing FET, Embryo Donor, Egg Donor Programs.

Sample size

Sample size was calculated by PS (power and sample size calculator Vs 3.1.2 a minimum of 30 subjects could be taken.

The sample size is 50.

Method of study

All patients with definite indication for ICSI were included in the study after fully written informed consent. A detailed medical, gynaecological history followed by thorough physical examination was done. Agonist, antagonist or ultra-long agonist protocol was followed according to individual patients. Gonadotropins (individualized dose) were started on day 2/3 of cycle, Timed hcg trigger was given when at least 3 follicle reached 17 mm diameter. On day 13 OPU done 36 hrs after trigger. All the oocytes obtained after retrieval screened, treated and then placed in the C02 incubators. Zygotes with 2PN were cultured in the fertilization media in the pre-equilibrated microdrops of 50 µL (sage) overlaid with 100µL of mineral oil in each well of the petri dishes at 37°C under 6% CO_2. After fertilisation standard D3 embryo transfer was done. The number of embryos transferred depended on factors such as patient's age, outcome in previous assisted reproductive technology attempts and patient's preference. The morphological grading of embryos was done. Basis of gradation was cleavage rates, percentage of fragmented blastomeres, and how uniform were the blastomeres. Good-quality embryos are defined as those of morphological grades I-II/V; embryos should have blastomeres of equal size with no or minor cytoplasmic fragmentation or blebs. Three optimum quality embryos were selected out of the lot of embryos and carefully transferred into the

uterus. All patients received luteal phase support after ART in combination of injectable progesterone 100 mg i/m on alternate days, oral estradiol valerate 2 mg thrice daily and micronized progesterone 300 mg intravaginal twice daily from the day of embryo transfer. Serum progesterone and estradiol levels were measured on D7 post ET. A confirmatory beta hcg was done 16 days after ET. All women with positive beta hcg >50 iu /l were called for TVS after one week. Ultrasonic scans were performed using a 7 mHz vaginal transducer. Luteal support was continued for those who became pregnant until 12 wks.

Hormone assays

Serum Estradiol- It is measured by ECLIA; roche (Electrochemiluminescence immunoassay) in pg/ml. The assay sensitivity was 5 pg /ml. Intra and inter assay cv was <20%.

Serum Progesterone- Its measuring range was 0.03 to 60 ng/ml by CMIA; Abbott (Chemiluscent magnetic microparticle assay). Samples with values >60 ng /ml, underwent dilution of (1:10). Electrolyte diluent was used for dilution. The dilution factor was used for calculation for multiplication to give the results. The intra assay CV were 1.5% to 2.7% and the interassay CV was 4.1% to 5.5% and this was already standardized.

However, large inter-method differences have been described for serum HCG, oestradiol and progesterone (Cole *et al*., 2004). Therefore, method-specific cut-off values are mandatory.

Amount of blood taken for the test is 3 ml by routine venipuncture and collected in red plain vacutainer and send within 1 hour of collection to the lab. Minimum processed serum sample should be 1 ml. Clotted blood specimens were taken and then were allowed to centrifuge at 3000g for 10 min such that the serum gets separated. Results were available within 2-6 hrs.

Outcomes:

Broadly classified into nonconception and conception outcomes.

Conception outcomes are further subcategorized as:

1) **biochemical pregnancy** – Beta Hcg value being >50 mIU/ml with no gestational sac in TVS.
2) **clinical pregnancy** – Beta Hcg value being >50mIU/ml with gestational sac seen in TVS.

Ethical consideration

- Consent (ANNEXURE 1, 2)
- Scientific committee at centre – cleared
- IEC IFS CLEARANCE: cleared

Financial consideration

Routine tests like serum estradiol and serum progesterone costs Rs 1,030/-. However, the burden of cost was borne by the centre**.**

Statistical analysis

The quantitative variables are expressed in terms of mean ±sd and compared between groups using Student's unpaired t test and Mann Whitney U test were used to assess the significance of difference between two groups. Correlation between quantitative variables is assessed using Pearson's correlation coefficient. A p-value < 0.05 holds a significant statistical value and derived as per chi square analysis. ROCs curves were derived in order to prove accuracy of serum biomarkers in predicting the outcomes most appropriately. The comparison between all the three biomarkers for predicting the outcome most accurately was also studied using these curves. ROC curve analysis was performed to determine optimal cut-off values. SPSS version 16.0 software© for windows™ Vs 17, IBM™ Corp NY and Microsoft excel™ 2007, Microsoft® Inc USA was used perform the statistical analysis.

Results

Table 1: General Characteristics of Study Subjects.

Characteristics		Value
Age (Yrs) (Mean± S.D.)		31.77 ± 4.6
Infertility N (%)	Primary	38 (76)
	Secondary	12 (24)
Protocol	Agonist	32 (64)
	Antagonist	16 (32)
	ultra-long agonist	2 (4)
POST ET D7 E2 (Median (range))		272.3 (34.7-2329.34)
POST ET D7 P4 (Mean± S.D.)		42.73 ± 33.9
BETA HCG (IU/ml) (Median (range))		24.71 (2-4708)
Outcome	Negative	27(54)
	Positive	23(46)

Table 1: Represents general characteristics of study subjects. Mean age was found to be 31.77 years. Primary infertility was present in 76% subjects while rest were having secondary infertility. Agonist protocol was used with highest frequency in 64% cases, while antagonist protocol was used in 32% subjects. Outcome was found to be positive in 46% subjects.

Table 2: Types of Infertility in Study Subjects.

Infertility	No. of subjects	Percentage
Primary	39	78
Secondary	11	22

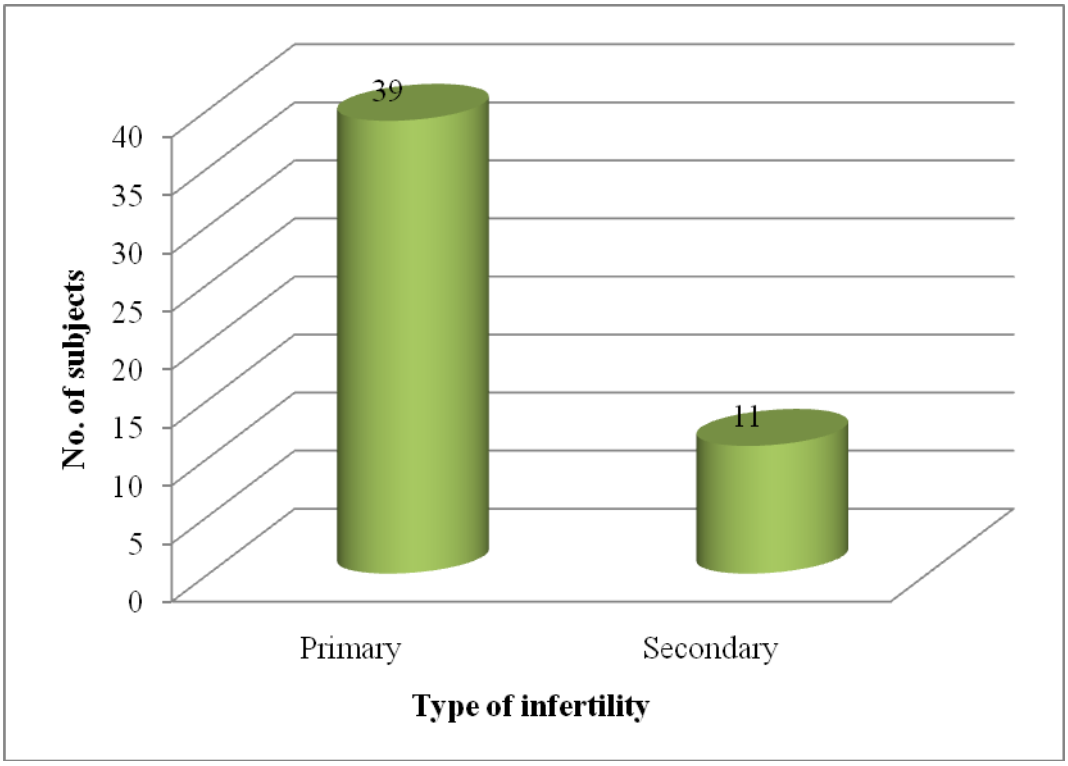


FIG 1: Types of Infertility in Study Subjects.

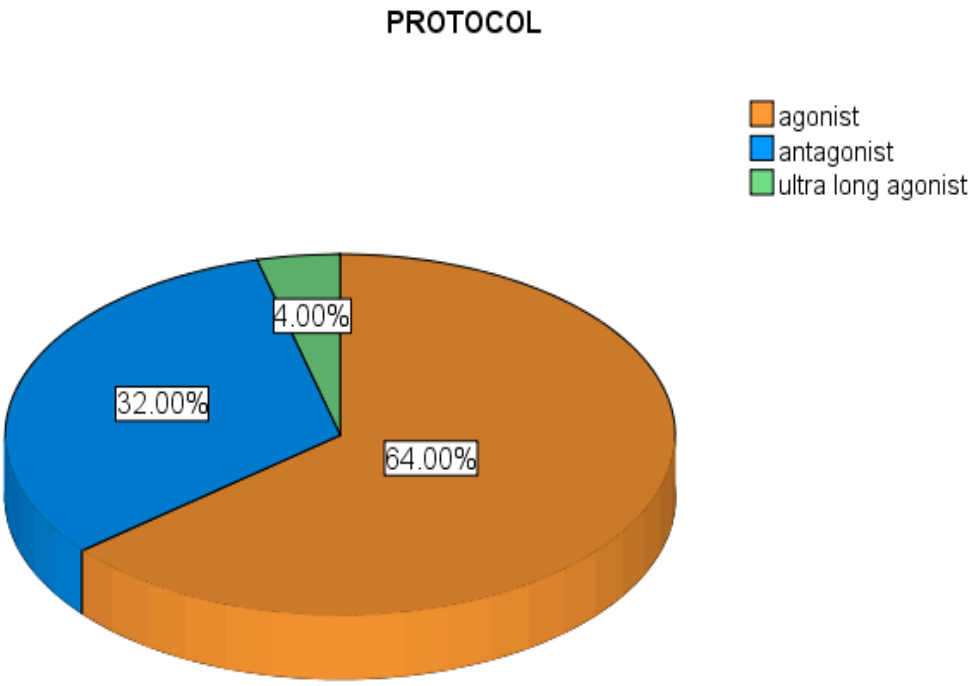


FIG 2: Distribution of Protocols used in subjects

Figure 2 showing 64% of the patients underwent long agonist protocol & 32% underwent ultralong agonist protocols.

Table 3: Comparison of Various Characteristics Between Positive and Negative Outcome.

Characteristics		Outcome		
		Negative	**Positive**	
Age (Yrs)		32.3 ± 5.6	31.05 ± 2.89	0.382
Infertility	Primary	18	20	0.089
	Secondary	9	3	
Protocol	Agonist	18	14	0.310
	Antagonist	9	7	
	Ultralong agonist	0	2	
POST ET D7 E2		221 (34.72-2329.34)	508.6 (132-1415.08).	0.043
POST ET D7 P4		24.8 (1.52-69.27)	53 (15.02-198.90)	0.001
Beta HCG (IU/ml)		2.00 (2.00-26.2)	711 (23.23-4708)	<0.0001

Table 3: Denotes Comparison of various characteristics between positive and negative outcome. It was found that both study groups were matched for age. No significant association of outcome with infertility, or protocol followed was detected. Subjects with positive outcome showed significantly higher POST ET D7 P4, post ET D7 E2 and significantly higher Beta HCG levels.

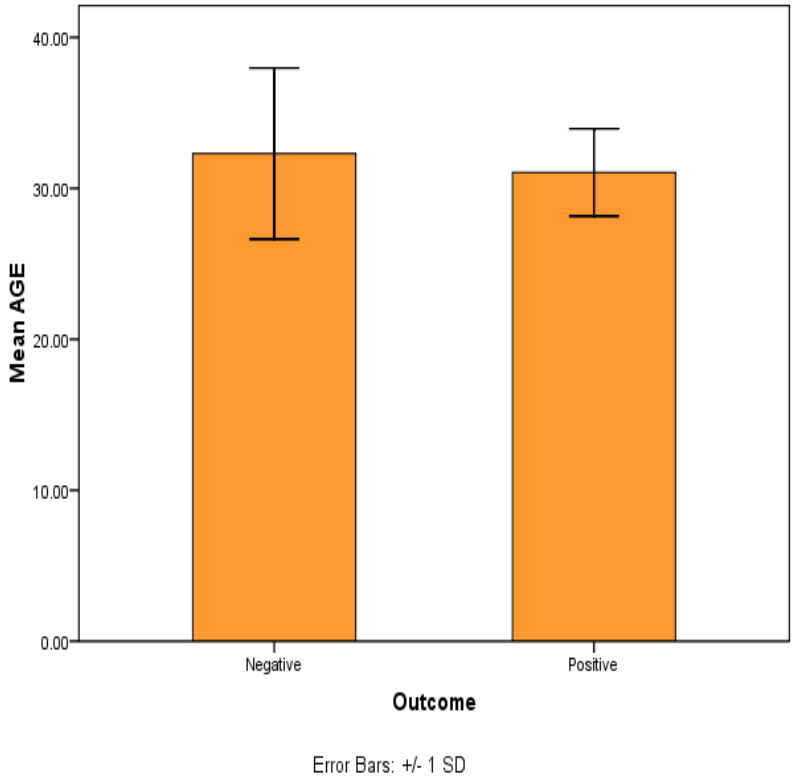


Fig 3: Mean Age in Both the Groups.

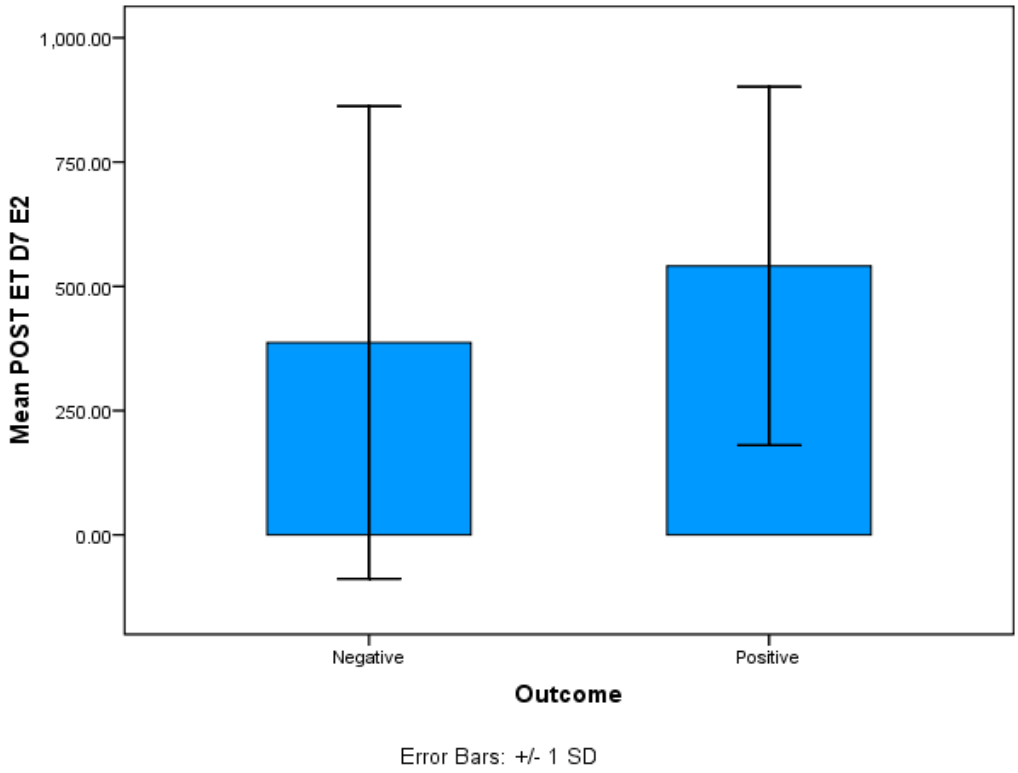


FIG 4: Mean E2 Level In Positive and Negative Outcome.

Figure 4 shows higher mean E2 level value in positive outcome group.

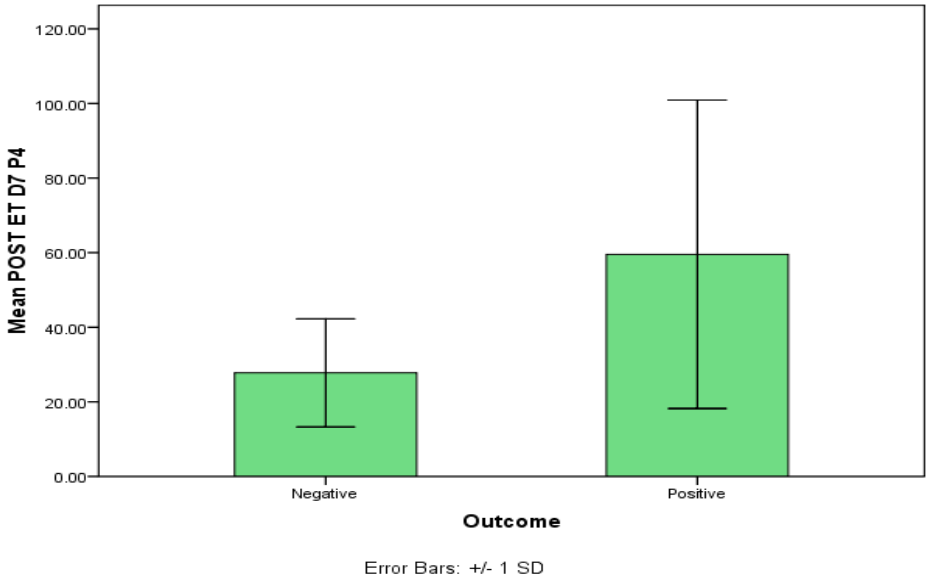


FIG 5: Mean P4 Level In Outcome Groups.

Figure 5 shows higher mean P2 level value in positive outcome group.

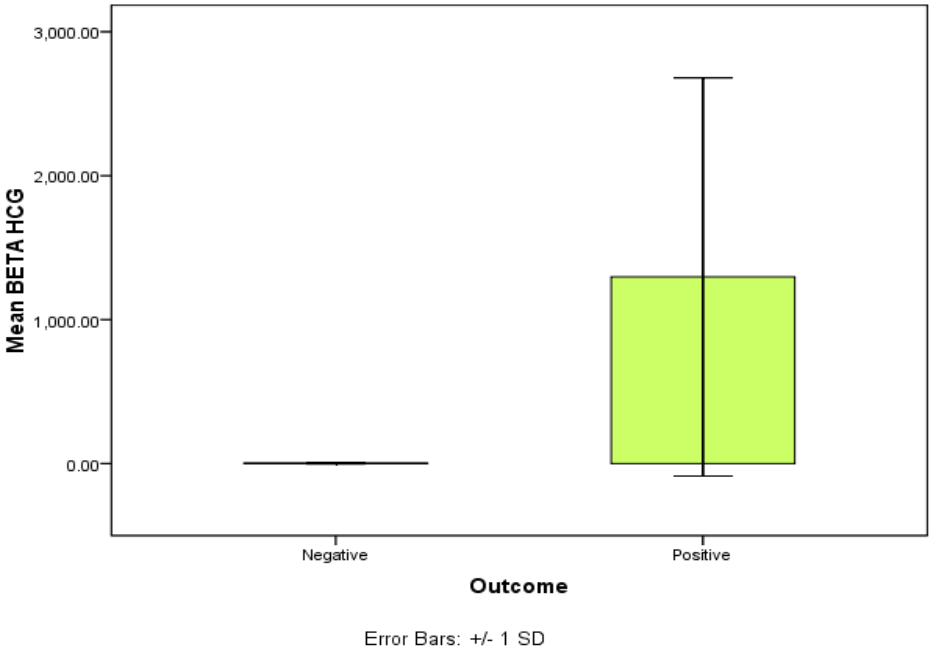


FIG 6: Mean Beta HCG In Outcome Groups.

Figure 6 showing higher mean Beta HCG level value in positive outcome group.

Table 4: Causes of infertility

Tubal	**27**	**54**
Endometrial	6	12
Dor	12	24
Sub-ovulation	11	22
POR	4	8

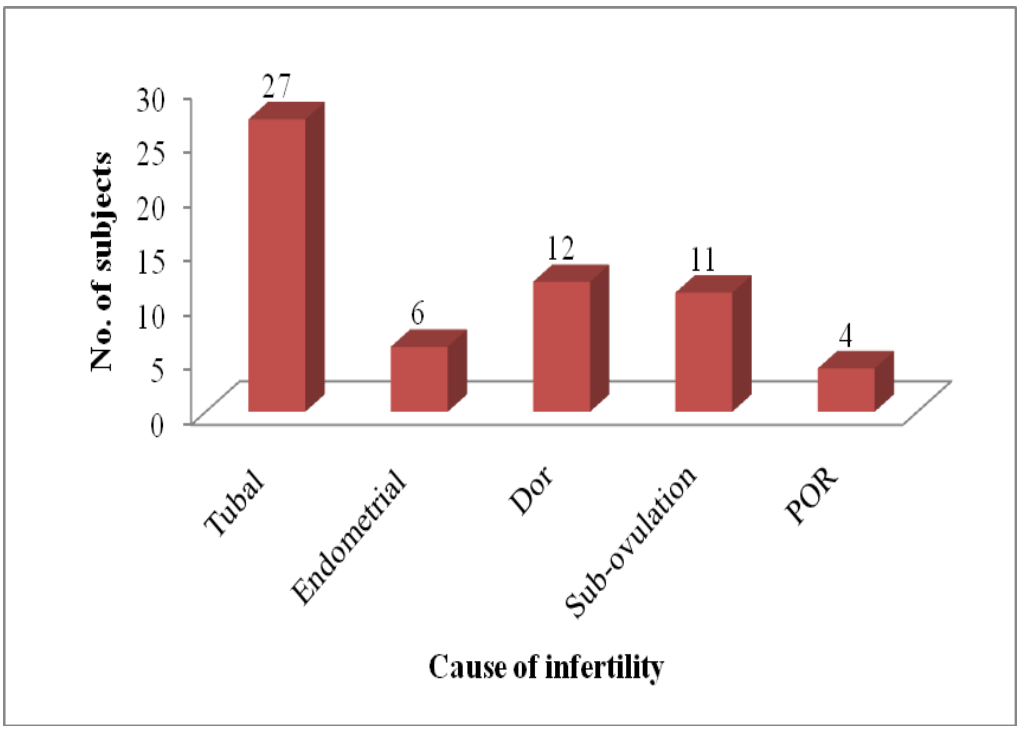


FIG 7: Causes of Infertility Among Subjects

Figure 7 is showing tubal factor as the most common cause of infertility.

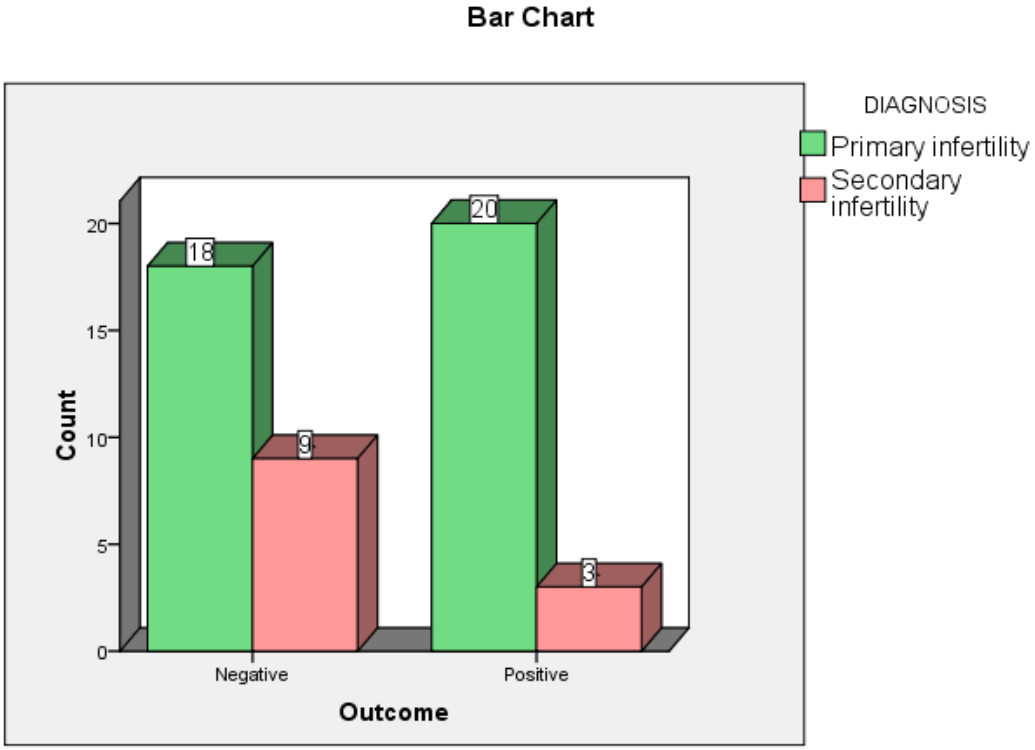


FIG 8: Outcomes In Primary And Secondary Infertility

Figure 8 shows the outcome among subjects with primary & secondary infertility. 20 subjects with primary infertility show positive outcome.

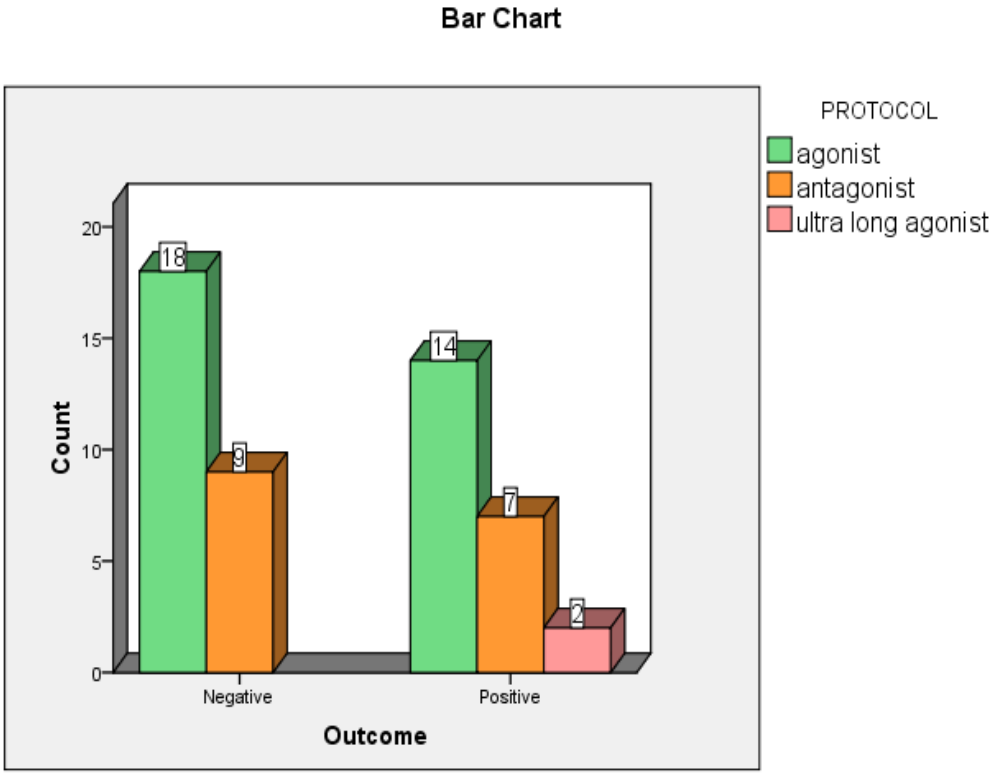
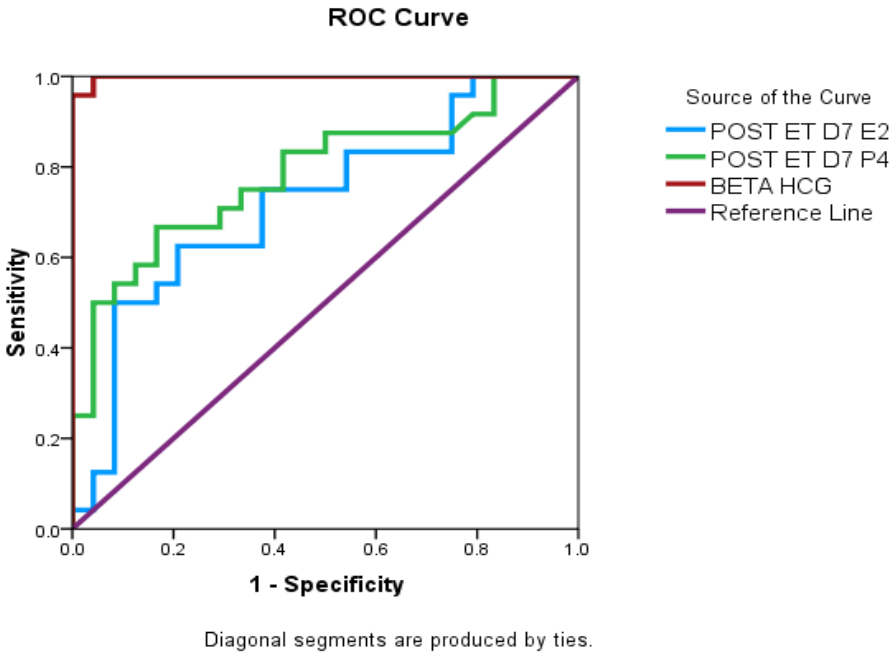


FIG 9: Distribution of Outcome in Subjects undergoing Different Protocols.

Figure 9 showing outcome in study subjects undergoing different protocols. 14 subjects undergoing agonist protocol show positive outcome.

Table 5: ROC curve analysis for predictive ability of various parameters to predict the outcome



Interpretation:
The test result variable(s): POST ET D7 P4 has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.
a. Under the nonparametric assumption;
b. Null hypothesis: true area = 0.5
ROC curve was plotted relative predictive value of various parameters. Beta HCG with maximum Area under curve was found to be best predictive parameter. POST ET D7 P4 was found to have AUC of 78.4% while POST ET D7 E2 showed AUC of 72.2%. The predictive cut off of various parameters and their sensitivity and specificity at that cut off value has been listed in table. Beta HCG shown maximum predictive potential with sensitivity of 100% and specificity of 95.8%.

Table 6: Outcome in study subjects

Outcome	Frequency	Percent
biochemical	3	6.0
clinical	19	38.0
negative	28	56.0
Total	50	100.0

Out of 19 clinical outcomes 9 were abortion

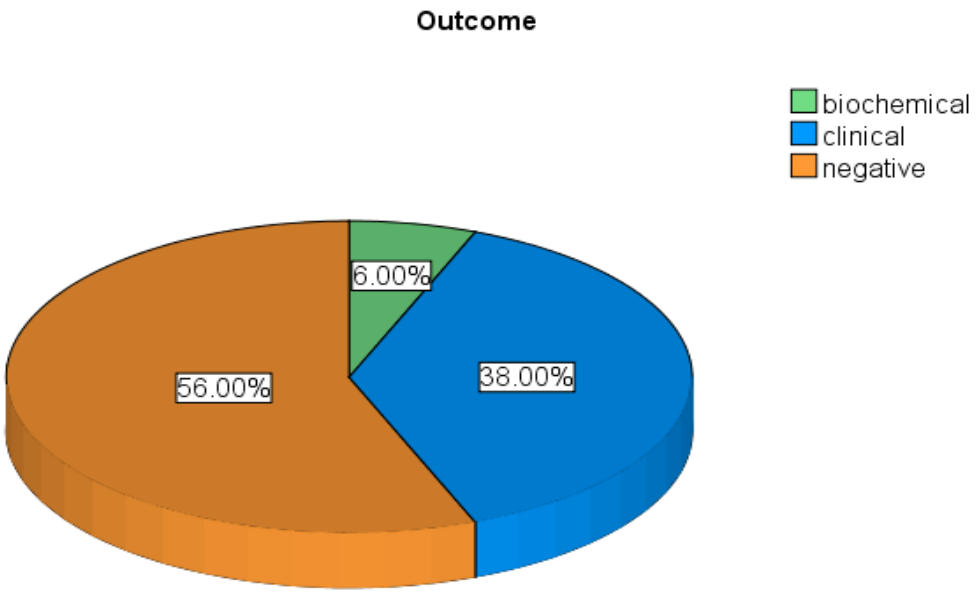


FIG 10: Outcome in study subjects

Figure 10 show 38% of positive clinical outcome in the study subjects.

Table 7: Correlation of E2 with P4 in subjects with different outcomes

Outcome	Pearson's r	P value	significance
Negative	0.247	0.213	No significant correlation
Positive	0.294	0.173	No significant correlation

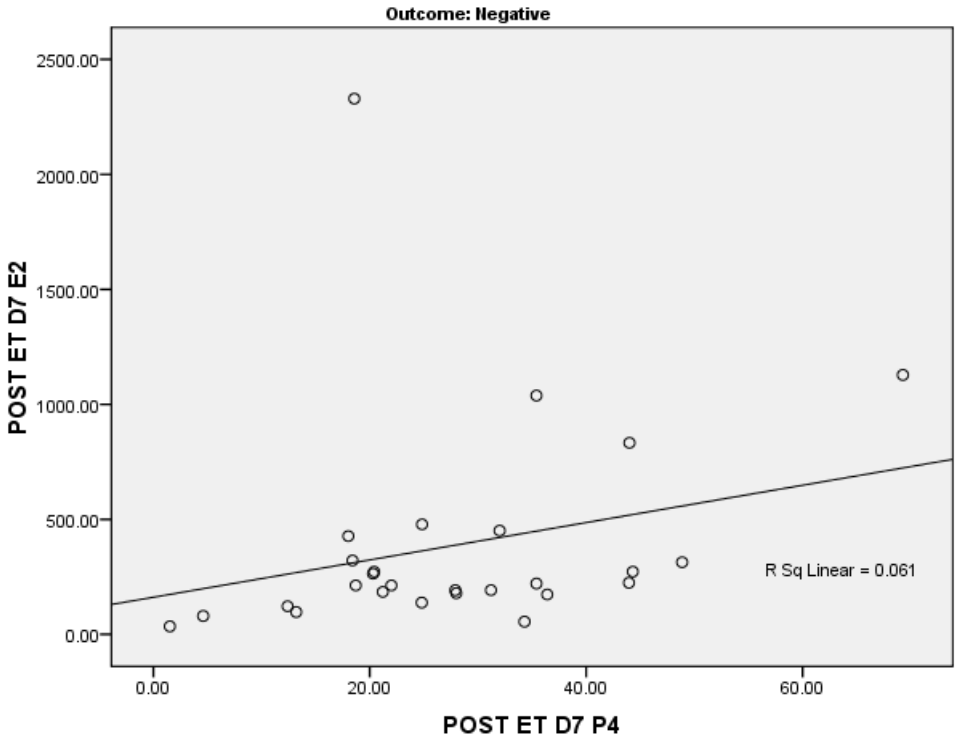


FIG 11: Correlation of E2 with P4 in subjects with negative outcomes.

Association of E2 cutoff with P4 cutoff was performed using pearsons correlation. No Significant association was found between two parameters for predicting negative outcomes.

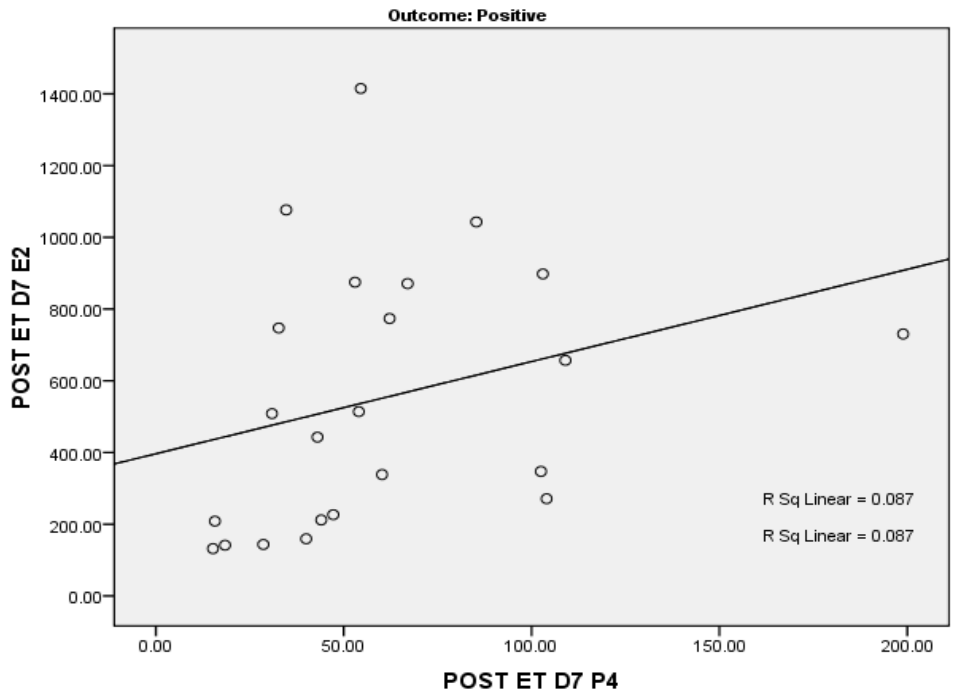


FIG 12: Correlation of E2 with P4 in subjects with positive outcomes.

Association of E2 cutoff with P4 cutoff was performed using pearsons correlation. No Significant association was found between two parameters for predicting positive outcomes.

Table 8: Association of E2 cutoff with outcome

E2	Outcome		Total
	Negative	Positive	
</=493.4	25	6	31
	80.6%	31.6%	62.0%
>493.4	6	13	19
	19.4%	68.4%	38.0%
Total	31	19	50
	100.0%	100.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	12.037[a]	1	.001		
Continuity Correction[b]	10.045	1	.002		
Likelihood Ratio	12.245	1	.000		
Fisher's Exact Test				.001	.001
Linear-by-Linear Association	11.797	1	.001		
N of Valid Cases[b]	50				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 7.22.
b. Computed only for a 2x2 table

Association of E2 cutoff with outcome was performed using Chi-square test. Significant association was found between two parameters indicating that higher E2 than cutoff was associated with positive outcome.

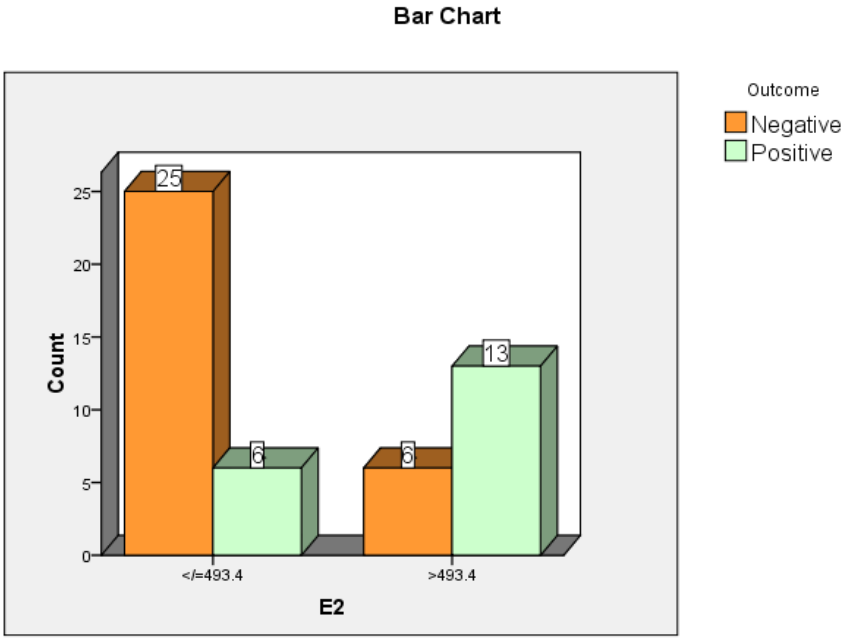


FIG 13: Association of E2 cutoff with outcome

Figure 13 shows Significant association between two parameters indicating that higher E2 than cutoff was associated with positive outcome.

Table 9: Association of P4 cutoff with outcome

P4	Outcome		Total
	Negative	Positive	
</=38.2	24	5	29
	77.4%	26.3%	58.0%
>38.2	7	14	21
	22.6%	73.7%	42.0%
Total	31	19	50
	100.0%	100.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	12.629[a]	1	.000		
Continuity Correction[b]	10.618	1	.001		
Likelihood Ratio	13.011	1	.000		
Fisher's Exact Test				.001	.000
Linear-by-Linear Association	12.376	1	.000		
N of Valid Cases[b]	50				

b. Computed only for a 2x2 table

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 7.98.

Association of P4 cutoff with outcome was performed using Chi-square test. Significant association was found between two parameters indicating that higher P4 than cutoff was associated with positive outcome.

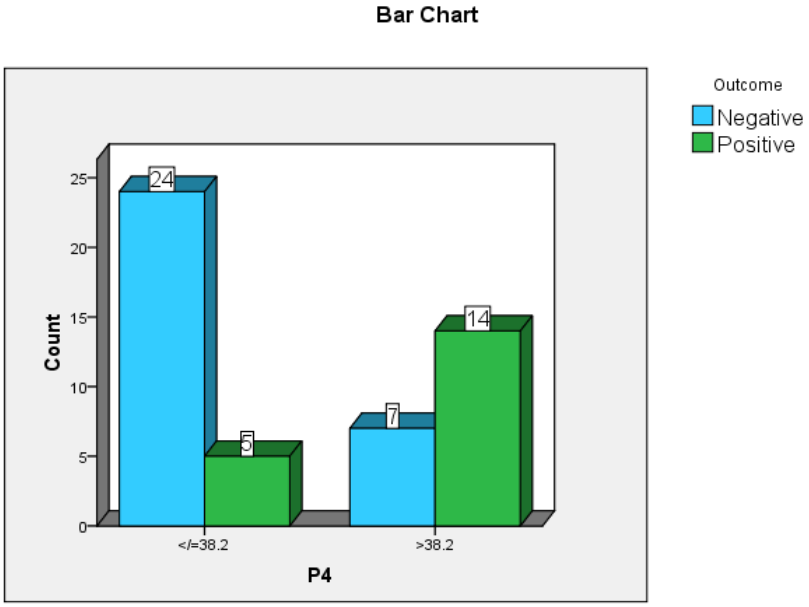


FIG14: Association of P4 cutoff with outcome

Association of P4 cutoff with outcome was performed using Chi-square test. Significant association was found between two parameters indicating that higher P4 than cutoff (>38.2) was associated with positive outcome.

Table 10: Association of cutoff for B HCG with outcome

Beta HCG	Outcome		Total
	Negative	Positive	
</=16.36	23	0	23
	95.8%	.0%	48.9%
>16.36	1	23	24
	4.2%	100.0%	51.1%
Total	24	23	47
	100.0%	100.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	43.165[a]	1	.000		
Continuity Correction[b]	39.415	1	.000		
Likelihood Ratio	56.821	1	.000		
Fisher's Exact Test				.000	.000
Linear-by-Linear Association	42.247	1	.000		
N of Valid Cases[b]	47				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 11.26.
b. Computed only for a 2x2 table

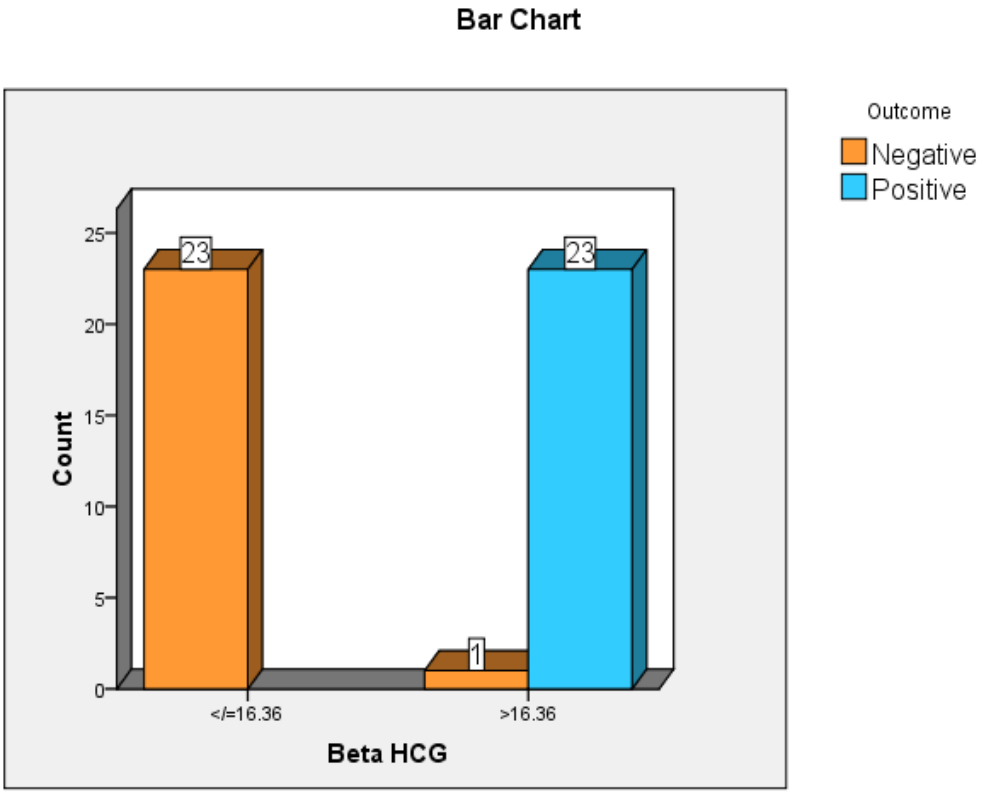


FIG 15: Association of Beta HCG cutoff with outcome

Association of cutoff for Beta HCG with outcome was assessed using Chi-square test. A significant correlation between two variables was derived indicating significantly higher frequency of positive outcome in subjects with Beta HCG higher than cutoff. The bar chart also show the higher positive outcome above the cutoff value of 16.36 for beta HCG (Figure 15).

Discussion

Day 7 median E2 was 508.6 (132-1415.08) and Day 7 mean P4 was 53 (15.02-198.90) in positive outcomes. D7 E2 was found to be significantly lower in negative outcome (p=0.043) and D7 P4 was found to be significantly lower in negative outcome (p=0.001) indicating a significant correlation between progesterone level d 7 and its predictive value on pregnancy outcome.

AUC of E2 is .722, AUC of P4 is .784 well within 95% confidence interval which emphasizes on significance of predicting the pregnancy outcome.

Cut off values

It is 493.4 ng/ml of serum E2 post D7 ET at which the sensitivity of predicting the outcome is 50% and specificity is 91.7% and 38.2 ng/ml of serum P4 post D7 ET at which the sensitivity of predicting outcome is 66.7% and specificity of 83.3%. suggesting a high significance in predicting the outcome.

Cut off levels of Progesterone on day 5 as >67.0ng /ml for pregnancy in a study [21]. This high value of cut off was because of administration of exogenous progesterone especially parentral progesterone that leads to a higher serum levels. However this was administered uniformally to all patients.

However there were 9 abortions in 19 of the clinical pregnancy outcomes out of which all showed either one parameter or both (E2 and P4) having decreased values below cut off levels, suggesting the need of having both the values being above the cut off for an ongoing pregnancy.

The statistical results even showed that both serum E2 and P4 values are independent predictors of pregnancy outcome. Since the study was of shorter duration suggesting the need of a prolonged study and detection of other factors affecting pregnancy resulting in abortion needs to be evaluated.

There have been few studies studying the predictive value of progesterone. Like most of the studies we found that serum progesterone and estradiol measurement has predictive value in predicting pregnancy outcome.

A similar study on serum biomarkers in predicting pregnancy outcome post ET by [19] were found to be higher in conception cycles than in non-conception for prediction of IU pregnancy. AUC of Progesterone was (.767; 95%CI.0.609-.926) in women with intrauterine pregnancies. Cut off being >32.1 ng/ml. However they had included Beta hcg in the study and found it to be the best predictor.

A study [17] where at D14, value of progesterone for predicting outcome in a pregnancy that occurs a result of post ICSI and found a similar significant correlation. Those women in which there was ongoing pregnancy had remarkably elevated values of serum progesterone levels (430 nmol/L),when compared to those with abnormal pregnancy, e.g., biochemical, abortions, (72 nmol/L) or the non-conception cycles (33 nmol/L). Their statistical analysis showed the corresponding values to my study (AUC, 0.927; 95% CI, 0.89-0.96; sensitivity 88.2%, specificity 84%). This result indicates that levels of serum progesterone are notably increased. The interpretation concludes that the progesterone level can be used to detect women bound to have viable intrauterine pregnancies despite supplementing progesterone exogenously.

In study [12], luteal serum oestradiol concentrations were shown to be statistically higher in conception IVF cycles compared with non-conception ones even as early as 4 days post-embryo transfer. They evaluated luteal oestradiol concentrations at different time points either before or after embryo

transfer and observed comparable oestradiol patterns before embryo transfer between conception and non-conception cycles, which indicated that higher early luteal oestradiol concentrations in conception cycles could be attributed to events after embryo transfer.

In another study, with small group of 22 IVF patients [15], conception cycles could be differentiated from non-conception ones by serum post-HCGday 8 oestradiol concentrations.

A similar study [5] had shown significantly higher post-embryo transfer day 8serum oestradiol concentrations in conception versus non-conception cycles, and also in ongoing versus nonviable pregnancies.

In another study [23] there was no established significant difference between the biomarkers and outcomes of pregnancies. The cut-off value >25.2 ng/mL for progesterone level at 14 days post retrieval of oocytes, demonstrated a better correlation than beta hcg in terms of AUC, sensitivity, and specificity. The beta hcg level had a cut-off value of >126.5 mIU/mL for forecasting to an extent the maintenance of pregnancy or whether it would end up in a miscarriage (AUC = 0.788 and 0.826; sensitivity = 0.788 and 0.723; specificity = 0.689 and AUC 0.833; $P <$ 0.0001 and $P <$ 0.0001, respectively).

In another study [20] a serum progesterone level of >22ng/ml is taken as cut off for pregnancy after superovulation after ART.

Mean progesterone level on 2 days after of ET were significantly high in pregnant female 401.6±118.21 as compared to non-pregnant 20.4±3.47 (p=0.001) [21] Cut off levels of Progesterone on day 5 as >67.0ng /ml for pregnancy. By the ROC Curves the sensitivity and specificity corresponding to these cut off both 100% for progesterone level at day 5 respectively depicting the role of progesterone in implantation potential.

Similarly in a study [1] median values for HCG, progesterone and oestradiol were higher in viable than non-viable pregnancies for all gestation times tested. The result hence confirmed these many previous studies showing similar results [8](Heiner *et al*., 1992; Keith *et al*., 1993; Schmidt *et al*., 1994; Fridstrom*et al*., 1995; Glatstein*et al*., 1995; al-Sebai*et al*., 1996; Bjercke*et al*., 1999; Urbancsek*et al*., 2002; Carmona *et al*, 2003).

Another study [9] saw that the mean serum progesterone value was remarkably elevated viable pregnancies (22.1 ng/mL) when it was compared to non-viable pregnancies (10.1 ng/mL) where the mean value was profoundly low thus establishing the fact even in spontaneous pregnancies, viability of a conception is largely] correlated to progesterone level. This study was purely done in non- stimulated cycles.

In another study [8], ROC for progesterone is 0.72 and 95% CI is 0.64-.85 and for estradiol is 0.60 and 95 % CI is 0.48 -0.73.Howeverthe study emphasized at beta hcg being the best predictor of all the three.

Conclusion

A significant correlation was found between D7 level of progesterone and estradiol and positive pregnancy outcome. A cut off value can be set above which more chance of a positive pregnancy might come. Apart from this, it can be used in therapeutic management of women having a lower progesterone level, where additional support of progesterone and estrogen would be given to maintain for any defect if at all.

It could also be used to improve patient counselling by giving quantitative and reliable predictive information instead of non-specific uncertainties**.**

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